Proteoglycans of the Human Periodontium

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A compilation of papers submitted for the degree of Doctor of Dental Science
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I. SIGNED STATEMENT

This collection of published works contains no material which has been accepted for the award of any degree or diploma in any university or tertiary institution and, to the best of my knowledge, contains no material previously published or written by another person, except where due reference has been made in the text.

For each paper where multiple authors are listed a summary statement is provided to indicate the contribution the candidate made to the publication.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

P. Mark Bartold
March, 1996

II. ACKNOWLEDGMENTS

For the development of my scientific career, I have been deeply influenced by three remarkable individuals - to each of whom I owe a great debt of gratitude. Professor John Thonard "collared" me as an undergraduate dental student and sowed the fertile seed of undertaking biological research on the tissues of the periodontium which ultimately led to my PhD studies. These early studies on the biochemistry of the connective tissues of the periodontium were also strongly influenced by Dr Ole Wiebkin who proved that science can be fun and rewarding. During my postdoctoral years in Seattle, I was particularly privileged to work in close association with Dr Roy Page, one of the truly great contributors to periodontal research over the past 25 years. To each of these individuals a heartfelt "thank you".

Of course, research by its very nature requires interaction with a great number of people, some of whom make a significant contribution to the development of one's projects. I have been lucky to have had the opportunity to work with many such individuals, many of whom have become collaborators, friends, and advisors. They include: Ted Cleary who provided much needed support following my return to Australia; Sampath Narayanan, from Seattle, who is a good friend and close collaborator; Chris Overall, who was my class mate through dental school, and later developed similar research interests to me, is a very special friend; Ulrich Schlagenhauf, whom I first met in Seattle and later developed a close friendship and collaborative interests; Barrie Vernon-Roberts, for his very important support during my return to Australia; and Marie Weger, who was my first research assistant and was a very important part of the "team" during the early establishment years.

I also wish to record my thanks to my family. To my parents who provided the important start in life and encouraged me to do my best at whatever I started. Of particular note here is the support my wife Mary has given me. She has always been supportive of my endeavors, and this has often been at the expense of her own desires.

Finally, to both Mary and my children, thank you. The work described in this collection of papers was often done largely in "your time".

III. PREFACE

This collection of 27 published journal articles represents work carried out between 1983 and 1995. The major thrust of these works is an extension of my PhD studies, and centres on detailed investigations into the nature of proteoglycans in various periodontal compartments and what factors might influence their structure and synthesis. To help this thesis read in a logical fashion the selected papers have been arranged, not in chronological order, but in a thematic form which covers the four main areas of research in this field that I have pursued over the past 13 years. These fields, together with the major sources of the subject matter used for the studies, are described below.

1. Identification of the cellular source of the proteoglycans in gingival connective tissues.

While my PhD studies had determined the presence of proteoglycans in human gingival tissues, their precise cellular source had not been determined. To do this, cell cultures were established from human gingival fibroblasts, lymphocytes and polymorphonuclear leukocytes. The tissues for these cell isolation studies were obtained from patients attending the Graduate Periodontics Clinic in Seattle, and the candidate's private practice in Adelaide. All of these cultures were established by the candidate. Healthy human volunteers, who were working in the Department of Pathology at The University of Adelaide with the candidate, provided the blood samples for isolation of the lymophocytes and polymorphonuclear leukocytes.

2. Isolation, identification and characterization of the proteoglycans found in the hard connective tissues of the periodontium.

Not only were proteoglycans identified in the soft connective tissues of the periodontium, but they were presumed to be present in the two hard tissues of the periodontium, namely cementum and alveolar bone. Thus, studies were undertaken to isolate, identify and characterize the proteoglycan content of these tissues. The human alveolar bone specimens were obtained from the Oral Surgery Clinic at the University of Adelaide following routine third molar surgical extractions. The cementum samples were provided by Dr Sampath Narayanan, University of Washington, Seattle, USA as part of a collaborative project investigating the biochemical composition of cementum.

3. Determination of the pathological changes which occur to the proteoglycans in human gingiva.

Once the proteoglycan composition of the "normal" periodontium had been established, it became necessary to investigate the changes which occurred during the development of inflammatory human periodontitis. The approaches used in these studies included studies on whole gingival biopsies as well as using cell culture. For these studies, inflamed human gingivae were obtained from the Graduate Periodontics Clinic at the University of Washington, Seattle, USA, and in subsequent years from the candidate's own specialist periodontal practice in Adelaide. The gingival fibroblast cultures derived from inflamed gingival biopsies were established by the candidate during his postdoctoral fellowship in Seattle.

4. Studying the effects of selected inflammatory and wound healing agents on proteoglycan synthesis.

Investigations into the molecular factors which might influence proteoglycan structure and synthesis were all carried out using cell culture. The gingival fibroblasts used in these studies included those derived from donors of a variety of ages, as well as those from normal and inflamed gingivae. All of the cultures were established by the candidate either in Seattle, Adelaide or Brisbane. The interleukin-1β was a generous gift from Dr Stephen Dower from Immunex Corporation, Seattle, Washington, USA; the lipopolysaccharide preparations from *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* were a gift from Dr Stephen Miller, State University at Buffalo, New York, USA; the culture supernatants from *Fusobacterium nucleatum* were provided by Dr Tony Rogers, The University of Adelaide; the platelet derived growth factor used was recombinant human of the BB isoform and was purchased from Genzyme Corporation, Boston, USA.

A more detailed description of the significance of these findings is provided at the commencement of each of the four sections. A statement regarding the candidate's contribution to any jointly authored papers is also detailed at the commencement of each section.

IV. OVERVIEW

Paper 1. Bartold, P.M.
Turnover in periodontal connective tissues. Dynamic homeostasis of cells, collagen and ground substances.
Oral Diseases **1:** 238-253; 1995

This paper provides a current overview of the biochemistry of the connective tissues which comprise the periodontium, and the changes which occur during inflammation. The complexity and unique features of the gingiva, periodontal ligament, cementum and bone are discussed with respect to their molecular composition and homeostasis. The author's studies are discussed in relation to the current literature, not only in the field of proteoglycan research, but in the more general terms of extracellular matrix biology. The importance of studies on the molecular composition of the periodontal connective tissues, and the factors which regulate their synthesis and degradation, is emphasized with respect to periodontal diagnosis and regenerative technologies.

Paper 1.

Bartold, P.M.
Turnover in periodontal connective tissues. Dynamic homeostasis of cells, collagen and ground substances.
Oral Diseases **1**: 238-253; 1995

Oral Diseases (1995) 1, 238–253

Turnover in periodontal connective tissues: dynamic homeostasis of cells, collagen and ground substances

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The connective tissues of the periodontium are composed of two soft tissues and two hard tissues—each of which has unique features. This review considers the constituents of normal, healthy periodontal connective tissues together with an appraisal of the changes in the connective tissue matrices of the periodontium which occur during the development of periodontitis. Recent developments in this field have paved the way for new and exciting vistas in periodontal diagnosis and regeneration which, ultimately, are two important goals in periodontal therapy.

Keywords: proteoglycans; collagens; connective tissue; periodontal diseases

Introduction

There are many diseases prevalent in our society which are associated with defects in either the connective tissue matrices, their resident cells, or both. A classic example of the relationship between connective tissue matrix and its cellular components is periodontitis.

The periodontal tissues normally exist in a steady state equilibrium oscillating between tissue degradation and tissue repair. Consequently, despite constant mechanical and chemical assault the periodontium manages, for the most part, to maintain its structural and functional integrity. Nonetheless, if the delicate balance between host response and bacterial virulence is disturbed then disease and associated tissue destruction results. Upon removal of the causative agent(s), tissue repair may proceed and ultimately health may be restored to the affected tissues.

Of course, the above is a simplistic approach to the problem. We now recognize at least four stages in the development of periodontitis (Page and Schroeder, 1976). The initial, early and established lesions refer to the onset and development of gingivitis. The advanced lesion is representative of deeper tissue destruction and indicates the onset of periodontitis. Thus there can be little doubt that the components of the extracellular matrix of the periodontium are significantly affected during inflammation. Of interest, however, is the recognition that gingivitis is very often a stable lesion which may never progress and develop into periodontitis. Hence in gingivitis, a balance exists between destruction and replacement of damaged matrix. If however, this balance is disrupted, then periodontitis results and the resultant advanced tissue destruction of both hard and soft connective tissues becomes evident and the resulting damage is, for the most part, irreversible.

This paper will be concerned with an overview of the normal constituents of the periodontal connective tissues together with an assessment of changes apparent with the development of periodontitis. Together, these findings pave the way for new and exciting vistas in periodontal diagnosis and regeneration which, ultimately are two important goals of periodontal therapy.

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The periodontal connective tissues

The periodontium is composed of a number of discrete and unique connective tissues, each of which confer upon the tissues their unique properties. Classically, the gingival connective tissue and periodontal ligament comprise the two principal soft connective tissues of the periodontium whereas the cementum and alveolar bone comprise the two hard connective tissues of the periodontium. Despite being in very close anatomic apposition, each of these tissues has a unique architecture, composition and function. Nontheless, there are some features which are characteristic of these collective tissues. In particular, all connective tissues are composed of three essential components, namely extracellular matrix, fibrous proteins and a variety of cells.

Extracellular components of connective tissues

Most connective tissues can be divided into fibrous and nonfibrous elements (Table 1). The fibrous elements include proteins such as collagen and elastin while the nonfibrous components include a variety of glycoproteins (laminin, fibronectin, proteoglycans, etc) as well as minerals, lipids, water and tissue-bound growth factors.

Table 1 Components of the extracellular matrix

Fibrous:	Collagens
	Elastin
Ground substance:	Proteoglycans
	Hyaluronan
	Non-fibrous proteins
	Lipids
	Minerals
	Water
Growth factors:	Fibroblast growth factors
	Transforming growth factors
	Platelet-derived growth factors

Collagens

The collagens are major constituents of many skeletal and soft connective tissues. Approximately 30% of the total protein in the human body is collagen (Nimni, 1980). These rigid proteins are responsible for the maintenance of the framework and tone of the tissues. To date at least 14 different collagens have been identified on the basis of their molecular composition and structure (van der Rest and Garrone, 1991). With such diversity it is apparent that the structure of collagens is not uniform and that there are several structural groups within the collagen family. Nonetheless, a reasonable definition of collagen would still be all encompassing such that collagen should be recognized as a structural protein which contains one or more domains having the conformation of a collagen triple helix.

The collagens may be grouped according to a variety of structural features as: (i) collagens participating in quarter staggered fibrils, (ii) fibril-associated collagens with interrupted triple helices (FACITS), (iii) collagens forming sheets, (iv) collagens forming beaded fibrils, (v) collagens forming anchoring fibrils, and (v) miscellaneous (Table 2).

The distribution of collagens varies considerably between different tissues as well as under a variety of normal and pathological conditions. For example the relative proportion and types of collagens can change according to the stage of development, morphogenesis, inflammation, wound healing, fibrosis and neoplasia (Adams and Watt, 1993). The substitution of one collagen for another and an anatomic redistribution of types has important ramifications for normal function and pathophysiology. In teeth, substantial changes in the distribution of collagens (as well as other extracellular matrix components) are associated with the cellular differentiation of teeth (Thesleff *et al*, 1991). In fetal tissues type III collagen predominates whereas in mature tissues type I is the predominant collagen. During inflammatory responses and early wound healing an increase in type III and type V collagens is seen (Gay *et al*, 1978, Narayanan and Page, 1983b).

Principally, collagens are considered to be structural proteins providing a rigid framework for tissues, however they do possess other functions. For example, collagenous surfaces (types I–IV) are a major substratum for cell adhesion *in vivo*, and this, together with collagen-mediated effects on cell growth and differentiation is likely to reflect a requirement for cell attachment to collagens (Hay, 1991). In addition, types I, II and III are chemotactic for fibroblasts and monocytes (Postlethwaite and Kang, 1976; Postlethwaite *et al*, 1978).

Table 2 Collagen classifications

Classification	*Collagen type*
Quarter staggered fibrils	I, II, III, V, XI
Fibril associated collagens with interrupted triple helices (FACITS)	XII, XIV, IX
Collagen sheets	IV, VIII
Beaded filaments	VI
Anchoring fibrils	VII
Miscellaneous	X, XIII

Elastin

Elastin is a very flexible and distensible protein which provides tissues the ability to stretch, bend and twist and is present in most vertebrate tissues to varying degrees (Mecham and Heuser, 1991). Elastin undergoes a number of changes in many diseases including atherosclerosis, emphysema and solar dermatosis and aging.

Elastin is extremely insoluble and this property has made it very difficult to isolate and characterize. Nonetheless recent data show that elastin fibers are composed of inner amorphous elastin components and an outer microfibrillar component (Cleary and Gibson, 1983). The molecule is stabilized by numerous crosslinks some of which are similar to those found in collagen and others, including desmosine and isodesmosine, are unique to elastin. With recent developments of molecular biology methodology, the structure and composition of elastin has become clearer. The precursor to elastin, tropoelastin is assembled into elastin fibers very rapidly extracellularly via efficient crosslinking reactions (Paz *et al*, 1982). Cell surface receptors (or binding proteins) for elastin have been identified and may be associated with cell chemotaxis (Senior *et al*, 1980).

Proteoglycans

Proteoglycans are large highly anionic glycoproteins ubiquitous to all connective tissues. They are located within the matrix as integral components of the matrix structure as well as on cell surfaces and within cell organelles (Gallagher, 1989). By virtue of their high charge they have been ascribed a variety of functions including tissue hydration, regulation of collagen fiber formation, growth factor binding, cell adhesion and growth (Ruoslahti, 1989; Ruoslahti and Yamaguchi, 1991; Scott, 1992; Yanagashita, 1993). By definition, a proteoglycan is composed of a single protein core to which one or more glycosaminoglycan side chains are covalently bound (Hardingham and Fosang, 1992). Historically, the classification of proteoglycans has been based on their glycosaminoglycan composition such that proteoglycans were given a nomenclature such as 'small dermatan sulfate proteoglycans' or 'large aggregating chondroitin sulfate proteoglycans' (Poole, 1986). However, in recent years, the classification system for proteoglycans has shifted from this simple approach to one more often based on core protein sequence or tissue location (Hardingham and Fosang, 1992). Irrespective of the names given to the proteoglycans, they can still be classified into at least three separate groups, based on their location, namely: (i) matrix organizers and tissue space fillers, (ii) cell surface proteoglycans or (iii) intracellular proteoglycans of the haemopoietic cells (Table 3).

Hyaluronan

This unsulfated, uronate-containing macromolecule is not found covalently bound to a protein core as are other glycosaminoglycans and thus does not exist as a proteoglycan (Mason *et al*, 1982; Laurent and Fraser, 1992). Hyaluronan also differs from proteoglycans in that it is synthesized in the plasma membrane by the addition of sugars to the reducing end of the molecule with the reducing end projecting into the pericellular environment (Prehm, 1984) to form a simple structure of repeating disaccharide units of

Table 3 Proteoglycan classification

Extracellular matrix proteoglycans:	Aggrecan
	Versican
	Perlecan
	Decorin
	Biglycan
	Centoglycan
Cell surface proteoglycans:	Syndecan-1
	Syndecan-2
	Syndecan-3
	Syndecan-4
	CD-44
	Betaglycan
Haemopoietic cell proteoglycans:	Serglycine

glucuronic acid and glucosamine. The distribution of this molecule is virtually ubiquitous to all tissues and it is synthesized by most cells. The functions of hyaluronan are many and varied being most importantly associated with tissue hydration, cell surface matrix interactions, cell migration, tissue development, aggregation with aggrecan, CD44 and other matrix components (Toole, 1990; Laurent and Fraser, 1992).

Noncollagenous proteins

Apart from the collagens, elastin and proteoglycans there are numerous other protein components of connective tissues (Yamada, 1991). In soft connective tissues these include matrix molecules such as fibronectin, laminin, vitronectin, thrombospondin, tenascin and entactin as well as the ubiquitous cell surface glycoproteins known as the integrins. In addition, several noncollagenous proteins have been identified in bone matrices and include osteocalcin, osteonectin, osteopontin and bone sialoproteins (Young *et al*, 1992). While the noncollagenous proteins are only beginning to be identified and studied in any detail, their role in regulation of tissue function and integrity will, no doubt, be just as critical as the better studied collagens and proteoglycans. As our understanding of the extracellular matrix develops it is becoming increasingly difficult to distinguish between matrix/structural proteins and matrix/cell surface molecules. The two components become inextricably intertwined to the point where it is difficult to distinguish between the two. Indeed it is clear that while the matrix may provide a substratum for cell surface attachment it also can dictate cell mobility, cell synthetic function and even cell shape (Hay, 1984).

Growth factors

Growth factors are very potent modulators of cell function and are produced by a wide variety of cells. Although growth factors are usually considered in isolation it is becoming apparent that these agents may be bound within the extracellular matrix acting as a reservoir for subsequent action and/or use (Ruoslahti and Yamaguchi, 1991). Many of the proteoglycans function as modulators of growth factors through their capacity to bind these agents (Table 4). These interactions may be mediated via the glycosaminoglycan chains or portions of the core proteins. For example heparan sulfate chains have high affinity for the fibroblast growth factors, GMCSF, and IL-3 (Roberts *et al*, 1988). A strong interaction between platelet factor 4 and chondroitin sulfate has also been reported with the chondroitin sulfate chains of serglycine being involved in this interaction (Ruoslahti and Yamaguchi, 1991). The core proteins of cell surface proteoglycan betaglycan and the matrix proteoglycan decorin can bind TGF-β (Andres *et al*, 1989; Yamaguchi *et al*, 1990).

Extracellular matrix composition of the healthy periodontium

Gingiva

The gingival tissues are comprised of an epithelial surface layer and an underlying stromal connective tissue. The epithelium is classically divided into oral, sulcular and junctional components which vary in their degree of keratinization, number of cells and presence of rete pegs. Both tissues have extracellular matrices but due to their vastly different structures and functions these matrices differ quite significantly.

Gingival epithelium

Despite its strategic position as the first line of defense against bacterial assault on the periodontal tissues, the extracellular matrix of gingival epithelium has been poorly studied. Histological assessment of gingival epithelium indicates that there are spaces between the epithelial cells, yet the nature of what 'fills' these spaces is not clear. It is clear that there is no fibrous protein component to the epithelial extracellular matrix but the nonfibrous components may include a variety of glycoproteins, lipids, water and proteoglycans as well as extensions of intercalated cell surface molecules (Bartold, 1987).

For many years the ability of epithelial cells to produce extracellular products such as proteoglycans was disputed (Pedlar, 1979) and it was suggested that the presence of any such material in the spaces between epithelial cells merely indicated an origin from the underlying mesenchymal tissues (Braun-Faulco, 1958). Early investigators tried to resolve this issue using histochemical techniques, however it was not until autoradiographic studies were developed that it was clearly demonstrated that gingival epithelial cells had a capacity to synthesize and secrete sulfated molecules interpreted to be proteoglycans (Wiebkin and Thonard, 1981).

More recently, using specific immunohistochemical and histochemical probes hyaluronan, decorin, syndecan and CD44 have all been identified in human gingival epithelium (Tammi *et al*, 1990; Häkkinen *et al*, 1993). While some site variation exists between junctional, sulcular and oral epithelium the precise significance of these findings is unclear (Kogaya *et al*, 1989; Takata *et al*, 1990; Oyarzun-Droguett, 1992).

Until recently the culture of oral epithelial cells was elusive. Following the development of improved methods of culturing oral keratinocytes (Oda and Watson, 1990; Willie *et al*, 1990), reports have indicated that gingival keratinocytes synthesize principally heparan sulfate proteoglycans with small amounts of chondroitin/dermatan sulfate (Rahemtulla *et al*, 1989; Potter-Perigo *et al*, 1994), con-

Table 4 Proteoglycans that bind growth factors

Name	*Growth factor bound*[a]	*Binding by*
Decorin	TGF-β	Protein core
Betaglycan	TGF-β	Protein core
Syndecan	bFGF;aFGF;GM-CSF CSF;IL-3;IFN-γ	Heparan sulfate
Cell surface heparan Sulfate proteoglycan	FGF	Heparan sulfate
Serglycine	Platelet factor-4	Chondroitin sulfate

[a]Abbreviations used: TGF-β = transforming growth factor beta; bFGF = basic fibroblast growth factor; aFGF = acidic fibroblast growth factor; GM-CSF = granulocyte-macrophage colony stimulating factor; CSF = colony stimulating factor; IL-3 = interleukin 3; IFN-γ = interferon gamma; FGF = fibroblast growth factor

firming previous biochemical analyses of whole extracts of human gingival epithelium (Bartold *et al*, 1981). To date the identity of specific proteoglycan molecular species associated with gingival epithelial cell cultures has not been elucidated.

Gingival connective tissue

The principal extracellular component of human gingival connective tissues is collagen. To date, six types of collagen have been identified in the gingival connective tissues with type I collagen being the predominant species (Narayanan and Page, 1983a). Type III collagen is also a principal component of the gingival connective tissues and together with type I collagen is uniformly distributed throughout the connective tissue (Rao *et al*, 1979; Wang *et al*, 1980; Chavrier *et al*, 1984; Narayanan *et al*, 1985; Romanos *et al*, 1991). Type IV collagen is found associated with blood vessels and basement membranes whereas type V and VI have a diffuse filamentous distribution (Narayanan *et al*, 1985; Romanos *et al*, 1991).

Numerous proteoglycans have been identified in gingival tissues and have been identified as decorin, biglycan, versican and syndecan (Bartold *et al*, 1983a; Pearson and Pringle, 1986; Bratt *et al*, 1992; Larjava *et al*, 1992). Immunohistochemical studies have identified a variety of proteoglycans within the gingival tissues which are closely associated with collagen fibers, blood vessels and cell surfaces (Shibutani *et al*, 1989; Bartold, 1992; Häkkinen *et al*, 1993).

In order to understand the biosynthesis of gingival connective tissue, fibroblasts which are the predominant cell type present and presumed to be the cell responsible for the production of the extracellular matrix have been used extensively to study collagen and proteoglycan synthesis (Narayanan and Page, 1976; Bartold and Page, 1987; Larjava *et al*, 1992). These studies have demonstrated that, *in vitro*, human gingival fibroblasts produce proteoglycans and collagens of a similar profile to those extracted from whole gingival tissue extracts.

Elastin is a minor constituent of gingival connective tissue, accounting for approximately 6% of the total tissue protein (Chavrier, 1990). There has been little consideration of this protein in the gingival tissues despite its important role in conferring distensible properties on tissues (Chavrier *et al*, 1988). Interestingly, elastin is present in relatively small amounts in the fixed, inflexible attached gingiva but it is more prominent in the submucosal tissues of the more movable and flexible alveolar mucosa (Bartold, 1991).

Other glycoproteins including fibronectin, tenascin, osteonectin and laminin have been identified in gingival connective tissues (Pitaru *et al*, 1987; Salonen *et al*, 1990; Steffensen *et al*, 1992).

Periodontal ligament

The principal collagen species in Sharpey's and other collagen fibers of the periodontal ligament is type I (Rao *et al*, 1979). Type III collagen appears to coat type I collagen of Sharpey's fibers. Together these two collagen types are codistributed with types V and XII, and fibronectin. Blood vessels within the periodontal ligament contain type I, III, IV and V collagens. The periodontal ligament also contains small amounts of elastin and tenascin, which are present in connective tissue and in zones along cementum and bone (Lukinmaa *et al*, 1991).

The proteoglycans in human periodontal ligament are composed of similar glycosaminoglycans to gingival tissue being hyaluronan, heparan sulfate, dermatan sulfate and chondroitin sulfate of which dermatan sulfate is the principal GAG (Pearson and Gibson, 1982; Gibson and Pearson, 1982). The finding that dermatan sulfate is the principal glycosaminoglycan in periodontal ligament is consistent with the highly collagenous nature of this tissue. Two principal proteoglycans have been identified in periodontal ligament as versican and decorin (Pearson and Pringle, 1986; Häkkinen *et al*, 1993). The distribution of proteoglycans in periodontal ligament is closely associated with collagen fibers, cell surfaces and blood vessels.

The use of cell cultures for studying *in vitro* synthesis of proteoglycans by periodontal ligament fibroblasts has not been utilized as much as one might expect. Indeed, relatively few groups have studied proteoglycan synthesis by periodontal ligament fibroblasts. Smalley *et al* (1984) identified the glycosaminoglycans synthesized by periodontal ligament fibroblasts and confirmed these were similar to those found *in vivo*. To date there appears to be only one detailed study on periodontal ligament fibroblast proteoglycans (Larjava *et al*, 1992). These cells, like their gingival counterparts, synthesize numerous types of proteoglycans of which decorin and versican have been identified. However it is likely that other proteoglycans are present and these will most likely include syndecan, glypican and betaglycan. Although biglycan has not been identified in cul-

tures of normal periodontal ligament it is noteworthy that exposure of these cells to TGF-β significantly stimulates the synthesis of this proteoglycan (Kähäri *et al*, 1991).

Cementum

Very little detailed information is available on the extracellular matrix of cementum. Histologically cementum is similar to bone and dentin. The inorganic matrix accounts for approximately 50% of the cementum content and is composed principally of hydroxyapatite. The major proportion of the organic matrix is composed of types I and III collagens (Birkadel-Hansen *et al*, 1977). In recent years interest in the noncollagenous matrix of cementum has intensified with the realization that many mineralized tissues have, within their organic matrix, many factors capable of inducing new mineralized tissues which are clearly of importance with respect to tissue regeneration (Reddi, 1992; Ripamonti and Reddi, 1994). Although difficulties have been encountered in obtaining suitable quantities of cementum for analysis, numerous studies in a variety of animals indicate that a variety of nonfibrous proteins are present in cementum. These include at least three RGD-associated adhesion proteins, bone sialoprotein, tenascin, osteonectin and proteoglycans (Somerman *et al*, 1990; 1991; MacNeil and Somerman, 1993). While the function of these molecules is still speculative some have been characterized in detail and appear to play a significant role in modulating fibroblast attachment and function (Somerman *et al*, 1987; Nakae *et al*, 1991).

Histochemical and biochemical studies have confirmed the presence of hyaluronan, dermatan sulfate and chondroitin sulfate in cementum with chondroitin sulfate being the predominant glycosaminoglycan (Bartold *et al*, 1988). Immunohistochemical studies have shown the distribution of proteoglycans to be closely associated with the cementoblasts and lightly distributed throughout the matrix (Bartold *et al*, 1990). Intact proteoglycans have been extracted from cementum and identified as predominantly a chondroitin sulfate proteoglycan—its identity awaits further work but on the basis of its size and similarity to bone proteoglycans it is most likely to be decorin. Due to the absence of any reliable culture techniques for cementoblasts no information is available for the biosynthesis of proteoglycans by cementoblasts.

Alveolar bone

Although better studied than cementum, alveolar bone has been rather neglected with respect to analyses of its extracellular matrix composition. Collagens type I and III comprise the bulk of the nonmineralized matrix of alveolar bone (Rao *et al*, 1979; Wang *et al*, 1980). Glycosaminoglycans have been identified by histochemical and biochemical means as predominantly chondroitin sulfate with minor proportions of heparan sulfate, dermatan sulfate and hyaluronan present (Waddington *et al*, 1988; Bartold, 1990). Immunohistochemical localizations have shown a distribution to the cells and to their lacunae as well as throughout the mineralized matrix (Bartold, 1990). Analysis of the proteoglycans of bone have been restricted to only a few studies with a chondroitin sulfate proteoglycan being the major species identified (Bartold, 1990; Waddington and Embery, 1991). This is most likely a mixture of decorin and biglycan.

Matrix changes with development of inflammation

Gingival epithelium

One of the earliest reported changes to occur in the periodontal tissues during development of plaque-induced periodontal inflammation is widening of the intercellular spaces between the epithelial cells of the sulcular epithelium (Thilander, 1968). Apart from a few early histochemical studies these changes have been poorly studied. What is interesting about these changes is that although widening occurs we have no idea what fills these widened spaces. Whether it is specific matrix components (perhaps proteoglycans, or hyaluronan), or merely water is an important consideration since the composition of these 'spaces' will have a significant bearing on how nutrients, metabolites and toxins may passage the epithelial barrier to initiate the inflammatory process (Bartold *et al*, 1983b). Indeed, the issue of matrix-controlled diffusion of molecules is very important in regulating the physiological properties of tissues. However, this area of physical biochemistry has been given little or no recognition in the periodontal literature. While it is recognized that diffusion occurs across the sulcular and junctional epithelia both from the connective tissue to the gingival sulcus and from the gingival sulcus to the connective tissue, the mechanisms are not understood. It has been proposed that the concentrated matrix of healthy epithelia may facilitate diffusion of molecules and even maintain tissue-destructive enzymes in a non-active conformation while the less concentrated matrices associated with widening of the spaces may be more conducive to ongoing tissue destruction (Bartold *et al*, 1983b).

Gingival connective tissue

During the development of gingivitis, the gingival connective tissues are disrupted within 3–4 days after plaque accumulation (Page and Shroeder, 1976). The destructive process begins at perivascular collagen bundles with up to 70% of the collagen within inflammatory foci being lost during the early stages of inflammatory periodontal disease (Payne *et al*, 1975). The observed increase in collagen solubility indicates either active synthesis of new collagen and/or impaired crosslinking. As the inflammatory process of gingivitis progresses, the amount of type V collagen increases, often exceeding that of type III collagen (Narayanan *et al*, 1983; 1985).

Numerous difficulties have been encountered in establishing the proteoglycan profile of normal and inflamed gingival tissues due to the large number of component cell types capable of synthesizing proteoglycans. For example, not only are fibroblasts present but, due to its high vascularity, gingival tissues also have a significant number of endothelial cells present. With the presence of a low grade inflammatory infiltrate, even in clinically healthy samples, numerous inflammatory cells (neutrophils, lymphocytes, mast cells etc) are also present in gingival connective tissues.

Early studies on inflamed periodontal tissues used histochemical changes and clearly showed that at foci of inflammation there was a loss of material which stained positively for glycosaminoglycans while at the periphery of the lesion there was a notable increase in staining intensity (Melcher, 1967). Additionally, histochemical dyes stained many of the inflammatory cells indicating them to have a significant component of proteoglycan (Melcher, 1967). Unfortunately these important observations were largely ignored as biochemical techniques were developed and studies became more focussed on the composition of the tissues rather than the dynamics of the events. Despite numerous studies, very few changes in the total amounts of proteoglycans have been noted in inflamed gingival tissues (Embery *et al*, 1979; Bartold and Page, 1986a). Nonetheless, some minor qualitative changes have been noted in which dermatan sulfate decreases and chondroitin sulfate increases. In addition, clear evidence has been reported on changes to the protein core of the tissue proteoglycans as well as significant depolymerization of hyaluronan (Bartold and Page, 1986b). These findings differ from those noted for the collagens where there appears to be a net loss of collagenous proteins from the inflamed tissues. Initially, the findings of little change with respect to proteoglycan content were perplexing. However, they can be explained in the light of the earlier histochemical studies alluded to above as well as more recent data on the ubiquitous cellular distribution of proteoglycans. Firstly, the findings should not have been surprising since earlier studies showed some loss of material at the inflammatory focus but increased staining at its periphery (Melcher, 1967). This observation indicates that, as part of the tissue response to inflammation, the resident cells, when trying to wall off the inflammatory lesion, increase their production of some components of the extracellular matrix. Furthermore, findings of inflammatory cell staining by histochemical dyes (Melcher, 1967) together with more recent detailed studies on the production of proteoglycans by numerous inflammatory cells (Bartold *et al*, 1989a; 1989b) explain why marked changes or depletion of proteoglycans in inflamed gingival tissue are unlikely to occur. In addition, since the principal proteoglycan produced by inflammatory cells is a chondroitin sulfate proteoglycan, and there is significant loss of proteoglycan (most likely dermatan sulfate) with the loss of fibrous tissue, the switch from a predominantly dermatan sulfate rich proteoglycan matrix to one richer in chondroitin sulfate with developing periodontal inflammation is explained.

Periodontal ligament

To date there have been few studies concerning the pathological changes occurring in the periodontal ligament during the development of periodontitis. Topographical studies in sheep have mapped the composition of the periodontal extracellular matrices in sheep (Kirkham *et al*, 1989) and determined the changes occurring in the periodontium during inflammatory mediated degradation (Kirkham *et al*, 1991; 1992). As noted for gingival tissues, the level of sulfated glycosaminoglycans in the periodontal ligament increases with increasing disease. This increase apppears to be specifically associated with an increase in chondroitin sulfate at the expense of dermatan sulfate. The significance of such findings is still unclear but is most likely related to an increase in cellularity of the tissue, release of proteoglycans from the alveolar bone, or altered metabolism of the sulfated glycosaminoglycans in the inflamed tissues. Evidence of structural changes to periodontal ligament proteoglycans has been noted in tissues undergoing orthodontic forces (Last *et al*, 1987). Although there appears to be little change in the types of glycosaminoglycans present in such tissues, there is a significant decrease in the amount of high molecular weight proteoglycans in periodontal ligaments subjected to a variety of tensional and compressive stresses.

Cementum

As noted above cementum has been poorly characterized with respect to its normal biochemical composition in relation to the other periodontal tissues. Virtually nothing is known of the biochemical changes occurring in cementum adjacent to tissues affected by periodontal inflammation. Apart from the obvious accrual of a variety of bacterial products either on the cementum surface or possibly within the cementum matrix (Hughes and Smales, 1986; Hughes *et al*, 1988), other changes in cementum associated with periodontal disease have included increased softness (Riffle, 1952), increased inorganic components (Selvig and Zander, 1962), decreased organic composition (Stepnick *et al*, 1975), all of which may contribute to the reported structural changes noted in exposed cementum. The collagens of periodontally affected cementum appear to be denatured and have a relatively high affinity for mineral adsorption (Furseth, 1971). It has been proposed that as periodontitis develops the cementum is exposed to four different environments (Armitage and Christie, 1977). In health the root surface is largely covered by the fibrous and non-fibrous matrix of the periodontal ligament and supracrestal gingival tissues. With development of gingival inflammation and loss of fiber attachment into the root surface, epithelium migrates along the root surface to cover the cementum. As a pocket begins to develop the cementum becomes exposed to the pocket environment and with subsequent recession (either as a result of therapy or continuing pathological events) the cementum may be exposed to the supragingival oral environment. There is clearly a need for further investigations into the chemical changes associated with exposed root surfaces and how these might be made more 'biologically acceptable'.

Alveolar bone

Changes to the matrix of resorbing alveolar bone are poorly understood. It is likely that the general mechanisms of bone resorption (Pierce *et al*, 1991) would apply to the alveolar bone affected by periodontitis. These mechanisms, require both decalcification as well as removal of the organic components and involve an interplay between osteoblasts and osteoclasts (Rodan and Martin, 1981; Wong, 1984). However, the precise sequence of these events is still the topic of debate. An important sequela to bone resorption is the release of organic components into the periodontal connective tissue matrices which may be detected subsequent to the resorptive process and used as potential markers of bone resorption (see later in this review).

Mechanisms of matrix disruption

As outlined above, there is little doubt that the components of the extracellular matrix of the periodontium are significantly affected during inflammation. However, the mechanisms of extracellular matrix disruption associated with microbial colonization and subsequent periodontal destruction are many and varied (Birkadel-Hansen, 1993a). Matrix changes noted in periodontitis may result via one of three principal pathways: (i) release of degradative enzymes by host or bacterial cells, (ii) release of reactive oxygen species, or (iii) alteration in matrix synthesis by fibroblasts, keratinocytes, endothelial cells and osteoblasts induced by a variety of cytokines and inflammatory mediators. As has been noted before, several of these pathways are indeed inflammation-independent (Birkadel-Hansen, 1993a) and thus the issue of tissue destruction, rather than inflammation alone, becomes a critical consideration in understanding the disease process associated with periodontitis (Figure 1).

Enzymatic degradation

Breakdown of the extracellular matrix in periodontal diseases can be caused by enzymes of either an intracellular or extracellular source. The extracellular pathways may involve mammalian interstitial enzymes or those of an exogenous source such as from bacteria.

Intracellular (phagocytic) degradation of extracellular matrix components is often overlooked as an important pathway of tissue remodelling (Melcher and Chan, 1981). The process of phagocytosis of collagen has been well described whereby collagen fibers have been noted in lysosomes where they undergo proteolytic digestion (Ten Cate, 1972; Listgarten, 1973). In order for phagocytosis to occur the collagen must first be partially digested extracellularly. The mechanism by which this occurs is not entirely clear but may be via cell surface associated or secreted proteases acting in the immediate pericellular environment (Svoboda *et al*, 1979). Once inside the cell the phagocytosed collagen collects in small vacuoles which appear to coalesce with lysosomes. Although numerous cathepsins are able to degrade the partially denatured collagen (Burleigh *et al*, 1974; Kirschke *et al*, 1982) cathepsin L appears to be the most effective (Kirschke and Barrett, 1985).

Extracellular degradation of the extracellular matrix may occur via the action of a number of metalloendopeptidases. Amongst these enzymes, interstitial collagenase (MMP-1), gelatinase (MMP-2), type IV collagenase (MMP-9), and the stromelysins (MMP-3, MMP-10, MMP-11) have been suggested to have a significant role in the initial destruction of periodontal extracellular matrix macromolecules (Birkadel-Hansen, 1993b). Subsequent to the initial depolymerization caused by these enzymes more complete degradation of the proteins involves the action of other less specific host and bacterial enzymes.

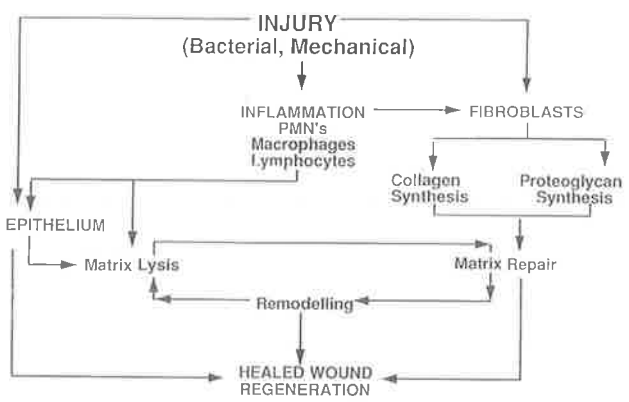

Figure 1 Schematic representation of the interplay between inflammation, matrix synthesis, matrix lysis and tissue repair

Although these enzymes possess the ability to degrade matrix components it must be remembered that most of them are secreted in a latent form and must be 'activated' before they can degrade their specific substrates. Proenzyme activators and specific inhibitors of metalloproteinases (TIMPs) are important regulators of enzyme activity. Activation of the enzyme involves a complex series of events closely linked to conformational changes leading to cleavage of the proenzyme sequence by exogenous proteinases such as trypsin, plasmin, chymotrypsin, neutrophil elastase, or plasma kallikrein (Nagase *et al*, 1990). The initial cleavage then permits a second, autolytic cleavage to occur leading to the mature and active form of the enzyme (Stricklin *et al*, 1983). In addition to endogenous control of latent matrix metalloproteinases, recent evidence indicates that proteases from periodontopathic bacteria may also activate latent collagenases (Sorsa *et al*, 1992). TIMPs form noncovalent complexes with the MMPs to inhibit the activity of the active enzyme or inhibit precursor activation. At least two members of the TIMP family have been identified (TIMP-1 and TIMP-2) with the likelihood of others being identified (Stettler-Stevenson *et al*, 1989).

The role of hydrolytic enzymes in matrix degradation is unclear. Many hydrolytic enzymes have been identified in inflamed periodontal tissues. The most notable of these enzymes identified in inflamed periodontal tissues include β-glucuronidase, aryl sulfatase and hyaluronidase (Goggins *et al*, 1968; Podhradsky *et al*, 1982; Lamster *et al*, 1985). The substrates for such enzymes would clearly be the carbohydrate components of the proteoglycans as well as hyaluronan. It would seem unlikely that these enzymes are responsible for the primary degradation of proteoglycans. More likely, such enzymes are involved in a secondary capacity, after initial proteolytic cleavage of proteoglycans, in the subsequent breakdown of glycosaminoglycan chains. However, in light of current biochemical analysis of inflamed periodontal tissues there is little evidence to support such a role for these enzymes in matrix degradation. No evidence has been found to indicate the products of their activity since glycosaminoglycans isolated from inflamed gingiva appear to remain relatively intact despite an abundance of hydrolytic enzymes (Embery *et al*, 1979; Bartold and Page, 1986b).

Bacterial enzymes

Another important source of degradative enzymes is from the microbial plaque in the gingival sulcus. Indeed, numerous enzymes associated with bacterial dental plaque have been implicated in the pathogenesis of inflammatory periodontal disease (Slots and Genco, 1984; Mayrand and Holt, 1988). In particular, the black pigmented *Bacteroides*

(Porphyromonas) species appear to synthesize numerous proteases with a potential to disrupt periodontal extracellular matrix (Mayrand and Holt, 1988; Greiner and Mayrand, 1993). In addition, *C. histolyticus* and some facultative *Bacillus* species from dental plaque also secrete enzymes which can degrade collagen (Makinen and Makinen, 1987). Other enzymes with trypsin-like activities have been described from *P. gingivalis, T. denticola* (and other spirochetes) and *B. forsythus* which may degrade type I collagen and plasma fibronectin (Mäkinen *et al*, 1986; 1990; Smalley *et al* 1988a; Söderling *et al*, 1991).

While there is no question that many oral bacteria secrete collagenases, their role in the primary destruction of periodontal collagens has been questioned (Overall *et al*, 1987). Evidence for a host, rather than bacterial, origin of collagen degradation comes from the observation that 3/4–1/4-fragments of collagen, indicative of mammalian collagenase digestion, can be identified in inflamed gingival extracts. Furthermore several investigators have found little evidence for the presence of bacterial collagenase in gingival crevicular fluid (Overall *et al*, 1991; Ingman *et al*, 1993). Thus, despite observations that periodontopathic bacteria such as *Porphyromonas gingivalis* and *Treponema denticola* contain and produce proteolytic enzymes (such as trypsin-like proteases and collagenase), there is little evidence to support their activity as first line initiators of collagen degradation *in vivo*. Nonetheless, it should be remembered that these enzymes may play an important role in degradation of collagen peptides subsequent to initial cleavage by mammalian collagenases.

Alternative mechanisms of action for bacterial enzymes may be via: (i) acting as antigens capable of stimulating cytokines from circulating mononuclear cells which in turn may activate MMPs, (ii) cleavage of host defence mediators such as immunoglobulins, plasma proteinases, and cytokines, (iii) cleavage and activation of procollagenase or (iv) inducing keratinocytes and fibroblasts to release matrix metalloproteinases (Birkadel-Hansen *et al*, 1993a; Nilson *et al*, 1985; Schenkhein, 1988; Sorsa *et al*, 1992). Thus, irrespective of their mode of action, bacterial enzymes are likely to be significant contributors to matrix disruption through either direct or indirect pathways.

With respect to proteoglycan destruction in the periodontium, bacterial enzymes may play a significant role. There is overwhelming evidence confirming that oral bacteria synthesize hyaluronidase (Nord *et al*, 1970; Tam *et al*, 1985), neutral proteinases (Uitto *et al*, 1986), heparinase (Okuda *et al*, 1985), chondrosulfatase (Tam *et al*, 1982) and chondroitinase (Tipler and Embery, 1985). All of these enzymes have the potential to degrade various structural elements of periodontal proteoglycans as well as influence the matrix indirectly through activation of interleukins or modulating fibroblast function. In addition to direct tissue damage, indirect effects of enzymes on proteoglycans has been noted whereby the enzyme released by *P. gingivalis* has been found to have a significant inhibitory effect on proteoglycan synthesis by periodontal ligament fibroblasts (Smalley *et al*, 1988b).

It is important to remember that in order for any tissue degradation to occur via enzymatic means, three mechanisms must operate. Firstly, the enzyme must be in an active form such that it is not complexed with an inhibitor or present in a pre-active form. Secondly, it must be in a conformational configuration which will allow it to be active—the quality and concentration of the matrix may have a significant effect on this (Bartold *et al*, 1983). Finally, the appropriate substrate must not only be available but must be in a configuration which will permit degradation. This final point is worthy of further consideration because, although enzymes can be shown to be active *in vitro* against specific substrates, one must always remember that such substrates rarely exist in isolation *in vivo* rather they are more likely complexed with other matrix proteins. Thus, the *in vivo* availability of collagen for degradation while tightly complexed with proteoglycans and other matrix components raises important issues as to the actual effectiveness of 'collagenases' in initiating tissue destruction without the additional help of other enzymes (stromelysins) to first 'unravel' or 'expose' the necessary substrate.

Oxygen-derived free radicals

Oxygen-derived free radicals, such as the superoxide radical and the hydroxyl radical, are integral reaction products of normal cellular metabolism but are elevated in cells undergoing active respiratory bursts (Baboir, 1984). In particular, tissues may be exposed to ODFR during inflammatory reactions, particularly where polymorphonuclear leukocytes and macrophages are in abundance. These phagocytic cells are very reactive in reducing molecular oxygen to superoxide radicals when exposed to stimuli which induce phagocytosis. For example, adhesion of particles to the cell surface of phagocytic cells results in an increase in oxygen consumption by the cell. The reduction of oxygen produces superoxide radicals. These superoxide radicals may then react with hydrogen peroxide in the presence of trace metals (iron) to produce hydroxyl radicals. Oxygen-derived free radicals are highly reactive, and if present in abundance in tissues, can cause extensive damage (Freeman and Crapo, 1982). Free radicals may disrupt cellular proteins, nucleic acids and membrane lipids as well as effecting depolymerization of matrix components such as collagen, hyaluronan and proteoglycans (Ward *et al*, 1988).

The role of oxygen-derived free radicals in periodontal tissue destruction has been largely overlooked in favour of the more commonly cited enzymatic degradation. However, given their highly reactive nature and likely abundance in inflamed periodontal tissues, their role in inflammatory mediated tissue destruction should not be discounted (Bartold *et al*, 1984; Sorsa *et al*, 1989). Studies on the effect of oxygen-derived free radicals on gingival proteoglycans and hyaluronan have demonstrated a susceptibility of these molecules to depolymerization by such reactive molecular species (Bartold *et al*, 1984). Molecular size is diminished significantly leading to a reduction of solution viscosity. If translated to an *in vivo* scenario, reduced matrix viscosity could be expected to have significant ramifications for the mechanical and biophysical properties of the periodontal tissues. A further role for oxygen-derived free radicals in early depolymerization of matrix macromolecules in inflamed periodontal tissues has been proposed whereby

neutrophil collagenase can be activated by reactive oxygen species (Sorsa *et al*, 1989).

Matrix changes associated with metabolic activity of fibroblasts

In addition to changes in the matrix brought about by degradation, significant changes may also result via the effects of microbial and host-derived mediators on the resident fibroblasts. Fibroblasts from normal and inflamed periodontal tissues have been analysed and indicate that stable phenotypic differences exist between these two conditions (Narayanan *et al*, 1978; Bartold and Page, 1986a; Häkkinen and Larjava, 1992). With respect to synthesis of matrix components, a good correlation has been noted with respect to the types of proteoglycans and collagens synthesized by gingival fibroblasts *in vitro* and the types present in either normal or inflamed tissues. Whether these represent the emergence of subpopulations within the tissues or permanent phenotypic change as a result of the inflammatory environment remains to be established (Bordin *et al*, 1984).

Irrespective of the above mechanisms of fibroblast phenotype expression it is clear that the inflammatory environment differs significantly from that of health. With the infiltration of plasma proteins, leukocytes and macrophages into the tissues a vast array of soluble mediators is available for interaction with the fibroblasts leading to changes in their mobility, synthetic activity, growth and life span (Irwin *et al*, 1994). Added to these soluble mediators of endogenous origin should be the numerous soluble products and fragments of the bacteria residing in the gingival sulcus. Taken together, the potential for significant effects on matrix synthesis, quality and quantity is very significant through altered metabolism of the matrix by the resident fibroblasts. As seen in Table 5 the variability of the effects of these agents on fibroblast function is high and highlights the complex interactions involving numerous different cells, systems and processes which are only beginning to be fully appreciated. Nonetheless critical to the emergence of periodontal pathology/destruction is a dynamic interaction of host and parasite mediators together with the nature of the extracellular matrix on the resident fibroblasts and other matrix-forming cells (Figure 2). Indeed, the role of pre-formed extracellular matrix and various cytokines in modulating periodontal fibroblast function in a manner quite different to that noted for monolayer cell culture has been reported (Irwin *et al*, 1994). In these studies proliferative responses and matrix production correlated well with the catabolic events noted *in vivo*.

Extracellular matrix components in gingival crevicular fluid

Given the significant amount of tissue destruction which occurs during development of periodontitis it is not surprising that attention has focussed on identifying tissue breakdown products as indicators of tissue damage for diagnostic purposes (Embery and Waddington, 1994). At present there is no single reliable diagnostic indicator for active periodontal destruction (Beck, 1995). Given the multifactorial nature of the disease it is unlikely that only one parameter will ever be used as a universal diagnostic aid. The events of periodontitis can be divided into bacterial etiology, metabolic events and anatomical changes each of which need to be taken into account when diagnosing periodontal conditions. Connective tissue markers of disease activity may be of use when considering the metabolic events of the disease process. Such connective tissue components may be

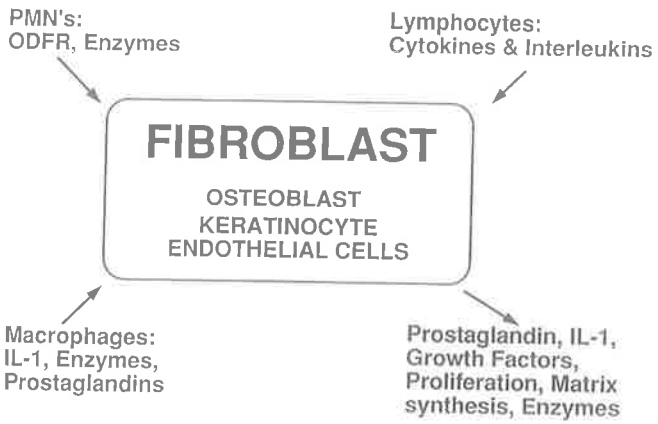

Figure 2 Interplay between cells which produce extracellular matrix and soluble mediators

Table 5 Effect of soluble mediators on fibroblast function

Agent	*Proliferation*	*Collagen*	*Proteoglycans*	*Hyaluronan*
PGE_2	−	−	−	+
IL-1	+	+	+/−	+
IL-6	??	+	+	+
ODFR	−	??	−	??
TGF	+	+	+	+
TNF	+	−	??	??
PDGF	+	+	+/−	+
IGF	+	+	nil	+
EGF	+	+	+/−	+
LPS	−	−	−	−

Abbreviations: PGE_2 = prostaglandin E_2; IL-1 = interleukin-1; IL-6 = interleukin-6; ODFR = oxygen-derived free radicals; TGF = transforming growth factor; TNF = tumor necrosis factor; PDGF = platelet-derived growth factor; IGF = insulin-like growth factor; EGF = epidermal growth factor; LPS = lipopolysaccharide
Symbols: '+' indicates stimulation; '−' indicates reduction; 'nil' indicates no effect; '??' indicates effect unknown; '+/−' indicates an equivocal effect

further divided into effectors of inflammation or products of inflammation. For the purposes of this paper only the connective tissue elements associated with the extracellular matrix and its degradation will be considered.

The gingival crevicular fluid contains many components of breakdown products from the periodontium arising from gingivitis and periodontitis. As a result, many studies have focussed on analysing components of this inflammatory exudate in the hope of finding an indicator of active periodontal breakdown (Lamster and Grbic, 1995). Specifically, plasma proteins, bacterial and mammalian enzymes, inflammatory mediators have been amongst the myriad components studied in detail. Unfortunately, a serious drawback of all of these 'potential markers' is that they reflect processes occurring in the gingival connective tissues and tell us little about attachment loss or bone loss. Thus, no distinction can be made between gingivitis and periodontitis. However, due to the site specificity of some matrix components (Table 6), the possibility exists to detect these and correlate them with specific tissue destruction.

Collagen components in gingival crevicular fluid

Since the major fibrous constituents of the periodontal connective tissues are collagens type I and III, and because of their hydroxyproline content, early studies on periodontal collagens focussed on quantitating the hydroxyproline levels in gingival tissue extracts and crevicular fluid in an attempt to monitor periodontal breakdown (Hara and Takahashi, 1975). However, due to the relative nonspecificity of such assays (ie all collagens have hydroxyproline), no correlation between the presence of hydroxyproline and its site or source of breakdown could be made. The hydroxyproline content of gingival crevicular fluid and serum has been monitored in patients before, one month and six months after periodontal surgery and found to be significantly lower in both serum and gingival crevicular fluid by six months. In a ligature-induced periodontitis model, collagen-derived hydroxyproline (total GCF minus serum value) was maximal in samples taken 4 days after ligature removal (Svanberg, 1987a; 1987b), however no indication was given regarding the extent of tissue destruction at this time point. Furthermore, the source of these collagenous components (ie hard or soft tissues) is unknown. Thus, further studies on the accuracy of hydroxyproline as a marker of periodontal breakdown are needed.

Other structural components of gingival collagens have been monitored in gingival crevicular fluid to evaluate collagen degradation and turnover. Detection of type I collagen carboxyterminal propeptide and type II collagen aminoterminal propeptide has been used to study collagen synthesis (Talonpoika and Hämäläinen, 1992; 1993). Other studies have described type I collagen carboxyterminal telopeptide in gingival crevicular fluid as a measure of collagen degradation (Talonpoika and Hämäläinen, 1994). However, as for hydroxyproline assays, the detection of such telopeptides is still relatively non-specific for the source of such degradation products, since measurement of type I collagen C-terminal telopeptide is unable to distinguish the source which may be soft tissue, periodontal ligament or alveolar bone.

In recent years interest has focussed on the development of methods to identify pyridinium crosslinks in collagens specific for bone (type I). Since these crosslinks result from post-translational modifications of collagen, and thus they cannot be reutilized during collagen synthesis, they represent a true indicator of bone resorption. Preliminary studies have identified pyridinium crosslinks in crevicular fluid and these may correlate with sites undergoing active bone resorption (Meng *et al*, 1991). If these observations are confirmed then the possibility of using the presence of pyridinium crosslinks in crevicular fluid as a marker of active bone destruction is very attractive.

Proteoglycans and glycosaminoglycans in gingival crevicular fluid

The measurement of proteoglycans and their glycosaminoglycan components in crevicular fluid shows some potential for monitoring active periodontal destruction (Embery *et al*, 1982; Last *et al*, 1985; Giannobile *et al*, 1993). The sulcular fluid appears to be quite rich in metabolic or degradative products of proteoglycans found in the various periodontal tissues. Some site specificity for various glycosaminoglycans in the periodontium has been noted with the gingival connective tissue being rich in dermatan sulfate while the alveolar bone is rich in chondroitin sulfate (see Table 6).

The application of histochemical dyes to exudate collected onto filter paper strips to indicate the presence of glycosaminoglycans in this fluid was first reported by Sueda *et al* in 1966. Chemical analysis identified uronic acid in sulcular fluid and thus confirmed the presence of glycosaminoglycans (Hara and Löe, 1969). More recently, these studies have been extended to include identification of the types of glycosaminoglycans present in crevicular

Table 6 Distribution of collagens and proteoglycans in the periodontium

Tissue	*Collagen*	*Glycosaminoglycan*	*Proteoglycan*
Healthy gingiva	I[a], III, IV, V, VI	HA, HS, DS[a], CS	Decorin[a], Versican, Syndecan, CD44
Periodontal ligament	I[a], III, V	HA, HS, DS[a], CS	Decorin[a], Versican
Cementum	I[a], III[a]	HA, HS, DS, CS[a]	??? CSPG[a]
Alveolar bone	I[a], III	HA, HS, DS, CS[a]	Decorin, Biglycan
Inflamed gingiva	I[a], III[a], IV, V, VI Type I trimer	HA, HS, DS[a], CS	Decorin[a], Versican, Syndecan, CD44

[a]Indicates the most abundant species present in the specified tissue
Abbreviations: HA = hyaluronan; HS = heparan sulfate; DS = dermatan sulfate; CS = chondroitin sulfate; ??? CSPG = unidentified sulfate proteoglycan

fluid. Hyaluronan is a ubiquitous component of sulcular fluid irrespective of the site it is sampled (inflamed or non-inflamed). Chondroitin sulfate is the principal sulfated glycosaminoglycan identifiable in crevicular fluid with minor proportions of heparan sulfate and dermatan sulfate being noted (Last *et al*, 1985; Shibutani *et al*, 1993). The presence of high amounts of chondroitin sulfate in crevicular fluid sampled from sites of active bone resorption has been interpreted to indicate that these components originate from the matrix of bone (Last *et al*, 1985; Samuels *et al*, 1993; Smedberg *et al*, 1993). However, the precise origin of this glycosaminoglycan has not been determined. An alternative source could be the relatively high cellular composition of inflamed tissues from which cell surface chondroitin sulfate may be sequestered. Nonetheless, the observation that high levels of chondroitin sulfate are noted in samples taken from non-inflamed sites undergoing orthodontic tooth movement does imply a close association between the appearance of chondroitin sulfate in crevicular fluid and bone resorption (Last *et al*, 1988; Samuels *et al*, 1993). While the usefulness of monitoring glycosaminoglycans in crevicular fluid awaits the appropriate longitudinal studies to test for correlation with disease progression, the potential for these as useful markers of active bone resorption is good and warrants further detailed investigation.

Other connective tissue markers in gingival crevicular fluid

The identification of other connective tissue proteins in gingival crevicular fluid specifically associated with bone continues to be of interest for development as markers of bone resorption associated with advancing periodontitis. In this light, osteocalcin has been correlated with increasing pocket depth (Bowers, 1989). However, these studies require further development and assessment as although osteocalcin may predominate in bone, it is not unique to bone alone, and thus difficulties in determining its precise source remain a problem.

Fibronectin has also been identified in crevicular fluid (Cho *et al*, 1984; Talonpoika *et al*, 1993). However, whether the appearance of fibronectin in gingival crevicular fluid reflects tissue remodelling or serum exudation is unclear.

Extracellular matrix, gene expression and periodontal regeneration

Repair of damaged tissues is a major biological response of all animals. However, the nature of the repair process may often lead to compromised function. In this respect the periodontium is no exception. Although tissue repair and regeneration of periodontal tissues affected by gingivitis will lead to full restitution of form and function, once the destructive phase of periodontitis reaches the deeper periodontal structures, then repair in the form of regeneration of the destroyed components is less likely to occur on a predictable basis (Melcher, 1976). Such compromised healing is not restricted to the periodontium since regeneration of organs or appendages in response to tissue destruction or loss is a general feature of mammalian healing. Commonly, the repair of injured tissues results in scarring and replacement with tissue which does not resemble the original tissues (Forrest, 1983). In this respect the periodontium differs in that parts of it have remarkable regenerative capacity. For example, the gingival tissues show little capacity to scar even following the most radical of surgical procedures (Melcher, 1976).

Despite the good reparative potential of the gingivae, the deeper periodontal structures (cementum, ligament and bone) have very poor regenerative capacity. Nonetheless, a principal aim of periodontics is to determine the mechanisms by which these tissues may be encouraged to repair and regenerate their original form, function and architecture following destructive episodes. At present significant advances are being made in this area but many of the procedures being used still lack clinical predictability. With a fuller understanding of the cell biology and physiology of the wound regenerative events these procedures should become more refined and based on sound biological principles.

Regenerating connective tissues are characterized by cell migration and proliferation, neovascularization and matrix synthesis. From this highly ordered sequence of events, evolve the molecular events which direct the wound healing process. The synthetic phase of healing is preceded by infiltration of inflammatory cells resulting from the original injury. This phase is not only protective for the host but instrumental in recruiting cells responsible for the reparative processes to the site. The organization of the new matrix follows several phases progressing from an immature matrix to a fully formed functional matrix complete with the fibrous and nonfibrous elements characteristic of the tissue prior to damage (Ivaska, 1981). With respect to matrix deposition the usual sequence of events is an initial deposition of a hyaluronan rich matrix together with plasma proteins. This provides a suitable matrix for cellular infiltration and further matrix development. Proteoglycan synthesis (rich in chondroitin sulfate) then appears closely followed by collagen deposition. At the same time that the collagen is deposited and matrix maturation takes place the types of proteoglycan begin to change with the levels of chondroitin sulfate decreasing and the levels of dermatan sulfate increasing. Little is known of the nature of the proteoglycans which these glycosaminoglycans constitute.

In the case of periodontal wound healing and regeneration, a significant confounding event is the rapid epithelialization of the wound surface. The epithelium rapidly establishes a surface covering between the root surface and the exposed connective tissue surface. Attempts have been made to impede this event by the utilization of barrier membranes and applying the principles of Guided Tissue Regeneration (Nyman *et al*, 1982). While the principles of this procedure are very sound, the clinical outcomes are still to some extent quite variable (Minabe, 1991). Although the histological and clinical events of GTR are well documented, the cell biology and molecular events associated with periodontal regeneration are still largely poorly understood. The precise importance of excluding gingival fibroblasts from the healing site to enable repopulation by fibroblasts derived from the periodontal ligament requires more rigorous testing. It is interesting to note that regeneration is possible in the absence of barrier exclusion tech-

niques by using selective tissue-promoting molecules known as growth factors (Lynch *et al*, 1989).

The role of growth factors in periodontal regeneration is becoming increasingly important. These extremely bioactive molecules have been isolated from repairing and developing tissues and are intimately associated in directing specific events associated with tissue repair and regeneration. For example some growth factors will promote specific cell migration (chemotaxis) to wounded sites while others will promote cell division and matrix synthesis. With the recent cloning and sequencing of many of the growth factor polypeptides, highly pure recombinant forms are now available for investigation. Of the growth factors studied to date, transforming growth factor, platelet-derived growth factor, platelet-factor-4 and fibroblast growth factor show great potential in being able to stimulate regenerative processes in periodontal tissues previously destroyed by inflammatory destruction (Lynch, 1994).

Thus the stage is now set. We know that it is technically possible to direct periodontal regeneration. However, we do not understand the underlying controlling mechanisms. Most likely the key will reside in the regulatory role that the extracellular matrix plays in cell function and gene expression. It is clear that the extracellular matrix significantly influences cell behaviour (Hay, 1984). Such influences begin during embryogenesis and continue throughout life whereby changes in matrix, as a result of aging, inflammation or other means, influence the synthetic, migratory and phenotypic behaviour of cells. While the mechanisms are poorly understood a reasonable working model is shown in Figure 3. In this model, the extracellular matrix macromolecules and bound tissue growth factors can bind to cell surface integrins or other cell surface receptor molecules (Bissell *et al*, 1982; Juliano and Haskill, 1993). Such interactions will, in turn, lead to rearrangements of cytoskeletal components (Hynes, 1981), or signal transduction pathways (Ullrich and Schlessinger, 1990) which subsequently lead to an effector system acting on the cell nucleus to allow specific gene expression (Streuli and Bissell, 1990). Such gene expression may well include new matrix synthesis or release of growth factors which in turn will feed back and lead to further regulation of gene expression and matrix modulation (Stoker *et al*, 1990). While simplistic in its outline, this model provides a working base upon which to further consider the intricate mechanisms of periodontal repair and regeneration.

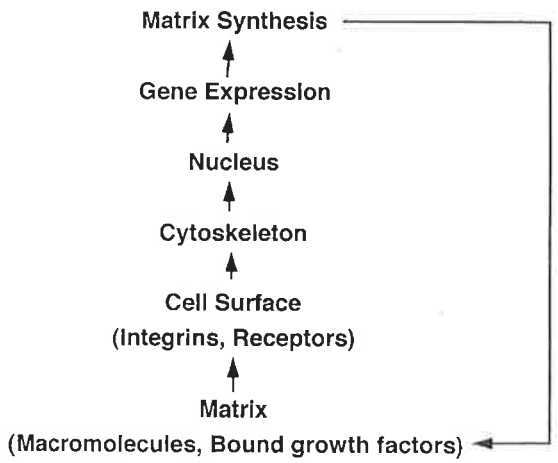

Figure 3 A model for the relationship between the extracellular matrix and gene expression

Concluding comments

The periodontal tissues are amongst the most metabolically active tissues in the entire human body. Given their strategic location and exposure to a wide variety of chemical and mechanical stresses it is remarkable how well these tissues remain intact. However, disease is still common in the periodontal tissues with all the classic signs and symptoms of chronic inflammatory disease. The delicate balance between tissue damage and tissue repair is well recognized (Figure 1). However, the mechanisms which govern these processes are still largely poorly understood. Nonetheless, by studying both tissue destruction as well as reparative processes we are assured of developing a deeper understanding of the periodontal tissues which will ultimately lead to improved therapeutic treatments based on a sound knowledge of tissue destruction and repair.

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V. CELLULAR SOURCE OF PROTEOGLYCANS IN THE GINGIVAL CONNECTIVE TISSUES

Paper 2. Bartold, P.M. and Page, R.C.
Isolation, identification and quantitation of glycosaminoglycans synthesized by human gingival fibroblasts *in vitro*.
Journal of Periodontal Research **20:** 284-292, 1985.

Paper 3. Bartold, P.M. and Page, R.C.
Isolation and characterization of proteoglycans synthesized by adult human gingival fibroblasts *in vitro*.
Archives of Biochemistry and Biophysics **253:** 399-412, 1987.

Paper 4. Bartold, P.M., Harkin, D.G. and Bignold, L.P.
Proteoglycans synthesized by human polymorphonuclear leukocytes *in vitro*.
Immunology and Cell Biology **67:** 9-17, 1989.

Paper 5. Bartold, P.M., Haynes, D.R. and Vernon-Roberts, B.
The effect of mitogen and lymphokine stimulation on proteoglycan synthesis by lymphocytes.
Journal of Cellular Physiology **140:** 82-90, 1989.

This section comprises a collection of papers dealing with the determination of the cellular source of proteoglycan synthesis within the periodontal tissues. Until these studies were carried out, few studies had addressed the ability of fibroblasts derived from periodontal tissues to synthesize and secrete proteoglycans, and none had carried out any sort of molecular characterization.

Paper 2 provided the first definitive statement on the qualitative and quantitative features of glycosaminoglycan and hylauronan synthesis by human gingival fibroblasts. Although hyaluronan was found to be synthesized in very large amounts, the levels of the sulfated glycosaminoglycans (heparan sulfate, dermatan sulfate and chondroitin sulfate) were found to be produced, *in vitro,* in similar quantities and proportions to those found *in vivo*. The high level of hyaluronan synthesis appears to be an artifact of cell culture and highlights the artificial nature of *in vitro* systems and the need for extreme caution in extrapolating from *in vitro* findings to *in vivo* conditions.

Paper 3 extended the above findings on the glycosaminoglycan synthesis of human gingival fibroblasts by considering the intact proteoglycan molecules synthesized and secreted by these cells. It was pioneering in its use of current cell culture techniques and recently developed techniques in proteoglycan biochemistry. This study provided the first serious attempt at isolating and characterizing all of the proteoglycans synthesized by gingival fibroblasts. It demonstrated that the proteoglycans synthesized in culture were very similar in composition and structure to those isolated from whole tissue extracts, and that multiple types of proteoglycans were likely to be synthesized by one cell type.

Papers 4 and 5 are particularly significant in that they are amongst the earliest papers demonstrating the ability of both lymphocytes and polymorphonuclear leukocytes to synthesize and secrete proteoglycans *in vitro*. These findings were interesting in that, at the time, it was generally considered that proteoglycan synthesis was the domain of mesenchymal and ectodermal cells. The issue that cells of the hemopoietic cell system might make significant quantities of these molecules had been largely ignored. These studies provided both qualitative and quantitative data concerning the proteoglycans made by these cells. The studies concerning the lymphocytes were more extensive and provided early documentation that proteoglycan synthesis was associated with mitogenic activation of the cells, yet was independent of cell proliferation. These studies also helped to explain, in part, why earlier results using extracts of inflamed gingivae had failed to show any remarkable differences in total amount of proteoglycan extractable from normal and inflamed tissues. On the basis that the inflamed tissues had a significant inflammatory cell infiltrate which contributed to the overall proteoglycan content of the tissues, it is hardly surprising that gross biochemical assessment of proteoglycan content of normal and inflamed tissues failed to uncover any identifiable differences.

Paper 2

Bartold, P.M. and Page, R.C.
Isolation, identification and quantitation of glycosaminoglycans synthesized by human gingival fibroblasts *in vitro*.
Journal of Periodontal Research **20:** 284-292, 1985.

Candidate's Contribution to this paper: 90%

P.M. Bartold's role in this study was:
Design of the experiments
Execution of all experimental work
Writing of the manuscript

R.C. Page's role in this study was:
Provision of research funds
Writing of the manuscript

Journal of Periodontal Research 1985: **20**: 284–292

Isolation, identification, and quantitation of glycosaminoglycans synthesized by human gingival fibroblasts in vitro

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The glycosaminoglycans synthesized by diploid fibroblasts obtained from healthy human gingivae of three donors were isolated, identified, and quantified. Degradation with specific enzymes identified the glycosaminoglycans as hyaluronic acid, chondroitin sulfate, dermatan sulfate, and heparan sulfate; hyaluronic acid predominating. The distribution of the sulfated glycosaminoglycans in the cell layer and the medium was not the same. The cells contained mainly heparan sulfate (48.3%) and the medium mainly dermatan sulfate (47%).

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Introduction

Proteoglycans are important extracellular nonfibrous macromolecules of connective tissues (Balazs 1973). Structurally, proteoglycans are arranged as a single protein core to which many sulfated glycosaminglycan side chains are covalently bound (Lindahl & Roden 1972). The glycosaminoglycans give the proteoglycans a highly anionic charge, making them capable of forming complexes with many components of the extracellular matrix (Comper & Laurent 1978) and of interaction at cell surfaces (Rollins, Cathcart & Culp 1982).

Three major molecular species of proteoglycans have been identified in soft connective tissues, each deriving its name from its principal glycosaminoglycan component: heparan sulfate proteoglycan, dermatan sulfate proteoglycan, and chondroitin sulfate proteoglycan (Kraemer & Smith 1974, Öbrink 1972, Damle et al. 1979). The other major glycosaminoglycan found in soft connective tissues is hyaluronic acid, which is unsulfated and is not found in association with a protein in the form of a proteoglycan (Varma et al. 1976). Biochemical analyses of enzyme digests of human gingivae have identified hyaluronic acid, heparan sulfate, dermatan sulfate, and chondroitin-4-sulfate as the resident glycosaminoglycans (Bartold, Wiebkin & Thonard 1981, 1982). Virtually nothing, however, is known about the biosynthesis of these four glycosaminoglycans in gingiva, either in vivo or in vitro.

This study was undertaken to determine the capacity of normal gingival fibroblasts to synthesize glycosaminoglycans, as well as to identify and quantitate the molecular specics of the glycosaminoglycans synthesized. The objective was to establish a baseline from which the factors modulating glycosaminoglycan synthesis could be further studied. In particular, this in vitro system could be used to study the effect of inflammatory agents on glycosaminoglycan production by gingival fibroblasts.

Material and Methods

Cell culture and labeling

The fibroblasts used were from 3 strains of normal human gingival fibroblasts obtained from donors with clinically and radiographically healthy periodontal tissues and were designated HGF_{22}, HGF_{25}, and HGF_{27}. Such cells have been well-characterized morphologically (Engel et al. 1980) as well as biochemically for collagen and protein synthesis (Narayanan & Page 1976). Only cells between the sixth and tenth transfer were used, and all experiments were done in triplicate.

Cells were plated into 75 cm^3 flasks (Falcon Plastics, Oxnard, CA) and cultured in 10 ml Dulbecco-Vogt medium supplemented with 10% fetal calf serum (Grand Island Biological Company, NY) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. On day 6 when the cultures were confluent (approximately 2.5×10^6 cells), the medium in each culture dish was replaced with 5 ml Dulbecco-Vogt medium in which $MgSO_4$ had been replaced with $MgCl_2$ and incubated for 1 h under the same conditions as described above. The sulfate-depleted medium was then removed and replaced with 5 ml of sulfate-depleted medium supplemented with 75 μCi/ml [^{35}S]-sulfate and 17.5 μCi/ml [^{3}H]-glucosamine (New England Nuclear, Boston, MA) and incubated for 48 h. To determine the rate of incorporation of [^{35}S]-sulfate, the sulfated glycosaminoglycans were harvested from the cells and the medium of triplicate cultures at 2, 6, 8, 24, 30, and 48 h after start of radiolabeling. The amount of radiolabel incorporated into the proteoglycans of the media and cell layer of each culture at the various time points was determined in triplicate. Aliquots of 100 μl from the media and cell layer were spotted onto Whatman 3MM chromatography paper, dried, immersed in 0.1% cetylpyridinium chloride over 5 changes of 1 h duration, dried, and counted in a liquid scintillation counter (Castor et al. 1981).

Isolation of glycosaminoglycans

On completion of labeling the cells for 48 h, the medium was removed and the cell layer was washed with 5 ml phosphate-buffered saline. Wash and medium were pooled and will henceforth be referred to as the "medium fraction." The cell layer of each culture was then extracted for 24 h at 4°C with 5 ml 4 M guanidine HCl in 0.05 M sodium acetate, pH 5.8, containing protease inhibitors (0.1 M 6-aminohexanoic acid, 5 mM benzamidine HCl, 50 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide) (Oegema, Hascall & Dziewiatkowski 1975) and scraped from the flasks. The flasks were then washed with 5 ml 4 M guanidine HCl in 0.05 M sodium acetate, pH 5.8. Wash and extract were pooled and will henceforth be referred to as the "cell fraciton."

The medium fraction and the cell fraction were then dialyzed against several changes of deionized water, and the retentates were lyophilized. The lyophilized fractions of the medium fraction and cell fraction were then separately digested with 1.0 ml papain (Sigma Chemicals, St. Louis, MO), 1 mg/ml in 0.2 M sodium acetate buffer, pH 5.7, containing 0.004 M EDTA and 0.02 M cysteine HCl (Sigma Chemicals, St. Louis, MO) for 48 h at 60°C (Bartold et al. 1981). Protein was precipitated with trichloroacetic acid and removed by centrifugation. The supernatant was dialyzed, concentrated by lyophilization, and the glycosaminoglycans recovered by precipitation with ethanol.

The ethanol precipitate was then reconstituted to a small volume (200 μl) with 0.2 M ammonium acetate, and 50 μl of 1 mg/ml each of standard glycosaminoglycans (hyaluronic acid, chondroitin 4-sulfate, and dermatan sulfate) (Miles Laboratories, El-

kart, IN) were added as carriers. These preparations were then chromatographed on columns (0.7 cm × 35 cm) of Sephadex G-50 (Pharmacia Inc., Piscataway, NJ) in the same buffer at 3 ml/h, and fractions of 0.5 ml were collected. Aliquots from each fraction of 50–150 μl were taken for radioactivity determination.

Glycosaminoglycan identification and quantitation

Individual glycosaminoglycans were identified and subsequently quantitated using a sequential series of species-specific degradative steps followed by column chromatography on Sephadex G-50 in 0.2 M ammonium acetate as described above (Pintar 1978). The degradative sequence used was *Streptomyces* hyaluronidase followed by chondroitinase AC II followed by chondroitinase ABC and finally degradation with nitrous acid. Each of these enzyme and chemical treatments is specific for the identification of hyaluronic acid, chondroitin sulfate, dermatan sulfate, and heparan sulfate and are listed in Table 1. The enzymes were purchased from Seikagaku Kogyo Co., Tokyo, through Miles Laboratories (Elkhart, IN).

Digestion with *Streptomyces* hyaluronidase (20 units/mg hyaluronic acid carrier) to specifically identify hyaluronic acid was carried out in 0.05 M sodium acetate, pH 5.0, for 3 h at 60°C (Ohya & Kancko 1970).

Table 1

Substrates of enzyme and chemical treatments

Enzyme/Chemical	Substrate
Streptomyces hyaluronidase	Hyaluronic acid
Chondroitinase AC II	Hyaluronic acid Chondroitin sulfates 4 and 6
Chondroitinase ABC	Hyaluronic acid Chondroitin sulfates 4 and 6 Dermatan sulfate
Nitrous Acid	Heparan sulfate

Chondroitin sulfate was identified by treatment with chondroitinase AC II (0.05 unit/0.5 mg chondroitin sulfate carrier) in 0.1 M tris-HCl, 0.1 M sodium acetate, pH 7.2, for 3 h at 37°C (Saito, Yamagata & Suzuki 1968). To identify dermatan sulfate, the remaining glycosaminoglycans were digested with chondroitinase ABC (0.2 unit/0.5 mg dermatan sulfate carrier) under the same conditions as described for chondroitinase AC II (Saito et al. 1968). Glycosaminoglycans containing N-sulfated amino sugars (heparan sulfate) were identified by their susceptibility to degradation by treatment with nitrous acid (Lagunoff & Warren 1962).

Chromatography was done after each degradative step. The percentage of total glycosaminoglycans either degraded by or resistant to such treatments could then be determined by directly computing the amount of radioactivity in each peak. Thus, the individual glycosaminoglycan species could be quantitated for both the cell and medium fractions.

Analysis for radioactivity

Aliquots (50–150 μl) from the fractions obtained by column chromatography were dissolved in 5 ml Aquamix (West Chem, San Diego, CA). A Beckman liquid scintillation counter (Model 3255) was used with the adjustable discriminators set for dual-label counting of ^{35}S and ^{3}H. In all instances, standard preparations at ^{35}S and ^{3}H of known radioactivity were counted to allow calculation of d.p.m. from c.p.m. by the channels ratio method.

Results

Synthesis of glycosaminoglycans

The amounts of [^{35}S]-sulfate-labeled macromolecules released into the medium by the gingival fibroblasts and present in the 4 M guanidine HCl extracts at various times

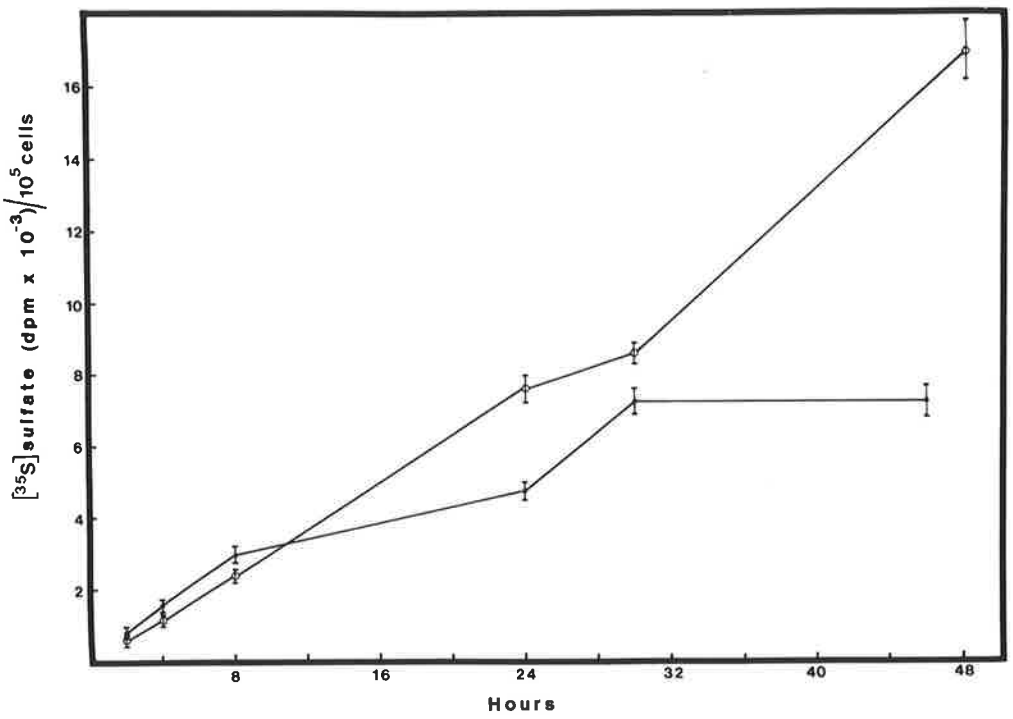

Fig. 1. Accumulation of ^{35}S-labeled macromolecules in the culture medium and cell layer of the HGF_{25} cell strain over a 48-h period. Medium ○—○; cell layer ●—●.

of continuous labeling were similar for all three cell strains studied. A typical incorporation profile is shown in Fig. 1. The release of [^{35}S]-labeled glycosaminoglycans into the medium was linear for at least 48 h, whereas the amount of incorporated radioactivity present in the 4 M guanidine HCl extracts of the cell layer first increased steadily over 30 h, then appeared to level off, staying fairly constant up to 48 h. By 48 h, approximately 70–80% of the [^{35}S]-labeled glycosaminoglycans was in the medium fraction and the remaining 20–30% was found in the cell fraction.

The incorporation of [^{3}H]-glucosamine over time was not measured because, as is shown later, hyaluronic acid is produced by fibroblasts in vitro in extremely large quantities which do not reflect in vivo hyaluronic acid production. Nonetheless, measurements of total [^{3}H]-glucosamine incorporation into glycosaminoglycans were made for all cultures at 48 h. The ratio of [^{35}S] to [^{3}H] incorporation for the cell-layer glycosaminoglycans was 1:1.3, whereas the ratio for the medium glycosaminoglycans was 1:3.0, indicating approximately 2.5 times more glucosamine-containing glycosaminoglycans in the medium than in the cell layer.

Identification of glycosaminoglycans

Individual glycosaminoglycan species were identified by specific enzyme and chemical degradation. The results of such treatments are shown in Fig. 2. The glycosaminoglycans isolated from both the medium and the cell layer demonstrated susceptibility to deg-

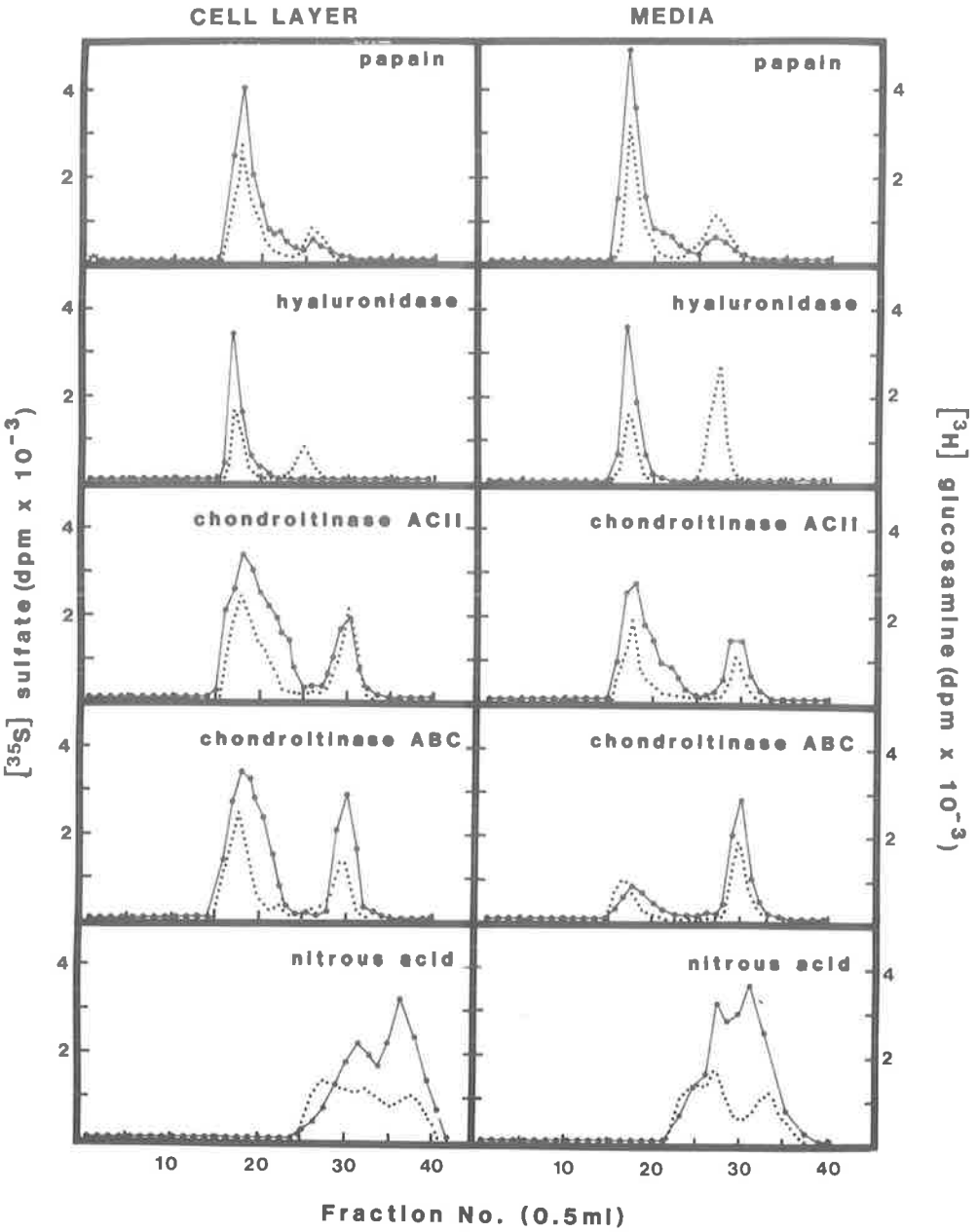

Fig. 2. Sephadex G-50 elution profiles of sequential enzyme and chemical degradation of glycosaminoglycans isolated from the medium and from the cell layer. [^{35}S]-sulfate ●——●; [^{3}H]-glycosamine ·····. The void volume (15 ml) and total volume (36 ml) were determined using Blue Dextran and Phenol Red, respectively.

Table 2

Proportions of glycosaminoglycans synthesized by gingival fibroblasts

	Cell Type				
	HGF_{22}	HGF_{22}**	HGF_{25}	HGF_{27}	Mean ± SD*
Cells					
HA	31.0 ± 8.0	63.0 ± 6.0	56.0 ± 9.0	50.0 ± 6.0	50.0 ± 13.0
Sulfated GAG					
ChS	16.0 ± 1.0	27.0 ± 1.0	23.0 ± 3.0	29.0 ± 1.0	23.6 ± 5.9
DS	27.0 ± 1.0	28.0 ± 1.0	26.0 ± 1.0	30.0 ± 3.0	27.8 ± 1.7
HS	56.0 ± 3.0	45.0 ± 2.0	51.0 ± 4.0	41.0 ± 2.0	48.3 ± 6.6
Medium					
HA	72.0 ± 5.0	90.0 ± 5.0	85.0 ± 7.0	80.0 ± 5.0	81.8 ± 7.6
Sulfated GAG					
ChS	28.0 ± 2.0	39.0 ± 2.0	25.0 ± 2.0	34.0 ± 2.0	31.5 ± 6.2
DS	50.0 ± 5.0	42.0 ± 2.0	25.0 ± 4.0	45.0 ± 3.0	47.5 ± 4.9
HS	22.0 ± 1.0	19.0 ± 1.0	22.0 ± 3.0	21.0 ± 2.0	21.0 ± 1.4

The values for HA are expressed as percentage of total GAG, while the values for the sulfated GAG are expressed as percentage of total sulfated GAG. The ± values represent standard deviation of the mean of triplicate cultures of each cell strain.

* Represents the mean value and its standard deviation for each of the glycosaminoglycans synthesized by all of the cell strains studied.

** This culture was labeled when noticeably subconfluent.

Abbreviations: HA, hyaluronic acid; ChS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; GAG, glycosaminoglycan.

radation by *Streptomyces* hyaluronidase, chondroitinase AC II, and chondroitinase ABC. In addition, a proportion of the glycosaminoglycans from both preparations could be degraded by treatment with nitrous acid. These findings indicated that the constituent glycosaminoglycans isolated from the medium and cell-layer preparations are hyaluronic acid, chondroitin sulfate, dermatan sulfate, and heparan sulfate. Keratan sulfate was considered not to be a component since none of the radiolabeled material was resistant to all four degradative steps.

Quantitation of glycosaminoglycans

The Sephadex G-50 chromatograms shown in Fig. 2 are representative of the profiles obtained for all three cell strains. Differences in the relative distribution of the various glycosaminoglycan species between the cell layer and the medium fractions can be seen. The total amount of radioactivity present in each peak allows quantitation of the proportion of the glycosaminoglycans either resistant to or degraded by the various degradative treatments. Data compiled from such calculations are shown in Table 2. Quantitatively, the major differences noted were that hyaluronic acid was the predominant glycosaminoglycan in both preparations. Furthermore, hyaluronic acid appeared to be present in greater proportions in the medium fractions rather than in the cell layer fractions. These observations corroborate the earlier finding of increased amounts of [^{3}H]-glucosamine labeled glycosaminoglycans in the medium after a 48 h incubation. With respect to the sulfated glycosaminoglycans, the predominant glycosaminoglycan present was identified as heparan sulfate (48.25%), whereas the predominant sulfated glycosaminoglycan in the

medium preparations was dermatan sulfate (47.5%).

Discussion

In this study, isolation, identification, and quantitation of the glycosaminoglycans synthesized by adult human gingival fibroblasts was achieved. Whilst data for one set of experiments are presented in the figures, these experiments have been repeated several times on the separate cell strains and a high degree of reproducibility has been achieved.

The glycosaminoglycans were identified using a sequential procedure of elimination of glycosaminoglycans by enzymes and chemicals of known specificity. Thus, hyaluronic acid, chondroitin sulfate, dermatan sulfate, and heparan sulfate were identified as the glycosaminoglycans synthesized by gingival fibroblasts in vitro. Qualitatively, these are the same molecular species as those previously identified in digests of human gingival tissue (Bartold et al. 1981, 1982). Quantitatively, the glycosaminoglycans synthesized by gingival fibroblasts in vitro show one major difference with respect to the glycosaminoglycans found in digests of gingival tissue. In culture, gingival fibroblasts appeared to synthesize large quantities of the unsulfated glycosaminoglycan hyaluronic acid compared with the other sulfated glycosaminoglycans. Nonetheless, while hyaluronic acid was the principal glycosaminoglycan synthesized in vitro (cell layer 50%; medium 82%), it is a relatively minor quantitative component of tissue extracts, comprising approximately 4% of the total glycosaminoglycans.

In one of the cultures studied (HGF_{22}), up to 90% of the total glycosaminoglycans in the medium and 63% of the total cell-associated glycosaminoglycan content were identified as hyaluronic acid. One explanation for such an elevated level of hyaluronic acid synthesis is that this culture was labeled when the cells were noticeably subconfluent (approximately 1×10^6 cells). Therefore, since hyaluronic acid is known to be associated with cell proliferation (Tomida, Koyama & Ono 1974, Hopwood & Dorfman 1977), such a finding of elevated hyaluronic acid synthesis in this particular culture was not surprising. Nonetheless, hyaluronic acid was routinely noted as the predominant glycosaminoglycan synthesized by gingival fibroblasts in all the strains studied and thus appears to corroborate other reports that elevated hyaluronic acid synthesis is a characteristic feature of fibroblasts in vitro (Wight & Ross 1975).

Because of the large proportion of hyaluronic acid synthesized, the data for quantitation of the sulfated glycosaminoglycans are presented as percentages of the total sulfated glycosaminoglycans rather than as percentages of the total glycosaminoglycans. The relative proportions of specific sulfated glycosaminoglycans in the medium and the cell layer were noted to differ. The sulfated glycosaminoglycans isolated from the medium were characterized by a predominance of dermatan sulfate, with chondroitin sulfate and heparan sulfate present in smaller quantities. Heparan sulfate was always present in the smallest proportion. On the other hand, glycosaminoglycans isolated from the cell layer consisted predominantly of heparan sulfate; dermatan sulfate and chondroitin sulfate were the minor components. These data further corroborate previous studies concerning the in vitro synthesis of glycosaminoglycans synthesized by fibroblasts from a variety of sources in which similar compartmentalization of glycosaminoglycans has been reported (Kresse et al. 1975, Vogel & Kendall 1980). In addition, the above findings support earlier biochemical studies concerning the glycosaminoglycan distribution in gingival tissue (Bartold et al. 1981). In that study, der-

matan sulfate was found to be the major connective tissue glycosaminoglycan and was presumed to be mainly associated with collagenous matrices, whereas heparan sulfate, which is a cell surface-associated glycosaminoglycan, was found in the highly cellular gingival epithelium. A similar distribution is seen in vitro; the cell layer (predominantly cells) has an elevated amount of heparan sulfate, while the medium (extracellular compartment) contains chiefly dermatan sulfate. Although the above observations compare two completely different tissues (epithelium and connective tissue) with a culture system that presumably resembles connective tissue, the study makes it clear that compartmentalization of extracellular matrix components occurs in vitro. Thus, while extrapolation from in vitro conditions to in vivo conditions must be made with caution, it seems likely that, in vivo, components of the extracellular matrix associated with the collagenous network are likely to be greatly different from the constituents of the pericellular environment.

In conclusion, this study has demonstrated that gingival fibroblasts in vitro express their phenotype for the same glycosaminoglycans as are found in vivo. These data therefore provide a background for future investigations concerning gingival glycosaminoglycans. In particular, the in vitro system described above should be useful for studying the physiological role of glycosaminoglycans produced by gingival fibroblasts as well as investigating the effects of factors associated with inflammation, on glycosaminoglycan synthesis.

Acknowledgments

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Isolation and Characterization of Proteoglycans Synthesized by Adult Human Gingival Fibroblasts *in Vitro*

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The proteoglycans synthesized by fibroblasts derived from healthy human gingivae were isolated and characterized. The largest medium proteoglycan was excluded from Sepharose CL-4B but not from Sepharose CL-2B; it was recovered in the most-dense density gradient fraction and identified as a chondroitin sulfate proteoglycan. The medium contained two smaller proteoglycans; one contained predominantly chondroitin sulfate proteoglycan, while the other was comprised predominantly of dermatan sulfate proteoglycan and was quantitatively the major species. The largest proteoglycan in the cell layer fraction, excluded from both Sepharose CL-2B and Sepharose CL-4B, was found in the least-dense density gradient fraction and contained heparan sulfate and chondroitin sulfate proteoglycan. It could be further dissociated by treatment with detergent, suggesting an intimate association with cell membranes. Two other proteoglycan populations of intermediate size were identified in the cell layer extracts which contained variable proportions of heparan sulfate, dermatan sulfate, or chondroitin sulfate proteoglycan. Some small molecular weight material indicative of free glycosaminoglycan chains was also associated with the cell layer fraction. Carbohydrate analysis of the proteoglycans demonstrated the glycosaminoglycan chains to have approximate average molecular weights of 25,000. In addition, N- and O-linked oligosaccharides which were associated with the proteoglycans appeared to be sulfated in varying degrees.

Gingival tissue, like skin, comprises a superficial epithelial layer and an underlying connective tissue. Since this tissue has the general features of many soft connective tissues and manifests a spontaneously occurring progressive inflammatory condition, it has served as a useful model for studying connective tissues in both health and disease (1).

The glycosaminoglycan composition of human gingivae is well established (2–6). Dermatan sulfate is the predominant glycosaminoglycan, accounting for 60% of the total; hyaluronic acid, heparan sulfate, and chondroitin sulfate are present as minor quantitative components (5, 6). With respect to the proteoglycans of this tissue, three types of proteoglycan which vary in molecular size as well as in glycosaminoglycan composition have been identified but their properties and structure are still unclear (3, 7, 8).

There have been many difficulties in obtaining samples of suitable size for the extraction and chemical characterization of soft connective tissue proteoglycans. Furthermore, analyses using whole tissue extracts provide little information regarding the cellular source and biosynthesis of these molecules. Consequently, fibroblasts from a variety of tissues, especially those

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of embryonic origin, have been studied *in vitro* for their proteoglycan synthetic capacity, and proteoglycans similar to those found in tissue are synthesized in culture (9–12). The classes of glycosaminoglycans synthesized by fibroblasts from a variety of tissue sources are similar; however, they vary in their size and tailoring. For example, proteoglycans synthesized by embryonic lung fibroblasts differ with respect to glycosaminoglycan composition and size distribution from those synthesized by embryonic skin fibroblast cultures (10, 12). Whether or not such differences reflect tissue specificity remains to be established.

In vitro studies can be useful for determining the molecular features of proteoglycans and providing information regarding their biosynthesis. However, because of the variability between the proteoglycans synthesized by fibroblasts from different sources, generalizations based on a single fibroblast type are questionable. In the present study, we have isolated and characterized the proteoglycans synthesized by fibroblasts from healthy human gingivae. The proteoglycans synthesized are unique for this cell type, and therefore presumably unique to the tissue of origin. Also they are very similar to those previously reported to be present in extracts of whole gingiva.

MATERIALS AND METHODS

Materials. Guanidine–HCl, 6-aminohexanoic acid, benzamidine hydrochloride, *N*-ethylmaleimide, phenylmethylsulfonylfluoride, cesium chloride, cysteine HCl, 3-[(3-chloamidopropyl)-dimethylaminio]-1-propanesulfonate (CHAPS),[2] sodium borohydride, and Type X neuraminidase (*Clostridium perfringens*) were all purchased from Sigma Chemical Co. (St. Louis, MO); sodium dodecyl sulfate (SDS) and Biogel P-10 were from Bio-Rad Laboratories (Richmond, CA); chondroitinase AC (*Arthrobacter aurescens*), chondroitinase ABC (*Proteus vulgaris*), *Streptomyces* hyaluronidase (*Streptomyces hyalurolyticus*), and keratanase (*Pseudomonas* sp. IFO-13309) were from Seikagaku Kogyo (Tokyo, Japan) through Miles Laboratories, Inc. (Elkhart, IN); Sephadex, Sepharose, and DEAE-Sephacel were from Pharmacia, Inc. (Piscataway, NJ); $Na_2[^{35}S]O_4$ (2 mCi/ml, 424.15 mCi/mmol) and D-[6-^{3}H]glucosamine HCl (1.0 mCi/ml, 31.3 Ci/mmol) were from New England Nuclear (Boston, MA); D-[2-^{3}H]mannose (1.0 mCi/ml, 16.0 Ci/mmol) was from Amersham (Arlington Heights, IL); Aquamix was from WestChem (San Diego, CA); Dulbecco–Vogt medium and fetal calf serum were from Grand Island Biological Co. (GIBCO, Grand Island, NY); sulfate-depleted medium was made from amino acids and vitamins purchased from GIBCO; and tissue culture flasks were from Falcon Plastics (Oxnard, CA).

Cell culture and radioisotope labeling. Fibroblasts were obtained from explants of human gingivae from individuals with clinically and radiographically healthy periodontal tissues (13). Three separate cell strains were initially studied and highly reproducible data were obtained for both radiolabel incorporation and gel filtration profiles. Therefore, representative data for only one cell strain are reported. Cells were allowed to grow to confluency in Dulbecco–Vogt modified Eagle's medium supplemented with 10% fetal calf serum, at which time they were trypsinized and passaged. The passaged cells were plated in 75-cm^2 flasks (approximately 1×10^6 cells per flask) in 10 ml medium and grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. By Days 3–5, the medium was then removed and replaced with 5 ml of sulfate-depleted medium containing 75 μCi/ml $Na_2[^{35}S]O_4$ and either 17.5 μCi/ml D-[6-^{3}H]glucosamine HCl or 50 μCi/ml D-[2-^{3}H]mannose, and incubation continued for 48 h.

Proteoglycan isolation. When labeling was complete, the media were removed and the cell layers were washed twice with phosphate-buffered saline. The media and washes were pooled and will be referred to as the medium extract. Solid guanidine–HCl (0.38 g/ml) was then added to the medium fraction to make it approximately 3 M in guanidine–HCl (14). The cell layers were immediately extracted with 4 M guanidine–HCl in 0.05 M sodium acetate containing protease inhibitors: 0.1 M 6-aminohexanoic acid, 5 mM benzamidine–HCl, 50 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM *N*-ethylmaleimide, pH 5.8. After extraction for 12 h at 4°C, the cell layers were scraped, the extract was removed, and the flasks were washed twice with 5 ml 4 M guanidine–HCl containing protease inhibitors. The extracts were pooled and centrifuged at 15,000 rpm for 20 min to remove any insoluble residue; the resulting supernatant will be referred to as the cell layer extract. Less than 3% of the total radioactivity was associated with the residue remaining after 4 M guanidine–HCl extraction. In the present study this fraction was not further analyzed.

Labeled macromolecules were separated from free label by eluting the medium and cell layer extracts from a Sephadex G-50 column (2.5 × 30 cm) that had been equilibrated with 4 M guanidine–HCl, in 0.05 M sodium acetate, pH 5.8, plus protease inhibitors.

[2] Abbreviations used: CHAPS, 3-[(3-chloamidopropyl)-dimethylaminio]-1-propanesulfonate; SDS, sodium dodecyl sulfate.

Fractions of 5.0 ml were collected at a flow rate of 15 ml/h and aliquots of each were assessed for radioactivity using a Packard Tri-Carb 3255 liquid scintillation counter.

Analytical column chromatography. Aliquots of the excluded Sephadex G-50 peak were chromatographed on columns of Sepharose CL-2B (0.7 × 100 cm), Sepharose CL-4B (0.7 × 100 cm), and Sepharose CL-6B (0.7 × 100 cm) using either dissociative conditions (4 M guanidine–HCl in 0.05 M sodium acetate, pH 5.8, containing the above-mentioned protease inhibitors) or detergent conditions (0.2% sodium dodecyl sulfate in 0.15 M sodium acetate, 1 mM magnesium chloride, and 1 mM calcium chloride, pH 5.8). Prior to chromatography on the SDS columns, the samples were dialyzed against acetate buffer, and then made 0.2% in SDS since SDS is insoluble in guanidine–HCl. Fractions of 0.5 ml were collected at a flow rate of 3 ml/h and assessed for radioactivity.

Ion-exchange chromatography. Aliquots of the excluded medium and cell layer extract peaks obtained after Sephadex G-50 chromatography were dialyzed against 7 M urea, 0.05 M Tris–HCl, and protease inhibitors, pH 7.0, and eluted from a DEAE-Sephadex column (0.9 × 7 cm) equilibrated in the same buffer. Prior to analytical ion-exchange chromatography, Triton X-100 was added to give a concentration of 0.5%. After elution of the unbound material, a continuous 0.1 to 0.8 M NaCl gradient was applied using a total of 140 ml. Fractions of 2 ml were collected at a flow rate of 5 ml/h and aliquots from each fraction were assayed for radioactivity. Ion-exchange chromatography was used as a preparative step as well as a method to analyze the proteoglycans (see later). In these cases the cells were incubated in the presence of [^{35}S]sulfate and either [^{3}H]glucosamine or [^{3}H]mannose, and the medium and cell layer extracts were desalted by elution from Sephadex G-50 columns in 7 M urea. The excluded peaks were then applied directly onto DEAE-Sephacel and eluted by a continuous 0.1 to 0.8 M NaCl gradient as described above, except that Triton X-100 was omitted from the eluting buffer.

Equilibrium density gradient centrifugation. Aliquots of the proteoglycans isolated by Sephadex G-50 chromatography were made to a starting density of 1.35 g/ml in 4 M guanidine–HCl buffer by the addition of solid cesium chloride. The samples were centrifuged for 60 h at 35,000 rpm in a Beckman SW 50.1 rotor and then divided into five fractions of approximately equal volume. These were termed D1 through D5 where D1 was the most dense fraction. The specific gravity of each fraction was determined using a 100-μl pipette. Portions of each fraction were assayed for [^{35}S]sulfate content by liquid scintillation counting. The remainder of each fraction was eluted from Sepharose CL-4B under dissociative conditions.

Proteoglycan analysis. The proteoglycan nature of the ^{35}S-labeled material in each of the peaks identified by Sepharose CL-4B chromatography was determined by chromatography on Sepharose CL-6B columns (0.7 × 100 cm) equilibrated in the SDS/acetate buffer both before and after digestion with papain (50 μg/ml) in 0.2 M sodium acetate, 0.004 M EDTA, and 0.02 M cysteine–HCl, pH 5.7, for 24 h at 60°C. Following papain digestion, the samples were dialyzed against acetate buffer prior to Sepharose CL-6B chromatography. The types of glycosaminoglycans and their relative proportions in each of the Sepharose CL-4B peaks were determined by treatment of the proteoglycans with either chondroitinase AC, chondroitinase ABC, or nitrous acid (15, 16). The enzyme digests were then chromatographed on Sepharose CL-6B columns (0.7 × 60 cm) using the same SDS/acetate buffer as described above. The relative proportions of each glycosaminoglycan species were determined by calculating the amount of ^{35}S-labeled material resistant to or degraded by each enzyme treatment.

Oligosaccharide analysis. Proteoglycans labeled with either [^{3}H]glucosamine and [^{35}S]sulfate, or [^{3}H]mannose and [^{35}S]sulfate, or [^{35}S]sulfate alone were isolated by elution from Sephadex G-50 followed by DEAE-Sephacel ion-exchange chromatography as described above. Aliquots from these preparations were treated with 0.05 M NaOH, 1 M $NaBH_4$ at 37°C for 12 h as previously described (17). The samples were then neutralized with glacial acetic acid and eluted from a Biogel P-10 column (0.7 × 60 cm) using 0.5 M pyridine acetate, pH 5.0, and the fractions were assessed for radioactivity. To determine whether the oligosaccharides contained sialic acid residues, aliquots of proteoglycans labeled with [^{35}S]sulfate and [^{3}H]glucosamine were treated with neuraminidase (20 milliunits/ml) in 0.05 M sodium acetate, pH 5.0, at 37°C for 3 h, followed by a 12-h incubation at 37°C in 0.05 M NaOH, 1 M $NaBH_4$. The digested samples were eluted from a Biogel P-10 column under the same conditions as described above. Oligosaccharide fractions eluted from Biogel P-10 were also analyzed for hexosamine and hexosaminitol content (18). Pooled oligosaccharide fractions which eluted from Biogel P-10 were hydrolyzed with 4 M HCl at 100°C for 10 h under nitrogen, then thoroughly dried, and analyzed with a Beckman amino acid analyzer Model 120C using a 0.2 M sodium citrate borate buffer, pH 5.28, and a column temperature of 50°C. Fractions of 0.5 ml were collected and counted for radioactivity. Since the above analyses were performed on crude proteoglycan preparations, the possibility of the presence of contaminating glycoproteins copurifying with the proteoglycans could not be excluded. Therefore, to further define the association of oligosaccharides with proteoglycans, we took aliquots of specific proteoglycan species isolated after sequential Sephadex G-50, DEAE-Sephacel, and Sepharose CL-4B purification. These isolates were then treated with alkaline sodium borohydride

and the reaction products were analyzed using Biogel P-10 as described above.

RESULTS

Extraction and Isolation of Proteoglycans

Gingival fibroblasts were incubated in the presence of medium containing serum and [^{35}S]sulfate for 48 h. Approximately 70–80% of the ^{35}S-labeled macromolecules were found in the medium; the remaining 20–30% remained associated with the cell layer and were recovered by guanidine–HCl extraction. The results reported were highly reproducible in the three cell strains examined, and the following are representative data from these experiments.

Analytical Column Chromatography: Dissociative Conditions

The molecular size distribution of medium- and cell layer-associated proteoglycans was assessed by Sepharose CL-2B and CL-4B gel filtration. In all cases, the recovery of labeled material from these columns was in the range of 75–80%. The ^{35}S-labeled proteoglycans from the medium eluted from Sepharose CL-4B into three well-defined peaks (Fig. 1a). The first peak (MI) eluted in the void volume and comprised approximately 5% of the total ^{35}S radioactivity. The second (MII) and third (MIII) peaks eluted with K_{av} values of 0.25 and 0.4 and accounted for 20 and 75% of the radiolabeled material, respectively. The cell layer extracts separated into four ^{35}S-labeled peaks upon elution from Sepharose CL-4B (Fig. 1c). Peak CI, which made up 25% of the total radioactivity, eluted in the void volume. The next peak (CII) eluted with a K_{av} of 0.1 and accounted for another 25% of the radioactivity. A third peak (CIII), which was often poorly defined, eluted at K_{av} 0.45 and comprised 10% of the radioactivity, with the remaining 40% eluting with a K_{av} of 0.6 (CIV).

Since some of the ^{35}S-labeled macromolecules from the medium and cell layer were excluded from Sepharose CL-4B, these preparations were also eluted from Sepharose CL-2B to determine if any of the labeled material could be excluded from this gel. The medium proteoglycans which originally separated into three peaks on Sepharose CL-4B eluted from Sepharose CL-2B as an asymmetrical peak of K_{av} 0.6 (Fig. 1b). The cell layer extracted material eluted as three peaks from Sepharose CL-2B (Fig. 1d). The first peak, which was excluded from the column, accounted for approximately 25% of the total ^{35}S counts. In addition, a second peak with a K_{av} of 0.4 and a third peak of K_{av} 0.7 were also noted; these accounted for 30 and 45% of the total radioactivity, respectively.

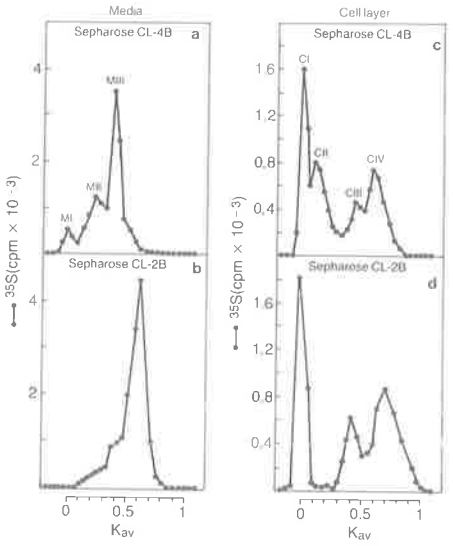

FIG. 1. Gel filtration profiles of gingival fibroblast medium and cell layer extracts (4 M guanidine–HCl). (a, c) Sepharose CL-4B; (b, d) Sepharose CL-2B. The peaks are labeled MI to MIII and CI to CIV for reference purposes and relate to their elution order. The void volume (V_0) and total volume (V_t) were identified with [^{3}H]DNA and $Na_2[^{35}S]O_4$, respectively.

Analytical Column Chromatography: Detergent Conditions

Medium and cell layer proteoglycans were also analyzed by gel filtration on Sepharose CL-4B eluted with an SDS/acetate buffer (Fig. 2). Recovery of labeled material under these conditions was slightly better than when guanidine HCl was used, and was in the range of 80–85%. Similar to the profiles observed under dissociative conditions, the medium ^{35}S-la-

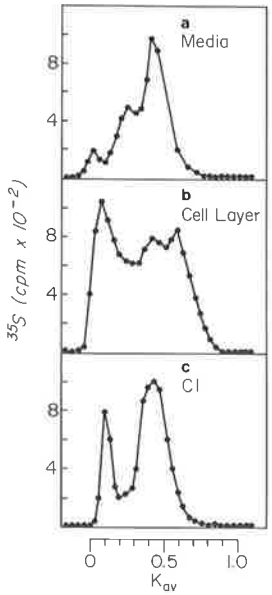

FIG. 2. Sepharose CL-4B chromatograms of medium and cell layer extract proteoglycans treated and eluted with SDS (Sepharose CL-4B/0.2% SDS): medium proteoglycans (a), cell layer proteoglycans (b), and cell layer proteoglycan peak CI (c). The V_0 and V_t were determined as described in Fig. 1.

beled proteoglycans eluted into three peaks (Fig. 2a) corresponding to MI, MII, and MIII seen in Fig. 1a. Under detergent conditions, the cell layer extract separated into three peaks with K_{av} values of 0.1, 0.45, and 0.6 (Fig. 2b). These corresponded to the peaks CII, CIII, and CIV seen in Fig. 1c. No material eluted in a position corresponding to peak CI under detergent conditions. In addition, the proportions of the peaks were different (compare Figs. 1c and 2b), indicating that in the presence of SDS, the material in peak CI may dissociate. Also, following SDS treatment, an increased amount of ^{35}S-labeled material was seen to elute between K_{av} 0.2 and K_{av} 0.4. To further analyze the nature of the cell layer proteoglycans eluting in the void volume, peak CI was obtained by dissociative Sepharose CL-4B chromatography and was chromatographed with buffer containing detergent. Under these conditions peak CI was resolved into two components of K_{av} 0.1 and 0.45 (Fig. 2c). The material eluting in each of these positions was pooled and kept for subsequent glycosaminoglycan analysis (see below). None of the other cell layer extract proteoglycans obtained by dissociative column chromatography (CII, CIII, and CIV) eluted in different positions after SDS treatment (results not shown).

Similar treatment of the medium and cell layer proteoglycans with the milder detergents, CHAPS, or Triton X-100 (0.2% w/v in 4 M guanidine–HCl/0.05 M sodium acetate, pH 5.8), failed to completely resolve peak CI into its separate components. However, if used at concentrations greater than 1%, these detergents were able to completely dissociate peak CI into its individual components (results not shown).

Since some contamination of peak CI by peak CII was likely, and could account for material eluting at position CII after SDS treatment of CI, we also isolated the material from the cell layer which eluted in the void volume on Sepharose CL-2B. This material was treated with SDS and eluted from Sepharose CL-4B. The results obtained confirmed our previous findings that this material was comprised of components which eluted with K_{av} values of 0.1 and 0.45 on Sepharose CL-4B (results not shown).

Ion-Exchange Chromatography

Proteoglycans were separated from other glycoproteins and hyaluronic acid by ion-exchange chromatography (Fig. 3). The profiles of radioactivity eluted from these columns were similar for both the medium and cell layer extracts even though the relative proportions of the various peaks differed somewhat (Fig. 3). In all cases four major peaks were noted. Peaks 1 and 2 contained mostly ^{3}H and were considered to be glycoprotein fractions because of their elution from the resin at low salt concentration. Peak 3 also contained only ^{3}H and the relative amounts of the total ^{3}H counts for the medium and cell layer extracts differed markedly. This peak was 80–90% susceptible to *Streptomyces* hyaluronidase digestion and therefore contained predominantly hyaluronic acid (results not shown). The remaining 10–20% was presumed to be a glycoprotein which coeluted with hyaluronic acid. However,

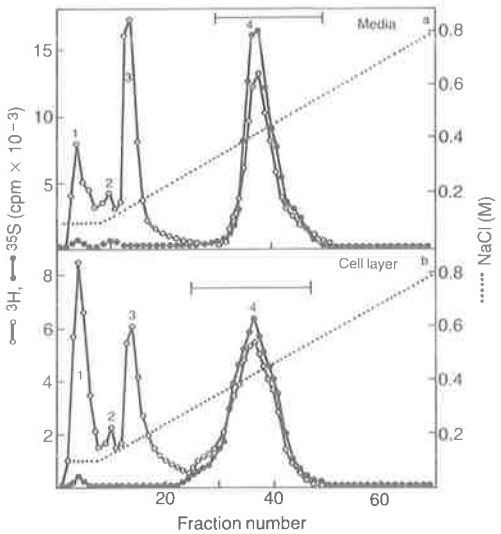

FIG. 3. DEAE-Sephacel ion-exchange profiles of [^{3}H]- and [^{35}S]-labeled macromolecules. (a) Medium; (b) cell layer proteoglycans were eluted with a linear NaCl gradient from 0.1 to 0.8 M, containing 0.5% Triton X-100. The major ^{35}S peak for each extract was pooled as indicated by the horizontal bar.

since it did not contain any [^{35}S]sulfate, and therefore was not likely a sulfated glycosaminoglycan/proteoglycan, it was not further analyzed. The major ^{35}S-containing peak, peak 4, was sometimes distorted by a leading edge which was always greater for the cell layer than for medium-derived preparations. Heparan sulfate was the major component of this leading portion of peak 4 for both medium and cell layer proteoglycans. Dermatan sulfate and chondroitin sulfate were the other components identified in peak 4 for both the medium and the cell layer. In none of these preparations was clear-cut separation of heparan sulfate, dermatan sulfate, and chondroitin sulfate achieved.

Density Gradient Ultracentrifugation

Proteoglycans labeled with [^{35}S]sulfate were further fractionated by density gradient ultracentrifugation (Fig. 4). In the medium proteoglycan preparation, 80% of the ^{35}S-labeled proteoglycans was found in the denser three-fifths, with 40% in the most dense fraction (D1). The cell layer extract proteoglycans were distributed somewhat differently throughout the gradient, with the D1 fraction containing approximately 30% of the ^{35}S activity and the remaining 70% of the radioactivity more evenly distributed through the D2–D5 fractions. The more even distribution of the cell layer proteoglycans throughout the density gradient is highlighted by the observation that the cell layer D5 fraction comprised slightly more than 10% of the total ^{35}S label whereas fraction D5 of the medium represented only 4% of the total radioactivity. Each of the fractions was concentrated and then chromatographed on a Sepharose CL-4B column in 4 M guanidine-HCl (Fig. 5). The D1 fraction of the medium proteoglycans contained all three components (MI, MII, and MIII) observed in the Sepharose CL-4B chromatogram of Fig. 1a. The D2-D4 fractions contained proteoglycans which eluted in a position similar to MIII with a K_{av} of 0.4 and minor components corresponding to MI and MII. Fraction D5 accounted for 4% of the total radioactivity and contained two molecular species, with K_{av} values of 0.2 and 0.4 which

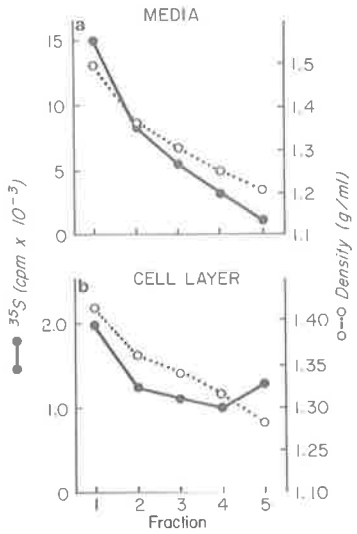

FIG. 4. Cesium chloride density gradient centrifugation. Aliquots of (a) medium and (b) cell layer proteoglycans were adjusted to a starting density of 1.35 g/ml with cesium chloride in 4 M guanidine-HCl, pH 5.8, and centrifuged at 35,000 rpm in a SW 50.1 rotor for 65 h. The fractions obtained were termed D1 to D5 in order of ascending density.

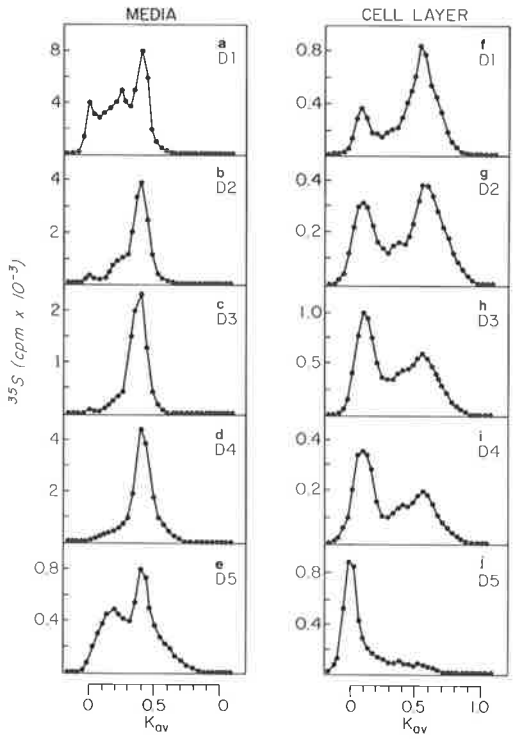

FIG. 5. Sepharose CL-4B chromatograms (Sepharose CL-4B/4 M guanidine HCl) of density gradient fractions of gingival fibroblast proteoglycans. Elution profiles for medium proteoglycans from D1 to D5 are shown in (a–e), and for the cell layer preparations in (f–j). The V_0 and V_t were identified as described in Fig. 1.

appeared to correspond to the peaks MII and MIII seen in Fig. 1. For the cell layer extract, fraction D5 eluted as a single peak in the void volume of the column, while fractions D1–D4 eluted in three principal regions corresponding to K_{av} values of 0.1, 0.4, and 0.6. The proportions of ^{35}S in the peaks differed for each density gradient fraction, with material in the peak of K_{av} 0.6 predominating in the D1 and D2 fractions. This component accounted for less of the total ^{35}S label in the D3 and D4 fractions, where material eluting at K_{av} 0.1 predominated.

Chemical Analyses of Proteoglycans

The proteoglycan nature of material eluted in the peaks following Sepharose CL-4B chromatography (MI-MIII and CI-CIV) was established by papain digestion. Material in all of the peaks, except peak CIV, was susceptible to papain (Fig. 6), indicating that peaks MI-MIII and CI-CIII contained ^{35}S-labeled proteoglycans. The resultant glycosaminoglycan chains eluted from Sepharose CL-6B with a K_{av} of 0.45. Based on previously reported data for calibrated Sepharose CL-6B columns (19), these glycosaminoglycans had an average molecular weight of 25,000.

Carbohydrate analysis of the various size classes of proteoglycans indicated them to have different glycosaminoglycan compositions (Table I). The larger proteoglycans of the medium (MI and MII) were comprised predominantly of chondroitin sulfate chains, while the smallest medium proteoglycan (MIII) contained mainly dermatan sulfate chains. The cell layer proteoglycans appeared to have a more heterogeneous glycosaminoglycan composition. The largest proteoglycans (CI and II)

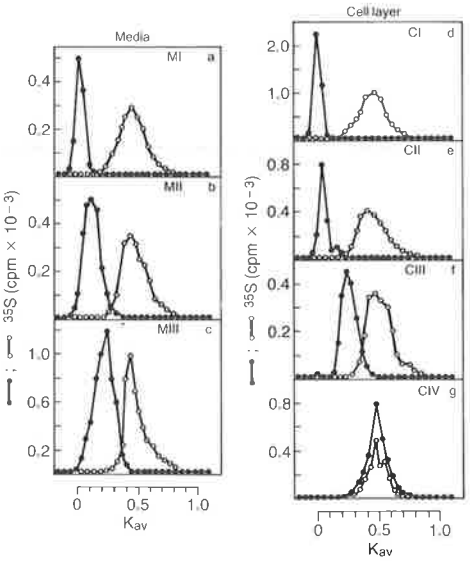

FIG. 6. Sepharose CL-6B chromatograms of papain-digested medium and cell layer proteoglycans (papain: Sepharose CL-6B/0.2% SDS). ^{35}S-labeled proteoglycans from medium (a–c) and cell layer (d–f) extracts obtained by Sepharose CL-4B chromatography (see Fig. 1) were eluted on Sepharose CL-6B both before (●) and after (○) digestion with papain. The V_0 and V_t were identified as described in Fig. 1.

TABLE I

GLYCOSAMINOGLYCAN COMPOSITION OF PROTEOGLYCAN COMPONENTS

Peak	% Fraction	Heparan sulfate	Dermatan sulfate	Chondroitin sulfate
Medium				
MI	5	15	0	85
MII	20	33	0	67
MIII	75	20	70	10
Cell layer				
CI	20	65	10	25
CII	30	61	0	39
CIII	15	36	54	10
CIV	35	70	30	0

Note. Glycosaminoglycan content was determined by sequential digestion with chondroitinase AC, chondroitinase ABC, and nitrous acid. Results are expressed as percentages of the total ^{35}S-activity remaining in each peak after digestion. Fractions refer to the peaks obtained from chromatography on Sepharose CL-4B, as shown in Fig. 1.

contained mainly heparan sulfate; however, some chondroitin sulfate was also identified in these populations. The smallest proteoglycan (CIII) contained predominantly dermatan sulfate with some heparan sulfate. From these data it is unclear whether heparan sulfate is located on the same protein core as chondroitin sulfate and dermatan sulfate, or is present as a separate heparan sulfate proteoglycan species. Cell layer peak CIV, which contained only glycosaminoglycan chains, was comprised of both dermatan sulfate (30%) and heparan sulfate (70%).

Since peak CI could be dissociated into two smaller proteoglycan components upon treatment with SDS (Fig. 2c), the glycosaminoglycan composition of these two proteoglycans was assessed to determine whether they were similar to the proteoglycans originally isolated from peaks CII and CIII. The first peak to elute under SDS conditions contained 90% heparan sulfate and 10% chondroitin sulfate, and thus closely resembled the composition of the proteoglycans isolated from peak CII. However, the second peak to elute after SDS treatment of proteoglycans in peak CI contained 80% heparan sulfate and 20% chondroitin sulfate. Thus the proteoglycans in this peak differed from the predominantly dermatan sulfate proteoglycans isolated from peak CIII.

Oligosaccharide Analyses

The oligosaccharide components of proteoglycans labeled with [^{35}S]sulfate and [^{3}H]glucosamine were identified by elution from Biogel P-10 (Fig. 7). Four peaks were seen for both the medium and cell layer extract proteoglycans following Biogel P-10 chromatography. Peak 1 (K_{av} 0) corresponded to glycosaminoglycan chains, and peaks 2 and 3 (K_{av} 0.45 and 0.65) were initially identified as oligosaccharides based on their elution position relative to previous reports of oligosaccharides associated with proteoglycans (14, 18). Peak 4 eluted at the V_t and presumably contained free ^{35}S and ^{3}H released by the chemical treatment. The proportions of the total ^{3}H activity accounted for 4 and 2%, respectively, for peaks 2 and 3 in the medium preparations and 3 and 4%, respectively, for the cell layer preparations. To further ascertain whether the oligosaccharides were sulfated, fibroblasts were incubated in the presence of [^{35}S]sulfate alone and the proteoglycans were isolated as described above. Following alkaline borohydride reduction and elution from Biogel P-10, four

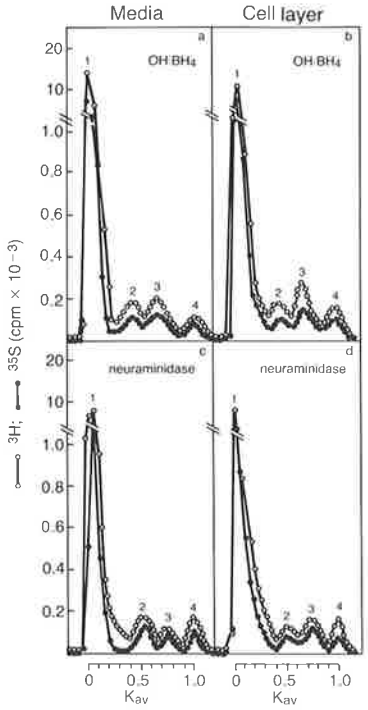

FIG. 7. Biogel P-10 chromatograms of alkaline borohydride-treated gingival fibroblast proteoglycans. Proteoglycans from (a) medium and (b) cell layer were treated with alkaline borohydride and chromatographed on Biogel P-10. Proteoglycans from medium (c) and cell layer (d) were also treated with neuraminidase prior to alkaline borohydride reduction and elution from Biogel P-10. The peaks are numbered to indicate their elution order. The V_0 and V_t were determined as described in Fig. 1.

peaks were identified corresponding to K_{av} values of 0, 0.45, 0.65, and 1.0 as was seen for the dual-label experiments (data not shown). The sulfate content of the oligosaccharides accounted for 2–3% of the total ^{35}S activity.

In order to further characterize these oligosaccharide units, proteoglycans were digested with neuraminidase followed by sodium borohydride reduction. The reaction products were then eluted from Biogel P-10 (Fig. 7). Four peaks were seen following such treatment. Peak 1 contained both ^{35}S and ^{3}H activity and presumably contained glycosaminoglycans. Peaks 2 and 3 were retarded by the gel, and eluted with higher K_{av} values than they did prior to enzyme treatment. The ^{35}S:^{3}H ratios within peaks 2 and 3 increased from 0.5 to 1.0 and from 0.56 to 0.69, respectively, and are indicative of loss of ^{3}H label most likely associated with sialic acid residues. In addition, the ^{35}S:^{3}H ratio within peak 4 decreased from 0.6 to 0.34, demonstrating an increase in ^{3}H activity eluting in the total volume and most likely came from sialic acid residues released by neuraminidase. The samples were also treated with keratanase to determine whether these oligosaccharides were keratan sulfate; no shift in K_{av} values on Biogel P-10 was noted (results not shown). Additionally, the cells were labeled in the presence of [^{3}H]mannose and [^{35}S]sulfate, and the labeled proteoglycans were eluted from Biogel P-10 (Figs. 8a, b). Three peaks demonstrating ^{3}H activity and four peaks of ^{35}S activity were identified. About 20% of the ^{3}H activity eluted in the void volume, while the bulk of the ^{3}H activity eluted as a single included peak of K_{av} 0.45 for both the medium and the cell layer preparations. No ^{3}H activity was detected in peak 3 which contained only ^{35}S activity. Thus only one of the oligosaccharides contained mannose residues. The ^{3}H-labeled material which eluted in the void volume of a Biogel P-10 column was presumed to be chondroitin sulfate or dermatan sulfate residues, since chondroitinase ABC treatment of the labeled proteoglycans resulted in a shift of

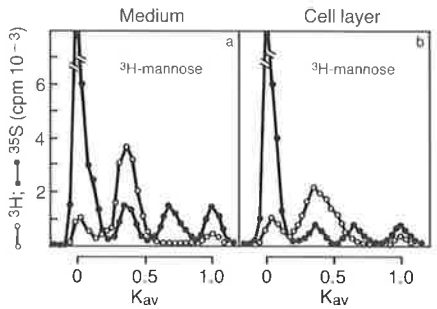

FIG. 8. Biogel P-10 chromatograms of [^{3}H]mannose- and [^{35}S]sulfate-labeled oligosaccharides. The medium (a) and cell layer (b) extracts were treated with alkaline sodium borohydride and then chromatographed on Biogel P-10. The V_0 and V_t were determined as described in Fig. 1.

20% of the ^{3}H activity to the total volume of a Sephadex G-50 column; nitrous acid treatment had no effect (results not shown).

The type of linkage between the carbohydrate chains and core protein was determined by sugar analysis for hexosamine and hexosaminitol in material associated with peaks 1, 2, and 3 seen in Figs. 7a and 7b. Peak 1 from the medium preparation, which had a predominance (86%) of galactosamine as well as a large amount of ^{35}S activity, could be digested substantially (80%) by chondroitinase ABC (results not shown). The material in cell layer peak 1 contained a large proportion of glucosamine and also had high levels of ^{35}S activity, but could only be partially digested with chondroitinase ABC (results not shown). These observations are consistent with the presence of glycosaminoglycan chains and correlate well with our observation of a predominance of dermatan sulfate in the medium and of heparan sulfate in the cell layer (Table I). The material eluting in peak 2 from Biogel P-10 was classified as N-linked oligosaccharide because no galactosaminitol was detected (Table II), indicating the absence of O-glycosidic bonds, since $NaOH/NaBH_4$ will cleave such linkages if present. In addition, this material could be radiolabeled using [^{3}H]mannose as a metabolic precursor (Fig. 8), a feature common to many N-linked oligosaccharides. Peak 3 was considered to contain O-linked oligosaccharides. This was based upon the presence of galactosaminitol residues (indicative of cleavage of O-glycosidic linkages to the core protein following $NaOH/NaBH_4$ treatment), as well as the absence of radiolabeling of these components using [^{3}H]mannose as a metabolic precursor. The relative recoveries of ^{3}H-labeled sugars in the oligosaccharide peaks were low and indicate that some ^{3}H activity may be in components other than the sugars analyzed. Since neuraminidase digestion of the oligosaccharides confirmed the presence of sialic acid residues, poor recovery of ^{3}H activity may be partially accounted for by degradation of sialic acid residues during the hydrolysis of the oligosaccharides prior to sugar analysis.

Thus, the above detailed analyses of the oligosaccharides associated with medium and cell layer proteoglycans indicated the presence of both N- and O-linked species which appear to be sulfated to varying degrees. Nonetheless, since these analyses had been performed on proteoglycans isolated by DEAE-Sephacel ion-exchange chromatography alone, we could not discount the presence of contaminating glycoproteins. Therefore, we isolated

TABLE II

HEXOSAMINE AND HEXOSAMINITOL ANALYSIS OF BIOGEL P-10 FRACTIONS

Peak	Galactosaminitol[a]	Glucosamine[a]	Galactosamine[a]	Recovery[b]
Medium				
1	ND[c]	14	86	90
2	ND	82	18	70
3	60	25	15	50
Cell layer				
1	ND	40	60	88
2	ND	71	29	55
3	51	22	27	34

[a] Values are expressed as percentages of the ^{3}H activity eluted from the column.

[b] Recoveries were measured relative to the recoveries of standard preparations of [^{14}C]galactosamine, [^{14}C]galactosaminitol, and [^{3}H]glucosamine applied to the column as internal standards.

[c] ND, not detected.

Note. Proteoglycans were treated with alkaline borohydride and eluted from Biogel P-10. The fractions in the resultant peaks were pooled, hydrolyzed, and analyzed for their hexosamine and hexosaminitol content.

[^{35}S]sulfate-labeled proteoglycans from peaks MII, MIII, CI, CII, and CIII after sequential Sephadex G-50, DEAE-Sephacel, and Sepharose CL-4B chromatography. These individual proteoglycan populations were then treated with $NaOH/NaBH_4$ and eluted from Biogel P-10. In all cases, two oligosaccharide peaks of K_{av} 0.45 and 0.65 were identified (Fig. 9), thus indicating that these oligosaccharides are intimately associated with the proteoglycans. The apparent increase in free sulfate release by such treatment is unclear, but it may relate to different degrees of sulfation of newly synthesized proteoglycans, dependent upon either the cellular source or cell transfer number.

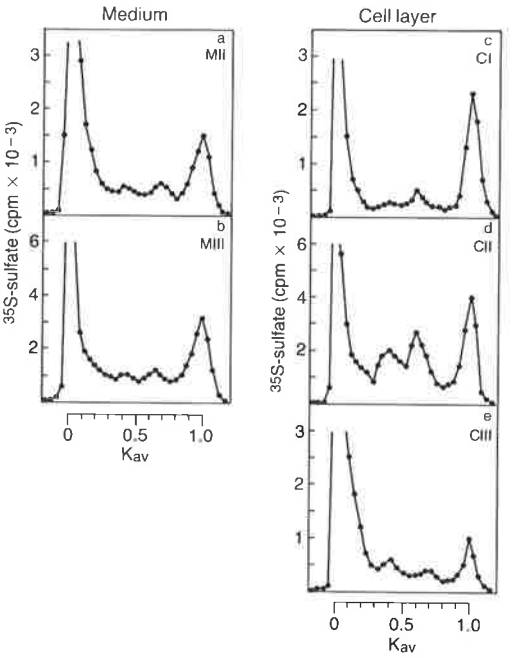

FIG. 9. Biogel P-10 chromatogram of ^{35}S-labeled oligosaccharides. Medium (a) and cell layer (b) proteoglycans isolated following Sephadex G-50, DEAE-Sephacel, and Sepharose CL-4B chromatography were subjected to alkaline sodium borohydride prior to chromatography on Biogel P-10. The abbreviations MII, MIII, CI, CII, CIII refer to the peaks identified in Figs. 1a and 1c. The V_0 and V_t were determined as described in Fig. 1.

DISCUSSION

Proteoglycans constitute the major proportion of [^{35}S]sulfate-labeled macromolecules synthesized by human gingival fibroblasts *in vitro*. Molecular sieve chromatography on both Sepharose CL-2B and CL-4B was used to initially separate the proteoglycans. Sepharose CL-4B gave the best resolution. The relative distribution of the various proteoglycans released into the culture medium was different from that reported for other fibroblasts grown under identical conditions. In our system, the bulk (75%) of the medium proteoglycans was represented by the small molecular size MIII peak which contained predominantly dermatan sulfate proteoglycan. These results differ from those obtained for skin fibroblasts, in which the quantitatively major component is a large chondroitin sulfate proteoglycan which excludes from Sepharose CL-4B (10). In addition, the profiles obtained from the gingival fibroblast proteoglycans differed from those of embryonic lung fibroblasts in that the chondroitin sulfate proteoglycan species comprised only 25% of the total medium proteoglycans, compared to 38% for the lung fibroblasts (12).

The cell layer extract proteoglycans contained a large proportion of heparan sulfate proteoglycans. This confirms our earlier observation that heparan sulfate is the quantitatively major glycosaminoglycan associated with the cell layer extract of gingival fibroblasts (20). Indeed, the association of heparan sulfate proteoglycans with cell membranes seems to be a general feature of most cultured cells (21). A significant proportion (20%) of the cell layer extract proteoglycans was excluded from both Sepharose CL-4B and Sepharose CL-2B (peak CI), while only 5% of the medium proteoglycans was excluded from Sepharose CL-4B and none from CL-2B. Following solubilization in SDS and subsequent chromatography in the presence of this detergent, peak CI could be separated into two components, indicating a likely association with lipid components of the cell membrane. The association seemed to be very strong since treatments with the milder detergents, CHAPS or Triton X-100 (results not shown), were unable to com-

pletely dissociate this peak at concentrations less than 1%. Thus, complete solubilization of the cell membrane appears to be necessary to adequately release the associated constituent proteoglycan monomers. This observation is very similar to that reported for cell layer-associated proteoglycans of mouse mammary epithelial cells (22). The fact that this material not only contained heparan sulfate proteoglycans, as reported for other fibroblasts (10, 12), but also chondroitin sulfate proteoglycans is of particular interest since it lends support to the concept that both of these proteoglycans may be associated with cell membranes and may indeed be integral plasma membrane components (23, 24). Whether these cell-associated proteoglycans are present as separate chondroitin sulfate and heparan sulfate proteoglycans, or hybrid species containing both glycosaminoglycan chains attached to the same protein core, remains to be established.

Additional analyses of the glycosaminoglycan composition of the SDS-dissociated components of peak CI revealed that the largest proteoglycans (K_{av} 0.1 in Fig. 2c) were similar to those isolated from peak CII (Fig. 1c). However, the smaller proteoglycans released by SDS treatment of peak CI (K_{av} 0.45 in Fig. 2c) differed in glycosaminoglycan composition from peak CIII (Fig. 1c) and may represent a different proteoglycan population. Thus, the differences between these two peaks could explain, in part, the poor resolution of proteoglycans eluting in the region between K_{av} 0.2 and K_{av} 0.4 when total cell layer proteoglycans were chromatographed in the presence of SDS (Fig. 2b).

The cell layer also contained a population of [^{35}S]sulfate-labeled molecules which eluted from Sepharose CL-4B with a K_{av} of 0.6. This material was resistant to papain digestion and was therefore considered to represent free glycosaminoglycan chains, presumably of intracellular origin. The glycosaminoglycans identified in this peak were heparan sulfate (70%) and dermatan sulfate (30%). This distribution is very similar to that of other fibroblast intracellular glycosaminoglycan pools (12).

Density gradient ultracentrifugation allowed further fractionation of the proteoglycans. Adequate separation of the gingival fibroblast proteoglycans was achieved at a much lower starting density of 1.35 g/ml than the 1.45 and 1.5 g/ml customarily used for soft tissue proteoglycan density gradient centrifugation (10, 12, 25, 26). The medium proteoglycans distributed throughout the gradient in a fashion similar to that observed for proteoglycans extracted from gingival tissue (8). Sepharose CL-4B chromatography of the gradient fractions revealed selective distribution of the proteoglycans of various molecular sizes on the basis of buoyant density. In particular, proteoglycans corresponding to those in the Sepharose CL-4B peak MIII predominated in the less dense fractions, thus suggesting that they have fewer carbohydrate chains per core protein than for the less buoyant, more dense MI and MII fractions.

Sepharose CL-4B elution profiles demonstrated that the bulk of the proteoglycans has a very small hydrodynamic size. Indeed, based on both their buoyant density and gel filtration profile it seems likely that most of the medium proteoglycans have a relatively short protein core (consistent with small hydrodynamic size), with only a few attached glycosaminoglycan chains (consistent with low buoyant density) compared to other proteoglycans which have a long protein core with many glycosaminoglycan chains. The cell layer extract proteoglycans (D1–D4) appeared to be distributed in a more even fashion throughout the density gradients than were the medium proteoglycans. The presence of most of the CI proteoglycan (13% of the total) in the least dense D5 fraction is interesting and further implies that peak CI contains lipid-associated proteoglycans.

Attempts to separate the proteoglycans on the basis of their charge by ion-exchange DEAE-Sephacel chromatography were unsuccessful. No separation of heparan sulfate proteoglycan from dermatan sulfate proteoglycan, as has been described in other systems (18, 27), was possible under the conditions used. In some cases with the cell layer extracts, a leading shoulder was seen before the major ^{35}S peak. Gly-

cosaminoglycan analysis of the shoulder region revealed heparan sulfate to be a major component, with smaller amounts of chondroitin sulfate and dermatan sulfate also identified. Heparan sulfate produced by gingival fibroblasts may therefore be more highly charged than the heparan sulfate made by some other cell types, and thus it may coelute with the more negatively charged chondroitin sulfate and dermatan sulfate species.

Analysis of the oligosaccharides released from proteoglycans revealed the presence of small sugars which were both N- and O-linked to the protein core. This is similar to other reports concerning proteoglycan oligosaccharides (14, 18, 28, 29). However, the oligosaccharides associated with gingival proteoglycans appeared to differ in one major respect from those isolated from most other proteoglycans in that they were sulfated to varying degrees. This is not a unique finding since sulfated oligosaccharides appear to be associated with a chondroitin sulfate isolated from brain (30). More recently, Gowda *et al.* have presented further evidence that oligosaccharides associated with proteoglycans may contain ester sulfate (31). Mannose and sialic acid residues were also associated with these oligosaccharides. The possibility that these oligosaccharides were keratan sulfate chains was ruled out by their resistance to keratanase. The extremely intimate association (presumably covalent) of these oligosaccharides with the proteoglycans has been highlighted by the observation that SDS dissociation of the proteoglycans in peak CI failed to release any small labeled molecular weight components which would have been indicative of contaminating glycoprotein (Fig. 2c). Furthermore, NaOH/$NaBH_4$ treatment of individually isolated proteoglycan populations revealed the presence of oligosaccharides in each of the preparations which eluted in identical positions on Biogel P-10. The significance of these components is not clear at this stage and must await further detailed analysis.

In conclusion, the proteoglycans synthesized by gingival fibroblasts in culture are very similar with respect to glycosaminoglycan composition, density gradient sedimentation, and molecular size to those present in extracts of whole gingiva (8). This study has demonstrated the capacity of human gingival fibroblasts *in vitro* to synthesize proteoglycans which appear to be characteristic for this cell type and its tissue of origin. Furthermore, it seems likely that fibroblasts derived from structurally similar but functionally different tissues (i.e., skin and gingiva) may have different proteoglycan synthetic capabilities, at least with respect to the relative types and proportions made.

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Paper 4

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Proteoglycans synthesized by human polymorphonuclear leukocytes *in vitro*.
Immunology and Cell Biology **67:** 9-17, 1989.

Candidate's Contribution to this paper: 75%

P.M. Bartold's role in this study was:
Design of the experiments
Provision of research funds
Supervision of research work
Writing of the manuscript

D.G. Harkin's role in this study was:
Summer Vacation Scholar
Execution of the experimental work

L.P. Bignold's role in this study was:
Advisory

Immunol. Cell Biol. (1989) **67**, 9–17

Proteoglycans synthesized by human polymorphonuclear leucocytes *in vitro*

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Summary Polymorphonuclear leucocytes (PMN) were assessed *in vitro* for their ability to synthesize and secrete proteoglycans. The PMN were isolated from human peripheral blood and were found to contain < 5% mononuclear cells. Following 24 h incubation in the presence of (^{35}S)-sulfate, significant quantities of ^{35}S-labelled macromolecules were detected both within the culture medium and cells. Although the PMN preparations contained some platelets (approximately five platelets : one PMN), culture of platelets alone did not result in the detection of any ^{35}S-labelled macromolecules in either the medium or platelets. ^{35}S/^{3}H-labelled macromolecules from the PMN cultures were identified as proteoglycans on the basis of their degradation by papain, alkaline sodium borohydride, chondroitinase ACII, chondroitinase ABC and nitrous acid. The labelled proteoglycans isolated from the medium and cells eluted from Sepharose CL-4B with a K_{av} of 0·63; this indicated a small size compared with many other proteoglycans. The glycosaminoglycans associated with the proteoglycans were identified as heparan sulfate, chondroitin sulfate and dermatan sulfate, with chondroitin sulfate being the principal component. The average molecular weight of the glycosaminoglycans was determined to be 16 000. Therefore, the data from this study demonstrate the ability of human PMN to synthesize and secrete proteoglycans *in vitro* which appear to differ from those synthesized by mesenchymal cells with respect to molecular size and glycosaminoglycan composition.

INTRODUCTION

Proteoglycans are large polyanionic molecules composed of a central protein core to which one or more glycosaminoglycan chains are covalently bound (1). They are widely distributed throughout mammalian tissues and are considered to be ubiquitous components of most, if not all, cells. For these reasons, proteoglycans are considered to play important roles in many cellular activities and interactions as well as being involved in the maintenance of normal tissue physiology.

Although mesenchymal cells are generally considered to produce most of the proteoglycans in tissues, it is becoming increasingly apparent that, especially in inflammation, other cells also synthesize and secrete proteoglycans. The presence of glycosaminoglycans in polymorphonuclear leucocytes (PMN) has been known since 1955 (2). Subsequent studies demonstrated some of the glycosaminoglycans to be sulfated and located within the cytoplasmic granules (3). Additional biochemical analyses have identified the PMN glycosaminoglycans as hyaluronic acid, heparan sulfate, dermatan sulfate and chondroitin sulfate (4–7). The synthesis of glycosaminoglycans by PMN has also been studied and the types of glycosaminoglycans produced have been suggested to vary depending upon the state of activation, maturation and adhesion of the PMN (8–10).

Despite such studies, few have addressed whether the glycosaminoglycans exist bound to a protein core in the form of proteoglycans or exist solely as glycosaminoglycan chains (9–11). This is an important consideration since, to date, hyaluronic acid is the only glycosaminoglycan believed to exist in tissues in a free form. Furthermore, most of the physiological properties of proteoglycans are derived from features of both the protein core and glycosaminoglycans and thus consideration of the glycosaminoglycans alone may oversimplify the possible reactivity of these molecules. Therefore, the aim of the present study was to isolate, identify and characterize the proteoglycans synthesized by human PMN cultured *in vitro*.

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Abbreviations used in this paper: PBS, phosphate-buffered saline; PMN, polymorphonuclear leucocytes.

MATERIALS AND METHODS

Materials

Guanidine HCl, L-glutamine, phenylmethylsulfonyl fluoride, 6-aminohexanoic acid, benzamidine HCl, 2-mercaptoethanol, N-ethylmaleimide were all purchased from the Sigma Chemical Company, St. Louis, MO; Na_2 (^{35}S)O_4 (1042 mCi/mmol) and L-(3,4,(n)-^{3}H) valine (1·26 TBq/mmol) were from Amersham Australia, Sydney, NSW; Ready Solv scintillation fluid was from Beckman Australia, Adelaide, SA; Chondroitinase ACII (*Arthrobacter aurescens*) and Chondroitinase ABC (*Proteus vulgaris*) were from Seikagaku Kogyo Co Ltd, Tokyo, Japan; all tissue culture plastic ware was from Nunc, Roskilde, Denmark; Dulbecco's modification of Eagle's Medium (DMEM), penicillin, streptomycin, non-essential amino acids and fetal calf serum were from Flow Laboratories, Irvine, Ayrshire, Scotland; Ficoll-Paque, Sepharose CL-4B, Sepharose CL-6B, Sephadex G-50 and DEAE-Sephacel were from Pharmacia Fine Chemicals, North Ryde, NSW.

Isolation of polymorphonuclear leucocytes

PMN were isolated from peripheral blood of healthy human donors by the method of Ferrante and Thong (12) as modified by Bignold and Ferrante (13). Briefly, blood samples were drawn into heparin (25 iu/ml) and 5 ml layered onto 3 ml Hypaque-Ficoll (8% w/v Ficoll 400, 5·6% (w/v) sodium diatrizoate and 11·3% (w/v) meglumine diatrizoate at pH 7·0; specific gravity of 1·114). This preparation was centrifuged at 500 *g* for 30 min in swing-out buckets at room temperature after which the PMN fraction between the mononuclear cell fractions and the erythrocytes was obtained. PMN were washed twice in Hank's balanced salt solution before culture. Cells were >95% viable as indicated by trypan blue exclusion and included <5% mononuclear cells. Viability remained constant throughout the incubation period. The ratio of platelets : PMN was determined to be less than 5 : 1.

Isolation of blood platelets

To heparinized blood buffered with Hepes (10 mmol/l), meglumine diatrizoate (22 mg/ml) was added to raise its specific gravity to approximately 1·03. This blood was then centrifuged for 15 min at 220 *g* after which the upper-half of the resultant platelet-rich plasma supernatant was removed and diluted 1 : 1 with 3·8% disodium hydrogen citrate solution. This dilute plasma was then centrifuged for 30 min at 800 *g* and the sediment then resuspended in culture medium (see below) to give a final concentration of 3×10^5 platelets/μl. Lymphocyte numbers in such preparations were less than 1/μl.

Cell culture and labelling

The PMN were adjusted to a concentration of 2×10^7 cells/ml in DMEM supplemented with 10% heat inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l glutamine, 10 mmol/l sodium pyruvate and non-essential amino acids (1% v/v). The cells were maintained in 25 cm^2 flasks in a total volume of 3 ml (6×10^7 cells) and cultured for up to 24 h. Incorporation of (^{35}S)-sulfate into proteoglycans was achieved by culturing the cells in the presence of 20 μCi/ml Na_2(^{35}S)O_4. In some experiments, the cells were cultured in the presence of 100 μCi/ml Na_2(^{35}S)O_4 and 10 μCi/ml (^{3}H)-valine.

Analysis of ^{35}S/^3H-labelled macromolecules

After incubating the cells in the presence of radiolabelled metabolic precursors for 24 h, the medium was removed from the flasks. The flasks were then rinsed out with 2 ml phosphate-buffered saline (PBS) and this was pooled with the medium. The medium and wash were centrifuged at 10 000 r/min for 5 min and the supernatant separated from the cell pellet. The supernatant fraction was adjusted to approximately 3 mol/l in guanidine HCl by adding solid guanidine HCl (0·38 g/ml), and then eluted from a Sephadex G-50 column (2·5 cm × 30 cm) in 4 mol/l guanidine HCl/0·05 mol/l sodium acetate containing the following as protease inhibitors: 0·1 mol/l 6-aminohexanoic acid, 5 mmol/l benzamidine HCl, 50 mmol/l EDTA, 0·1 mmol/l phenylmethylsulfonyl fluoride and 10 mol/l N-ethylmaleimide, pH 5·8 (14). Fractions of 1·5 ml were collected and 100 μl aliquots were taken for determination of radioactivity in a Beckman LS-2800 liquid scintillation counter. The material eluting in the void volume was well separated from unincorporated radioisotope and was pooled, dialysed against deionized water and lyophilized in preparation for further analyses. The cell pellets were extracted with 4 mol/l guanidine HCl/0·05 mol/l sodium acetate containing protease inhibitors for 24 h at 4°C and centrifuged at 15 000 r/min for 20 min to remove any insoluble residue. Less than 5% of the total radioactivity remained with the residue and this was not characterized any further in the present study. The radiolabelled macromolecules associated with the cell extract were obtained in a similar manner to the medium fraction. Recoveries of radioactivity from these columns ranged between 75 and 85%.

Analytical column chromatography

The molecular size distribution of ^{35}S-labelled proteoglycans was analysed by gel filtration from Sepharose CL-4B columns (0·7 cm × 100 cm) with 4 mol/l guanidine HCl/0·05 mol/l sodium acetate containing protease inhibitors, pH 5·8. Lyophilized material obtained from the void volume of Sephadex G-50 chromatography was made to 0·5 ml and 200 μl aliquots were applied to the Sepharose columns. Fractions of 0·5 ml were collected at a flow rate of 3 ml/h and the radioactivity of all of the fractions was measured. Recovery of radiolabelled material from these columns ranged between 80 and 85%.

The size of the glycosaminoglycan chains was assessed following digestion of the material which was excluded from Sephadex G-50 with papain (50 μg/ml) in 0·2 mol/l sodium acetate, 0·004 mol/l EDTA, and 0·02 mol/l cysteine HCl, pH 5·7, for 24 h at 60°C. Following digestion, the samples were boiled and

applied onto a column of Sepharose CL-6B (0·7 cm × 50 cm) and eluted with 4 mol/l guanidine HCl/0·05 mol/l sodium acetate, pH 5·8. The whole fractions were analysed for radioactivity. Glycosaminoglycan chain size was also assessed following treatment of the labelled macromolecules with 0·05 mol/l NaOH, 1 mol/l $NaBH_4$ at 45°C for 24 h. The released glycosaminoglycans were assessed in a similar manner to the papain digest.

Ion-exchange chromatography

A portion of the material which was excluded from Sephadex G-50 was dialysed exhaustively against water, lyophilized and resolubilized in 7 mol/l urea containing 0·1 mol/l NaCl and 0·05 mol/l Tris HCl, pH 7·0. This sample was then applied onto a column (0·8 cm × 7·0 cm) of DEAE-Sephacel which had been equilibrated in the same buffer. Following elution of all unbound radiolabelled material a 0·1–0·8 mol/l NaCl gradient was applied to the column. Fractions of 0·3 ml were collected and radioactivity was determined by liquid scintillation counting. Recoveries of radioactivity from these columns were between 75 and 85%.

Glycosaminoglycan analysis

The types of glycosaminoglycans and their relative proportions in proteoglycans which eluted in the void volume from Sephadex G-50 for both the medium and cell pellet were determined after pre-treatment with either chondroitinase ACII, chondroitinase ABC, or nitrous acid (15,16). The enzyme digests were then chromatographed on Sephadex G-50 (1·5 × 30 cm) eluted with 0·2% sodium dodecyl sulfate in 0·15 mol/l sodium acetate, 1 mol/l magnesium chloride, 1 mol/l calcium chloride, pH 5·8. Fractions of 0·5 ml were collected at a flow rate of 3 ml/h and all of the fractions were assayed for radioactivity. The relative proportion of each glycosaminoglycan species was determined by calculation of the amount of radiolabelled material resistant to (void volume) or degraded by (included volume) each treatment.

RESULTS

Extraction and isolation of proteoglycans

Human neutrophils were obtained from human peripheral blood and incubated in Dulbecco's modification of Eagle's medium containing 10% fetal calf serum and (^{35}S)-sulfate for 24 h. Initial isolation of ^{35}S-labelled macromolecules indicated 67% (s.d. = 6) were recovered from the medium and the remaining 33% (s.d. = 6) were associated with the cells and recovered by extraction with guanidine HCl. When purified platelets were cultured in a similar fashion, no ^{35}S-labelled macromolecules were detectable in either the medium or cell layer fractions.

Analytical column chromatography

The molecular size distribution of ^{35}S-labelled medium- and cell-associated proteoglycans was initially assessed by Sepharose CL-4B gel filtration. The ^{35}S-labelled proteoglycans isolated from the medium and cells eluted within the included volume of the column as single polydisperse peaks with K_{av} values of 0·63 (s.d. = 0·08) and 0·65 (s.d. = 0·03) respectively (Fig. 1). In addition, samples which had been labelled with both ^{3}H and ^{35}S were also eluted from Sepharose CL-6B (Fig. 2). Molecules which had been labelled with (^{3}H)-valine eluted from Sepharose CL-6B in three peaks for the medium and four peaks for cell layer-associated material. In both the medium and cell layer material the ^{35}S-labelled material co-eluted with material eluting in a ^{3}H-labelled peak at a K_{av} of 0·25.

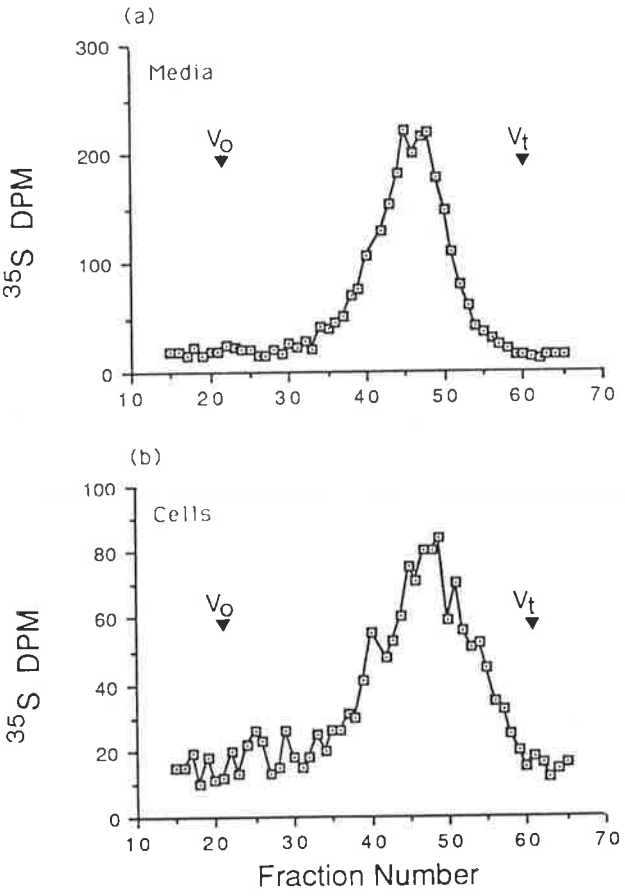

Fig. 1. Gel chromatography of proteoglycans isolated from (a) medium and (b) cells of PMN cultures. Samples were chromatographed on Sepharose CL-4B with 4 mol/l guanidine HCl containing protease inhibitors. The void volume (V_0) and total volume (V_t) were determined by the elution of Blue Dextran and [^{35}S]-sulfate, respectively.

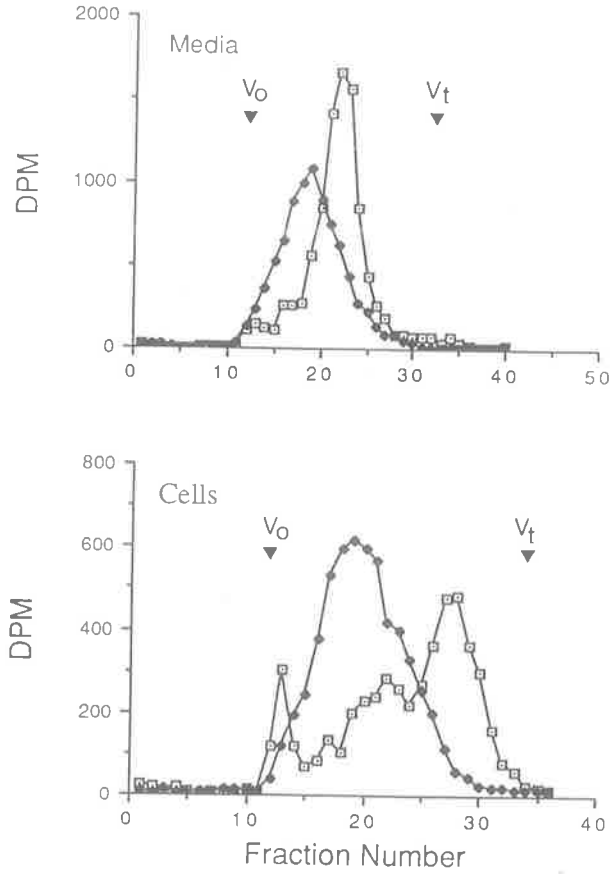

Fig. 2. Molecular sieve chromatography of $^{35}S/^{3}H$-labelled macromolecules on Sepharose CL-6B: ^{35}S (—◆—), ^{3}H (—⊡—). Cells were incubated in the presence of $Na_2[^{35}S]O_4$ and [^{3}H]-valine and the labelled macromolecules from the medium and cell layer were isolated by Sephadex G-50 chromatography. The material which eluted in the void volume of Sephadex G-50 was then chromatographed on Sepharose CL-6B. The V_0 and V_t were determined as described in Fig. 1.

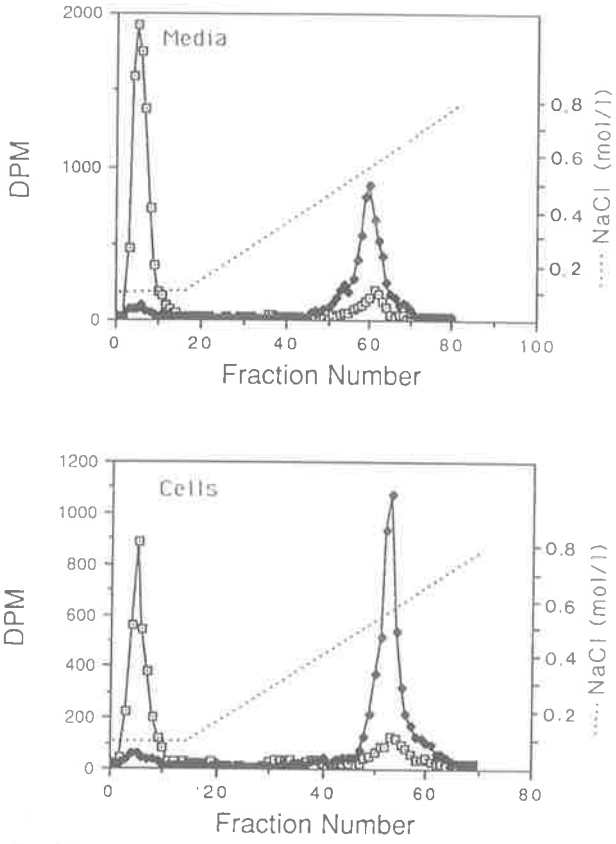

Fig. 3. Ion-exchange chromatography of $^{35}S/^{3}H$-labelled macromolecules: ^{35}S (—●—), ^{3}H (—⊡—). Cells were incubated in the presence of $Na_2[^{35}S]O_4$ and [^{3}H]-valine and the labelled macromolecules from the medium and cell layer were isolated by chromatography from Sephadex G-50.

Ion-exchange chromatography

Proteoglycans were separated from other glycoproteins by ion-exchange chromatography (Fig. 3). The profiles of radioactivity eluted from these columns were similar for both the medium and cell layer extracts. In both cases, two major peaks were noted. The first peak eluted was considered to be a glycoprotein fraction because of its elution from the resin at a low salt concentration and being principally comprised of ^{3}H-labelled material. Although some ^{35}S-labelled molecules eluted in this peak, they were a minor component and accounted for 6% and 5% respectively of the medium and cell layer-associated labelled molecules. The second peak eluted from the columns at 0·6 mol/l NaCl and, although it contained both ^{35}S- and ^{3}H-labelled molecules, it was characterized by a predominance of ^{35}S.

Chemical analyses of proteoglycans

The proteoglycan nature of the $^{3}H/^{35}S$-labelled macromolecules isolated in the void volume of Sephadex G-50 was established by papain digestion as well as alkaline sodium borohydride reduction followed by chromatography on Sepharose CL-6B. Material from both the medium and cells was susceptible to papain as evidenced by an increase in K_{av} values following papain digestion (Fig. 4). The ^{35}S-labelled glycosaminoglycan chains released by papain eluted from Sepharose CL-6B with K_{av} values of 0·4. Most of the ^{3}H-label eluted close to the V_t indicating digestion of proteins by the papain. However, some ^{3}H-labelled material co-eluted with the ^{35}S-labelled molecules and indicated the possibility of small peptides remaining associated with the glycosaminoglycans released by papain digestion. Therefore the proteogly-

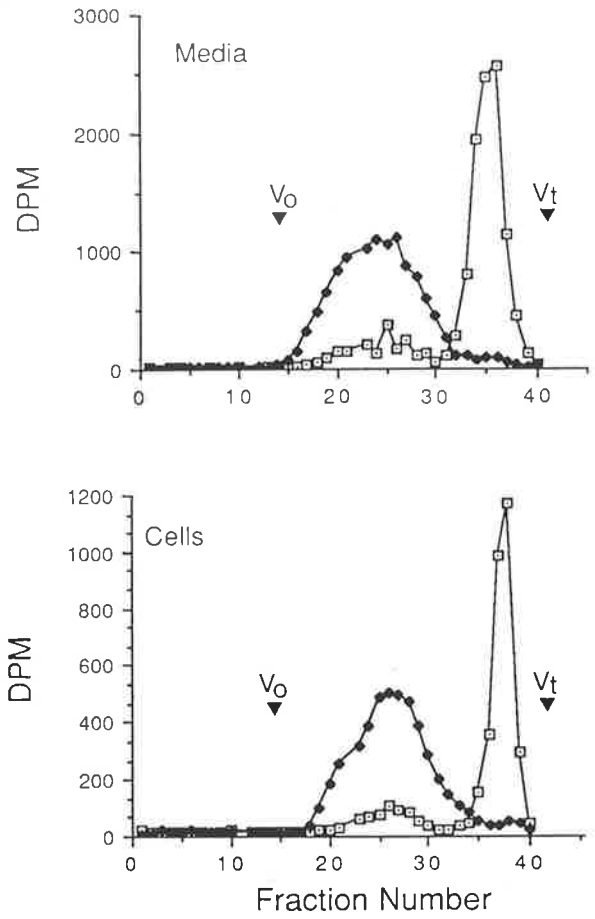

Fig. 4. Molecular sieve chromatography on Sepharose CL-6B of $^{35}S/^{3}H$-labelled macromolecules after digestion with papain: ^{35}S (—●—), ^{3}H (—⊡—). Labelled macromolecules were isolated from the medium and cell layer by Sephadex G-50 chromatography. The material which eluted in the void volume of Sephadex G-50 was digested with papain prior to elution from Sepharose CL-6B. The V_0 and V_t were determined as described in Fig. 1.

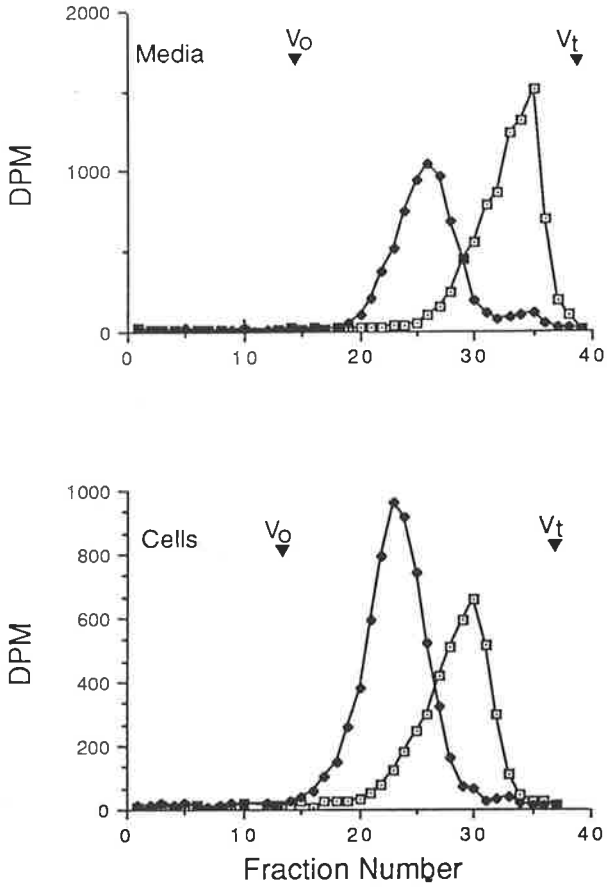

Fig. 5. Molecular sieve chromatography on Sepharose CL-6B of $^{35}S/^{3}H$-labelled macromolecules after treatment with alkaline sodium borohydride: ^{35}S (—●—), ^{3}H (—⊡—). Labelled macromolecules from the medium and cell layer were isolated by chromatography from Sephadex G-50. The material which eluted in the void volume of Sephadex G-50 was treated with 1 mol/l $NaBH_4$ in 0·05 mol/l NaOH prior to elution from Sepharose CL-6B. The V_0 and V_t were determined as described in Fig. 1.

cans were also treated with alkaline sodium borohydride to allow complete removal of peptides from the glycosaminoglycans (Fig. 5). This treatment resulted in the elution of ^{35}S-labelled molecules from Sepharose CL-6B at K_{av} of 0·5. Based on previously reported data for calibrated Sepharose CL-6B columns (17) these glycosaminoglycans may be assumed to have an average molecular weight of approximately 16 000.

Analysis of the glycosaminoglycan composition of proteoglycans can be achieved by selective digestion with chondroitinase ACII, chondroitinase ABC and nitrous acid and permits the identification of chondroitin sulfate, dermatan sulfate and heparan sulfate respectively. As can be seen in Fig. 6, such treatments allowed the identification of these glycosaminoglycans as the constituent components of the medium and cell proteoglycans. In addition the quantitative analyses are shown in Table 1. Chondroitin sulfate was the principal component in both the medium and cell proteoglycans accounting for 76% and 86% respectively.

Table 1. Glycosaminoglycan quantitation.

	Media	Cell
Heparan sulfate	21·3 ± 3·9	21·0 ± 3·2
Chondroitin sulfate	76·3 ± 9·7	85·7 ± 3·0
Dermatan sulfate	15·7 ± 8·3	2·3 ± 2·3

Medium and cell proteoglycans were subjected to nitrous acid, chondroitinase ACII and chondroitinase ABC degradation to determine the relative contribution of heparan sulfate, chondroitin sulfate and dermatan sulfate. The data (percentages of the total) are represented as the mean and s.d. of the mean from triplicate experiments.

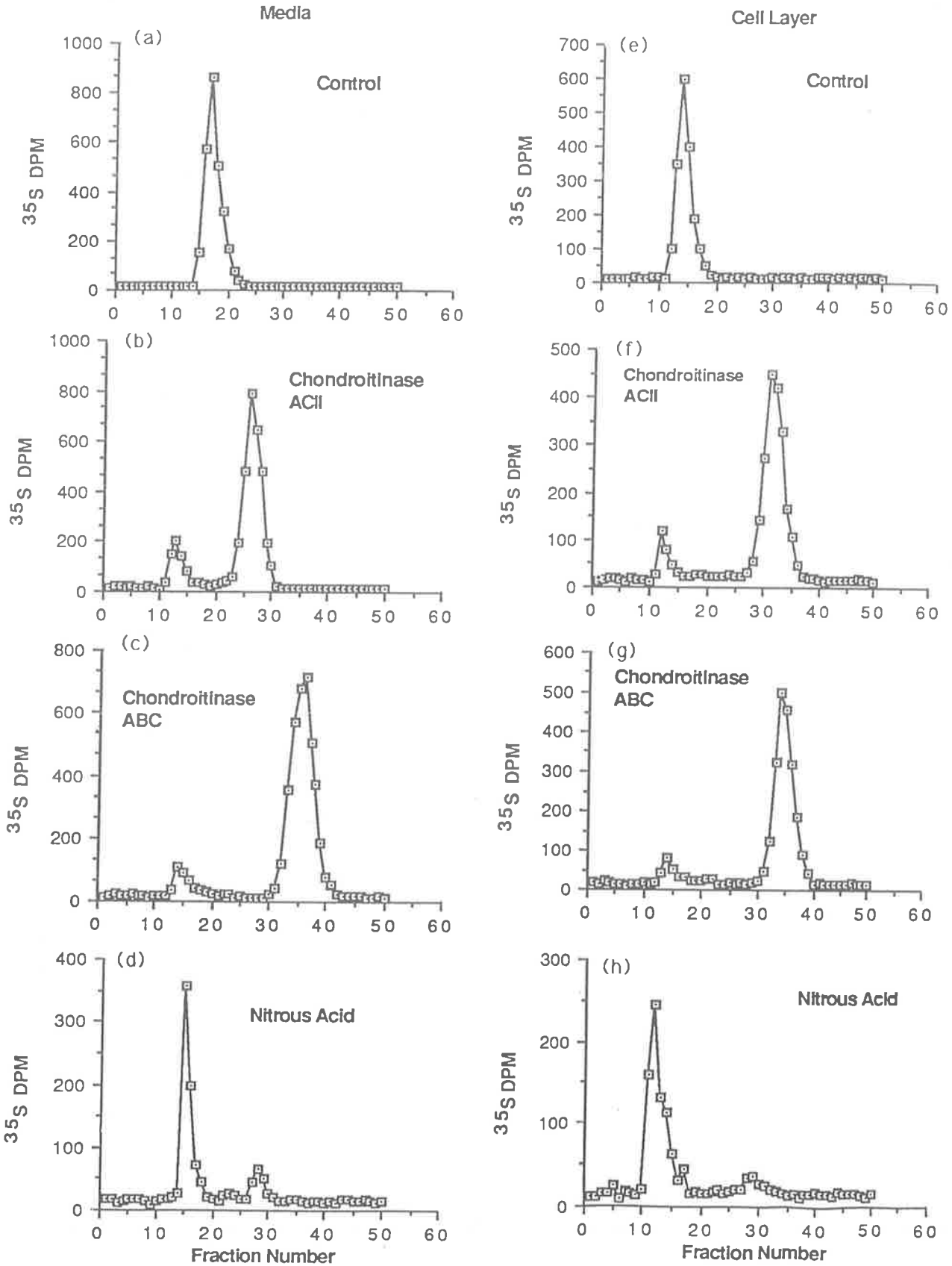

Fig. 6. Selective digestion of PMN medium proteoglycans. Medium (a–d) and cell (e–h) proteoglycans were incubated in the presence or absence of chondroitinase ACII, chondroitinase ABC or nitrous acid prior to elution from Sephadex G-50. The V_0 and V_t were determined as described in Fig. 1.

DISCUSSION

In previous histochemical, electronmicroscopic and biochemical studies, glycosaminoglycans have been demonstrated both within the cytoplasmic granules and within the cell membrane of PMN (4–7). In addition, recent *in vitro* biochemical studies have demonstrated that PMN, from a variety of sources, can secrete glycosaminoglycans into culture media (5,9,10). From these observations it has been proposed that glycosaminoglycans may play a role in PMN function. With the possible exception of hyaluronic acid, however, glycosaminoglycans rarely appear *in vivo* in a free state but rather exist complexed to a protein core in the form of proteoglycans. Therefore, we have extended the previous studies to consider the proteoglycan nature of the sulfated macromolecules synthesized by human PMN.

After incubating PMN *in vitro* for 24 h, sulfated macromolecules were isolated from the medium and cells. These were most likely proteoglycans on the basis of (i) their susceptibility to protease digestion resulting in the release of macromolecular sulfated polysaccharides indicative of glycosaminoglycans; (ii) release of sulfated polysaccharides from the intact macromolecules by alkaline sodium borohydride which were smaller than those released by papain digestion; (iii) co-elution of ^{35}S-labelled molecules with ^{3}H-labelled molecules from molecular sieve columns; and (iv) co-elution of ^{35}S- and ^{3}H-labelled molecules at high salt concentration from an anion-exchange column.

Since platelets were the only contaminating component in our preparations, and these were demonstrated not to synthesize proteoglycans in culture, the present data indicate the source of ^{35}S-labelled proteoglycans to be the PMN. The complete digestion of these polysaccharides by chondroitinase ACII, chondroitinase ABC and nitrous acid confirmed that the ^{35}S-labelled macromolecules were indeed proteoglycans. In addition, such treatments indicate the proteoglycans synthesized by human PMN are composed of heparan sulfate, dermatan sulfate and chondroitin sulfate. Whether these glycosaminoglycans are all present on the same core protein, attached to different core proteins or exist in various hybrid forms remains to be established.

To date, proteoglycans isolated from a variety of cells and tissues have shown great heterogeneity in terms of molecular size ranging from $2{\cdot}5 \times 10^6$ for the large chondroitin sulfate proteoglycans in cartilage to 70 000–100 000 for the small dermatan sulfate proteoglycans found in skin. In the present study, the elution position from Sepharose CL-4B of the proteoglycans isolated from human PMN indicates them to be smaller than the dermatan sulfate proteoglycans of skin. Such a small size may be accounted for, in part, by the relatively small size of the glycosaminoglycan chains. In our study we determined them to have an average molecular weight of 16 000. This compares with other reports of 25 000 and 11 000 for guinea-pig and human PMN respectively (9,11). Therefore, assuming the PMN proteoglycans are smaller than the dermatan sulfate proteoglycans of skin (approximately 70 000) then possible configurations for the PMN proteoglycans could include one of a core protein of approximately 40 000 to which one 16 000 glycosaminoglycan chain could be attached or a shorter protein core of 30 000 with two 16 000 glycosaminoglycan chains attached. It is important to note that these are estimations based on data obtained from the average elution positions of heterogeneous preparations of proteoglycans and glycosaminoglycans isolated from human PMN. For this reason, it is necessary to recognize the likelihood of the presence of several populations of proteoglycans being present which may differ in both size and glycosaminoglycan composition. Thus, further detailed analyses of these molecules will be required before the precise structure of PMN proteoglycans will become evident.

Our finding that the proteoglycans isolated from the medium of PMN cultures were of similar size to those from the cells is in contrast to that of a recent study by Levitt *et al.* (11). They found that the cell-associated proteoglycans were larger than those isolated from the medium. Although non-specific proteolytic degradation of the proteoglycans isolated from the cells should not be discounted, care was taken in the present study to minimize this possibility by the use of protease inhibitors during all preparative stages. A more likely explanation may be the different elution buffers used for chromatography. To eliminate non-specific aggregation occurring we used strong dissociating buffers whereas Levitt *et al.* (11) used low-salt buffers which may permit aggregation or incomplete solubilization of cell membrane associated material.

Many of the early studies concerning PMN glycosaminoglycans detected only chondroitin sulfate (2,3). With improved techniques for glycosaminoglycan identification, some reports

suggested that heparan sulfate and dermatan sulfate may also be synthesized by PMN (4–6). Nonetheless, additional reports have not been in agreement with such observations (4–6). Therefore, we have reassessed the characterization of glycosaminoglycans associated with proteoglycans associated with medium and cell proteoglycans. By using specific enzyme and chemical degradation in conjunction with chromatographic monitoring of the digestion products we clearly identified heparan sulfate, chondroitin sulfate and dermatan sulfate as the constituent glycosaminoglycans of PMN proteoglycans. In this respect our findings most closely agree with those of Parmley *et al.* (7), and Ohhayashi *et al.* (9).

Although the functional significance of proteoglycan synthesis by PMN remains to be established, it seems likely that, in the cell membrane, they are involved in cell adhesion. Indeed, it has been reported that upon stimulation of PMN with caseinate solution or adherence to plastic the relative distribution of specific glycosaminoglycans varies between the cell associated and medium content of glycosaminoglycans (6,8). The fact that proteoglycans have been reported to have a role in the adhesion, mobility and shape of mesenchymal cells (19) lends support to the concept that such molecules could be of importance in these fundamental events associated with PMN function.

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Paper 5.

Bartold, P.M., Haynes, D.R. and Vernon-Roberts, B.
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Provision of research funds
All cell culture work and proteoglycan assays
Writing of the manuscript

D.R. Haynes' role in this study was:
Isolation of cells and cytokine assays

B Vernon-Roberts' role in this study was:
Provision of funds
Writing of the manuscript

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Effect of Mitogen and Lymphokine Stimulation on Proteoglycan Synthesis by Lymphocytes

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The ability of mouse thymocytes and peripheral blood lymphocytes from rats to synthesize and secrete proteoglycans in the presence of a variety of mitogens and lymphokines was studied in vitro, and it was confirmed that such lymphocytes synthesize and secrete significant quantities of proteoglycans. Mitogenic stimulation of the cells with phytohaemagglutanin (PHA) induced a fourfold increase in proteoglycan synthesis; stimulation with interleukin-1 stimulated proteoglycan synthesis up to fivefold. Proteoglycan synthesis could also be stimulated by culturing the cells in the presence of interleukin-2. To determine if this response was related to cell proliferation, the cells were cultured in the presence of PHA and either cyclosporine or prostaglandin E_2, two agents that inhibit lymphocyte proliferation. Under these conditions, proteoglycan synthesis remained elevated, indicating that this effect may be independent of cell proliferation. Chemical analysis of the proteoglycans indicated them to be composed of chondroitin sulfate and heparan sulfate. Their molecular size was small compared with cartilage proteoglycans but similar to the small dermatan sulfate proteoglycans synthesized by fibroblasts. On the basis of molecular size, three proteoglycan populations were identified, and their relative proportions were altered by mitogenic stimulation of the cells. Taken together, these findings imply that proteoglycan synthesis is intimately associated with lymphocyte activation and may be related to cellular function in immune responses.

Many cellular activities and interactions appear to be regulated by secreted or cell surface-associated molecules (Comper and Laurent, 1978). Among such molecules, proteoglycans have received considerable attention (Höök et al., 1984; Iozzo, 1985). These highly anionic macromolecules, which are heterogeneous in both their structure and distribution, are defined as being composed of a single protein core to which one or more glycosaminoglycan chains are covalently bound (Hascall and Hascall, 1981). Many of the biological properties of these molecules can be attributed to either the protein core or glycosaminoglycan composition (Lindahl and Höök, 1978). Although lymphocytes are not regarded as cells having a significant role in proteoglycan synthesis, it is now evident that some lymphocytes are capable of synthesizing and secreting significant amounts. Hart (1982) first described the biosynthesis of glycosaminoglycans by thymus-derived lymphocytes and not only characterized the different types of glycosaminoglycans produced, but also demonstrated that synthesis was increased with mitogenic stimulation of the cells. Levitt and Ho (1983) subsequently demonstrated that these glycosaminoglycans were secreted as proteoglycans and also found that synthesis was increased upon mitogenic stimulation.

Although the functions of lymphocyte-produced proteoglycans have not been elucidated, it is of interest to note that B cells are stimulated to proliferate by a factor that coisolates with a T-cell proteoglycan (Levitt and Olmstead, 1986). In addition, the invariant chain associated with the immune-associated (Ia) antigens of various lymphoid-like cells has been demonstrated to be the protein core of a chondroitin sulfate proteoglycan (Giacoletto, et al., 1986; Sant et al., 1985) and is intimately associated with antigen presentation (Sivak et al., 1987). Furthermore, proteoglycans have been extensively characterized from cultures of large granular lymphocytes with natural killer function (Bland et al., 1984; MacDermott, et al., 1985). Such proteoglycans have been related to the specific activity of target lysis by these cells (Schmidt et al., 1985).

Because cellular interactions are dependent upon direct contact, or mediated through soluble factors, highly charged molecules such as proteoglycans seem likely candidates for involvement in the cellular events associated with immune responses. Therefore, this investigation aimed to study the effects of both mitogen and lymphokine stimulation on proteoglycan synthesis by lymphocytes.

MATERIALS AND METHODS

Materials

Guanidine HCl, L-glutamine, phenylmethylsulfonyl fluoride, 6-aminohexanoic acid, benzamidine HCl, 2-

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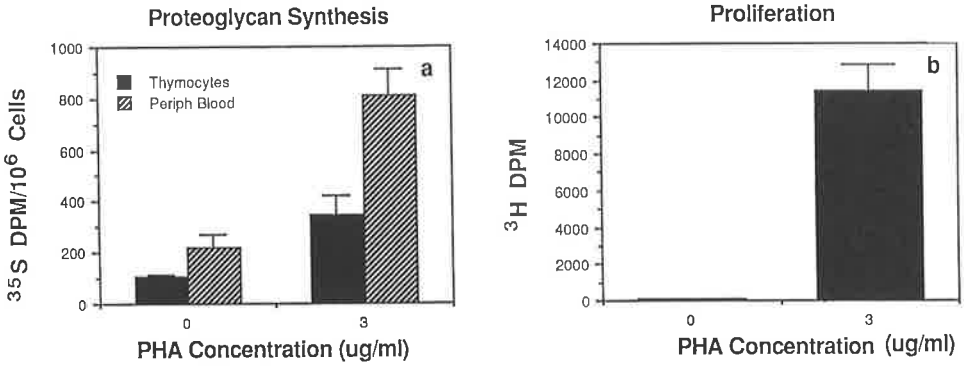


Fig. 1. **a:** Effect of phytohaemagglutanin on proteoglycan synthesis **b:** Effect on proliferative activity of lymphoid cells. Mouse thymocytes and rat peripheral blood lymphocytes were cultured in the presence or absence of 3 μg/ml PHA. Proteoglycan synthesis was assessed by ^{35}S-sulfate incorporation into macromolecules, and proliferative activity was assessed by uptake of ^{3}H-thymidine by the cells. Data are expressed as the mean and standard deviation of the mean from a representative experiment.

mercaptoethanol, N-ethyl maleimide, lipopolysaccharide (*E. coli* 0111:B4), and prostaglandin E_2 were all purchased from the Sigma Chemical Co., St. Louis, MO; phytohaemagglutanin was from Wellcome Reagents, Beckenham, England; recombinant interleukin-1β was a generous gift from Immunex Corporation, Seattle, WA; purified interleukin-2 was purchased from Boehringer-Mannheim Australia Pty. Ltd., North Ryde, NSW; cyclosporine was donated by Sandoz Australia Pty. Ltd., North Ryde, NSW; [6-^{3}H]-thymidine (23 Ci/mMol), $Na_2[^{35}S]O_4$ (1042 mCi/mMol), and D-[6-^{3}H]-glucosamine (33 Ci/mMol) were from Amersham Australia, Sydney, NSW; Ready Solv scintillation fluid was from Beckman Australia, Adelaide, SA; chondroitinase ACII (*Arthrobacter aurescens*) and chondroitinase ABC (*Proteus vulgaris*) were from Seikagaku Kogyo Co. Ltd., Tokyo, Japan; all tissue culture ware was from Nunc, Roskilde, Denmark; medium RPMI-1640, penicillin, streptomycin, and fetal calf serum (FCS) were from Flow Laboratories, Irvine, Ayrshire, Scotland; Ficoll-Paque, Sepharose CL-4B, Sephadex G-50, and Sephadex G-25 (PD-10) were from Pharmacia Fine Chemicals, North Ryde NSW.

Isolation of lymphocytes

Mouse thymocytes were isolated by passing the thymus glands of 9-11 week old C3H/HeJ mice through a fine wire mesh and collecting the cells in Hank's balanced salt solution (HBSS). Adherent cells were removed by incubating the cells in a plastic Petri dish at 37°C for 60 minutes in medium RPMI-1640 supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol (complete medium).

Peripheral blood lymphocytes were isolated by collecting 5 ml venous blood from male hooded Wistar rats in heparinized tubes. Samples were diluted with an equal volume of HBSS and 3 ml Ficoll-Paque and then centrifuged at 400g for 40 minutes at 37°C (Boyum, 1968). The lymphocyte fraction was removed, washed three times with HBSS to decrease the platelet contamination, and then placed into a plastic Petri dish in complete medium for 60 minutes at 37°C to remove adherent cells. The supernatant was removed, centrifuged, and the cell pellet washed twice in HBSS.

Mononuclear cell supernatant

Heparinized blood was taken from healthy human donors and the mononuclear cells isolated by Ficoll-Paque sedimentation as described above. A suspension of these cells (2×10^6 cell/ml) in 10 ml complete medium was placed into a 25 cm^2 flask and incubated at 37°C. One hour later, the nonadherent cells were removed and the adherent cells overlaid with fresh complete medium supplemented with 20 μg/ml lipopolysaccharide (*E. Coli* 0111:B4). After incubation for 24 hours, the supernatant was collected and centrifuged at 400g for 10 minutes to remove any cells. It was then filtered through a 0.22 μm Millipore filter, and an aliquot was tested in a lymphocyte activation (LAF) assay (Haynes, et al., 1988); the remainder was stored frozen until required.

Cell cultures and radiolabelling

The cells were adjusted to a concentration of 1×10^7 cells/ml and maintained in complete medium. To determine the effect of lymphocyte stimulation on proteoglycan synthesis, the cells were plated, in triplicate, into a 24-well plate at a density of 10^6 cells/well. The cells were cultured for up to 48 hours in complete medium containing: 1) phytohaemagglutanin (PHA): 3 μg/ml; 2) LPS-stimulated mononuclear cell supernatant: 1/4-1/2056 vol/vol dilution; 3) recombinant interleukin-1β (rIL-1β): 10^{-9}-10^{-14} M; 4) interleukin-2 (IL-2): 1/10-1/1,000 vol/vol dilution; 5) cyclosporine 1-1000 ng/ml; 6) prostaglandin E_2 (PGE_2): 10.0.001 μM. Incorporation of [^{35}S]-sulfate into proteoglycans was achieved by culturing the cells in the presence of 20 μCi/ml [^{35}S]-sulfate. The proliferative activity of the cells was assessed by culturing the cells in the presence of 1 μCi/ml [^{3}H]-thymidine for the final 3 hours of a 48 hour culture. For proteoglycan biochemical analyses, 40×10^6 cells were cultured in 5 ml complete medium in 25 cm^2 flasks.

Cell proliferative activity

Following incubation in the presence of [^{3}H]-thymidine, the cells were harvested onto glass filters in the

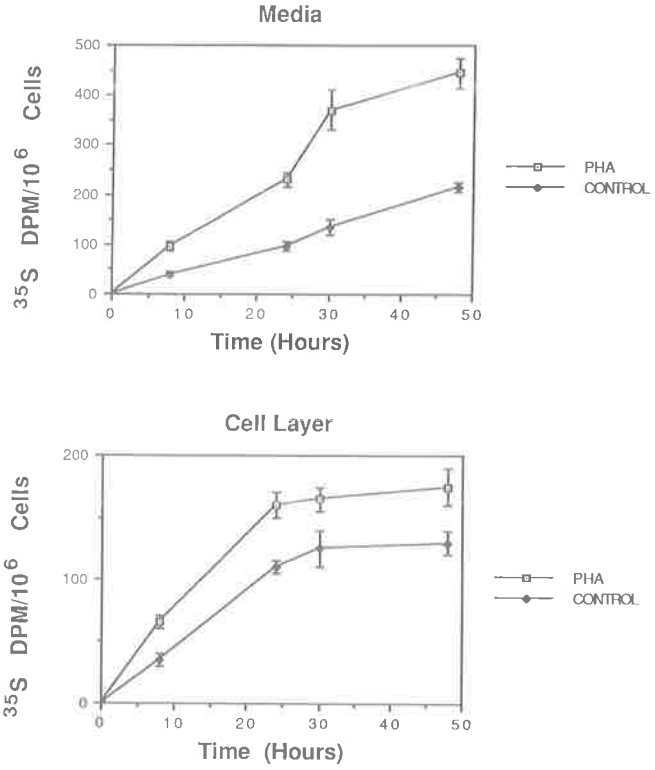


Fig. 2. ^{35}S-sulfate incorporation into proteoglycans with time. Mouse thymocytes were cultured in the presence or absence of 3 μg/ml PHA and proteoglycans associated with the medium or cell layer fractions were monitored by assessing the incorporation of ^{35}S-sulfate into macromolecules over a 48-hour period. Data are expressed as the mean and standard deviation of the mean from a representative experiment.

presence of 5% trichloroacetic acid (TCA) using an automated cell harvester (Titre Tek). The TCA precipitated material then was counted for radioactivity in a Beckman LS-2800 liquid scintillation counter.

Analysis of [^{35}S]-labelled macromolecules

After incubation of the cells in the presence of [^{35}S]-sulfate, the medium was removed from the wells, placed into Eppendorf microfuge tubes, and centrifuged for 5 minutes. The medium fraction was removed and applied onto Sephadex G-25 (PD-10) columns and eluted with 4 M guanidine HCl. Radioactivity was determined in 0.4 ml effluent fractions by liquid scintillation counting. The amount of radioactivity recovered in the void volume provided a measure of the incorporation of [^{35}S]-sulfate into newly synthesized macromolecules (Yanagashita and Hascall, 1979). Recoveries of radioactivity from these colums ranged between 80 and 90%. The [^{35}S]-labelled macromolecules associated with the cell pellets were assessed in a similar fashion after the addition of 0.5 ml 4 M guanidine HCl.

Proteoglycan analysis

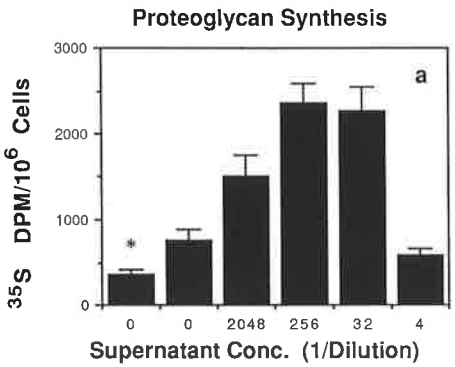
Following incubation for 48 hours in 25 cm^2 flasks, the medium and cells were separated by centrifugation. Cell pellets were washed twice with HBSS, and these washes were pooled with the medium fraction. The cell pellets were extracted with 4 M guanidine HCl/0.05 M sodium acetate containing the following as protease inhibitors: 0.1 M 6-aminohexanoic acid, 5 mM benzamidine HCl, 50 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM N-ethylmaleimide, pH 5.8 (Oegema et al., 1979). The pooled medium fraction was adjusted to approximately 3 M in guanidine HCl by adding solid guanidine HCl (0.38 g/ml), and then eluted from a Sephadex G-50 column (2.5 cm × 30 cm) in 4 M guanidine HCl/0.05 M sodium acetate containing protease inhibitors. Fractions of 1.5 ml were collected, and 100 μl aliquots were taken for determination of radioactivity. The material eluting in the void volume was well separated from unincorporated radioisotope and was pooled, dialysed against deionized water, and lyophilized in preparation for further analyses. The radiolabelled macromolecules associated with the cell extracts were obtained in a similar manner. Recoveries of radioactivity from these columns ranged between 85 and 90%.

The molecular size distribution of [^{35}S]-labelled proteoglycans was analyzed by gel filtration from Sepharose CL-4B columns (0.7 × 100 cm) with 4 M guanidine HCl/0.05 M sodium acetate containing protease inhibitors, pH 5.8. Fractions of 0.5 ml were collected at a flow rate of 3 ml per hour, and all of the fractions were assayed for radioactivity. Recovery of radiolabelled material from these columns ranged between 80 and 85%.

The types of glycosaminoglycans associated with the proteoglycans isolated from the medium and cell pellets were determined following selective enzyme digestion (chondroitinase ACII and chondroitinase ABC) (Saito et al., 1968) and chemical (nitrous acid) degradation (Lagunoff and Warren, 1962). Portions of the excluded material from Sephadex G-50 were dissolved in 0.05 M Tris HCl, 0.06 M sodium chloride, and 0.04 M sodium acetate, pH 8.0, and then subjected to one of the above degradative procedures. The reaction products were eluted from Sephadex G-50 columns (0.7 cm × 30 cm) with 0.2% sodium dodecyl sulfate (SDS) in 0.15 M sodium acetate, 1 mM magnesium chloride, 1 mM calcium chloride, pH 5.8. Fractions of 0.5 ml were collected at a flow rate of 3 ml per hour, and all of the fractions were assayed for radioactivity. The relative proportion of each glycosaminoglycan species was determined by calculation of the amount of radiolabelled material resistant to (void volume) or degraded by (included volume) each treatment.

Specific activity determinations

Because stimulation of the cells may alter the degree of sulfation of newly synthesized proteoglycans, the ratio of ^{35}S/^{3}H was assessed (Bartold and Page, 1986). Cells were incubated in the presence or absence of 3 μg/ml PHA and labelled in the presence of both [^{35}S]-sulfate (20 μCi/ml) and [^{3}H]-glucosamine (17.5 μCi/ml) for 24 hours. The disaccharides released by chondroitinase ABC digestion of the isolated proteoglycans were assayed for their ^{35}S/^{3}H ratio by liquid scintillation counting.



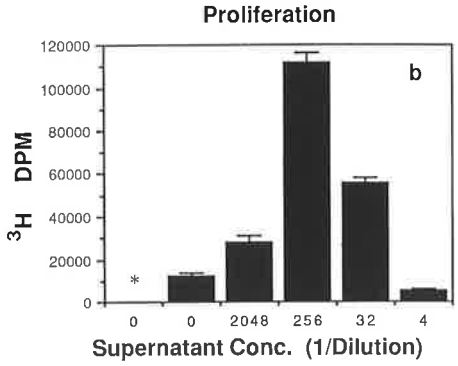
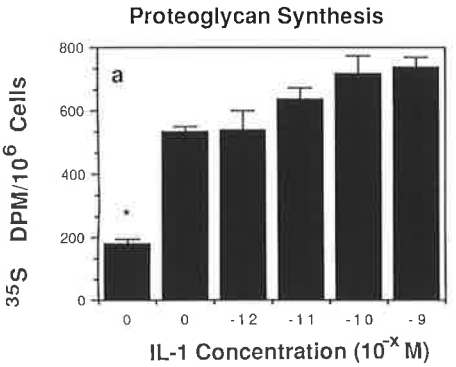


Fig. 3. **a:** Effect of mononuclear cell supernatant on proteoglycan synthesis; **b:** Effect on proliferative activity of mouse thymocytes. Cells were cultured in the presence of increasing concentrations of supernatant from stimulated mononuclear cells and 3 μg/ml PHA. *Represents cells cultured in the absence of both PHA and supernatant. Proteoglycan synthesis and synthetic activity were assessed as described in Figure 1. Data represent the mean and standard deviation of the mean from a representative experiment.



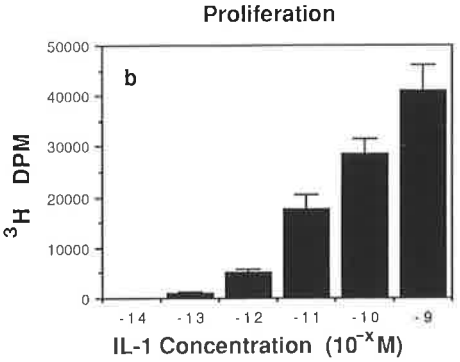
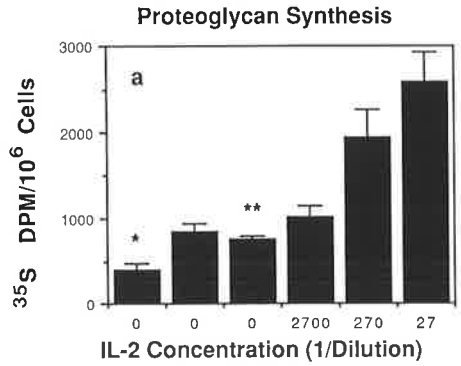


Fig. 4. **a:** Effect of recombinant IL-1β on proteoglycan synthesis; **b:** Effect on proliferative activity of mouse thymocytes. Cells were cultured in the presence of 3 μg/ml PHA and increasing concentrations of rIL-1β. *Represents cells cultured in the absence of both rIL-1β and PHA. Proteoglycan synthesis and proliferative activity were assessed as described in Figure 1. Data represent the mean and standard deviation of the mean of a representative experiment.



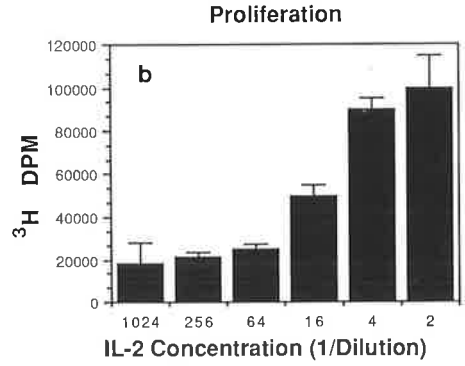


Fig. 5. **a:** Effect of purified interleukin-2 on proteoglycan synthesis; **b:** Effect on proliferative activity of mouse thymocytes. Cells were cultured in the presence of 3 μg/ml PHA and increasing concentrations of purified IL-2. *Represents cells cultured in the absence of both purified IL-2 and PHA. **Represents cells cultured in the presence of IL-2 (1/27) alone (i.e., no PHA present). Proteoglycan synthesis and proliferative activity were assessed as described in Figure 1. Data represent the mean and standard deviation of the mean from a representative experiment.

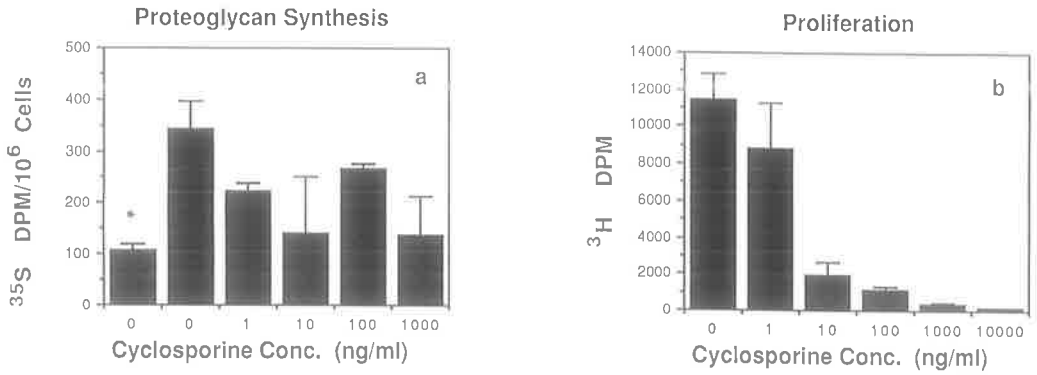


Fig. 6. a: Effect of cyclosporine on proteoglycan synthesis; b: Effect on proliferative activity of mouse thymocytes. Cells were cultured in the presence of 3 μg/ml PHA and increasing concentrations of cyclosporine. *Represents cells cultured in the absence of both cyclosporine and PHA. Proteoglycan synthesis and proliferative activity were assessed as described in Figure 1. Data represent the mean and standard deviation of the mean from a representative experiment.

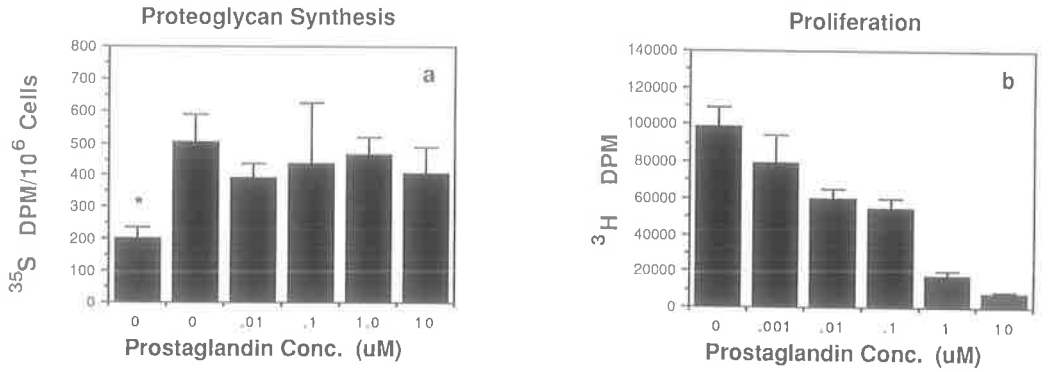


Fig. 7. a: Effect of prostaglandin E_2 on proteoglycan synthesis; b: Effect on proliferative activity of mouse thymocytes. Cells were cultured in the presence of 3 μg/ml PHA and increasing concentrations of PGE_2. *Represents cells cultured in the absence of both PGE_2 and PHA. Proteoglycan synthesis and proliferative activity were assessed as described in Figure 1. Data represent the mean and standard deviation of the mean from a representative experiment.

Statistical analyses

All data were subjected to statistical analysis using the method of analysis of variance.

RESULTS

To confirm previous reports that lymphoid cells synthesize and secrete proteoglycans, the ability of both mouse thymocytes and rat peripheral blood lymphocytes to synthesize ^{35}S-labelled macromolecules in vitro was analyzed first. As shown in Figure 1, nonstimulated lymphocytes synthesized detectable amounts of sulfated macromolecules. Upon stimulation to proliferate with PHA, this synthesis increased threefold to fourfold.

Figure 2 shows the results of time-course experiments following the incorporation of ^{35}S-sulfate into proteoglycans, which were either released into the culture medium or retained within the cell layers. The stimulatory effect was evident by 8 hours ($P < 0.05$) and was most noticeable for the medium fraction.

To determine if this apparent increase in proteoglycan synthesis was a general feature of lymphocyte activation, we studied the effect of three lymphokines on proteoglycan synthesis by mouse thymocytes (Figs. 3–5). Supernatants from stimulated mononuclear cells, which stimulated thymocyte proliferation, caused a stimulation of proteoglycan synthesis that closely followed the proliferation curve of thymocytes (Fig. 3). To investigate if this response could be related to IL-1 activity in the mononuclear cell supernatant, we tested the ability of rIL-1β to affect proteoglycan synthesis by thymocytes. Although the increase in proteoglycan synthesis was not as great as that noted for the crude IL-1 preparations, a 30% increase in proteoglycan synthesis above that seen for PHA alone was noted (Fig. 4). This elevation in proteoglycan synthesis was still evident at dilute concentrations of rIL-1β that did not affect thymocyte proliferation. Because rIL-1β exerts its effect on lymphocyte proliferation by enhancing IL-2 secretion by these cells, we investigated the effect of IL-2 on proteoglycan synthesis by thymocytes. As shown in Figure 5, IL-2 stimulated thymocyte proliferation in a dose-dependent fashion. The addition of IL-2 alone to the cultures caused a marginal increase in proteoglycan synthesis over nonstimulated cells. However, when IL-2 was added to the cultures in the presence of PHA, a marked increase in the synthesis of proteoglycans became evident. This increase in synthesis was still evident at concentrations of IL-2 that failed to stimulate thymocyte proliferation.

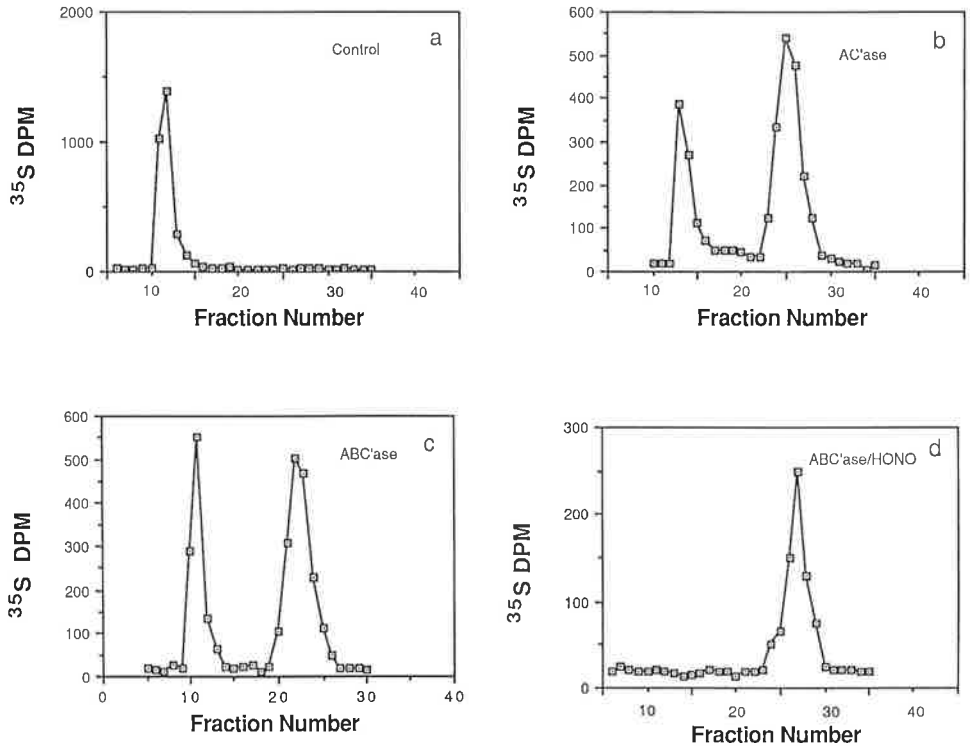


Fig. 8. Confirmation of proteoglycan nature of ^{35}S-labelled macromolecules. **a:** Papain digestion; **b:** Chondroitinase ACII digestion, **c:** Chondroitinase ABC digestion, **d:** Chondroitinase ABC followed by nitrous acid degradation. All samples were chromatographed on Sephadex G-50 following the specified treatment.

TABLE 1. Glycosaminoglycan Quantitation[1]

	AC	ABC	HNO_2
Media			
Control	62.5 (± 5.0)	59.5 (± 4.9)	40.0 (± 14.0)
PHA	58.5 (± 12.0)	55.3 (± 4.0)	40.5 (± 9.1)
Cells			
Control	48.0 (± 8.4)	49.3 (± 5.5)	55.5 (± 4.9)
PHA	39.5 (± 12.5)	33.6 (± 9.8)	64.0 (± 1.4)

[1]Glycosaminoglycans were quantitated after selective degradation with chondroitinase ACII (AC), chondroitinase ABC (ABC), or nitrous acid (HNO_2). Data are expressed as percentages (mean ± standard deviation of the mean from triplicate experiments) of the total radiolabelled material digested by each degradative procedure.

Because all three lymphokines tested influenced proteoglycan synthesis at concentrations that did not noticeably alter thymocyte proliferation, the elevation of proteoglycan synthesis did not appear to be related solely to cell proliferation. Therefore, the effect of cyclosporine and PGE_2, two agents that inhibit mitogen-stimulated proliferation of lymphocytes, on proteoglycan synthesis in the presence of PHA was tested. As expected, cyclosporine caused a dose-dependent decrease in proliferation (Fig. 6). However, although proteoglycan synthesis was not as great as in the presence of PHA alone, it remained elevated over the levels of synthesis by cells cultured in the absence of any mitogens. Treatment of the cells with PGE_2, which also inhibited thymocyte proliferation in a dose-dependent manner, did not affect the influence of PHA on proteoglycan synthesis (Fig. 7). Taken together, these results indicate that the increase of proteoglycan synthesis may be partially independent of cell proliferation and may be a more general feature of lymphocyte activation.

To confirm that the newly synthesized ^{35}S-labelled macromolecules were proteoglycans, and not sulfated glycoproteins, the isolated macromolecules were digested with papain and chromatographed on Sephadex G-50. As shown in Figure 8, the ^{35}S-labelled material remained in the excluded volume of the column, indicating that the sugar units of these macromolecules are greater than 10,000. Complete digestion of this material with specific glycosaminoglycan lyases and nitrous acid identified chondroitin sulfate and heparan sulfate as the constituent glycosaminoglycans (Fig. 8). Although no remarkable quantitative differences between the glycosaminoglycans in the medium fractions were evident, an increase in the amount of heparan sulfate was noted in the cell pellet from cultures exposed to PHA (Table 1).

Chondroitinase ABC digestion of glycosaminoglycans labelled with ^{35}S-sulfate and ^{3}H-glucosamine indicated that the $^{35}S/^{3}H$ ratios in the sulfated disaccharides from PHA treated cells (0.23) were twice those for the disaccharides from control cultures (0.13).

An assessment of proteoglycan molecular size distribution (Figs. 9, 10) revealed that the medium proteoglycans eluted from Sepharose CL-4B in two peaks of K_{av} 0 and 0.45. The proteoglycans from the cell pellets

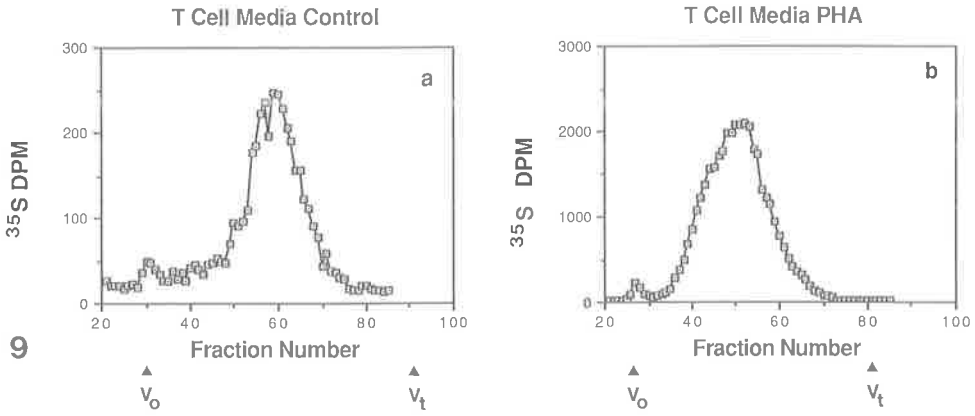


Fig. 9. Gel chromatography on Sepharose Cl-4B of proteoglycans isolated from the medium of mouse thymocytes cultured in the presence or absence of 3 μg/ml PHA. The void volume (V_o) and total volume (V_t) were determined with ^{3}H-DNA and ^{35}S-sulfate respectively.

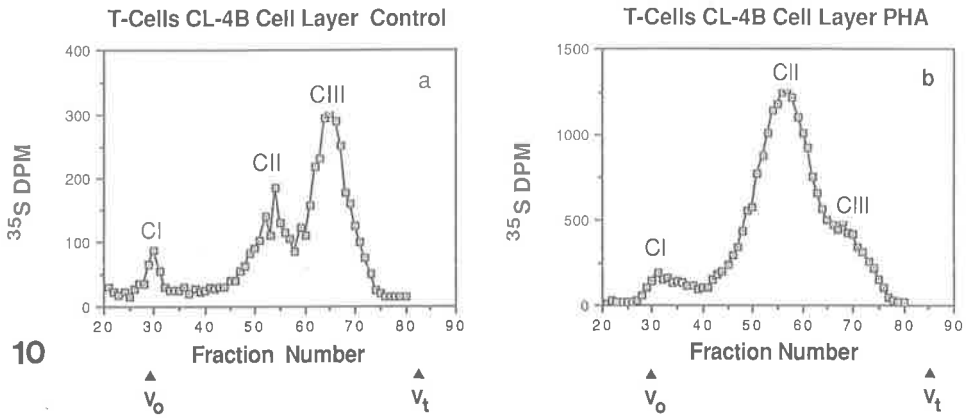


Fig. 10. Gel chromatography on Sepharose Cl-4B of proteoglycans isolated from the cell pellets of lymphocytes cultured in the presence or absence of 3 μg/ml PHA. The V_o and V_t were determined as described in Figure 8.

eluted in three peaks designated CI, CII, and CIII with K_{av} values of 0, 0.45, and 0.65, respectively. Exposure of the cultures to PHA resulted in a change in the relative distribution of the cell pellet proteoglycan peaks designated CII and CIII. The majority of the proteoglycans isolated from unstimulated cells eluted at position CIII, whereas stimulated cells synthesized proteoglycans that eluted principally at position CII.

DISCUSSION

The synthesis of proteoglycans is classically considered to be the domain of extracellular matrix producing cells (Hascall and Hascall, 1981). However, there have been several reports concerning the ability of lymphoid cells to synthesize proteoglycans. In particular, peripheral blood lymphocytes (Levitt and Ho, 1983), thymocytes (Hart, 1982), natural killer cells (Bland et al., 1984; MacDermott, 1985), and specific cell lines (Sivak et al., 1987) have been studied. The present study has confirmed that lymphoid cells possess the ability to synthesize and secrete proteoglycans and attempted to relate this to lymphocyte activation.

The proteoglycans synthesized by lymphoid cells were found to be composed of heparan sulfate and chondroitin sulfate. Whether these glycosaminoglycans are present on the same core protein or attached to different protein cores remains to be established. The isolation of proteoglycans from both the culture medium and cell pellet indicates that the cells not only secrete newly synthesized proteoglycans but also retain a certain percentage (10–20%) closely associated with the cell. The precise location of the cell-associated proteoglycan awaits further investigation. However, it is presumed that it will be located both within the cells as well as associated with the cell membranes in a fashion similar to that described for natural killer cells (Parmley et al., 1985).

In addition to analysis of proteoglycans synthesized by quiescent lymphocytes, we also addressed the question of cell activation and its relationship to proteoglycan synthesis by thymocytes. Although an increase in proteoglycan synthesis upon mitogenic stimulation of thymocytes has been reported previously (Hart, 1982; Levitt and Ho, 1983), the present findings relating to the effects of various lymphokines are new. The initial finding that supernatants from stimulated monocytes

stimulated thymocyte proteoglycan synthesis implicated a possible role for interleukin-1, as these supernatants have been demonstrated previously to stimulate thymocyte proliferation via an IL-1-like factor (Haynes et al., 1988). Therefore, the effect of rIL-1β in our system was studied and duly found to stimulate proteoglycan synthesis. The effect of IL-2 also was assessed, because it is synthesis of this hormone that is principally responsible for the increase in lymphocyte proliferation following stimulation with IL-1. This agent also stimulated proteoglycan synthesis by thymocytes.

Although the three agents tested would normally be considered to act principally on thymocytes by stimulating proliferation, effects on proteoglycan synthesis were still evident at concentrations too low to evoke any detectable proliferative response. This could be due to proteoglycan synthesis being more sensitive to lymphokine stimulation than is proliferation. Alternatively, proteoglycan synthesis may be totally unrelated to proliferative response, and thus the lymphokines act via several unrelated subcellular pathways. To test the second alternative, we studied the effect of mitogen stimulation on proteoglycan synthesis by thymocytes cultured in the presence of cyclosporine or PGE_2, two agents known to inhibit mitogen-induced thymocyte proliferation (Lillehoj et al., 1984; Elliot, 1984; Niebergs et al., 1985). However, in the presence of mitogen (PHA) and either cyclosporine or PGE_2, the thymocytes exhibited elevated levels of proteoglycan synthesis. These data indicate that proteoglycan synthesis by thymocytes may not be related solely to proliferative activity.

When analyzing data obtained from in vitro experiments, caution is required in interpreting an observed increase in sulfate levels in proteoglycans as equating with an increase in proteoglycan synthesis. Thus, assessment of the $^{35}S/^{3}H$ ratio was made on dual-labelled glycosaminoglycans. Mitogenic stimulation of the cells caused a twofold increase in this ratio and indicates that either the specific activity of the sugar nucleotide precursor pool was altered by such treatment, or the degree of sulfation of glycosaminoglycans increases upon stimulation of the cells. The latter alternative is currently under further investigation. Regardless of the outcome of these experiments, the present data strongly imply that mitogen or lymphokine exposure alters proteoglycan synthesis by thymocytes, because the alteration in $^{35}S/^{3}H$ ratio is insufficient to account for the observed fivefold increase in sulfate levels in proteoglycans synthesized by cells exposed to mitogens or lymphokines.

Although lymphocytes have been known for some time to synthesize proteoglycans, few systematic studies of the intact proteoglycans have been carried out. In the present studies, the proteoglycans isolated from thymocytes appear to differ from those synthesized by natural killer cells (Schmidt et al., 1985) in that they are digested by proteases. They are therefore like "conventional" proteoglycans. When chromatographed on Sepharose CL-4B, the proteoglycans synthesized by thymocytes were heterogeneous with respect to molecular size. However, in general terms, these proteoglycans were of quite small molecular size and differed markedly from the predominantly large proteoglycans found in cartilage. However, their size distribution is similar to that of the small proteoglycans (dermatan and chondroitin sulfate proteoglycans) found in the soft connective tissues (e.g., skin, gingiva, ligament, etc.) (Cöster et al., 1984; Bartold and Page, 1987; Vogel and Evanko, 1987). The significance of the observation that the proportions of various proteoglycans associated with the cell pellets varied depending upon the state of activation is not yet clear; however, it does imply a functional relationship between cell-associated proteoglycans and lymphocyte activation.

In conclusion, this study supports the hypothesis that synthesis of proteoglycans may be a ubiquitous property of all mammalian cells. In addition, the data present interesting ramifications in terms of the functions of lymphocyte proteoglycans and indicate a possible role for proteoglycans in the immune system. Such a role has been highlighted by recent reports that a factor that coisolates with T-cell proteoglycan as well as the T-cell proteoglycan itself is capable of stimulating B-cells (Levitt, 1987; Levitt and Olmstead, 1986).

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VI. PROTEOGLYCANS IN THE HARD CONNECTIVE TISSUES OF THE PERIODONTIUM

Paper 6. Bartold, P.M., Miki, Y., McAllister, B., Narayanan, A.S. and Page, R.C.
Glycosaminoglycans of human cementum
Journal of Periodontal Research **23:** 13-17, 1988

Paper 7. Bartold, P.M., Reinboth, B., Nakae, H., Narayanan, A.S. and Page, R.C.
Proteoglycans of bovine cementum. Isolation and characterization.
Matrix **10:** 10-19, 1990.

Paper 8. Bartold, P.M.
A biochemical and immunohistochemical study of the proteoglycans of alveolar bone.
Journal of Dental Research **69:** 7-19; 1990.

The purpose of these studies was to focus on the proteoglycan content of the two hard tissues of the periodontium, namely cementum and alveolar bone. At the time of carrying out these studies little attention had been given to the matrix composition of these two tissues. The studies concerning cementum were of particular interest since this is a unique tissue associated only with the dentition. The studies concerning the alveolar bone were based on previous studies on skeletal tissues found elsewhere in the body, but were still considered to be important in terms of defining the molecular composition of the human periodontium.

Papers 6 and 7 followed a classical approach to the problem of characterizing the proteoglycans of cementum. First, an analysis of the glycosaminoglycan content was carried out, followed by a detailed biochemical and immunohistochemical characterization of the proteoglycans in cementum. These studies provided the first evidence of multiple glycosaminoglycan species within the matrix of cementum, as well as providing clear data concerning their molecular identification, composition and structure. Using monoclonal antibodies and immunohistochemical techniques, the proteoglycan studies on cementum were the first to show that intact proteoglycans were the principle nonfibrous macromolecules located in the matrix of cementum.

Paper 8 was the first to document the isolation and characterization of intact proteoglycans from alveolar bone. It extended earlier work from Graham Embery's laboratory in the U.K. which had, up until this time, considered only the glycosaminoglycan content of alveolar bone. This study was the first to demonstrate the tissue localization of proteoglycans within the extracellular matrix of human alveolar bone using monoclonal antibodies and immunohistochemistry.

An interesting feature of these studies was that the principle type of glycosaminoglycan and proteoglycan isolated from both cementum and alveolar bone was chondroitin sulfate-rich, and differed from the dermatan sulfate-rich material isolated from the soft connective tissues of the periodontium. These studies highlighted the important nature of site specificity of matrix composition with the periodontium. The presence of chondroitin sulfate in periodontal hard tissues was subsequently exploited by Embery's group to develop diagnostic markers of bone remodeling based on the identifiable presence of chondroitin sulfate in gingival crevicular fluid.

Paper 6.

Bartold, P.M., Miki, Y., McAllister, B., Narayanan, A.S. and Page, R.C.
Glycosaminoglycans of human cementum
Journal of Periodontal Research **23:** 13-17, 1988.

Candidate's Contribution to this paper: 80%

P.M. Bartold's role in this study was:
Design of the experiments
Provision of research funds
Execution of all proteoglycan assays
Writing of the manuscript

Y. Miki's role in this study was:
Provision of cementum samples

B. McAllister's role in this study was:
Provision of cementum samples

A.S. Narayanan's role in this study was:
Advisory
Writing of the manuscript

R.C. Page's role in this study was:
Provision of funds
Writing of the manuscript

Glycosaminoglycans of human cementum

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Bartold PM, Miki Y, McAllister B, Narayanan AS, Page RC: Glycosaminoglycans of human cementum. J Periodont Res 1988; 23: 13–17.

The glycosaminoglycans in human cementum have been studied. Following proteolytic digestion of guanidine/EDTA and collagenase extracts of cementum, glycosaminoglycans were isolated and then separated by cellulose acetate membrane electrophoresis. After specific elimination by enzymatic and chemical treatments the glycosaminoglycans were identified as hyaluronic acid, chondroitin sulfate and dermatan sulfate. Neither heparan sulfate nor keratan sulfate were observed. Quantitation of the glycosaminoglycans in both extracts revealed chondroitin sulfate to represent the major species present. Hyaluronic acid was observed predominantly in the guanidine/EDTA extract while dermatan sulfate was a quantitative minor component of both extracts.

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Introduction

Cementum is one of two hard tissues which comprise the periodontium. Although its precise function is unclear, its anatomical features indicate a primary role in the attachment of the periodontal ligament fibers to the tooth root surface. Histologically, cementum is very similar to bone and dentin. However, chemical and physical analysis of cementum have indicated it to be softer than dentin (1). Analysis of the organic components of cementum has been achieved following decalcification. Glimcher, Friberg & Levine (2) demonstrated that approximately 90% of the organic matrix of cementum had a chemical composition characteristic of collagen. This observation was later confirmed by Rodriguez & Wilderman (3, 4). Additional analyses have revealed type I collagen to be the principal collagen type present in cementum although some type III may also be present (5, 6).

With respect to the non-collagenous components of the organic matrix of cementum, little is known. Smith, Leaver & Smith (7) demonstrated that while the non-collagenous matrix of human cementum accounts for a larger proportion of the total organic matrix than in bone or dentin, the amino acid composition of cementum organic matrix is similar to that of bone and dentin. On the basis of histochemical evidence, Vidal, Mello & Valdrighi (8) postulated that both carboxylated and sulfated mucopolysaccharides (glycosaminoglycans) may be components of human cementum.

To date, the predictability of new connective tissue attachment to root surfaces of teeth treated for periodontal disease has been elusive. Therefore, further assessment of the components of cementum which are likely to be involved in such regenerative processes is warranted. Since proteoglycans are known to interact specifically with collagens in a variety of tissues (9, 10) and are integral components of cell substratum attachment matrices (11) the aim of the present study was to isolate, identify and quantitate the glycosaminoglycan components of the proteoglycans in human cementum.

Material and Methods

Materials

Alcian blue 8GX, cysteine HCl, N-ethylmaleimide, pepstatin, phenylmethyl sulfonyl fluoride, papain EC 3.4.22.2 (Type III 2X crystalized from papaya latex; activity 10–15 U/mg) were all purchased from Sigma Chemical Co., St. Louis, MO. Bacterial collagenase EC 3.4.24.3 (Form 3) was from Advance Biofactures Co. Lynbrook, NY. *Streptomyces* hyaluronidase EC 4.3.3.1. (*Streptomyces* hyalurolyticus), chondroitinase ACII EC 4.2.2.5 (*Arthrobacter* aurescens) and chondroitinase ABC EC 4.2.2.4 (*Proteus* vulgaris) were purchased from Seikagaku Kogyo Ltd., Tokyo, Japan. Sepraphore III cellulose acetate electrophoresis membranes were from Gelman Sciences, Ann Arbor, MI. Ultrafiltration membranes (YM-10) were purchased from Amicon corporation (Danvers, MA). The GAG standards were obtained from Seikagaku Kogyo Ltd., Tokyo, Japan.

Extraction of cementum

Cementum was obtained from freshly extracted teeth which showed no signs of inflammation. The procedure used was essentially that described by Somerman et al. (12) and Miki, Narayanan & Page (13). Briefly, adhering periodontal ligament and other soft connective tissues were removed under a dissecting microscope. The cementum was then scraped from the root surface using curettes under the microscope. The shavings were immediately placed into 50 mM Tris-HCl buffer (pH 7.5) containing the proteinase inhibitors 1 mM phenylmethylsulfonyl fluoride 5 mM, N-ethylmaleimide and 25 mM EDTA. Extraction of the cementum was performed in 500 mM acetic acid containing 1 μg/ml each of pepstatin A and leupeptin at 4°C for 7 d. The extract was clarified by centrifugation and the pellet was extracted with 50 mM Tris-HCl (pH 7.5) containing 4 M guanidine

HCl and 500 mM EDTA at 4°C for 4 d. The residue left after this extraction was then incubated in Tris-HCl buffer (pH 7.5) containing 250 Units/ml bacterial collagenase, 1 mM N-ethylymaleimide and 5 mM calcium chloride for 2 d at 37°C. The solubilized material was again separated from the residue by centrifugation. The acetic acid extract contained no glycosaminoglycan components, based on uronic acid analysis, and therefore was not used for the present studies. The other two extracts, guanidine/EDTA extract and collagenase digest, were concentrated by ultrafiltration through Amicon YM-10 membranes, dialysed against water and lyophilized.

Extraction and isolation of glycosaminoglycans

Samples of the guanidine/EDTA extract or collagenase digest were preincubated in 1 ml 0.1 M sodium dihydrogen phosphate, pH 6.5 for 60 min at 60°C after which 125 μl of activated papain (3 mg of papain/ml of 0.1 M sodium dihydrogen phosphate. 0.1 M EDTA, 0.3 M cysteine HCl, pH 6.5) was added. Digestion was allowed to proceed for 24 h after which the samples were centrifuged and the residue digested in 1 ml of activated papain at 60°C for a further 24 h. Trichloroacetic acid was then added to the digests to give a final concentration of 10% and allowed to stand for 3 h at 4°C. The samples were then centrifuged and the supernatants dialysed exhaustively against deionized water. The retentates were then lyophilized, reconstituted to 100 μl and 10 volumes of 1% sodium acetate in ethanol was added and the samples were allowed to stand overnight at 4°C. The precipitated glycosaminoglycans were recovered by centrifugation, dried and then solubulized in 50 μl water in preparation for electrophoretic analysis.

Cellulose acetate membrane electrophoresis

Electrophoresis of standard glycosaminoglycans and glycosaminoglycans extracted from cementum was carried out on cellulose acetate membranes. The strips were soaked for 30 min in 0.2 M calcium acetate, pH 7.2, lightly blotted between filter paper and assembled in a Gelman electrophoresis chamber (Model PN51211). Samples (2 μl) were applied to the strips, allowed to penetrate the membrane and then electrophoresed for 4 h at 1.2 mAMP/strip. After completion of the electrophoresis, the strips were stained for 30 min with Alcian blue in 0.05 M magnesium chloride and 0.025 M sodium acetate buffer, pH 5.8 in 50% (v/v) ethanol/water. The strips were then destained in 0.05 M magnesium chloride, 0.025 M sodium acetate and 50% ethanol.

Since the above system does not adequately separate all known mammalian glycosaminoglycans, a second electrophoresis system was used to verify the findings obtained with calcium acetate. This system relies on a discontinuous buffer approach as described previously by Capelletti, Del Rosso & Chiarugi (14) and modified by Hopwood & Harrison (15).

Identification of glycosaminoglycan species

Samples of standard glycosaminoglycans as well as those isolated from the cementum extracts were digested with *Streptomyces* hyaluronidase, chondroitinase ACII, chondroitinase ABC or nitrous acid as described previously (16–19). These selective degradation steps permit the identification of hyaluronic acid, heparan sulfate, chondroitin sulfate and dermatan sulfate.

Quantitative analyses

For quantitating glycosaminoglycans, they were separated by cellulose acetate membrane electrophoresis, stained with Alcian Blue, solubilized in dimethyl sulfoxide buffer containing 0.025 M magnesium chloride, 0.025 M sodium acetate and 0.1 M acetic acid and read in a spectrophotometer (19, 20, 21). Since the various mammalian glycosaminoglycans vary in their coefficient of binding for Alcian blue (19), they were quantitated from standard curves determined for each glycosaminoglycan (21).

Results

Following extensive papain digestion of the guanidine/EDTA and collagenase extracts of human cementum the glycosaminoglycan content was determined to be 0.2% and 0.1% of the dry weights of these samples respectively.

Electrophoretic separation of the glycosaminoglycans isolated in the guanidine/EDTA and collagenase extracts, together with that of a mixture of standard glycosaminoglycans, is shown in Fig. 1. Three discrete regions which stained with Alcian blue were identified. On the basis of their relative mobilities they were deduced to correspond to hyaluronic acid/heparan sulfate, dermatan sulfate and chondroitin sulfate.

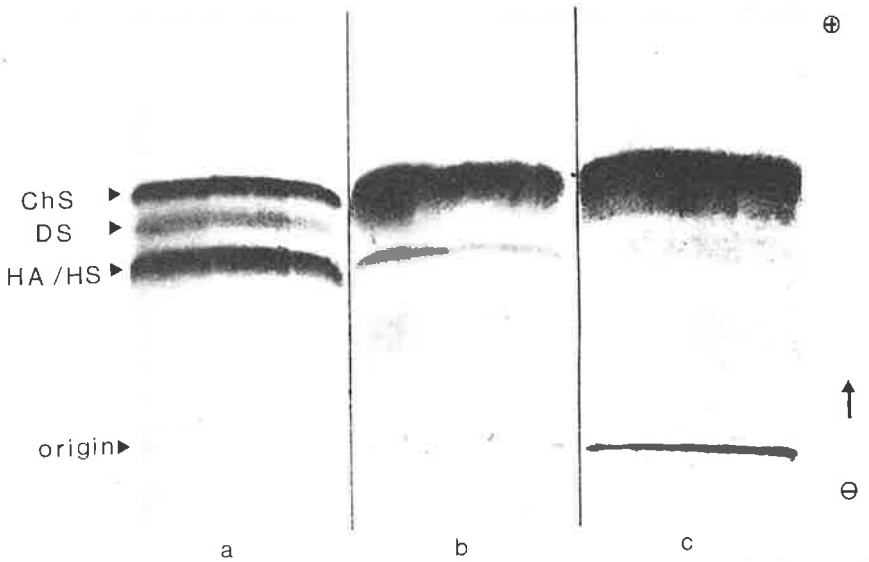

Fig. 1. Cellulose acetate membrane electrophoresis of cementum extracts. a) mixture of standard glycosaminoglycans; b) guanidine HCl/EDTA extract; c) collagenase digest. Abbreviations: HA (hyaluronic acid), HS (heparan sulfate), DS (dermatan sulfate), ChS (chondroitin sulfate).

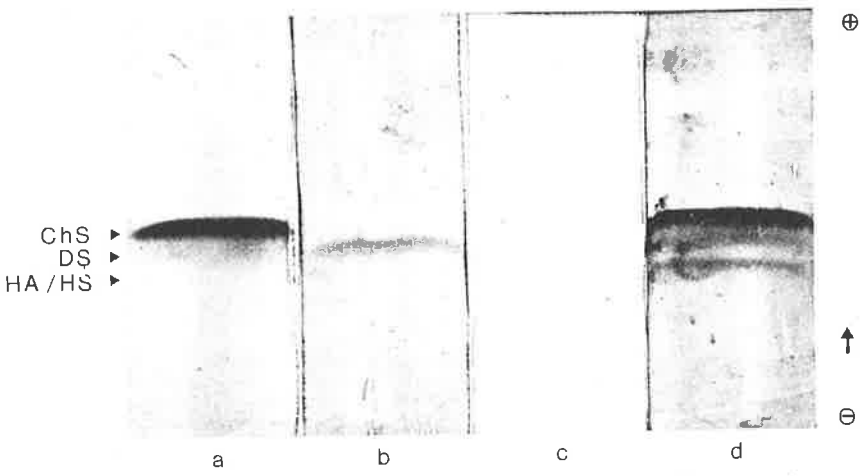

Fig. 2. Cellulose acetate membrane electrophoresis of guanidine HCl/EDTA extract of cementum after degradation with a) *Streptomyces* hyaluronidase; b) chondroitinase ACII; c) chondroitinase ABC; d) nitrous acid. The arrows indicate the migration positions of hyaluronic acid (HA), heparan sulfate (HS), dermatan sulfate (DS) and chondroitin sulfate (ChS).

Since electrophoresis in calcium acetate does not adequately separate hyaluronic acid from heparan sulfate or keratan sulfate from dermatan sulfate, the samples were also electrophoresed in a discontinuous buffer system (14, 15). The results obtained using this method confirmed the presence of hyaluronic acid, dermatan sulfate and chondroitin sulfate in both samples (results not shown), and no heparan sulfate or keratan sulfate was noted.

Because comparisons of the electrophoretic mobility of standards with tissue extracts to identify glycosaminoglycans is not dependable due to possible variations in charge distribution, selective elimination of glycosaminoglycans by enzymatic and chemical treatments followed by electrophoresis was used to confirm the identity of specific glycosaminoglycans initially identified by electrophoresis alone (Figs. 2 and 3). Treatment of the samples with *Streptomyces* hyaluronidase, which will digest hyaluronic acid alone (16), resulted in two bands in the chondroitin sulfate and dermatan sulfate region. This indicated that hyaluronic acid was present in the original extracts and that it was eliminated by the enzyme.

Chondroitinase ACII will digest not only chondroitin sulfate but also hyaluronic acid (22). After treatment of the samples with this enzyme only one band remained in the dermatan sulfate region. This indicated that both hyaluronic acid and chondroitin sulfate were present in the extracts and were digested by the enzyme. Digestion of glycosaminoglycans with chondroitinase ABC will eliminate both chondroitin sulfate and dermatan sulfate as well as hyaluronic acid (22). Since no bands were seen following treatment of the samples with this enzyme we deduce that dermatan sulfate, chondroitin sulfate and hyaluronic acid were originally present. Treatment with nitrous acid will degrade those glycosaminoglycans which are N-sulfated (i.e. heparan sulfate). This treatment of the samples resulted in no change in the distribution of three bands, which migrated in positions corresponding to hyaluronic acid dermatan sulfate and chondroitin sulfate (compare Fig. 2d and 3d with Fig. 1). Taken together, these findings confirm that hyaluronic acid, dermatan sulfate and chondroitin sulfate are the constituent glycosaminoglycans in human cementum and that keratan sulfate is not present.

The relative distribution of the various glycosaminoglycan components in the guanidine/EDTA and collagenase extracts is compared in Table 1. It is evident that chondroitin sulfate is the predominant glycosaminoglycan in both samples and dermatan sulfate is also a significant contributor to both. Although hyaluronic acid could be localized visually in electrophoresed and stained paper strips in both samples, it was noticeably diminished in the collagenase digest and could not be extracted and quantified reliably in this sample. This may be because it is not as extractable as the other GAGs after electrophoresis.

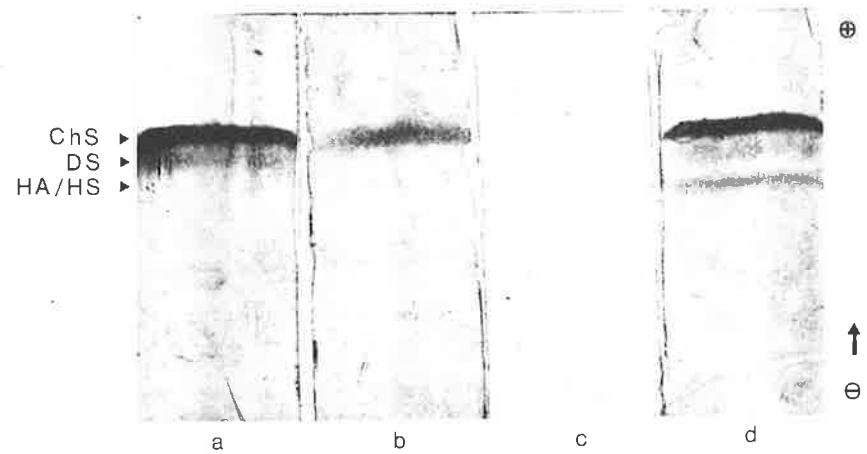

Fig. 3. Cellulose acetate membrane electrophoresis of glycosaminoglycans isolated from the collagenase digest of cementum after degradation with a) *Streptocomyces* hyaluronidase; b) chondroitinase ACII; c) chondroitinase ABC; d) nitrous acid. The arrows and abbreviations are the same as described in Fig. 2.

Discussion

In this study the organic matrix of human cementum was isolated by sequential guanidine/EDTA and collagenase treatments. Collagenase extraction was carried out to determine and study additional GAG components which may remain bound to collagens and not extracted by the guanidine. Following papain digestion of these samples, the glycosaminoglycans present were isolated, identified and quantified. Electrophoretic separation together with specific elimination of the glycosaminoglycans with enzyme and chemical treatments were used to identify hyaluronic acid, dermatan sulfate and chondroitin sulfate as the constituent glycosaminogly-

Table 1. Glycosaminoglycan composition of cementum extracts

	A_{678}	μg GAG*	% Total†
Guanidine HCl/EDTA			
Hyaluronic Acid	0.054	5	16
Dermatan Sulfate	0.113	10	31
Chondroitin Sulfate	0.178	17	53
Collagenase Digest			
Hyaluronic Acid	ND§	ND§	ND§
Dermatan Sulfate	0.136	11	31
Chondroitin Sulfate	0.190	25	69

* Glycosaminoglycan. The amounts given were derived from 0.179 g and 0.08 g of guanidine/EDTA and collagenase extracts, respectively.

† Represents the contribution of each glycosaminoglycan to the total material identified in each extract.

§ ND: not determined.

cans of human cementum. No keratan sulfate or heparan sulfate were detected. Due to the limited amount of material available for analysis, chondroitin sulfate was not analyzed further to determine whether it was present in the 4 or 6 isomeric form.

The quantitative distribution of glycosaminoglycans in cementum appears to be quite different from that reported for gingival connective tissue and periodontal ligament in which dermatan sulfate predominates (19, 23). This may reflect differences in function and composition between hard and soft tissue. For example, it has been proposed that proteoglycans inhibit calcification of collagen by occupying strategic locations normally destined to be filled with hydroxyapatite (24). Thus the proteoglycan content of mineralized tissues is notoriously low and therefore it is not surprising that we found the uronic acid content of the cementum extracts to be quite low.

Whether the glycosaminoglycan molecules in cementum are arranged within the extracellular matrix in a similar fashion to those in bone and dentin remains to be established. Nonetheless, it is interesting to note the different proportions of glycosaminoglycan species identified in the guanidine/EDTA extract and collagenase digest of cementum. The collagenase digest contained approximately one-half as much glycosaminoglycan as did the guanidine/EDTA extract (0.1% vs. 0.2%); this could indicate a variable degree of association of these components with the collagenous matrix of cementum. In addition, while chondroitin sulfate was the predominant glycosaminoglycan in both extracts, there were marked differences in the amount of hyaluronic acid present. Hyaluronic acid was easily identifiable in the guanidine/EDTA extract but only barely visible in the collagenase digest and could not be reliably quantitated in this extract.

These observations are consistent with the general arrangement of proteoglycans in bone in which dermatan sulfate proteoglycans are oriented parallel to the collagen fiber axis with chondroitin sulfate proteoglycan and hyaluronic acid occupying the interfibrillar region in a space-filling capacity (24).

In conclusion, it must be noted that glycosaminoglycans (apart from hyaluronic acid) do not exist *in vivo* as separate entities, but rather are covalently complexed to protein in the form of proteoglycans. Thus it is the whole proteoglycan unit which is the functional component of these macromolecules. While this study has identified their glycosaminoglycan composition, more definitive studies regarding the structure of the proteoglycans will provide important information relative to the complex nature of the organic matrix of cementum.

Acknowledgments

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Paper 7

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P.M. Bartold's role in this study was:
Design of the experiments
Provision of research funds
All proteoglycan assays
Writing of the manuscript

B. Reinboth's role in this study was:
Assistance with high performance liquid chromatographic analyses

H. Nakae's role in this study was:
Provision of cementum samples

A.S. Narayanan's role in this study was:
Advisory
Writing of the manuscript

R.C. Page's role in this study was:
Provision of funds
Writing of the manuscript

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Proteoglycans of Bovine Cementum: Isolation and Characterization

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Abstract

The proteoglycans associated with the mineralized matrix of bovine cementum have been studied biochemically and their distribution within this tissue localized immunohistochemically. Both hyaluronate and proteoglycans were fractionated by DEAE-Sephacel ion-exchange chromatography. The proteoglycans eluted in three separate peaks of which two contained alkali labile protein associated with glycosaminoglycans, and one appeared as free glycosaminoglycan chains. Analysis of the glycosaminoglycans identified chondroitin sulfate as the predominant species, although minor quantities of dermatan sulfate and heparan sulfate were also identified. Agarose-acrylamide gel electrophoresis and Sepharose CL-6B molecular sieve chromatography of the proteoglycans indicated them to be smaller in size with respect to periodontal ligament and gingival proteogylcans, but similar to bone and dentine proteoglycans. Amino acid analyses indicated subtle differences between cementum and bone proteoglycans. Using a monoclonal antibody (9-A-2) which recognizes the unsaturated disaccharide of chondroitinase ACII-digested glycosaminoglycans, chondroitin sulfate was identified in the pericellular environment within the lacunae housing the cementoblasts as well as in the extracellular matrix of cementum.

Key words: bovine cementum, monoclonal antibodies, proteoglycans.

Introduction

Cementum is one of five calcified tissues found in mammals and it forms the interface between the periodontal ligament and dentin allowing anchorage of teeth within the alveolar bone. Despite its strategic anatomical position with respect to tooth retention, this has been a poorly studied tissue.

Histologically, cementum is similar to bone and dentin containing an organic matrix composed primarily of dense collagen fibers embedded within a granular matrix (Selvig, 1964; 1965). However, physically it is the softest of all mineralized tissues (Selvig and Selvig, 1962) and therefore has been regarded as a modified form of bone. More recently, four types of cementum have been described on the basis of their histological appearance with respect to the presence or absence of cells and the source of the constituent collagen fibers (Schroeder, 1986).

Biochemical analyses have indicated approximately 90% of the organic matrix of cementum has the chemical characteristics of type I collagen, although some type III collagen may also be present (Birkedal-Hansen, 1974; Birkedal-Hansen, 1977; Christner et al., 1977). The non-collagenous component of human cementum accounts for a greater proportion of the total organic matrix than it does in bone or dentine and is due mainly to the presence of more collagenase-resistant material (Smith et al., 1983; Chovelon et al., 1975). Although these biochemical studies did not definitively identify or characterize any specific non-collagenous glycoproteins, early histochemical studies of this matrix indicated the likely presence of carboxylated and sulfated mucopolysaccharides (Vidal et al., 1974). This

observation has been confirmed recently by the extraction and biochemical identification of hyaluronate, chondroitin sulfate and dermatan sulfate as the constituent glycosaminoglycans in extracts of human cementum (Bartold et al., 1987).

The interactions of proteoglycans with a variety of hard and soft connective tissue components (e.g. collagen, fibronectin, laminin etc.) and cell surfaces implicates them in many biological functions (Ciba Foundation Symposium, 1986). Since regeneration of the periodontium following inflammatory disruption involves cellular repopulation of the root surface as well as new matrix formation for all components of the periodontium, the role of proteoglycans in these processes should not be overlooked. It was, therefore, the purpose of this study to extract, isolate and characterize the proteoglycans in the organic matrix of bovine cementum.

Materials and Methods

Materials

Agarose was purchased from SeaKem, FMC Corporation, Rockland, ME; Acrylamide (ultrapure grade) was from Pierce, Rockford, IL; Alcian blue 8GX, Toluidine blue, guanidinium HCl, *N*-ethylmaleimide, phenylmethylsulfonyl fluoride, pepstatin, leupeptin and urea were purchased from Sigma chemical Co, St. Louis, MO; Cellulose acetate membranes (Cellogel) were from Gelman Sciences, Ann Arbor, MI; Chondroitinase ACII *(Arthrobacter aurescens)* and chondroitinase ABC *(Proteus vulgaris)* were from Seikagaku Kogyo Pty. Ltd., Tokyo; Monoclonal antibody 9-A-2 against chondroitin sulfate was purchased from ICN Biochemicals Lisle, IL; Biotinylated species specific anti-mouse Ig and biotinylated horseradish peroxidase-Streptavidin complex were purchased from Amersham, Australia, North Ryde, NSW; Sepharose CL-6B and DEAE Sephacel were obtained from Pharmacia (Australia), North Ryde, NSW, and hydroxylapatite (Bio-Gel HTP) from BioRad Laboratories (Australia), Hornsby, NSW.

Bovine cementum was obtained from the molar teeth of young, freshly slaughtered cows using the method described by Miki et al. (1988). The teeth were cleaned thoroughly and all adherent periodontal ligament was scraped off the root surface with curettes and placed immediately into 50 mM Tris-HCl, pH 7.5 containing the following as protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 5 mM *N*-ethylmaleimide, and 25 mM EDTA. The samples were then extracted first in 0.5 M acetic acid containing 1 μg/ml each of pepstatin and leupeptin at 4 °C for 7 days. This extract, which has been shown previously to contain very little uronic acid containing macromolecules (Bartold et al., 1988), was separated by centrifugation and the residue extracted with 50 mM Tris-HCl, pH 7.5 containing 4 M guanidine HCl at 4 °C for 4 days. The second extract, which contains the bulk of extractable uronic acide containing macromolecules associated with bovine cementum (Bartold et al., 1988), was dialyzed against water, lyophilized and used for the experiments described in this study.

Since cementum has been reported to have a biochemical composition similar to bone, many of the experimental procedures used in this study were based upon previously reported studies on bone proteoglycans (Franzén and Heinegård, 1984 a, b; Goldberg et al., 1988).

Sepharose CL-6B Chromatography

For the initial preparation of cementum proteoglycans, 120 mg of the lyophilized extract was dissolved in 7 M urea in 50 mM Tris-HCl, pH 8.0 containing protease inhibitors and chromatographed with the same buffer at room temperature on a column (1.5 cm × 90 cm) of Sepharose CL-6B. A flow rate of 20 ml/h was used and 6 ml fractions were collected. Aliquots were taken from each fraction and analyzed for, (1) proteoglycan content by immobilization on cellulose acetate, staining with Alcian blue and measurement of dye concentration at A_{678} (Bartold and Page, 1985), and, (2) protein content by UV absorbance (A_{280}). Fractions enriched in proteoglycans were pooled and subjected to anion-exchange chromatography.

DEAE-Sephacel Ion-Exchange Chromatography

Ion-exchange chromatography of the proteoglycan-enriched fraction eluted from Sepharose CL-6B was performed on DEAE-Sephacel (1.5 cm × 10 cm) at room temperature with 7 M urea in 50 mM Tris-HCl buffer containing proteinase inhibitors, pH 8.0 using a linear 0–1.5 M sodium chloride gradient at a flow rate of 0.5 ml/min. The fractions were monitored for proteoglycans (A_{678}) and protein (A_{280}) and those which were enriched in proteoglycans were pooled as individual peaks or collectively. Those pooled collectively were subjected to further chromatography on hydroxyapatite, while those pooled according to peaks were characterized further according to molecular size, glycosaminoglycan composition and amino acid composition.

Hydroxylapatite Chromatography

The samples pooled for hydroxylapatite chromatography were dialyzed against 7 M urea containing 10 mM Tris-HCl and 10 mM disodium hydrogen phosphate, pH 7.4. The sample was then loaded onto a hydroxylapatite column (1 cm × 7 cm) and washed until the A_{280} reading returned to zero. The bound proteoglycans were eluted with a linear gradient of disodium hydrogen phosphate (10 mM–50 mM) in the above buffer at a flow rate of

0.25 ml/min. The proteoglycans which eluted were pooled and kept for subsequent analyses.

Analytical Sepharose CL-6B Chromatography

The molecular size distribution of proteoglycans extracted from cementum was assessed by molecular sieve chromatography on Sepharose CL-6B. The Alcian blue positive peaks from DEAE-Sephacel ion-exchange chromatography were pooled separately, dialysed against water, lyophilized and then reconstituted to 200 µl in 4 M guanidine HCl. The samples were then eluted from Sepharose CL-6B (0.8 × 100 cm) at a flow rate of 3 ml/h.

Agarose/Acrylamide Gel Electrophoresis

Aliquots from the proteoglycans separated on DEAE-Sephacel were subjected to agarose/acrylamide gel electrophoresis using a combination of conditions described by Heinegård et al. (1985) and Stanescu and Chaminade (1987). This consisted of pouring horizontal slab gels of 0.6% agarose and 1.2% acrylamide and running the gel submerged in 0.04 M Tris-acetate, pH 6.8. The samples to be analysed (10 µl) were placed into preformed wells and after 15 min of premigration at 40 mAmp the current was increased to 65 mAmp. The whole procedure was carried out at 4 °C using a water cooled electrophoresis unit. The gels were fixed with methanol:acetic acid:water (50:7:43) for 1 h and then stained with Toluidine blue (0.2 g/l in 3% acetic acid) and destained with 3% acetic acid. The gels could be viewed either wet or dried upon cellophane.

Cellulose Acetate Membrane Electrophoresis

The glycosaminoglycans associated with the proteoglycans isolated from DEAE-Sephacel were obtained following papain digestion, removal of peptides and nucleic acids with trichloroacetic acid and precipitaion in 10 volumes of 1% sodium acetate in 10% ethanol (Bartold et al., 1979). The precipitated glycosaminoglycans were recovered by centrifugation, resolubilized in deionized water and adjusted to a concentration of 1 µg uronic acid/ml. Aliquots (1 µl) of these samples were then applied onto presoaked, Titan III Zip Zone cellulose acetate membranes and electrophoresed using the discontinuous buffer system described by Capelletti et al. (1979). Quantitation of the glycosaminoglycans in the visualized bands was carried out by cutting the bands out and solubilizing in dimenthylsulfoxide and reading the absorbance at 678 nm (Newton et al., 1974). Since Alcian blue has a variable coefficient of binding for individual glycosaminoglycans, readings were normalized against reference absorbance curves using individual standard glycosaminoglycans (Bartold et al., 1981).

Glycosaminoglycan Identification

An aliquot of glycosaminoglycan from each peak eluted from DEAE-Sephacel (approximately 50 µg uronic acid) was subjected to the following treatments: (a) digestion with 0.2 U chondroitinase ACII overnight at 37 °C in 70 mM sodium acetate, pH 6.0; (b) digestion with 0.2 U chondroitinase ABC overnight at 37 °C in 50 mM Tris-HCl, 60 mM sodium acetate and 50 mM sodium chloride, pH 8.0; (c) digestion with 20 TRU hyaluronidase overnight at 60 °C in 20 mM sodium acetate, pH 6.0; (d) nitrous acid treatment for 2 h at room temperature using the low pH method of Shively and Conrad (1976). Amino Acid Analysis.

Samples (1 mg) of the proteoglycans isolated from DEAE-Sephacel ion-exchange chromatography to be analyzed for amino acids were dissolved in 6 N HCl, frozen and thawed under vacuum to remove dissolved oxygen, sealed under vacuum and hydrolyzed for 72 h at 110 °C. These samples were analyzed using a Waters high performance liquid chromatography system with *o*-phthaldialdehyde post column derivatization according to the manufacturers instructions. Assay of hydroxyproline and proline was carried out concurrently using hypochlorite oxidation (Waters Manual).

Immunohistochemistry

Bovine teeth were removed from the jaws of young cows and fixed in 10% buffered formalin, decalcified in Perenyi's solution (10% nitric acid:absolute alcohol:0.5% chromic acid; 4:3:3) and embedded in paraffin. Sections (5 µm) were cut and stained with haematoxylin and eosin for routine light microscopic examination. For immunostaining, the sections were pretreated with chondroitinase ACII (0.5 units/ml) for 3 h, washed with PBS and incubated with the monoclonal antibody 9-A-2 diluted 1:400 (Couchman et al., 1984). The sections were then exposed to a second biotinylated antibody (1:100; species specific sheep anti mouse), and then reacted with biotinylated peroxidase-conjugated streptavidin prior to color development with diaminobenzidine.

Results

Initial separation of the bovine cementum proteoglycans from proteins in the cementum extract was achieved by molecular sieve chromatography on Sepharose CL-6B (Fig. 1). Two well separated protein peaks (A_{280}) were identified which eluted with K_{av} values of 0.13 and 0.78. The proteoglycans (A_{678}) eluted from this gel in a broad and polydisperse peak between the two protein peaks (K_{av} = 0.26).

Further purification of the proteoglycans was achieved by DEAE-Sephacel ion-exchange chromatography (Fig. 2).

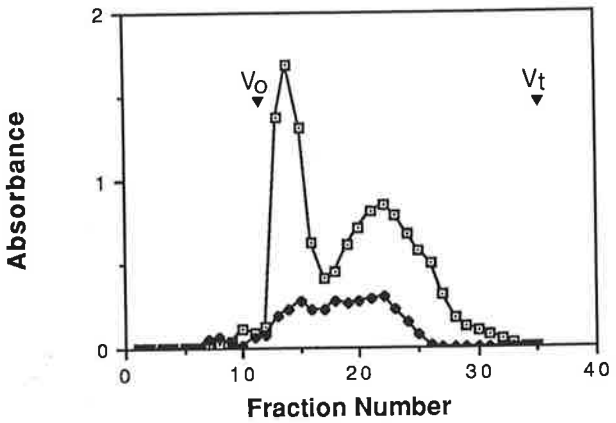

Fig. 1. Sepharose CL-6B chromatography of crude cementum extract. Samples were solubilized in 7 M urea in 50 mM Tris-HCl, pH 8.0 containing protease inhibitors and eluted from Sepharose CL-6B with the same buffer. The vold volume V_o and the total volume V_t were determined with [^{3}H]-DNA and $Na_2[^{35}SO_4]$ respectively. The fractions were assayed for protein (A_{280}; □—□) and proteoglycans (A_{678}; •—•).

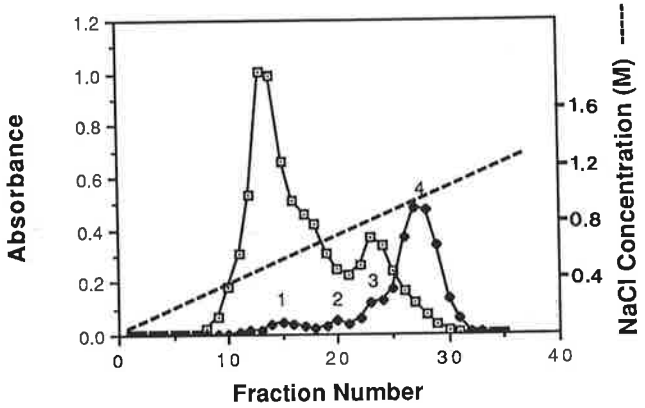

Fig. 2. DEAE-Sephacel ion-exchange chromatography of cementum extracts partially purified by Sepharose CL-6B chromatography. Alcian blue-positive material eluted from Sepharose CL-6B was pooled, applied to DEAE-Sephacel and eluted with a linear 0–1.5 M NaCl gradient in 7 M urea containing 50 mM Tris-HCl and protease inhibitors, pH 8.0. The fractions were monitored for protein (A_{280}; □—□) and proteoglycans (A_{678}; •—•).

Bound material was eluted from the column with a 0–1.5 M NaCl gradient and was identified in two protein peaks and four Alcian blue-positive peaks. The first three Alcian blue-positive populations to elute from DEAE-Sephacel co-eluted with protein components while the Alcian blue-positive material which eluted in the last peak did not contain a significant amount of protein.

Adsorption of proteoglycans to hydroxyapatite and elution with a salt gradient has been successfully used in the purification of bone-derived proteoglycans Goldberg et al. (1988). Therefore, this method was investigated as a means

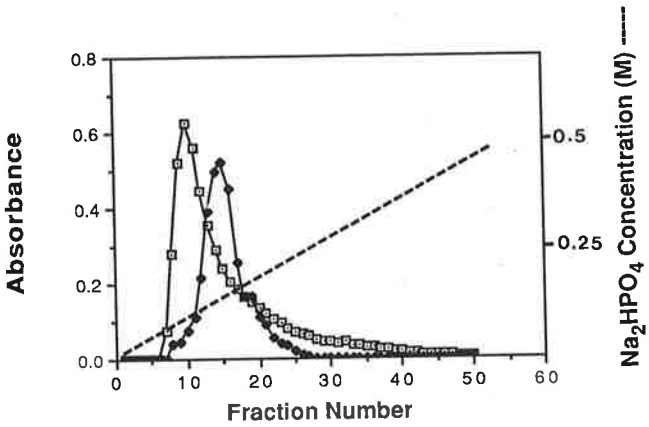

Fig. 3. Hydroxylapatite chromatography of cementum extracts partially purified by Sepharose CL-6B chromatography. Alcian blue-positive material eluted from Sepharose CL-6B was pooled, applied onto hydroxyapatite and eluted with a linear 10 mM – 500 mM Na_2HPO_4 gradient in 7 M urea containing 10 mM Tris-HCl, pH 7.4. The fractions were monitored for protein (A_{280}; □—□) and proteoglycan (A_{678}; •—•).

of isolating cementum proteoglycans free from contaminating proteins (Fig. 3). Although a significant amount of protein did not bind to the hydroxyapatite (not shown), negligible proteoglycans (A_{678}) were detected in the fractions obtained during loading or washing prior to commencement of the gradient elution. Bound material eluted from the hydroxyapatite in two principal, but not co-incident, peaks. A large protein peak with a long trailing end eluted prior to a moderately sharp and well-defined peak containing the proteoglycans. This separation was not considered adequate as a means of isolating proteoglycan populations from cementum extracts and therefore material obtained from DEAE-Sephacel was used for further analyses.

Glycosaminoglycan analysis of the material in the four Alcian blue-positive peaks eluted from DEAE-Sephacel by cellulose acetate membrane electrophoresis indicated that their glycosaminoglycan composition is different (Fig. 4). In peak 1 hyaluronate and minor amounts of heparan sulfate and chondroitin sulfate were identified. Peak 2 contained significant quantities of dermatan sulfate and chondroitin sulfate, and lesser amounts of heparan sulfate and hyaluronate. Peaks 3 and 4 contained predominantly chondroitin sulfate with smaller quantities of dermatan sulfate (Fig. 4). Enzymatic and chemical treatments of these samples with *Streptomyces* hyalurondidase, chondroitinase ACII, chondroitinase ABC and nitrous acid confirmed the glycosaminoglycans present as hyaluronate, chondroitin sulfate, dermatan sulfate and heparan sulfate respectively (Fig. 5). Under the conditions used *Streptomyces* hyaluronidase digests only hyaluronate, chondroitinase ACII digests hyaluronate and chondroitin sulfate, chondroitinase ABC digests hyaluronate, chondroitin sulfate and dermatan sulfate and nitrous acid eliminates heparan

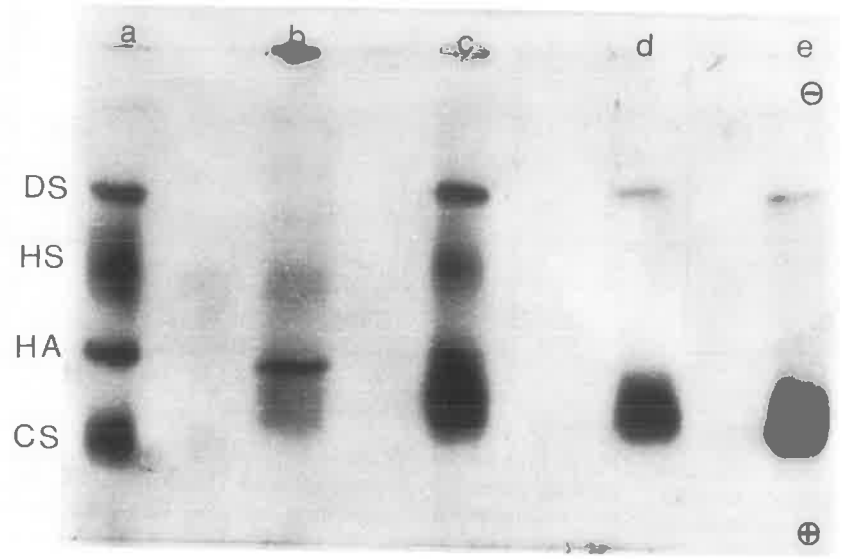

Fig. 4. Cellulose acetate membrane electrophoresis of glycosaminoglycans associated with material eluted from DEAE-Sephacel. Glycosaminoglycans were obtained by papain digestion of material in DEAE peaks 1–4, electrophoresed on cellulose acetate and visualized following Alcian blue staining. (a) Standard glycosaminoglycans; (b) DEAE-peak 1; (c) DEAE-peak 2; (d) DEAE-peak 3; (e) DEAE-peak 4. (Abbreviations: HA (hyaluronate), DS (dermatan sulfate); CS (chondroitin sulfate); HS (heparan sulfate).

sulfate. Quantitation of the individual glycosaminoglycans present in these peaks was also carried out and is listed in Table I.

To assess the proteoglycan nature of the material, peaks 2, 3 and 4 obtained from DEAE-Sephacel were chromatographed on Sepharose CL-6B (Fig. 6). As expected from the data shown in Figure 2, material from DEAE peaks 2 and 3 co-eluted from Sepharose CL-6B with material identifiable as protein and had K_{av} values of 0.36 and 0.39 (Fig. 6 a and b). However, no protein was detected in peak 4 (Fig. 6 c).

The proteoglycan nature of the material in these peaks was further assessed by alkaline borohydride reduction followed by Sepharose CL-6B chromatography (Fig. 7). Molecules from peaks 2 and 3 demonstrated a shift in K_{av} values following such treatment and indicated a reduction in molecular size following removal of the proteins associated with the glycosaminoglycans. However, peak 4 did not demonstrate any evidence of reduction in size by the alkaline borohydride treatment.

Table I. Relative Proportion of Glycosaminoglycans in Proteoglycan Peaks.

	Hyaluronate	Heparan Sulfate	Dermatan Sulfate	Chondroitin Sulfate
Peak 2	10	19	26	45
Peak 3	0	0	31	69
Peak 4	0	0	9	91

Proteoglycans isolated from DEAE-Sephacel (Peaks 2, 3 and 4; Fig. 2) were subjected to papain digestion and the recovered glycosaminoglycans were electrophoresed on cellulose acetate membranes, Quantitation was achieved by eluting bound Alcian blue and reading the absorbance against calibrated curves for standard glycosaminoglycans. Data are expressed as percentages of the total glycosaminoglycans in each peak.

Agarose/acrylamide gel electrophoresis indicated that the three proteoglycans eluted from DEAE-Sephacel had different electrophoretic mobilities (Fig. 8). Peak 2 separated into two components which stained in approximately equal densities. Peak 3 migrated as one band which had an intermediate mobility. Peak 4 also had two components, the bulk of which co-migrated with the standard chondroitin sulfate glycosaminoglycan sample.

Amino acid analyses were performed on the proteoglycans which eluted from DEAE-Sephacel in peaks 2, 3 and 4 (Table I). Amino acids were detected only in peaks 2 and 3; this also indicated the absence of protein in peak 4. Aspartic acid, glutamic acid and glycine were present in greatest proportions, no methionine or hydroxyproline and only trace amounts of hydroxylysine were detected.

Reaction of sections of bovine teeth with the monoclonal antibody 9-A-2 after digestion with chondroitinase ACII revealed dense staining in the periodontal tissues (Fig. 9). The periodontal ligament stained intensely along the collagen fibers as well as some apparent nonspecific cellular staining. A different pattern of staining was evident within the cementum. Both the cell surface and pericellular environment within the lacunae housing the cementoblasts stained positively and throughout the extracellular matrix a fine granular meshwork of staining was evident. No antibody localization in the cementum was seen in control sections reacted with the monoclonal antibody in the absence of enzyme digestion.

Discussion

To date many different types of proteoglycans have been identified, some of which possess features specific for the tissues from which they are obtained (see reviews by Poole, 1986; Bartold, 1987). For example, in cartilage the princi-

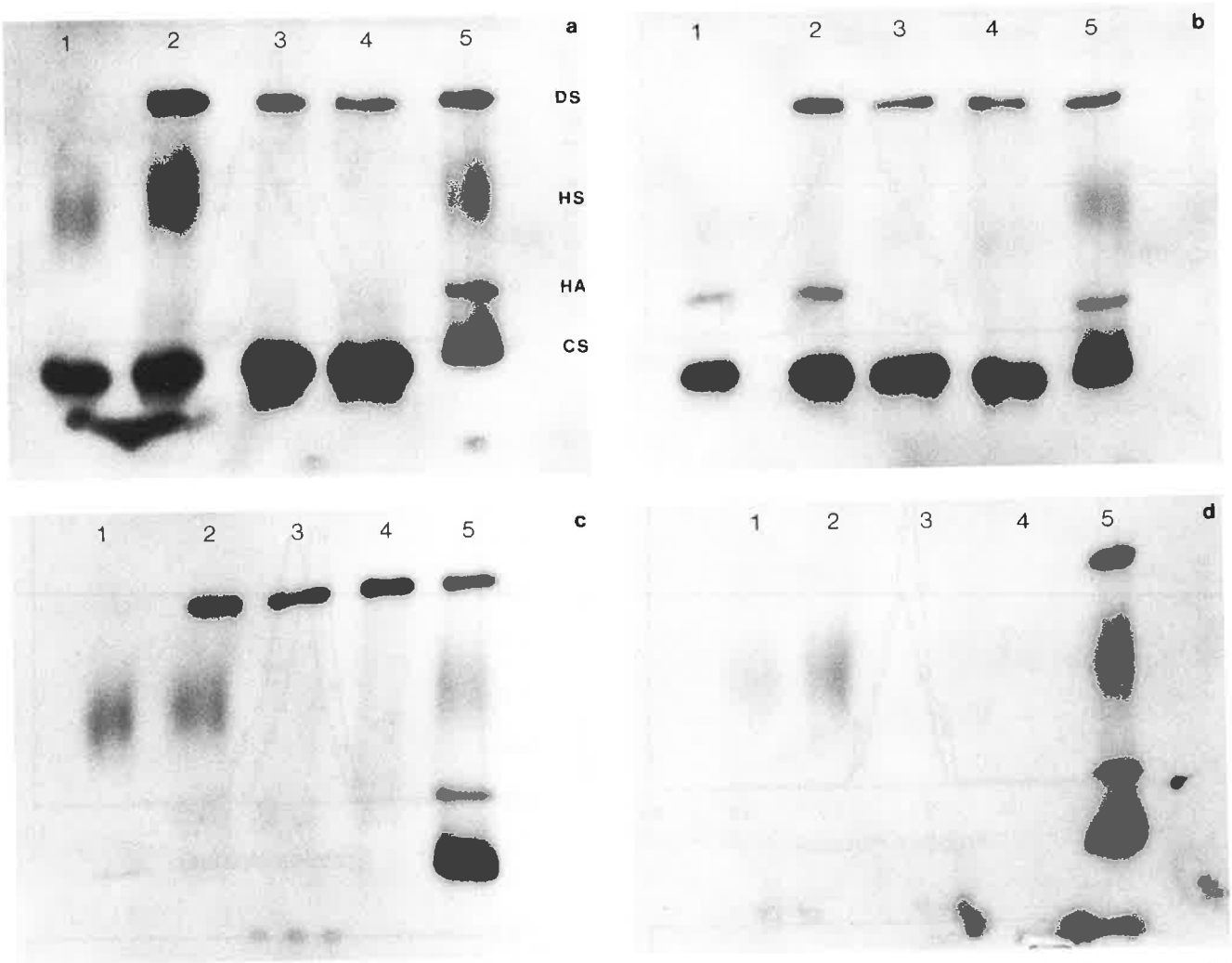

Fig. 5. Selective enzymatic and chemical elimination of glycosaminoglycans. Samples of glycosaminoglycans isolated from material eluting in peaks 1, 2, 3, and 4 from DEAE-Sephacel (Fig. 4) were exposed to (a) *Streptomyces* hyaluronidase, (b) low pH nitrous acid, (c) chondroitinase ACII, and (d) chondroitinase ABC to identify the molecular species as hyaluronate, heparan sulfate, chondroitin sulfate and dermatan sulfate respectively. Lanes 1, 2, 3, and 4 correspond to material from DEAE-Sephacel peaks 1–4 and lane 5 represents undigested samples of a mixture of glycosaminoglycans containing dermatan sulfate (DS), heparan sulfate (HS), hyaluronate (HA) and chondroitin sulfate (CS).

pal proteoglycan is a species of molecular mass up to 2 500 000 daltons, which is composed of a core protein ($M_r \sim 400\,000$) and over 100 chondroitin sulfate and keratan sulfate glycosaminoglycan chains ($M_r \sim 35\,000$). In contrast, the principal proteoglycan found in soft connective tissues (eg. skin, gingiva and tendon) is much smaller (average size approximately 100 000 daltons) and it contains only a few dermatan sulfate chains ($M_r \sim 25\,000$) on a protein core of $\sim 45\,000$. Two small proteoglycans have been isolated from the mineralized matrix of bone; one is composed of a single chondroitin sulfate chain bound to a small protein core, while the other contains two chondroitin sulfate chains bound to a protein core of slightly different amino acid composition but similar size (Fisher et al., 1983).

In the present study, we have identified three populations of proteoglycans within the matrix of bovine cementum which share some common features with bone proteoglycans. For example, although three populations of sulfated proteoglycans were identified by their affinity for Alcian blue, only two of these qualify as proteoglycans on the basis of the presence of an alkali labile protein associated with glycosaminoglycan chain(s). The third population appeared to be present unassociated with any protein component and was extracted as alkali insensitive glycosaminoglycans. Two features common to both of the cementum proteoglycans were their very small size and the predominance of chondroitin sulfate as the principal glycosaminoglycan. Both of them eluted from Sepharose CL-6B with a smaller size than that of small dermatan sulfate

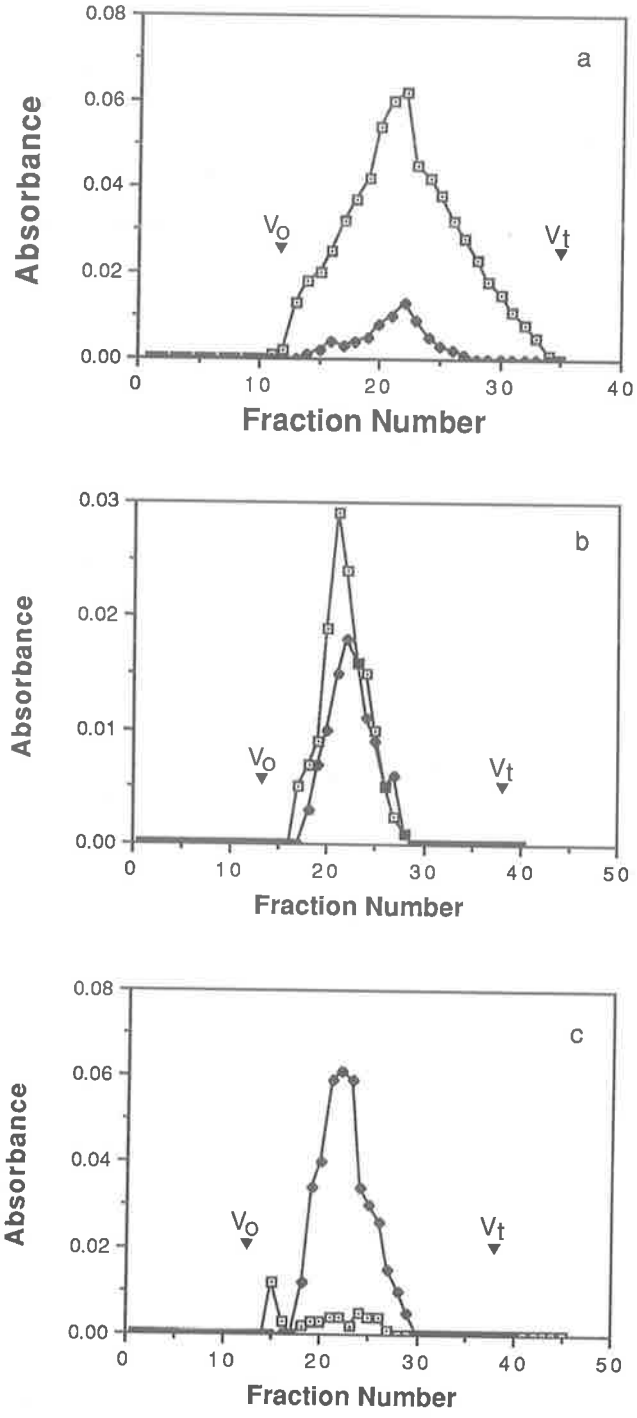

Fig. 6. Sepharose CL-6B chromatography of bovine cementum proteoglycans isolated from DEAE-Sephacel. Peaks 2–4 from DEAE-Sephacel chromatography contained sulfated glycosaminoglycans and therefore most likely represented proteoglycans. These samples were eluted from Sepharose CL-6B with 4 M guanidine HCl in 50 mM sodium acetate containing protease inhibitors, pH 5.8. The fractions were monitored for protein (A_{280}; □—□) and proteoglycans (A_{678}; •—•). a: peak 2; b: peak 3; c: peak 4.

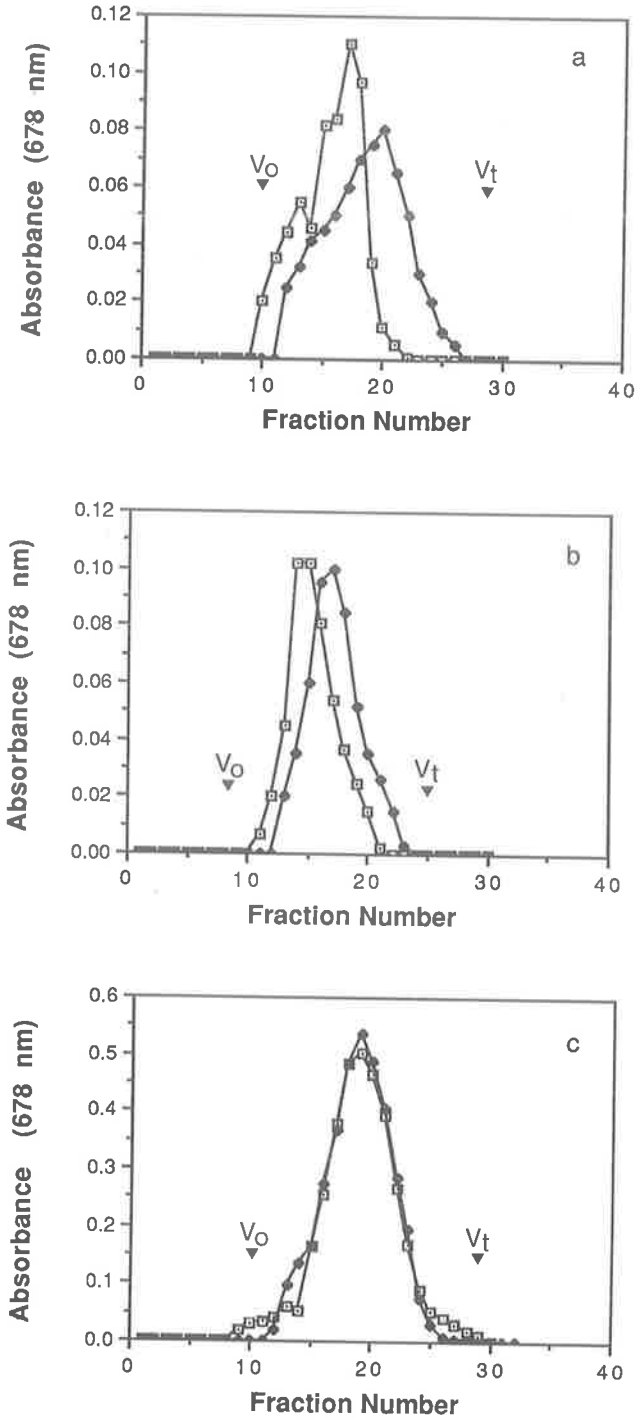

Fig. 7. Sepharose CL-6B chromatography of protecglycans treated with alkaline sodium borohydride. Samples of the proteoglycans eluted from DEAE-Sephacel (DEAE-peaks 2, 3 and 4) were treated with alkaline sodium borohydride and then eluted from Sepharose CL-6B with 4 M guanidine HCL in 50 mM sodium acetate, pH 5.8. The fractions were monitored for Alcian blue affinity only (A_{678}). The V_o and V_t were determined as described in Fig. 1. a: peak 2; b: peak 3; c: peak 4. Control; □—□ Treated; •—•.

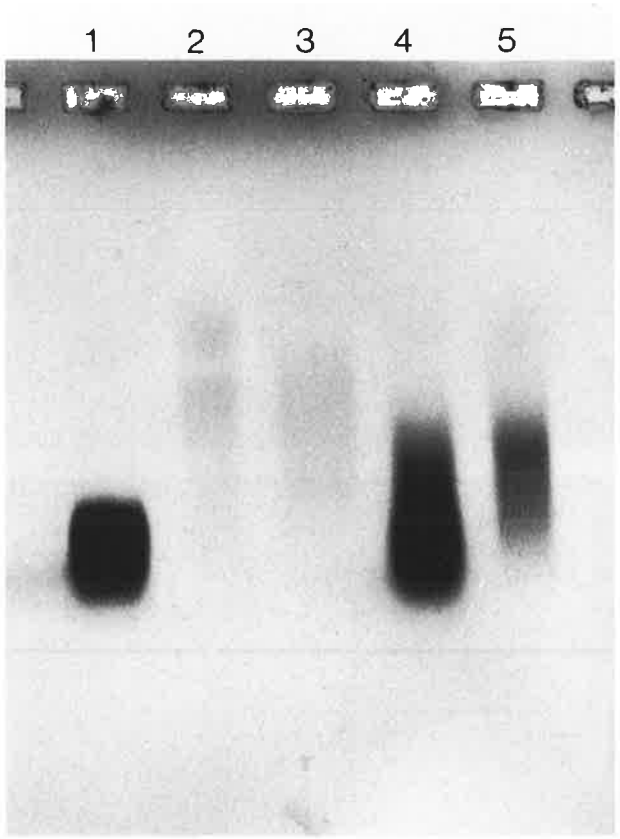

Fig. 8. Agarose acrylamide gel electrophoresis of proteoglycans extracted from cementum. Samples of proteoglycans obtained by DEAE-Sephacel chromatography (DEAE-peaks 2, 3 and 4) were electrophoresed on submerged slab gels of 0.6% agarose and 1.2% acrylamide. The proteoglycans were visualized by staining with Toluidine blue. Lane 1, standard chondroitin sulfate glycosaminoglycan; lane 2, DEAE-peak 2; lane 3, DEAE-peak 3; lane 4, DEAE-peak 4; lane 5, gingival dermatan sulfate proteoglycan.

proteoglycans of gingiva and periodontal ligament (Bartold et al., 1979; Pearson and Gibson, 1982). Nonetheless, their size is comparable with the proteoglycans identified in bone and dentine (Goldberg et al., 1988; Rahemtulla et al., 1984) and therefore this may be a general feature of mineralized tissue proteoglycans (apart from those in cartilage). Although dermatan sulfate is not considered a major component of bone, small amounts of iduronate-containing polymers have been isolated from both bone and dentin (Prince et al., 1983; Hjerpe et al., 1983). In this regard, cementum is similar, in that a small presence of dermatan sulfate was identified in the proteoglycan extracts. This finding is also consistent with our previous report on the glycosaminoglycan content of human cementum (Bartold et al., 1988). It is likely that this glycosaminoglycan may reflect the presence of remnants of Sharpey's fibres (collagen bundles inserted into the cementum during development and serving as anchorage for the periodontal ligament), since dermatan sulfate appears to be very closely associated with collagen fibres in both hard and soft connective tissues (Scott, 1988). The presence of large amounts of free glycosaminoglycan chains in cementum is an interesting finding since, apart from hyaluronate, the sulfated glycosaminoglycans are usually found in tissues bound to protein in the form of a proteoglycan (Hascall and Hascall, 1981). However, recent evidence indicates that free glycosaminoglycans may also be components of mineralized tissues and that these arise from protease removal of the proteoglycan core proteins leaving the chondroitin sulfate chains behind (Fisher, 1985).

Despite the above similarities, the amino acid composition of cementum and bone proteoglycans do not closely resemble each other. The two proteoglycans identified in bone differ in their amino acid composition; one being rich in glutamic acid and glycine residues while the other being rich in leucine residues (Fisher et al., 1983). However, both of the proteoglycans identified in the cementum extracts were rich in glutamic acid and glycine residues and relatively poor in leucine residues. This apparent difference between cementum and bone proteoglycans is not immediately explicable but may represent slight tissue to tissue variation. This is supported by the observation that the amino acid composition of proteoglycans isolated from dentine differs from both bone and cementum. Interestingly, the amino acid profile of cementum proteoglycans do

Table II.

	C #2	C#3	Bone #1[a]	Bone #2[a]	Gingiva[c]	PDL[b]
Cysteic Acid	19	15	ND	ND	ND	ND
Aspartic Acid	101	100	126	138	113	125
Threonine	41	45	45	52	52	39
Serine	71	100	70	77	66	74
Glutamic Acid	161	183	94	127	129	108
Proline	85	71	73	67	86	67
Glycine	144	169	84	95	120	80
Alanine	80	76	51	49	77	49
Cysteine	7	3	ND	ND	ND	ND
Valine	50	37	62	54	48	58
Methionine	0	0	8	6	Trace	9
Isoleucine	27	23	54	46	43	57
Leucine	65	47	132	102	93	123
Tyrosine	16	16	30	34	20	29
Phenylalanine	24	16	34	28	27	33
Histidine	26	38	32	25	11	75
Hydroxylysine	Trace	Trace	ND	ND	ND	ND
Lysine	37	28	57	56	63	27
Arginine	43	30	50	44	52	31
Hydroxyproline	0	0	ND	ND	ND	ND

C#2 = Cementum DEAE Peak 2; C#3 = Cementum DEAE Peak 3; ND = Not Determined.

[a] Values from results Fisher et al. (1983)

[b] Values from Pearson and Gibson (1982)

[c] Values from Bartold et al. (1983)

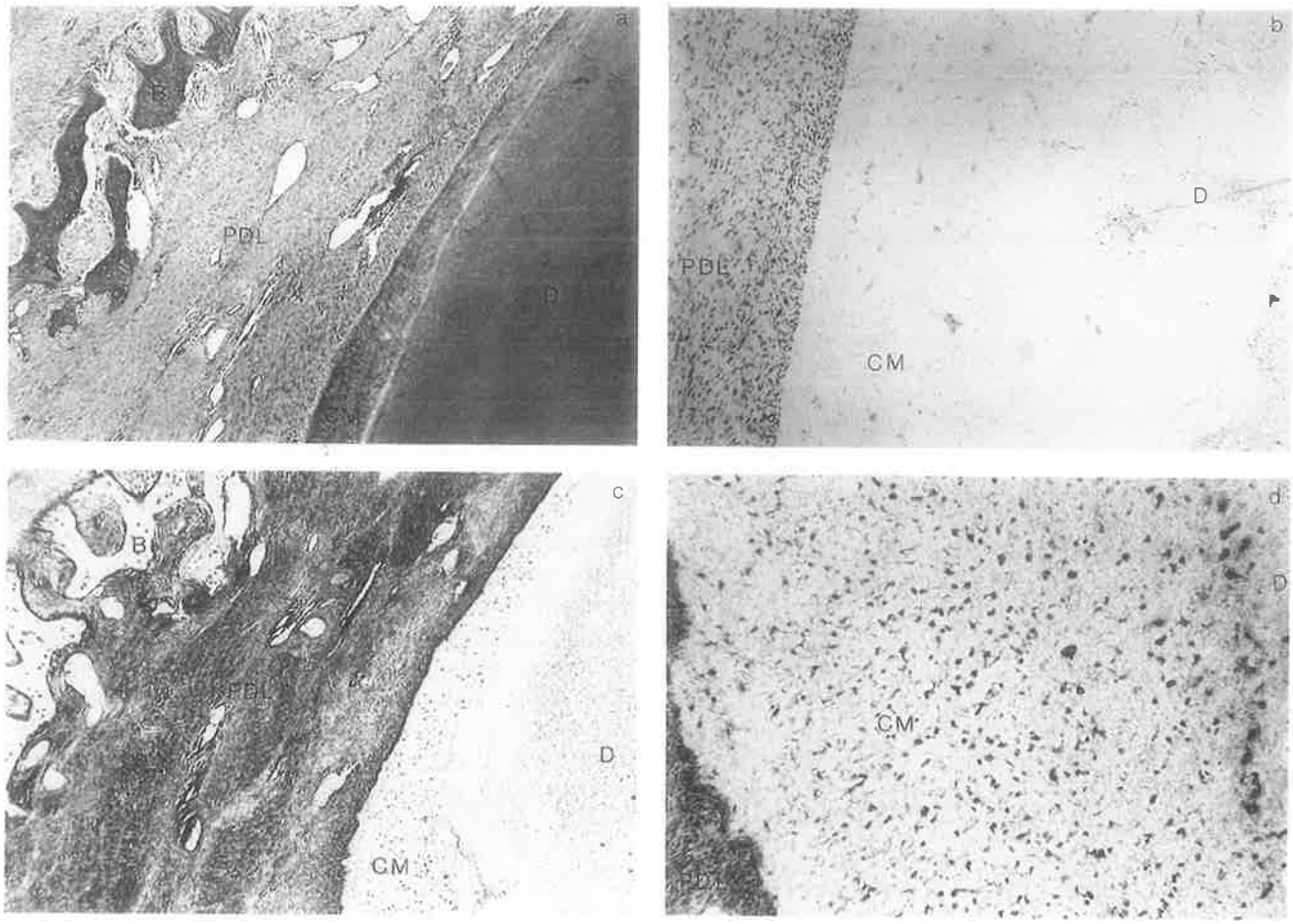

Fig. 9. Immunohistochemical localization of proteoglycans in cementum. Sections of bovine teeth were reacted with a monoclonal antibody which recognizes the unsaturated disaccharides of digested chondroitin sulfate glycosaminoglycans.
(a) Haematoxylin and Eosin. Magnification X40.
(b) Control (no enzyme digestion). Magnification X100.
(c) Antibody 9-A-2. Magnification X40.
(d) Antibody 9-A-2. Magnification X100.
Abbreviations: PDL (periodontal ligament); CM (cementum) D (dentine); B (bone).

not appear to differ significantly from the proteoglycans isolated from gingiva and periodontal ligament. Whether this reflects a common lineage of the cells responsible for proteoglycan synthesis in these tissues, and thus similar matrix expression in terms of their core proteins, remains to be established.

The role of proteoglycans in mineralized tissues is not clear. However, a close association between proteoglycans and mineralization has been observed recently (Fisher et al., 1983). The bone proteoglycans appear to diffuse rapidly to the mineralized zone immediately following biosynthetic release from the cells, to interact with the osteoid and to become embedded into the mineralized compartments. Our findings indicate that the cementum proteoglycans, which appear to be a major, identifiable component of the noncollagenous matrix in this tissue, may be involved in processes similar to those described above. Further biochemical characterization of these macromolecules will be essential to understand their role in mineralization and how they participate in periodontal disease and new cementum formation during periodontal regeneration.

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Paper 8.

Bartold, P.M.
A biochemical and immunohistochemical study of the proteoglycans of alveolar bone.
Journal of Dental Research **69:** 7-19; 1990.

A Biochemical and Immunohistochemical Study of the Proteoglycans of Alveolar Bone

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The purpose of this investigation was to study the proteoglycans in alveolar bone of three animal species. Alveolar bone was obtained from humans, pigs, and rabbits. Portions were fixed, sectioned, and stained with monoclonal antibodies against keratan sulfate and chondroitin sulfate. In other samples, biochemical analyses were performed. After removal of the organic matrix by 4 mol/L guanidinium HCl extraction in the presence of proteinase inhibitors, proteoglycans in the mineralized matrix were extracted with 4 mol/L guanidinium HCl/0.5 mol/L EDTA/proteinase inhibitors, and characterized on the basis of their glycosaminoglycan content (cellulose acetate membrane electrophoresis), charge (DEAE-Sephacel and hydroxylapatite chromatography), size (Sepharose CL-6B chromatography and agarose/polyacrylamide gel electrophoresis), and amino acid content. The results indicated that keratan sulfate could be detected immunohistochemically and biochemically in rabbit bone only. The predominant glycosaminoglycan in pig and human alveolar bone was chondroitin sulfate, although some hyaluronate, dermatan sulfate, and heparan sulfate were also detected. The proteoglycans were found to be slightly smaller than gingival proteoglycans, but similar to those in cementum, dentin, and other bones. In addition to intact proteoglycans, some free glycosaminoglycan chains were also extracted from the mineralized matrix. Amino acid analyses showed some subtle differences between alveolar bone proteoglycan and those of the soft tissues of the periodontium.

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Introduction.

Proteoglycans appear to be ubiquitous components of both hard and soft connective tissues (Poole, 1986; Bartold, 1987). Structurally, these non-fibrous macromolecules are composed of a central protein core to which one or more glycosaminoglycans are covalently bound. By virtue of the highly anionic nature of their glycosaminoglycans, proteoglycans are believed to play critical roles in maintaining a wide range of physiological properties of connective tissues, as well as regulating the behavior of the resident cells in these tissues.

The glycosaminoglycan composition of bone has been studied in a variety of different species, including dog, ox, sheep, and man, and chondroitin sulfate was found to be the predominant glycosaminoglycan in these species (Herring, 1968; Hjertquist and Vejlens, 1968; Vejlens, 1971; Dickson, 1974; Engfeldt and Hjerpe, 1976). Despite this apparent uniformity, rabbit bone differs in that it contains a significant amount of keratan sulfate in addition to chondroitin sulfate (Masubuchi *et al.*, 1975; Diamond *et al.*, 1982; Fisher *et al.*, 1987). Further to these glycosaminoglycan analyses, the intact proteoglycans isolated from bone have also been studied. The major proteoglycans associated with the mineralized matrix of bone are small glycoconjugates (molecular weight, M_r:80,000–120,000) containing 1 -2 chondroitin sulfate chains (Fisher *et al.*, 1983). These may be fractionated into several populations of which the molecular dimensions of the protein core and glycosaminoglycans—as well as the amino acid compositions—differ (Franzén and Heinegård, 1984a,b). In addition, a large proteoglycan (molecular weight, M_r:1,000,000) has been isolated from newly-forming bone and is located in the soft connective tissue between growing trabeculae, and not within the mineralized matrix (Fisher *et al.*, 1983, 1987). The small proteoglycans of the mineralized matrix appear to be immunologically distinct from the larger proteoglycans of the organic matrix, which are immunologically similar to the cartilage proteoglycans (Fisher *et al.*, 1983; Franzén and Heinegård, 1984b). Biosynthesis studies, with cultured bone tissue and isolated bone cells, have indicated that the small proteoglycans of bone diffuse to the mineral front and, with time, the protein cores of the proteoglycans are degraded. These events lead to the resultant glycosaminoglycan chains becoming embedded within the mineralized bone matrix (Fisher, 1985).

Alveolar bone is anatomically unique in that it houses the dentition and, because of the constant application of changing forces through occlusal loads, this spongy bone is continually undergoing remodeling processes. Although, as outlined above, proteoglycans have been recognized as significant components of bone for over 20 years (Herring, 1968), the contribution of these macromolecules to the composition of alveolar bone has been largely neglected. Thus, apart from one histochemical study in which chondroitin sulfate, dermatan sulfate, and hyaluronate were localized within the extracellular matrix of rat alveolar bone (Johnson, 1988), two biochemical analyses of the glycosaminoglycans of rabbit and human alveolar bone (Waddington *et al.*, 1988, 1989), and one study of a proteoglycan isolated from rabbit alveolar bone (Oosawa, 1986), those interested in the biochemical composition of alveolar bone proteoglycans have had to rely upon information obtained largely from studies concerned with bones from extra-oral sources.

In the present study, the location of various proteoglycans in the alveolar bone of three different species (pig, rabbit, and human) was studied by immunohistochemistry. On the basis of similar proteoglycan species being identified in pig and man, more detailed biochemical analyses were carried out on proteoglycans in extracts of pig alveolar bone.

Materials and methods.

Materials.—Agarose was purchased from SeaKem, FMC Corporation, Rockland, ME; acrylamide (ultrapure grade) was from Pierce, Rockford, IL; Alcian blue 8GX, toluidine blue, guanidinium HCl, N-ethylmaleimide, phenylmethylsulfonyl fluoride, and urea were purchased from Sigma Chemical Co., St. Louis, MO; cellulose acetate membranes (Cellogel) were from Gelman Sciences, Ann Arbor, MI; chondroitinase ACII (*Arthrobacter aurescens*) was from Seikagaku Kogyo Pty. Ltd., Tokyo; monoclonal antibodies 9-A-2 against chondroitin sulfate and 5-D-4 against keratan sulfate were purchased from ICN Biochemicals, Lisle, IL; biotinylated species-specific anti-mouse immunoglobulin and biotinylated horseradish peroxidase-Streptavidin complex were purchased from Amersham,

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North Ryde, NSW, Australia; Sepharose CL-6B and DEAE-Sephacel were obtained from Pharmacia (Australia), North Ryde, NSW; hydroxylapatite was from BioRad Laboratories (Australia), Hornsby, NSW; and Titan III Zip Zone cellulose acetate membranes were purchased from Helena Laboratories, Beaumont, TX.

Porcine alveolar bone was obtained from freshly slaughtered pigs, rabbit alveolar bone was obtained from freshly killed New Zealand White rabbits, and human alveolar bone was obtained from patients undergoing orthognathic surgery. Block segments of bone were transported on ice to the laboratory, where the teeth and adherent soft tissues were immediately removed.

Immunohistochemistry.—Portions of alveolar bone from the three species under study were fixed in 10% buffered formalin, decalcified in Perenyi's solution (10% nitric acid: absolute alcohol:0.5% chromic acid; 4:3:3), and embedded in paraffin. Sections (5 μm) were cut and stained with hematoxylin and eosin for routine light microscopic examination. For the detection of chondroitin sulfate, the sections were pre-treated with chondroitinase ACII (0.5 units/mL) for three h, washed with phosphate-buffered saline, and incubated with the monoclonal antibody 9-A-2 (Couchman *et al.*, 1984). Keratan sulfate was localized by undigested sections being reacted with the monoclonal antibody 5-D-4 (Caterson *et al.*, 1983). The sections were then reacted with biotinylated peroxidase-conjugated streptavidin prior to color development with diaminobenzidine. For the localization of keratan sulfate, control sections were incubated in the absence of the primary antibody. Control sections for the localization of chondroitin sulfate were incubated in the absence of chondroitinase ACII.

Glycosaminoglycan extraction.—Portions of alveolar bone from all three species (10 g) were pulverized in liquid nitrogen and digested in 100 mL of activated papain (1 mg/mL papain in 100 mmol/L NaH_2PO_4, 100 mmol/L EDTA, 30 mmol/L cysteine HCl, pH 6.5) at 65°C for 24 h. The residue was removed and digested in fresh papain for another 24 h. Trichloroacetic acid was then added to the pooled digests (to make a final concentration of 10%) and allowed to stand at 4°C for 12 h. The precipitated material was then removed by centrifugation, and the supernatant dialyzed exhaustively against water prior to lyophilization. The samples were resolubilized in a small volume of water, and the glycosaminoglycans precipitated with 10 volumes of ethanolic 1% sodium acetate.

Cellulose acetate membrane electrophoresis.—The glycosaminoglycans were resolubilized in water and adjusted to an approximate concentration of 1 mg uronic acid/mL. Aliquots (1 μL) of these samples were then applied onto pre-soaked, Titan III Zip Zone cellulose acetate membranes, and electrophoresed with use of the discontinuous buffer system described by Capelletti *et al.* (1979). Where keratan sulfate was suspected to be present, both 25% and 50% ethanol precipitations were performed. Quantitation of the glycosaminoglycans was achieved by the bands being cut out, the membrane being solubilized in dimethylsulfoxide, and the absorbance being read at 678 nm (Newton *et al.*, 1974). Since Alcian blue has different affinities for various glycosaminoglycans (Bartold *et al.*, 1981), readings were normalized against reference absorbance curves obtained with use of standard glycosaminoglycans.

Extraction of proteoglycans from alveolar bone.—Protocols for the extraction of proteoglycans from bone have been variously described (Termine *et al.*, 1981; Franzén and Heinegård, 1984a,b; Goldberg *et al.*, 1988), and these have formed the basis upon which the protocols in the following experiments were used.

Since rabbit bone appears to be unusual because of the presence of keratan sulfate, and because the pig bone contained similar glycosaminoglycans that were distributed throughout the matrix in a fashion similar to that of human bone, as well as because of the very limited amounts of human bone available for biochemical analyses, the remainder of the experiments described were performed with use of pig alveolar bone only.

Alveolar bone (100 mg) was pulverized in liquid nitrogen, and the non-mineralized connective tissue components extracted at 4°C with four daily one-liter changes of 4 mol/L guanidinium HCl, 50 mmol/L Tris-HCl, pH 7.4, buffer containing the following as proteinase inhibitors: 1 mmol/L phenylmethylsulfonyl fluoride, 100 mmol/L 6-amino-hexanoic acid, and 5 mmol/L N-ethylmaleimide. Material in the remaining mineralized matrix was then extracted at 4°C with three daily one-liter changes of 50 mmol/L Tris-HCl/0.5 mol/L EDTA (tetrasodium salt), pH 7.4, containing proteinase inhibitors. This extract was then concentrated to 30 mL by ultrafiltration through a PM-10 Amicon filter, dialyzed against de-ionized water, and lyophilized. The demineralized residue was re-extracted with 4 mol/L guanidinium HCl in 50 mmol/L Tris-HCl, pH 7.4, concentrated, dialyzed, and lyophilized. In the following experiments, only the EDTA extract of the alveolar bone was studied, since this has been shown to contain the bulk of the mineralized tissue-associated proteoglycans (Goldberg *et al.*, 1988). The initial extract and the last 4 mol/L guanidinium HCl extracts were kept and are currently under further investigation.

Preparative column chromatography.—Chromatographic preparation of alveolar bone proteoglycans was carried out by 120–200 mg of the lyophilized extract being dissolved in 2 mL of 7 mol/L urea in 50 mmol/L Tris-HCl, pH 8.0, containing proteinase inhibitors, and elution, with the same buffer, from a column (1.5 cm × 90 cm) of Sepharose CL-6B. A flow rate of 20 mL/h was used, and 6-mL fractions were collected. Aliquots were taken from each fraction and analyzed for protein content by ultra-violet absorbance (A_{280}), and for proteoglycan content by immobilization on cellulose acetate, staining with Alcian blue, and measurement of dye concentration at A_{678} (Bartold and Page, 1985). The fractions which were enriched in proteoglycans were pooled and subjected to further analyses.

DEAE-Sephacel ion-exchange chromatography.—Proteoglycans eluted from the preparative Sepharose CL-6B column were loaded onto DEAE-Sephacel (1.5 × 10 cm) at room temperature, and unbound material was removed by the column being washed with 7 mol/L urea in 50 mmol/L Tris-HCl buffer containing proteinase inhibitors, pH 8.0, until the absorbance at 280 nm returned to a baseline level. A linear gradient of 0–1.5 mol/L sodium chloride in the above buffer was used for elution of the bound proteoglycans at a flow rate of 0.5 mL/min. The fractions were monitored for protein (A_{280}) and proteoglycans (A_{678}), and those that were enriched in proteoglycans were pooled as individual peaks prior to further characterization according to molecular size, glycosaminoglycan, and amino acid composition.

Hydroxylapatite chromatography.—A portion of the proteoglycan fractions that eluted from the preparative Sepharose CL-6B column was dialyzed against 7 mol/L urea containing 10 mmol/L Tris-HCl and 10 mmol/L disodium hydrogen phosphate, pH 7.4. The sample was then loaded onto a hydroxylapatite column (1 cm × 7 cm) and was washed with the urea/phosphate buffer until the absorbance at 280 nm returned to baseline. The bound proteoglycans were then eluted from the column with a linear gradient of disodium hydrogen phosphate (from 10 mmol/L to 500 mmol/L) in the above buffer at a flow rate of 0.25 mL/min. The fractions were monitored for proteoglycans (A_{678}) and protein (A_{280}), and those that contained

the proteoglycans were pooled as various peaks prior to subsequent glycosaminoglycan analyses.

Analytical Sepharose CL-6B column chromatography.—The molecular size of proteoglycans derived from DEAE-Sephacel ion-exchange chromatography was assessed by molecular sieve chromatography from Sepharose CL-6B. The various proteoglycan populations were dialyzed against water and lyophilized, and 2-mg portions of dried material were solubilized in 4 mol/L guanidinium HCl. The samples were eluted from Sepharose CL-6B (0.8 × 100 cm) at a flow rate of 3 mL/h. Following elution, each proteoglycan population was pooled, dialyzed against water, and lyophilized. The molecular size of the glycosaminoglycan chains associated with these proteoglycans was then assessed after alkaline sodium borohydride reduction (Lohmander *et al.*, 1980). Lyophilized proteoglycans were treated with 0.05 mol/L sodium hydroxide in 1.0 mol/L sodium borohydride for 24 h at 45°C, dialyzed against water, and lyophilized prior to elution from the analytical Sepharose CL-6B columns.

Agarose polyacrylamide gel electrophoresis.—Aliquots of the proteoglycans isolated by either DEAE-Sephacel or hydroxylapatite chromatography were subjected to agarose/polyacrylamide gel electrophoresis by a combination of the conditions described by Heinegård *et al.* (1985) and Stanescu and Chaminade (1987). This was done by horizontal slab gels (14 × 11 × 0.4 cm) of 0.6% agarose and 1.2% acrylamide being poured and the gel being run submerged in 0.01 mol/L Tris-acetate buffer, pH 6.8. The samples to be analyzed (10 μL) were placed into pre-formed wells, and after 15 min of pre-migration at 40 mA, the current was increased to 65 mA. The whole procedure was carried out at 4°C in a water-cooled electrophoresis unit. The gels were fixed with methanol:acetic acid:water (50:7:43) for several hours, stained with Toluidine blue (0.2 g/L in 3% acetic acid), and then de-stained with 3% acetic acid. The gels were viewed either wet or dried on cellophane.

Amino acid analyses.—Samples (1 mg) of the proteoglycans obtained from DEAE-Sephacel ion-exchange chromatography were prepared for analysis of their amino acid content by being dissolved in 6 mol/L HCl. They were then frozen and thawed under vacuum for removal of any dissolved oxygen, sealed under vacuum, and hydrolyzed for 72 h at 110°C. Amino acid content of the samples was assessed with use of a Waters high-performance liquid-chromatography system with o-phthaldialdehyde post-column derivatization, according to the manufacturer's instructions. Assay of hydroxyproline and proline was carried out concurrently by means of hypochlorite oxidation (Waters Manual).

Results.

Immunolocalization of chondroitin sulfate and keratan sulfate in sections of alveolar bone from pigs, humans, and rabbits was achieved with use of the monoclonal antibodies 9-A-2 and 5-D-4. After pre-treatment of the sections with chondroitinase ACII, the presence of chondroitin sulfate was demonstrated in alveolar bone from all three species (Figs. 1–3). This component was localized both on the cell surface and in the pericellular environment within the lacunae housing the osteocytes. Moreover, the mineralized matrix of alveolar bone demonstrated a fine granular meshwork of positive staining for chondroitin sulfate. Although some non-specific staining was sometimes noted in the periodontal ligament, no antibody localization was seen in the bone matrix of control sections in which the antibody was reacted with sections incubated in the absence of chondroitinase ACII.

Positive staining for keratan sulfate in alveolar bone matrix was noted only in the sections obtained from rabbits (Figs. 4 and 5). The pattern of staining for keratan sulfate differed from that seen for chondroitin sulfate. The immediate periphery of the osteocyte lacunae showed positive staining, and the matrix of the mineralized tissue appeared to have a "lamellar" deposition of keratan sulfate. Control sections for all species demonstrated negative staining of the bone matrix.

For confirmation of the absence of keratan sulfate in human and pig alveolar bone, and confirmation of its presence in rabbit bone, whole portions of alveolar bone from these species were digested with papain, and the recovered glycosaminoglycans were subjected to cellulose acetate membrane electrophoresis (Fig. 6). As expected from the previous immunohistochemical data, keratan sulfate was identified only in the rabbit bone digests. Hyaluronate, dermatan sulfate, heparan sulfate, and chondroitin sulfate were identified and present in all species, and only slight species variation in glycosaminoglycan content was noted. Disregarding keratan sulfate in rabbit (which contributed approximately 20% to the total glycosaminoglycan content), chondroitin sulfate predominated (accounting for approximately 80% of the total), with dermatan sulfate (10%), heparan sulfate (5%), and hyaluronate (5%) accounting for the remainder of the glycosaminoglycan content. Confirmation of the identity of these molecular species was achieved by means of specific enzymatic and chemical eliminations (results not shown).

Biochemical analyses of the proteoglycans in the mineralized matrix of alveolar bone first required initial separation from other proteins. This was achieved by molecular sieve chromatography on Sepharose CL-6B, from which the proteoglycans (A_{678}) eluted in a polydisperse peak (K_{av} = 0.33) between two well-defined protein peaks (A_{280}) with K_{av} values of 0.1 and 0.5 (Fig. 7).

Additional purification of the proteoglycans was achieved following elution from DEAE-Sephacel (Fig. 8). Bound material eluted from the column and separated into two protein peaks and three Alcian-blue-positive peaks. The first two Alcian-blue-positive populations to elute from the ion-exchange resin co-eluted with the protein components. The Alcian-blue-positive material that eluted in the last peak did not coincide with a detectable protein peak. Glycosaminoglycan analysis of the material in the three Alcian-blue-positive peaks was achieved by cellulose acetate membrane electrophoresis (Fig. 8, inset). In all three peaks, heparan sulfate, dermatan sulfate, and chondroitin sulfate were identified, but their relative proportions varied. Peak 1 contained principally chondroitin sulfate (95%); peak 2 contained the most glycosaminoglycan species, with dermatan sulfate (10%), heparan sulfate (5%), hyaluronate (15%), and chondroitin sulfate (75%) being detected; peak 3 contained only dermatan sulfate (10%) and chondroitin sulfate (90%).

Elution with a salt gradient of proteoglycans adsorbed to hydroxylapatite was also investigated as a means of isolation of alveolar-bone proteoglycans (Fig. 9). As for the ion-exchange chromatography, some fractionation of the molecules was achieved. The proteoglycans eluted from this column in three major peaks, while the material identified as protein (A_{280}) eluted in one early sharp peak and a later broad and polydisperse peak. Glycosaminoglycan analysis of the material in these peaks was also performed (Fig. 9, inset). In this case, only dermatan sulfate and chondroitin sulfate were observed, with an apparent loss of material (identifiable as hyaluronate or heparan sulfate) resulting from poor recoveries. Peak 2 and peak 3 contained principally chondroitin sulfate, while peak 1 contained both dermatan sulfate and chondroitin sulfate.

On the basis of the better quantitative recovery of material

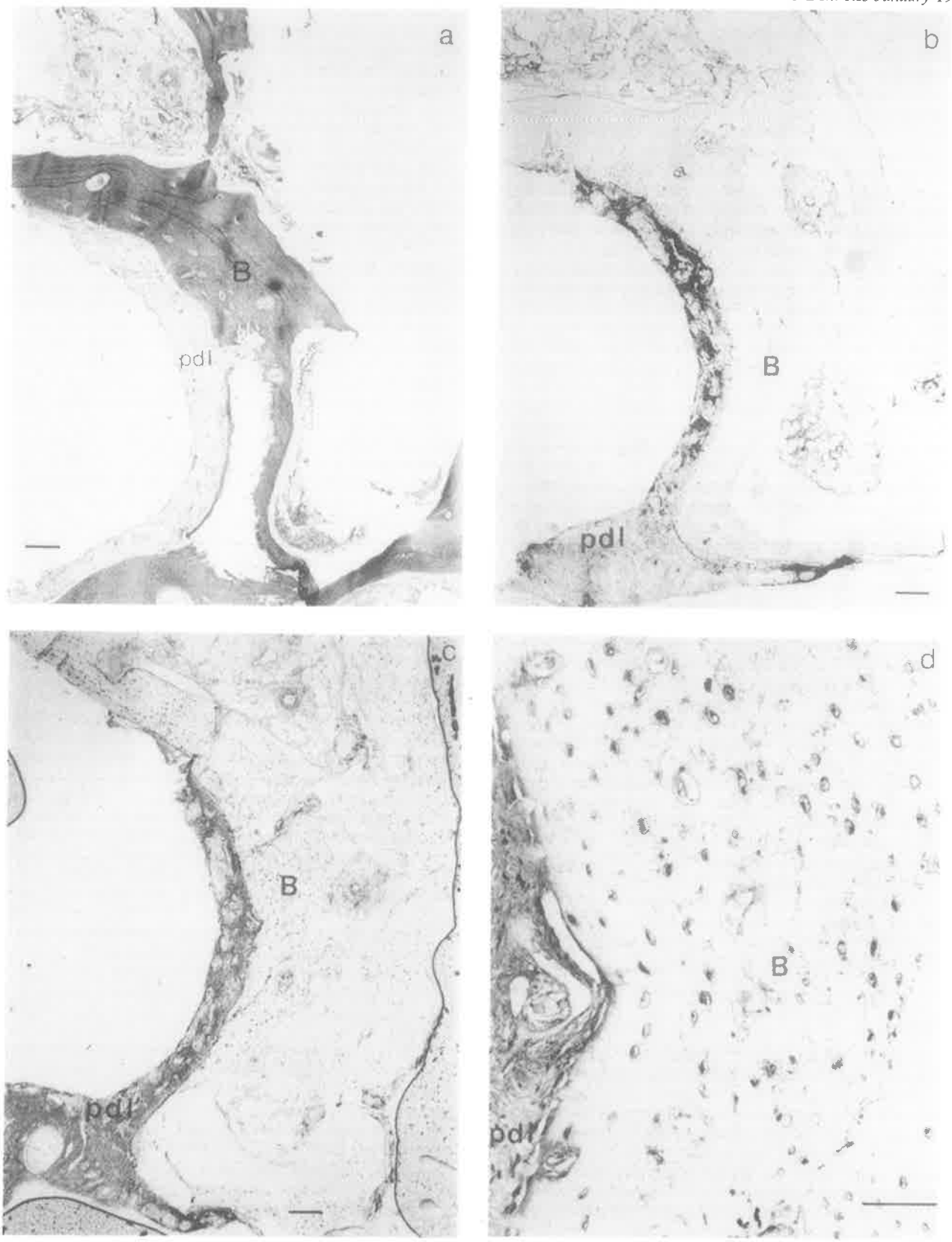

Fig. 1—Immunohistochemical localization of chondroitin sulfate in sections of human alveolar bone. (a) Section stained with hematoxylin and eosin; (b) control section incubated with antibody 9-A-2 in the absence of pre-treatment with chondroitinase ACII; (c) section incubated with antibody 9-A-2 after digestion with chondroitinase ACII; (d) section incubated with antibody 9-A-2 after digestion with chondroitinase ACII. Bar = 100 μm. Abbreviations: B, bone; pdl, periodontal ligament.

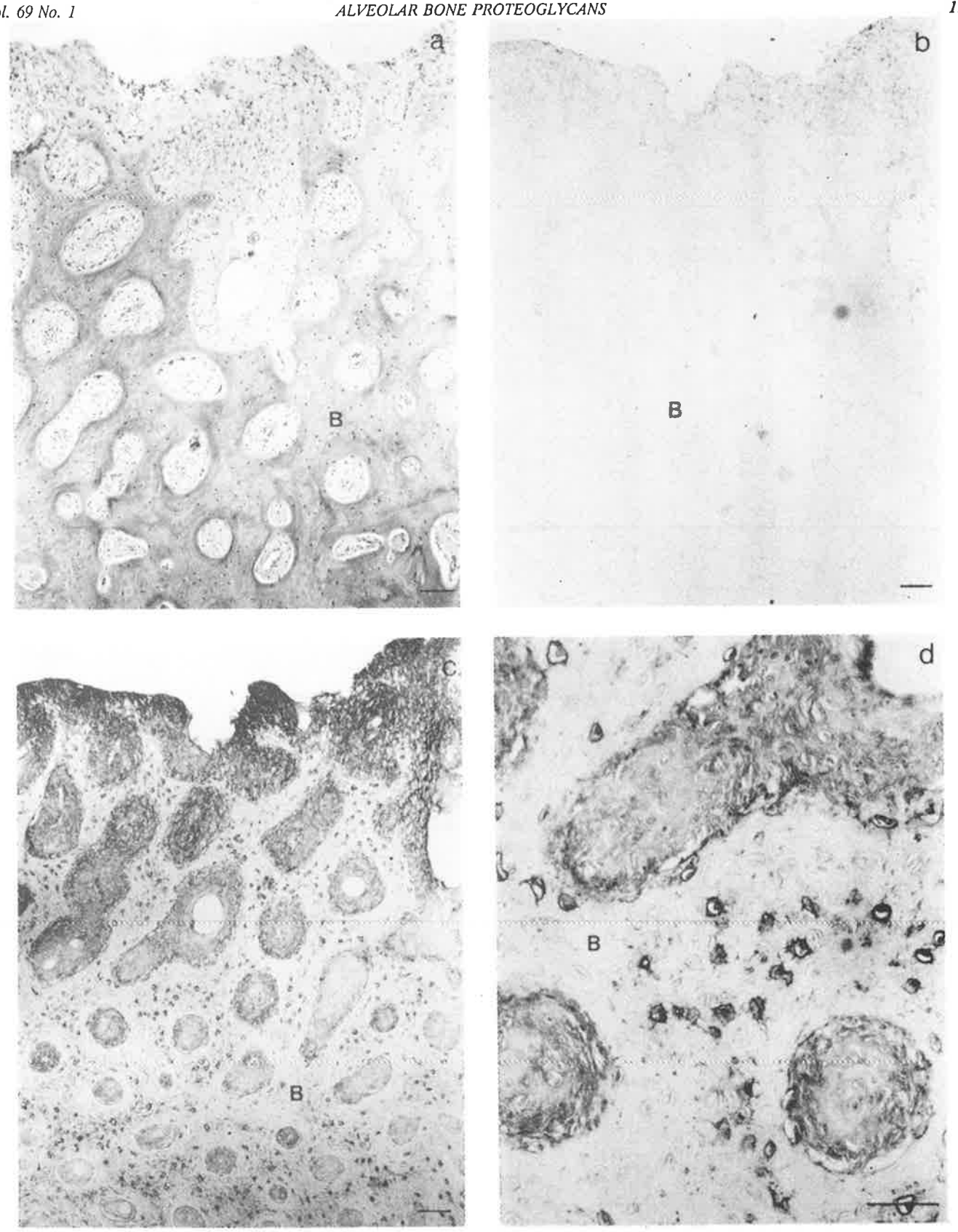

Fig. 2—Immunohistochemical localization of chondroitin sulfate in porcine alveolar bone. (a) Section stained with hematoxylin and eosin; (b) control section incubated with antibody 9-A-2 in the absence of pre-treatment with chondroitinase ACII; (c) section incubated with antibody 9-A-2 after digestion with chondroitinase ACII; (d) section incubated with antibody 9-A-2 after digestion with chondroitinase ACII. Bar = 100 μm. Abbreviation: B, bone.

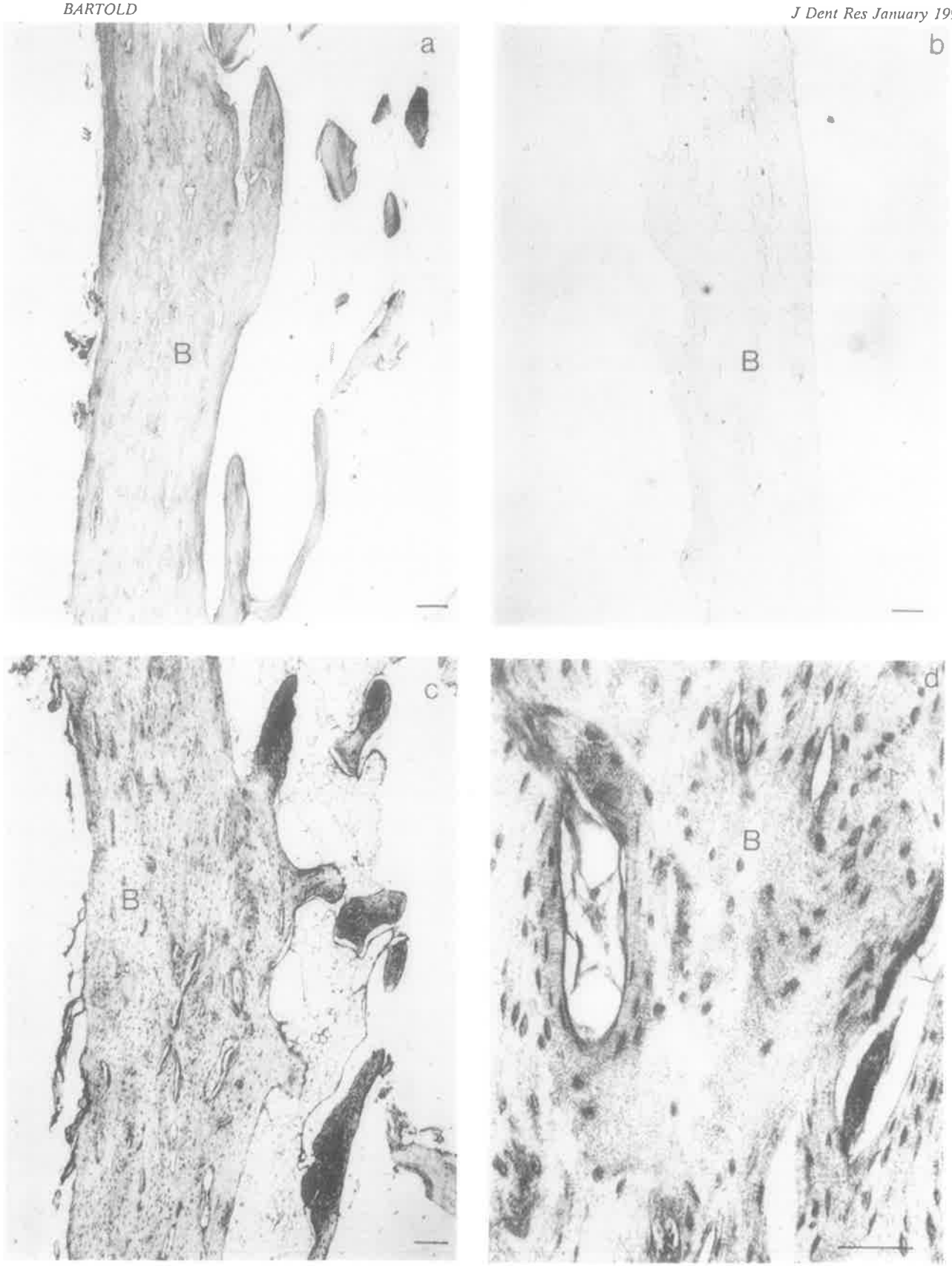

Fig. 3—Immunohistochemical localization of chondroitin sulfate in rabbit alveolar bone. (a) Section stained with hematoxylin and eosin; (b) control section incubated with antibody 9-A-2 in the absence of pre-treatment with chondroitinase ACII; (c) section incubated with antibody 9-A-2 after digestion with chondroitinase ACII; (d) section incubated with antibody 9-A-2 after digestion with chondroitinase ACII. Bar = 100 μm. Abbreviation: B, bone.

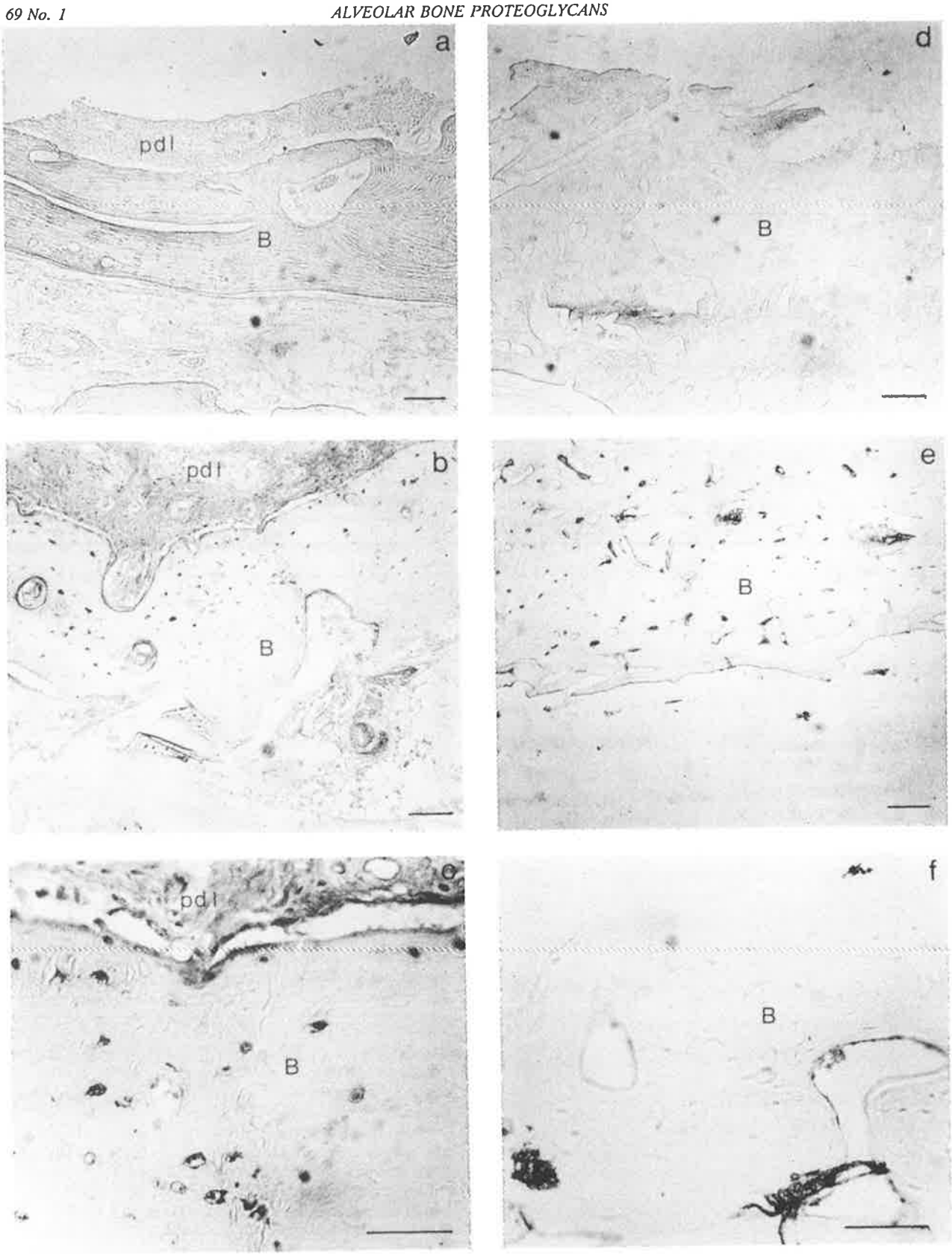

Fig. 4—Immunohistochemical localization of keratan sulfate in human and pig alveolar bone. (a) Section of human alveolar bone incubated in the antibody of 5-D-4; (b) section of human alveolar bone incubated with antibody 5-D-4; (c) section of human alveolar bone incubated with antibody 5-D-4; (d) section of pig alveolar bone incubated in the absence of antibody 5-D-4; (e) section of pig alveolar bone incubated with antibody 5-D-4; (f) section of pig alveolar bone incubated with antibody 5-D-4. Bar = 100 μm. Abbreviations: B, bone; pdl, periodontal ligament.

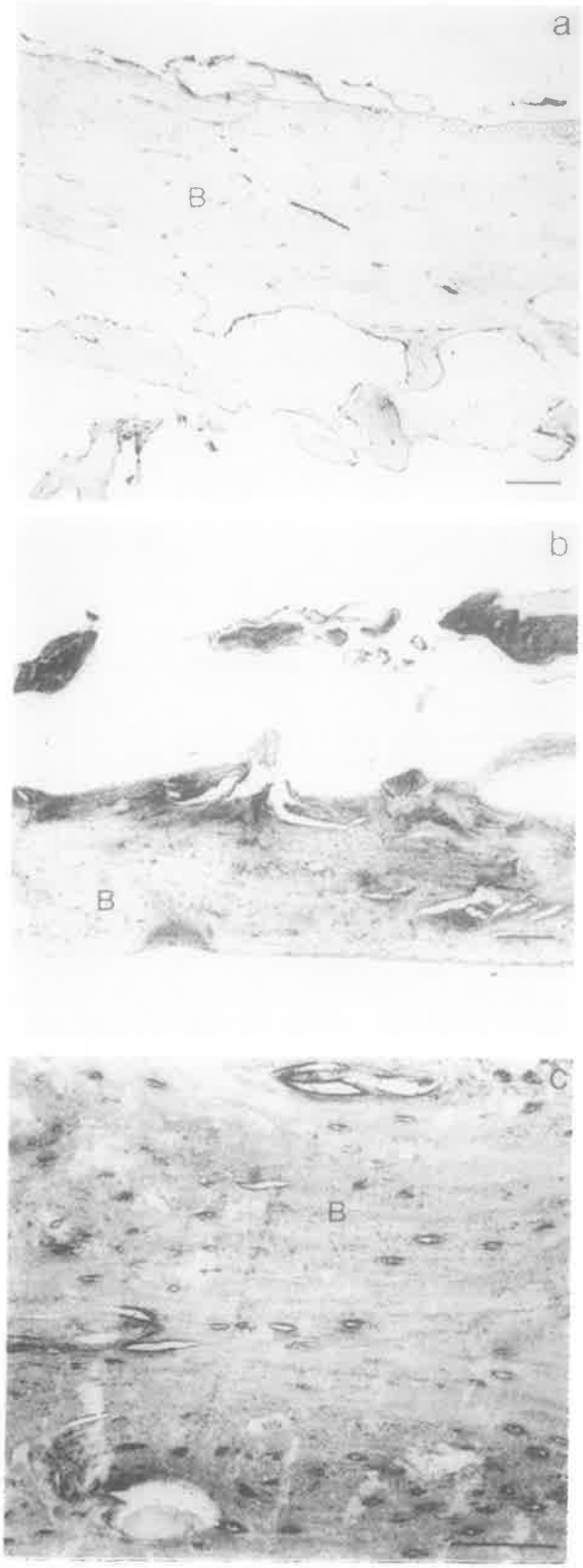

Fig. 5—Immunohistochemical localization of keratan sulfate in rabbit alveolar bone. (a) Section of rabbit alveolar bone incubated in the absence of antibody 5-D-4; (b) section of rabbit alveolar bone incubated with antibody 5-D-4; (c) section of rabbit alveolar bone incubated with antibody 5-D-4. Bar = 100 μm. Abbreviation: B, bone.

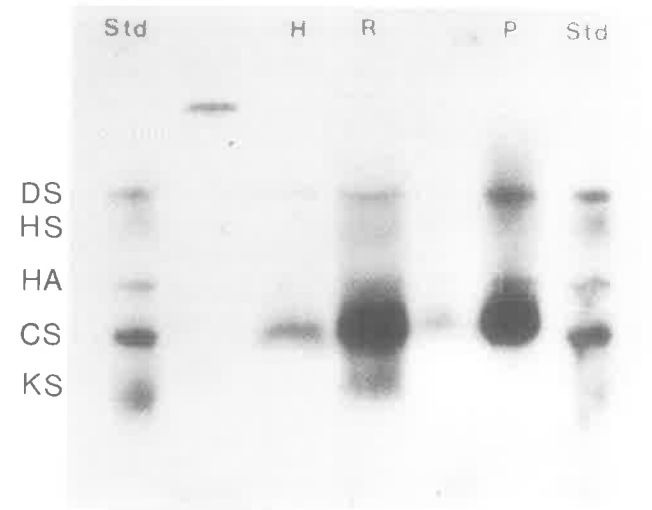

Fig. 6—Cellulose acetate membrane electrophoresis of glycosaminoglycans isolated from human, rabbit, and pig alveolar bone. (Abbreviations: Std, standard glycosaminoglycans; H, human glycosaminoglycans; R, rabbit glycosaminoglycans; P, pig glycosaminoglycans; HA, hyaluronate; CS, chondroitin sulfate; HS, heparan sulfate; DS, dermatan sulfate.)

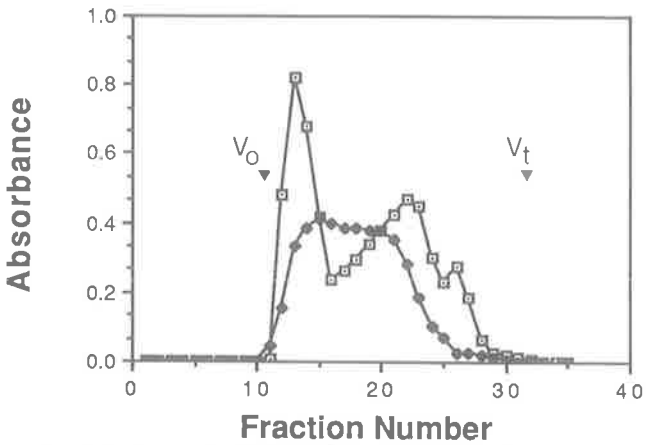

Fig. 7—Sepharose CL-6B chromatography of crude alveolar-bone extract. Samples were solubilized in 7 mol/L urea in 50 mmol/L Tris HCl, pH 8.0, containing proteinase inhibitors and eluted from Sepharose CL-6B with the same buffer. The void volume (V_o) and the total volume (V_t) were determined with ^3H DNA and $Na_2{}^{35}SO_4$, respectively. The fractions were assayed for protein (A_{280}; □—□) and proteoglycans (A_{678}; ●—●).

from ion-exchange chromatography, the remainder of the analyses were performed on proteoglycans isolated from pig alveolar bone after DEAE-Sephacel ion-exchange chromatography.

For assessment of the molecular size of the intact proteoglycans, as well as their constituent glycosaminoglycans, material eluted from DEAE-Sephacel in peaks 1, 2, and 3 was chromatographed on Sepharose CL-6B before and after alkaline sodium borohydride reduction (Fig. 10). Peak 1 and peak 2 eluted from this gel with K_{av} values of 0.4, while peak 3 had a K_{av} value of 0.5. Following degradation with alkaline borohydride, only material from peaks 1 and 2 demonstrated a shift in K_{av} (from 0.4 to 0.5), while material from peak 3 did not change its elution position (K_{av} = 0.5).

Additional analyses of the proteoglycans were achieved by agarose polyacrylamide gel electrophoresis (Fig. 11). These

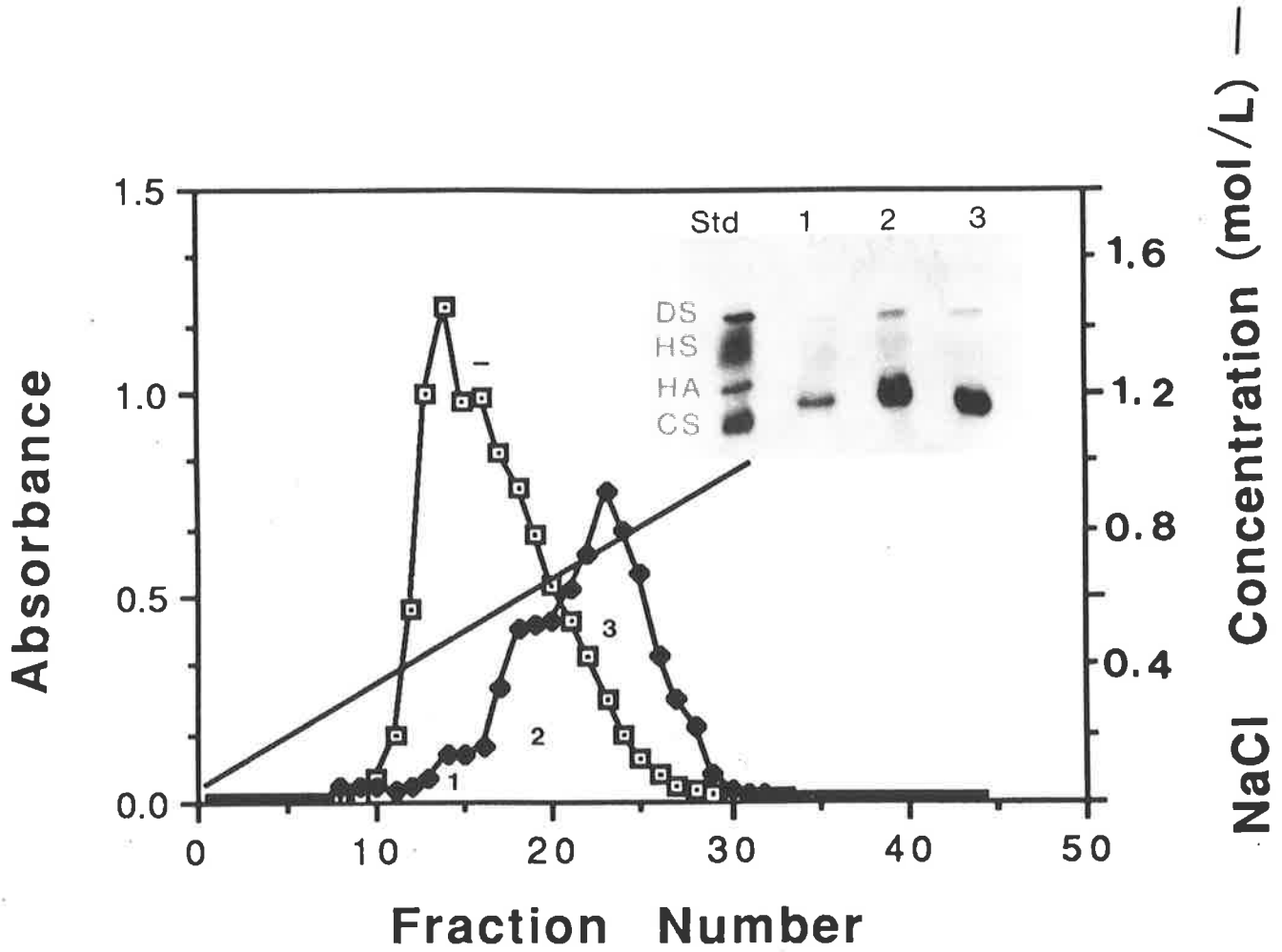

Fig. 8—(a) DEAE-Sephacel ion-exchange chromatography of alveolar-bone extracts partially purified by Sepharose CL-6B molecular-sieve chromatography. Alcian-blue-positive material that eluted from Sepharose CL-6B was pooled and applied onto DEAE-Sephacel. Bound material was eluted with a linear 0–1.5 mol/L NaCl gradient in 7 mol/L containing 50 mmol/L Tris HCl and proteinase inhibitors, pH 8.0. The fractions were monitored for protein (A_{280}; □—□) and proteoglycans (A_{678}; ●—●). Inset: Cellulose-acetate-membrane electrophoresis of glycosaminoglycans associated with material eluted from DEAE-Sephacel in peak 1 (lane 1), peak 2 (lane 2), and peak 3 (lane 3). (Abbreviations: Std, standard glycosaminoglycans; DS, dermatan sulfate; HS, heparan sulfate; HA, hyaluronate; CS, chondroitin sulfate.)

studies indicated that the proteoglycans that eluted from DEAE-Sephacel had different electrophoretic mobilities. As expected from the above gel filtration data, material from peaks 1 and 2 had similar mobilities, while material from peak 3 moved faster and co-migrated with the chondroitin sulfate standard glycosaminoglycan preparation.

Amino acid analyses were performed on material eluted from DEAE-Sephacel (Table). In most cases, only material from peaks 1 and 2 had sufficient protein present to allow analysis. However, by pooling several preparations of peak 3, we obtained enough protein to conduct an amino acid analysis on this material. Aspartic acid, glutamine, and serine were the major contributors in all three peaks, with aspartic acid and glutamine being present in significantly higher proportions in peaks 2 and 3, which had nearly identical profiles. Indeed, given the similar composition between peaks 2 and 3, it would appear that the amino acids detected in peak 3 represent a "carry-over" from peak 2 resulting from incomplete separation on DEAE-Sephacel. No residues of methionine, cysteine, hydroxylysine, or hydroxyproline were detected.

Discussion.

Although the presence of proteoglycans in bone has been recognized for many years, it has not been until the development of non-denaturing extraction procedures and appropriate proteinase inhibitors that these macromolecules could be studied confidently. In the present investigation, proteoglycans in the mineralized matrix of alveolar bone from humans, pigs, and rabbits have been studied.

The glycosaminoglycan composition was determined both biochemically and immunohistochemically. In accordance with studies on other types of bones (Engfeldt and Hjerpe, 1976; Prince *et al.*, 1983), chondroitin sulfate was found to be the predominant glycosaminoglycan species present in alveolar bone. In addition to chondroitin sulfate, minor amounts of hyaluronate, dermatan sulfate, and heparan sulfate were also detected. In an early study of the proteoglycans in rabbit alveolar bone, no keratan sulfate was detected (Oosawa, 1986). However, the data obtained in the present report indicate that rabbit bone differs from pig and human bone due to the presence of keratan sulfate, as well as the other glycosaminoglycans mentioned

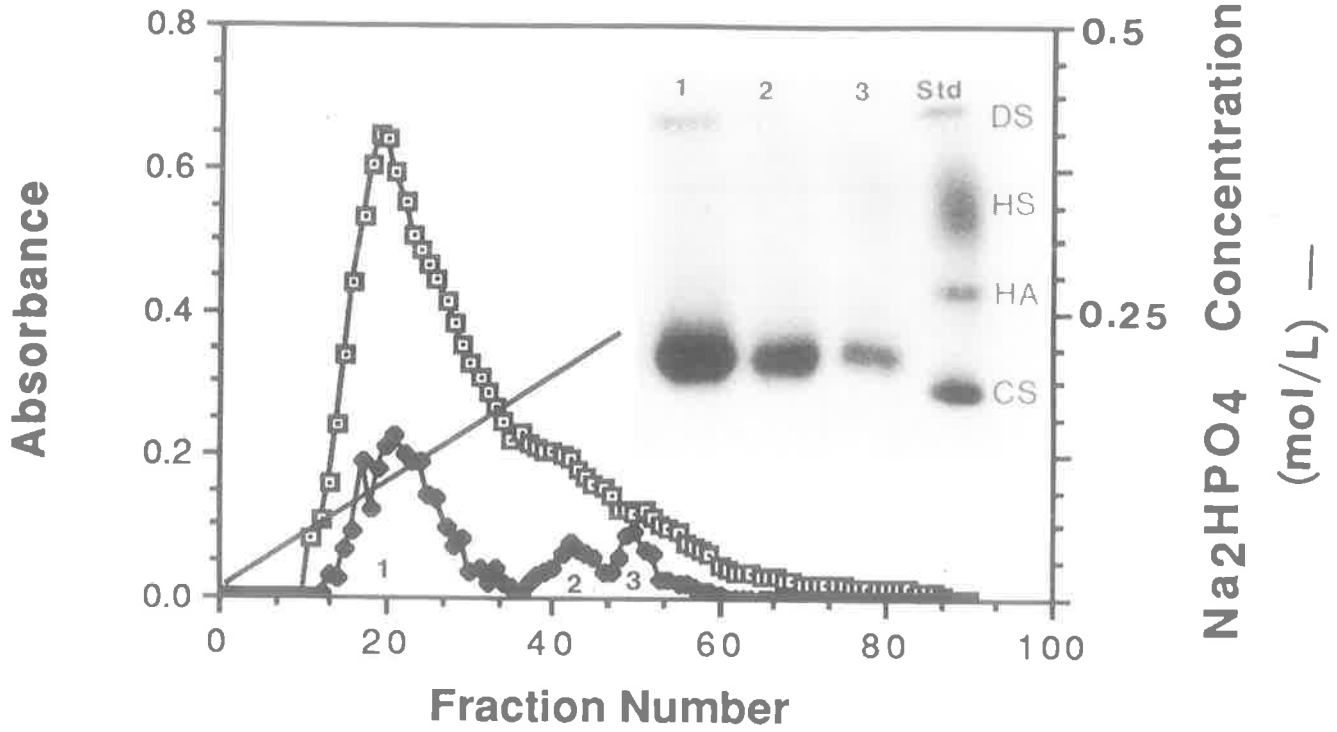

Fig. 9—(a) Hydroxylapatite chromatography of alveolar-bone extracts partially purified by Sepharose CL-6B chromatography. Alcian-blue-positive material that was eluted from Sepharose CL-6B was pooled, dialyzed into 7 mol/L urea containing 10 mmol/L Tris HCl and 10 mmol/L Na_2HPO_4, and applied onto hydroxylapatite. The bound material was eluted with a linear 10 mmol/L-500 mmol/L Na_2HPO_4 in urea buffer. The fractions were monitored for protein (A_{280}; □—□) and proteoglycans (A_{678}; •—•). Inset: Cellulose-acetate-membrane electrophoresis of glycosaminoglycans associated with material eluted from hydroxylapatite in peak 1 (lane 1), peak 2 (lane 2), and peak 3 (lane 3). (Abbreviations: Std, standard glycosaminoglycans; DS, dermatan sulfate; HS, heparan sulfate; HA, hyaluronate; CS, chondroitin sulfate.)

above. The findings are in agreement with those recently reported by Waddington *et al.* (1988, 1989). Although the significance of the presence of keratan sulfate is unclear, it appears certain that rabbit bone differs significantly from other mammalian bones with respect to the presence of proteoglycans containing keratan sulfate chains (Masubuchi *et al.*, 1975; Diamond *et al.*, 1982). A possible explanation for its occurrence may lie in different post-translational modifications of bone matrix macromolecules in different species, since the core protein of bone sialoprotein II appears to be identical to the core protein of rabbit bone keratan sulfate proteoglycan (Kinne and Fisher, 1987).

Due to the limited amount of human bone available for analysis, and to the presence of keratan sulfate in rabbit bone (and apparent absence in pig and human bone), biochemical studies on proteoglycans associated with the mineralized matrix of alveolar bone were carried out on specimens derived from pigs. Although care must be taken when comparisons between different species are made, it is likely that pig oral tissues resemble human tissues more closely than do those of rabbits or other experimental animals (Weaver *et al.*, 1962; Jump and Weaver, 1966). Thus, the pig may be a useful model for further analytical investigations into the macromolecular composition of alveolar bone.

In the present study, three populations of sulfated proteoglycans were identified by their affinity for Alcian blue. However, on the basis of alkali-sensitive protein associated with glycosaminoglycan chains, only two of these populations could be classified as proteoglycans. The third did not contain any appreciable alkali-labile material and appeared to be present as glycosaminoglycan chains only. In light of the routine use of proteinase inhibitors and non-denaturing extraction conditions, the presence of free glycosaminoglycan chains in this tissue is interesting. It is generally recognized that, with the possible exception of hyaluronate, the sulfated glycosaminoglycans are usually covalently bound to protein in the form of proteoglycans (Hascall and Hascall, 1981). Nonetheless, recent evidence has indicated that free glycosaminoglycans may form part of the extracellular matrix of bone and cementum (Franzén and Heinegård, 1984a,b; Fisher, 1985; Bartold *et al.*, 1989). Their occurrence is believed to arise because of the large amount of proteinase activity in bone, which is capable of causing *in situ* degradation of proteins within the mineralized matrix. Thus, prior cleavage of the core proteins of embedded proteoglycans could lead to the appearance of free glycosaminoglycans in extracts of these tissues. Multiple proteoglycan populations within the mineralized matrix of bone have been reported previously (Franzén and Heinegård, 1984a,b; Fisher *et al.*, 1983; Goldberg *et al.*, 1988). The size of alveolar bone proteoglycans appears to be comparable with that of other bone proteoglycans in that they are very small and most likely contain either one or two glycosaminoglycan chains. Glycosaminoglycan chain size of alveolar bone proteoglycans was established at an approximate M_r of 25,000, and compares with an approximate M_r of 40,000 for the glycosaminoglycans of proteoglycans in other bones (Fisher *et al.*, 1983). Since much of the previously published data have been obtained from fetal or developing bones (Fisher *et al.*, 1983; Goldberg *et al.*, 1988) or tissue culture (Hunter *et al.*, 1983; Beresford *et al.*, 1987; Fisher *et al.*, 1987), it is possible that such differences reflect changes in glycosaminoglycan chain size during maturation and turnover of bone (Franzén and Heinegård, 1984a).

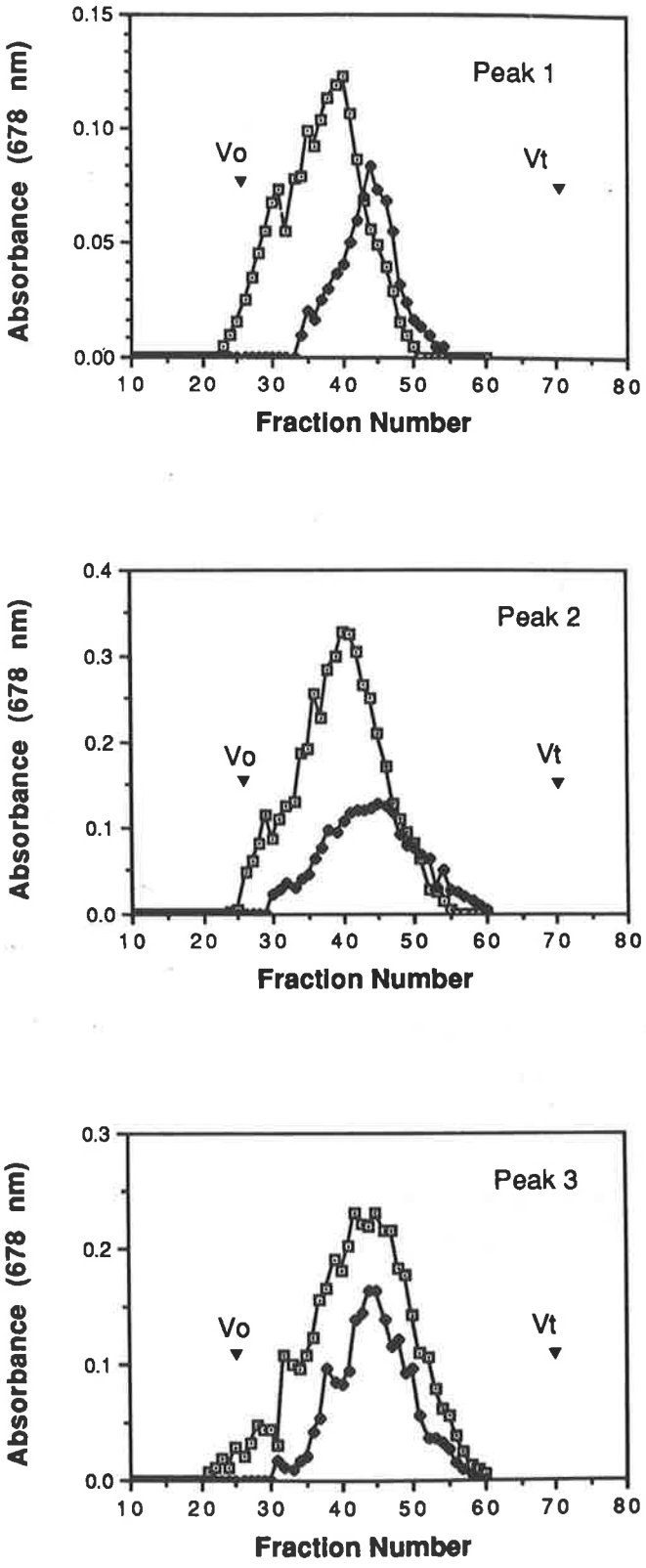

Fig. 10—Sepharose-CL-6B chromatography of proteoglycans isolated from DEAE-Sephacel. Peaks 1, 2, and 3 from DEAE-Sephacel ion-exchange chromatography were eluted from Sepharose CL-6B before (□—□) and after (●—●) treatment with alkaline sodium borohydride. The fractions were monitored for proteoglycans (A_{678}). The V_o and V_t were determined as described in Fig. 1.

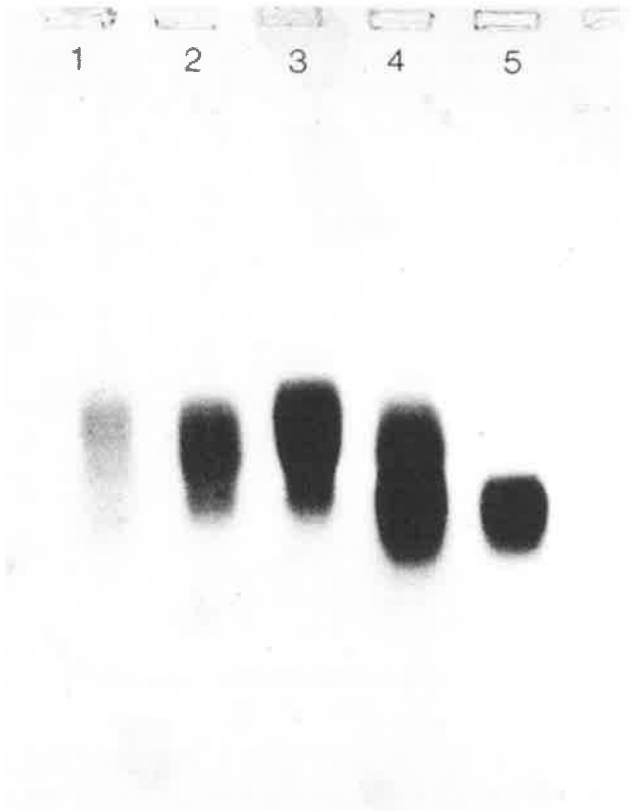

Fig. 11—Agarose polyacrylamide gel electrophoresis of alveolar-bone proteoglycans. Samples obtained from DEAE-Sephacel ion-exchange chromatography (DEAE-peaks 1, 2, and 3) were electrophoresed on submerged slab gels of 0.6% agarose and 1.2% polyacrylamide. The proteoglycans were visualized by being stained with Toluidine blue. Lane 1, gingival proteoglycan; Lane 2, DEAE-Sephacel peak 1; Lane 3, DEAE-Sephacel peak 2; Lane 4, DEAE-Sephacel peak 3; Lane 5, standard chondroitin sulfate glycosaminoglycan.

The proteoglycans isolated in the present study differed in their amino acid composition, whereby the material that eluted first from DEAE-Sephacel was rich in glutamine and aspartic acid residues, while the more anionic proteoglycans contained elevated proportions of glycine and leucine. Similar differences in bone proteoglycan amino acid composition have been noted by Fisher *et al.* (1983). The amino acid composition of alveolar bone proteoglycans appeared to differ from those derived from the soft tissues of the periodontium (gingiva and periodontal ligament). In these tissues the proteoglycans are relatively poor in serine and glutamic acid residues (Pearson and Gibson, 1982; Bartold *et al.*, 1983). Whether these represent significant differences between bone and soft-tissue proteoglycans remains to be established, because, although some immunological evidence suggests that the core proteins of the small proteoglycans of bone differ from their soft-tissue counterparts (Fisher *et al.*, 1983), recent data indicate that the deduced protein sequences of bone and proteoglycans from other tissues show some degree of homology (Fisher *et al.*, 1989).

The present findings are of significance in light of current interest in the presence of glycosaminoglycans in sulcular fluid (Last *et al.*, 1985). The presence of keratan sulfate in rabbit bone has been reported to be a feature unique to alveolar bone (Waddington *et al.*, 1988). However, it is now evident, from the present study and another recent report (Waddington *et al.*,

TABLE
AMINO ACID COMPOSITION OF PROTEOGLYCANS*

	Bone #1¶	Bone #2¶	Bone #3¶	Gingiva§	PDL‡
Cysteic Acid	14	8	7	ND	ND
Aspartic Acid	107	152	152	113	125
Threonine	41	64	66	52	39
Serine	103	109	103	66	74
Glutamine	169	232	252	129	108
Proline	61	54	52	86	67
Glycine	96	67	90	120	80
Alanine	61	54	48	77	49
Cysteine	2	0	0	ND	ND
Valine	42	44	40	48	58
Methionine	0	0	0	Trace	9
Isoleucine	32	15	15	43	57
Leucine	71	52	42	93	123
Tyrosine	14	13	21	20	29
Phenylalanine	24	14	11	27	33
Histidine	91	45	43	11	75
Hydroxylysine	0	0	0	ND	ND
Lysine	40	45	39	63	27
Arginine	32	32	23	52	31
Hydroxyproline	0	0	0	ND	ND

*Values expressed as residues *per* 1000 residues.
¶Refers to peaks 1, 2, and 3 obtained from DEAE-Sephacel ion-exchange chromatography.
§Values taken from results published by Bartold *et al.* (1983).
‡Values taken from results published by Pearson and Gibson (1982).
ND = Not Determined.

1989), that this is a property peculiar to rabbit bone. Thus, keratan sulfate will not be a useful marker of alveolar bone resorption in humans. Nevertheless, the core protein of bone sialoprotein II (which is ubiquitous and specific to bone) is the same as the core protein of rabbit bone keratan sulfate proteoglycan. Therefore, the present observation—that the core proteins of bone proteoglycans differ from those in the surrounding soft tissues—indicates that the core proteins of bone proteoglycans may supply the information necessary for provision of a bone-specific marker.

Although the association between proteoglycans and mineralization is far from clear, a close association between proteoglycans and mineralization process has been suggested (Fisher, 1985). It appears that following synthesis by the osteoblast, proteoglycans rapidly diffuse to the mineralized zone, where they become embedded in the mineralized compartment. During the maturation process, the protein core may be degraded, leaving behind glycosaminoglycan chains embedded in the matrix. Therefore, the role of these macromolecules in the formation, remodeling, and destruction of alveolar bone during both normal function and pathological processes cannot be discounted and should not be overlooked.

Acknowledgments.

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VII. PATHOLOGICAL CHANGES IN PROTEOGLYCANS OF HUMAN GINGIVA

Paper 9. Bartold, P.M. and Page, R.C.
The effect of chronic inflammation on gingival connective tissue proteoglycans and hyaluronic acid.
Journal of Oral Pathology **15:** 367-374. 1986.

Paper 10. Bartold, P.M., Wiebkin, O.W. and Thonard, J.C.
The effect of oxygen-derived free radicals on gingival proteoglycans and hyaluronic acid.
Journal of Periodontal Research **19:** 390-400, 1984

Paper 11. Bartold, P.M. and Page, R.C.
Proteoglycans synthesized by cultured fibroblasts derived from normal and inflamed human gingiva.
In Vitro Cellular and Developmental Biology **22:** 407-417, 1986.

Paper 12. Bartold, P.M. and Page, R.C.
Hyaluronic acid synthesized by fibroblasts from normal and chronically inflamed gingiva.
Collagen and Related Research **6:** 365-377, 1986

Paper 13. Bartold, P.M.
Distribution of chondroitin sulfate and dermatan sulfate in normal and inflamed human gingiva.
Journal of Dental Research **71:** 1587-1593, 1992.

Paper 14. Bartold, P.M.
Biochemical and immunohistochemical studies on overgrown gingival tissues associated with mannosidosis.
Virchows Archiv B **62:** 391-399; 1992.

The papers presented in this section focus on the changes which occur to the proteoglycan and hyaluronan content of inflamed human gingiva. These were a logical extension of earlier work which had focused predominantly on the features of the normal, healthy gingival tissues. Several approaches were taken for these studies which included an analysis of extracts of inflamed tissues, the use of cells derived from inflamed tissues, as well as an assessment of tissues derived from an individual with a genetic defect affecting proteoglycan synthesis.

Paper 9 describes the nature of proteoglycans and hyaluronan extracted from inflamed human gingivae. These studies showed that the total amount of proteoglycan extracted from inflamed tissues did not differ substantially from that extracted from normal tissues. For some time this was a most perplexing problem, and it was not until the studies on proteoglycan synthesis by lymphocytes and polymorphonuclear leukocytes (see section VI), and studies on the effects of inflammatory mediators and cytokines on proteoglycan synthesis (see section VII) were carried out, that these findings could be fully appreciated. Although no quantitative changes were identified, some qualitative changes in the proteoglycan and hyaluronan content of inflamed tissues were noted. Hyaluronan was found to be depolymerized in inflamed tissue and significant alterations to proteoglycan structure was noted. However, no evidence of sulfated glycosaminoglycan degradation could be found.

Paper 10 describes an *in vitro* study on hyaluronan concerning its depolymerization by oxygen derived free radicals. This was the first paper in the periodontal literature to consider the role that free radicals might play in the process of matrix destruction. It was found that oxygen derived free radicals caused efficient and rapid depolymerization of hyaluronan. It was proposed that this might be one mechanism by which tissue destruction could occur in periodontitis.

Papers 11 and 12 formed a two part study using gingival fibroblasts isolated from normal and inflamed human gingiva. These studies demonstrated that cells isolated from inflamed tissues exhibited different phenotypic expression of matrix synthesis. Hyaluronan synthesis was elevated as was the synthesis of dermatan sulfate proteoglycans. These were the first studies to consider fibroblast heterogeneity in terms of proteoglycan and hyaluronan synthesis, and demonstrated that chronically inflamed gingival tissues contained fibroblasts which manifested a heritable phenotype differing from fibroblasts isolated from normal gingival connective tissues.

Paper 13 provided further evidence of regional variation of proteoglycan distribution in both normal and inflamed gingival tissues. Using immunohistochemical techniques, chondroitin sulfate and proteoglycan sulfate proteoglycans were located within the gingival tissues. Of particular note was the localization of dermatan sulfate to the immediate subepithelial layers of the gingival connective tissue and the very significant increase in staining for chondroitin sulfate at the sites of inflammatory cell infiltration. This latter finding further corroborated the earlier findings concerning proteoglycan synthesis by lymphocytes. These findings also served to confirm that there is no loss of material identifiable as proteoglycan in inflamed tissues.

Paper 14 describes a study using tissues derived from a patient suffering from mannosidosis. This is a rare lysosomal enzyme storage disease in which there is an accumulation of mannose-containing oligosaccharides within various cells. The presentation of a patient with gingival overgrowth associated with this condition provided a unique chance to investigate the effect of a defect in mannose metabolism on proteoglycan synthesis. Gingival biopsies were studied immunohistochemically and cultures from the resident fibroblasts were also established. A particular histological feature of the excised gingivae was the presence of large and highly vacuolated lymphocytes which stained intensely for chondroitin sulfate. This observation provided further support to the importance of proteoglycan synthesis by lymphocytes since these cells, due to the retention of large amounts of mannose containing carbohydrates, have a compromised function which was surmised to be partly responsible for the events associated with gingival overgrowth. The fibroblasts isolated from this patient's gingival tissues showed no discernible differences with respect to growth and matrix production compared to an age- and sex-matched control cell strain.

Paper 9.

Bartold, P.M. and Page, R.C.
The effect of chronic inflammation on gingival connective tissue proteoglycans and hyaluronic acid.
Journal of Oral Pathology **15:** 367-374. 1986.

Candidate's Contribution to this paper: 95%

P.M. Bartold's role in this study was:
Design of the experiments
Execution of all experimental work
Writing of the paper

R.C. Page's role was:
Provision of research funds
Writing of the paper

The effect of chronic inflammation on gingival connective tissue proteoglycans and hyaluronic acid

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Proteoglycans have been isolated and analysed from extracts of normal and chronically inflamed human gingiva in order to determine the effects of chronic inflammation on these important soft connective tissue extracellular macromolecules. The uronic acid content of glycosaminoglycans isolated by papain digestion of normal and inflamed gingiva did not differ significantly. Likewise, electrophoretic analysis revealed that the content of hyaluronic acid, heparan sulfate, dermatan sulfate and chondroitin sulfate was similar. The sulfated glycosaminoglycans from both sources eluted from a Sepharose Cl-6B column with a Kav of 0.45 (approximate M, 25,000). However, hyaluronic acid from normal gingiva was predominantly of a large size eluting in the void volume of a Sepharose. CL-6B column, while that isolated from inflamed tissue was mostly a small molecular weight species which eluted in the included volume of a Sepharose CL-6B column. Using dissociative conditions, intact proteoglycans could be more readily extracted from inflamed tissues (90% of the total tissue uronic acid) than from normal tissues where only 80% of the total tissue uronic acid was extractable. Even though DEAE-Sephacel ion-exchange chromatography revealed no differences in charge between normal and inflamed gingival proteoglycans, Sepharose CL-4B chromatography revealed more molecular size polydispersity in samples from inflamed tissue than from normal tissue. Taken together, these results indicate that while hyaluronic acid is depolymerized in inflamed tissue, no evidence of sulfated glycosaminoglycan degradation was found. Therefore, the most likely cause for disruption to the molecular integrity of the proteoglycans is via proteolytic alteration to the proteoglycan core protein.

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Chronic inflammation of soft connective tissues is characterized by infiltration of inflammatory cells, tissue destruction and scarring. Associated with the tissue destruction is a concomitant loss of structural integrity and impairment of normal physiological function of the extracellular matrix. Therefore, it has been of interest to study the specific effects of inflammation on various components of the extracellular matrix to determine what particular molecular alterations occur and how these might arise.

Gingiva, a tissue which possesses the morphological features of most soft connective tissues, has served as a useful model for studying inflammation because it manifests a naturally occurring progressive chronic inflammatory lesion (1). Treatment of this condition often requires surgical removal of the affected tissue, and, therefore, tissue specimens are readily available for study.

Recently, proteoglycans, which are the major extracellular non-fibrous macromolecules present in gingiva (and indeed all connective tissue), have received attention as being important components of this tissue (2–7). Indeed, these complex macromolecules are no longer considered to be an inert cementing substance, but rather are thought to be responsible for maintaining a wide variety of tissue and cellular functions (8). Therefore, an assessment of their types and structures in both health and disease is now recognized as being essential for understanding the mechanisms associated with inflammation-mediated tissue destruction (9).

Since there is an abundance of metabolic and catabolic activity associated with inflammation, one would expect breakdown of proteoglycans to occur as is seen for the collagens (10). Indeed, many enzymes capable of proteoglycan depolymerization under optimal conditions are released by inflam-

matory cells and fibroblasts at sites of inflammation (11–15). In addition, oxygen-derived free radicals produced by polymorphonuclear leukocytes in response to inflammatory stimuli are also capable of initiating matrix destruction (16–19).

This expected loss and destruction of proteoglycans at the inflammatory sites has been reported consistently in studies of rheumatoid arthritis (20–22). Studies on the proteoglycans of normal and diseased cartilage are abundant and have provided most of the current data concerning the effects of inflammation on proteoglycans. However, cartilage is structurally very different from soft connective tissues, and it is likely that the sequence of events associated with inflammation and their sequelae between soft and hard connective tissues will be different. Despite this, there are few reports dealing with the effects of soft connective tissue inflammation on proteoglycans (23). Therefore, using gingiva as a model, we have studied the effect of chronic inflammation on proteoglycans in soft connective tissues. The results demonstrated inflammation-associated disruption to both proteoglycans and hyaluronic acid; these may be partially responsible for the loss of tissue integrity seen in most inflammatory lesions.

Material and methods

Alcian blue 8GX, 6-amino hexanoic acid, benzamidine HCl, cysteine HCl,ethylenediaminetetraacetic acid (EDTA) disodium salt, D-glucuronic acid, guanidine HCl, N-ethylmaleimide, phenylmethylsulfonylflouride, papain (EC 3.4.22.2), sodium acetate and magnesium chloride were all purchased from Sigma Chemical Co., St. Louis, MO. Dimethylsulfoxide was from Mallinkrodt Inc., Paris KY. Glycosaminoglycan standards (hyaluronic acid, heparan sulfate dermatan sulfate and chondroitin-4-sulfate), chondroitinase AC (*Athrobacter aurescens*), chondroitin sulfate ABC (*Proteus vulgaris*) and hyaluronidase (*Streptomyces hyalurolyticus*) were from Seikagaku Kogyo, Tokyo through Miles Laboratories Inc., Elkhart, IN. Sepharose CL-4B, Sepharose CL-6B and DEAE-Sephacel were purchased from Pharmacia, Inc., Piscataway, NJ. Cellogel cellulose acetate electrophoresis membranes were obtained from Gelman Sciences, Ann Arbor, MI.

Gingival tissue

Tissue samples were obtained from patients with chronic periodontitis of varying severity. Surgical specimens from each donor were assessed as being derived from normal or inflamed sites by their clinical appearance. Representative portions of each specimen were dissected, fixed in formalin (4%), embedded in paraffin, sectioned (5 μm) and stained with haematoxylin and eosin. From their histological appearance, the clinical assessment of the gingival specimens as "normal" or "inflamed" was confirmed.

Glycosaminoglycan extraction

The gingival specimens were pooled in acetone and stored at 4°C until sufficient quantities were available for analysis. In general, as little as 0.3 gm (dry weight) of gingiva could be used for glycosaminoglycan analysis. The glycosaminoglycans were isolated as previously described (4, 5). Briefly, the tissue was digested with papain (1 mg/ml buffer) in 0.2 M sodium acetate, 0.004 M EDTA, 0.02 M cysteine HCl, pH 5.7, for 48 h at 60°C. Following digestion, proteins and nucleic acids were precipitated with trichloroacetic acid (10% final concentration) for 1 h at 4°C, then separated by centrifugation. The supernatant was then dialysed (molecular weight cut-off of 3,500) against deionized water and concentrated to one-tenth voloume in the same dialysis membrane against Aquacide. Four volumes of 1% sodium acetate in 95% ethanol were added to the concentrated retentate and allowed to stand for 24 h at 4°C. The resultant precipitated glycosaminoglycans were recovered by centrifugation and resolubilized in deionized water. The uronic acid content of each sample was determined by the method of Blumenkrantz and Asboe-Handen (24) using D-glururonic acid as the reference standard.

Cellulose acetate electrophoresis

Glycosaminoglycans isolated from gingiva were initially identified by cellulose acetate electrophoresis in 0.3 M cadmium acetate buffer, pH 4.1, for 4 h at 1.2 mAmp per strip (25). The strips were then immersed in 95% ethanol for 5 min and subsequently stained with 0.2% Alcian blue in 0.05 M magnesium chloride, 0.025 M sodium acetate, pH 5.8 in 50% v/v ethanol-water, each of 30 min duration. Clearing of the strips was achieved by dehydrating in methanol and then washing for 5 min in methanol-acetic acid (86:14 v/v) followed by dry heating at 60°C for 5 min on glass plates.

The glycosaminoglycan bands stained with Alcian blue were quantitated by scanning with a Helena densitometer. Quantitation was achieved by reference to standard curves generated by plotting known standard glycosaminoglycan concentrations against areas under the densitometric curves (4, 27).

Confirmation of glycosaminoglycan identity

To confirm the identity of all the separated glycosaminoglycan bands, 15 μl aliquots of the samples were subjected to the following treatments: (a) hyaluronic acid was eliminated by digestion with 6 TRU of *Streptomyces* hyaluronidase in 0.05 M sodium acetate, pH 6.0 for 1 h at 60°C (4); (b) chondroitin 4- and 6-sulfates were eliminated by digestion with 0.06 units of chondroitinase AC in enriched 0.25 M Tris HCl buffer, pH 8.0, for 3 h at 37°C (28); (c) dermatan sulfate, together with the chondroitin sulfates, was eliminated by digestion with 0.02 units of chondroitinase ABC in the same buffer as (b) for 3 h at 37°C (28). Heparan sulfate was identified by its susceptibility to nitrous acid treatment, as described by Shiveley and Conrad (29).

Proteoglycan extraction and isolation

Proteoglycans were extracted from gingiva by treatment of the tissue with 5 volumes of 4 M guanidine HCl in 0.05 M sodium acetate, pH 5.8, at 4°C for 24 h. The following were added to the buffer as protease inhibitors: 50 mM EDTA, 0.1 M 6–aminohexanoic acid. 5mM benzamidine HCl, 0.1 mM phenylmethylsulfonylflouride, and 10 mM N-ethylmaleimide. The tissue was then separated from the extract by centrifugation. The supernatant was removed and another 5 volumes of 4 M guanidine HCl in the same buffer containing protease inhibitors as described above was added to the residue. The tissue in this buffer was then homogenized and the extraction was allowed to proceed for another 24 h at 4°C. Residual tissue particles were removed from the second extract by centrifugation and washed several times in 0.05 M sodium acetate, pH 5.8, and then digested with papain to recover any unextracted gly-

cosaminoglycans. The two guanidine HCl extracts were pooled and exhaustively dialysed (molecular weight cut-off 3,500) against 7 M urea in 0.05 M Tris HCl containing 0.1 M sodium chloride, pH 7.0, in preparation for ion-exchange chromatography.

DEAE-Sephacel ion-exchange resin was equilibrated in the same 7 M urea buffer used for the dialysis of the guanidine HCl extracts. The retentate from the dialysis step was applied to an equilibrated ion-exchange column (1.0 cm × 10 cm) and thoroughly washed with 7 M urea in 0.05 M Tris HCl containing 0.1 M sodium chloride, pH 7.0. The column was monitored for protein by assessing the absorbance at 280 nm (A_{280}) of the eluent. Once the absorbance reading had returned to baseline, a linear 0.1 M to 0.8 M sodium chloride gradient in 7 M urea/0.05 M Tris HCl, pH 7.0, was applied to the column using a total volume of 150 ml at a flow rate of 10 ml/h. After completion of the salt gradient, any residual bound material was eluted from the column by washing with 10 ml of 4 M guanidine HCl/0.05 M sodium acetate. Fractions of 2.0 ml were collected, and each fraction was assayed for glycosaminoglycans using a new method of detection (30). Briefly, 20 µl of each fraction were spotted onto cellulose acetate electrophoresis membranes, stained with Alcian blue, and then destained using the same buffers described above in the electrophoretic techniques. The strips were then cut at each application point, solubilized in 2.0 ml dimethylsulfoxide, and after 30 min. absorbance was determined at 678 nm (A_{678}). This method was used as it requires much less material than the currently used method of Blumenkrantz and Asboe-Hansen (24). The fractions which constituted the Alcian blue positive peak were pooled, dialysed against deionized water and then lyophilized.

Column chromatography

Glycosaminoglycan molecular size was assessed by molecular sieve chromatography on a column of Sepharose CL-6B (50 cm × 0.7 cm) eluted with 4 M guanidine HCl/0.05 M sodium acetate pH 5.8, at a flow rate of 3 ml/h. Fractions of 0.5 ml were collected and the eluted glycosaminoglycans identified using the Alcian blue binding method described above. Similarly, proteoglycans extracted and isolated from normal and inflamed gingiva were analyzed for structural integrity and molecular size on columns of Sepharose CL-4B (50 cm × 0.7 cm) eluted with the same guanidine HCl buffer as described above, but also containing the protease inhibitors used during the extraction procedures. Fractions of 0.5 ml were collected and the proteoglycans eluted from the column were monitored by their ability to complex with Alcian blue upon localization on cellulose acetate membranes.

Results

Samples of normal and inflamed gingival tissue were digested with papain and the recovered glycosaminoglycans were analyzed for their uronic acid content on a dry weight of tissue basis. The amount of uronic acid recovered from the normal tissue specimens was 0.27% (±0.05) of the dry weight (n = 7). A similar value of 0.25% (±0.1) was obtained for the uronic acid content of the inflamed gingival tissue (n = 5).

Following isolation of the glycosaminoglycans, identification of the individual species was achieved by cellulose acetate electrophoresis (Fig. 1). The presence of each species identified (hyaluronic acid, heparan sulfate, dermatan sulfate and chondroitin sulfate) was confirmed by selective elimination with *Streptomyces* hyaluronidase, chondroitinase AC, chondroitinase ABC and nitrous acid treatment (results not shown). Quantitation of each glycosaminoglycan and its relative contribution to the total glycosaminoglycan pool in either normal or inflamed gingiva was achieved by densitometric scanning of the electrophoretic strips and is listed in Table 1. There appeared to be few differences (none of which was significant) in the relative proportion of each glycosaminoglycan species between the two types of tissue studied. In both normal and inflamed gingiva, dermatan sulfate was the predominant glycosaminoglycan, representing approximately 51% and 55% respectively, of the total. Heparan sulfate was the quantitative minor component accounting for 4% and 3% of the total glycosaminoglycan of normal and inflamed gingiva, respectively. Hyaluronic acid and chondroitin sulfate were found to account for the remaining 45–50% of the total glycosaminoglycans. The amount of hyaluronic acid reported is greater than previously noted (4). In the present study epithelium and connective tissue were not separated and thus hyaluronic acid loss prior to analysis was eliminated. The present values agree with other reports for whole gingival digests (2).

Sepharose CL-6B chromatography of the glycosaminoglycans isolated from gingiva was performed to determine whether inflammation had any effect on their molecular size (Fig. 2). The glycosaminoglycans from normal tissue eluted into 2 peaks (NG1 and NG2) from Sepharose CL-6B and accounted for 21% and 79%, respectively, of the

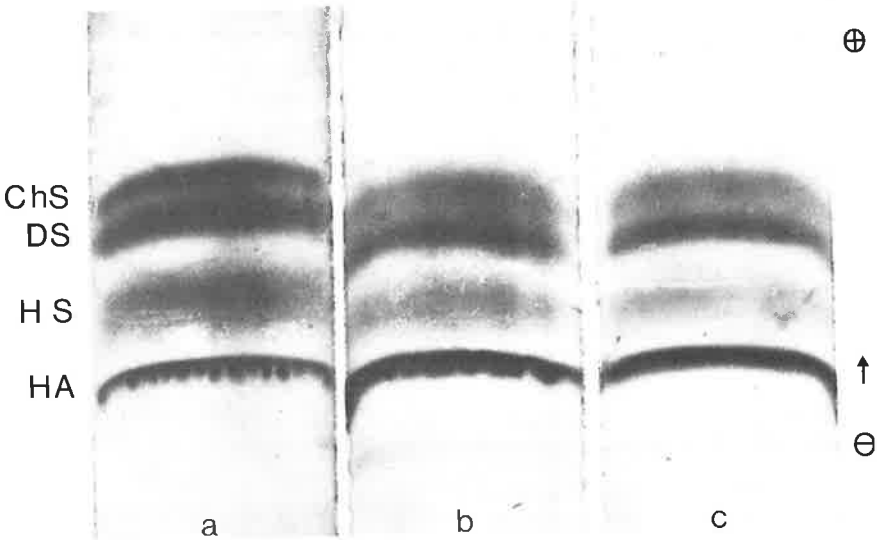

Fig. 1. Cellulose acetate electrophoretograms of glycosaminoglycans. Electrophoresis was performed in 0.3 M cadmium acetate, pH 4.1, for 4 h at a constant current of 1.2 mAmp per strip. Staining with Alcian blue allowed identification of glycosaminoglycans. (a) standard glycosaminoglycans; (b) normal gingival glycosaminoglycans; (c) inflamed gingival glycosaminoglycans. Abbreviations: HA, hyaluronic acid: HS, heparan sulfate; DS, dermatan sulfate; ChS chondroitin-4-sulfate.

Table 1. Glycosaminoglycans in normal and inflamed gingiva. Relative proportions of individual glycosaminoglycan species in each sample extracted from either normal or inflamed gingiva were determined by integration of densitometric scanning of Alcian blue-stained electrophoretograms. Values are expressed as a percentage of the total glycosaminoglycans. Mean values are expressed as ± standard deviation of the mean.

Specimen	Hyaluronic Acid	Heparan Sulfate	Dermatan Sulfate	Chondroitin Sulfate
normal				
1	24	4	50	22
2	20	3	49	12
3	20	1	58	20
4	33	3	51	11
5	24	7	40	11
6	23	4	52	20
7	19	4	50	19
Mean SD	23±4.7	4.1±2.0	50.0±5.3	20.0±5.6
Inflamed				
1	23	0	47	28
2	17	1	54	28
3	20	0	56	24
4	16	5	43	34
5	24	7	58	17
Mean SD	20.0±3.5	2.8±3.0	51.6±6.3	26.0±9.0

total glycosaminoglycans eluted (Fig. 2a). Analysis of the glycosaminoglycans by cellulose acetate electrophoresis (Fig. 3b), revealed the presence of hyaluronic acid exclusively in peak NG1, whereas peak NG2 contained heparan sulfate, dermatan sulfate and chondroitin sulfate, together with some hyaluronic acid (Fig. 3c). The inflamed tissue glycosaminoglycans also eluted from Sepharose CL-6B into 2 peaks (IG1 and IG2); however, the relative proportions of these peaks differed from the normal glycosaminoglycans (10% and 90% respectively for IG1 and IG2). Peak IG1 contained exclusively hyaluronic acid; however, peak IG2 contained heparan sulfate, dermatan sulfate and chondroitin sulfate, as well as an elevated amount of hyaluronic acid (15%) compared to peak NG2 which contained only 4% as hyaluronic acid (Fig. 3 d,e). Taken together, these results indicate a sizeable proportion of hyaluronic acid to be present in inflamed gingiva in a smaller molecular weight form.

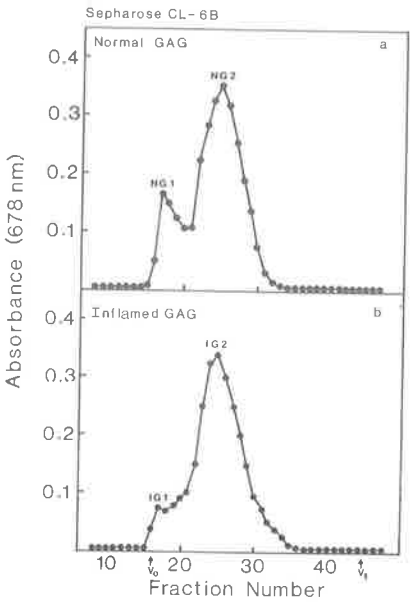

Fig. 2. Gel filtration of normal and inflamed gingival glycosaminoglycans. Glycosaminoglycans isolated from (a) normal and (b) inflamed human gingiva were eluted from a Sepharose CL-6B column in the presence of 4 M guanidine HCl/0.05 M sodium acetate, pH 5.8. The void volume (V_o) and total volume (V_t) were identified with [^{3}H]-DNA and $Na_2[^{35}S]O_4$, respectively. Fractions of 0.5 ml were collected at a flow rate of 3 ml/h and the eluted glycosaminoglycans identified as Alcian blue complexes at A_{678}.

Since the glycosaminoglycans represent only the carbohydrate moiety of the proteoglycans, intact proteoglycan molecules were also studied. They were extracted from normal and inflamed gingiva with 4M guanidine HCl in the presence of protease inhibitors to minimize potential degradation by co-extracted enzymes. After extraction of the tissue was completed the residue was digested with papain to release any unextracted glycosaminoglycans. Uronic acid analysis of the glycosaminoglycans recovered from the residue revealed approximately 20% of the total uronic of normal tissue could not be extracted by guanidine HCl compared to about 10% of the total from the inflamed tissue. Electrophoretic analysis of the glycosaminoglycans in these residues revealed no particular glycosaminoglycan had been extracted at the preference of another, and that a small portion of all 4 glycosaminoglycan species resisted extraction (data not shown).

Following guanidine HCl extraction, the proteoglycans were purified from the bulk of co-extracted matrix proteins and assessed for their charge characteristics by DEAE-Sephacel ion-exchange chromatography (Fig. 4). Material from both normal and inflamed gingiva which had an absorbance at 280 nm eluted from the ion-exchange resin

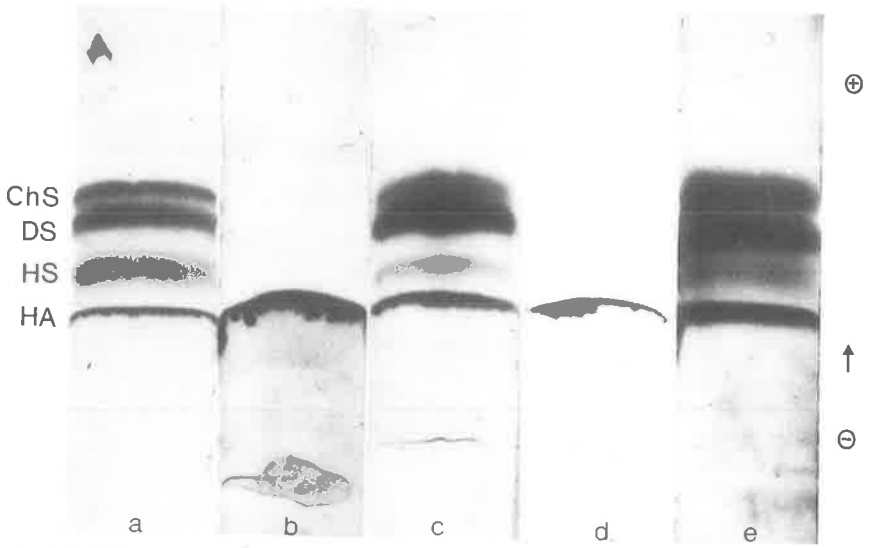

Fig. 3. Cellulose acetate electrophoretograms of glycosaminoglycans separated on Sepharose CL-6B. (a) standard glycosaminoglycans; (b) normal glycosaminoglycans Peak 1; (c) normal glycosaminoglycan Peak 2; (d) inflamed glycosaminoglycan Peak 1; (e) inflamed glycosaminoglycan Peak 2. The electrophoretic separations and abbreviations are the same as in Fig. 1.

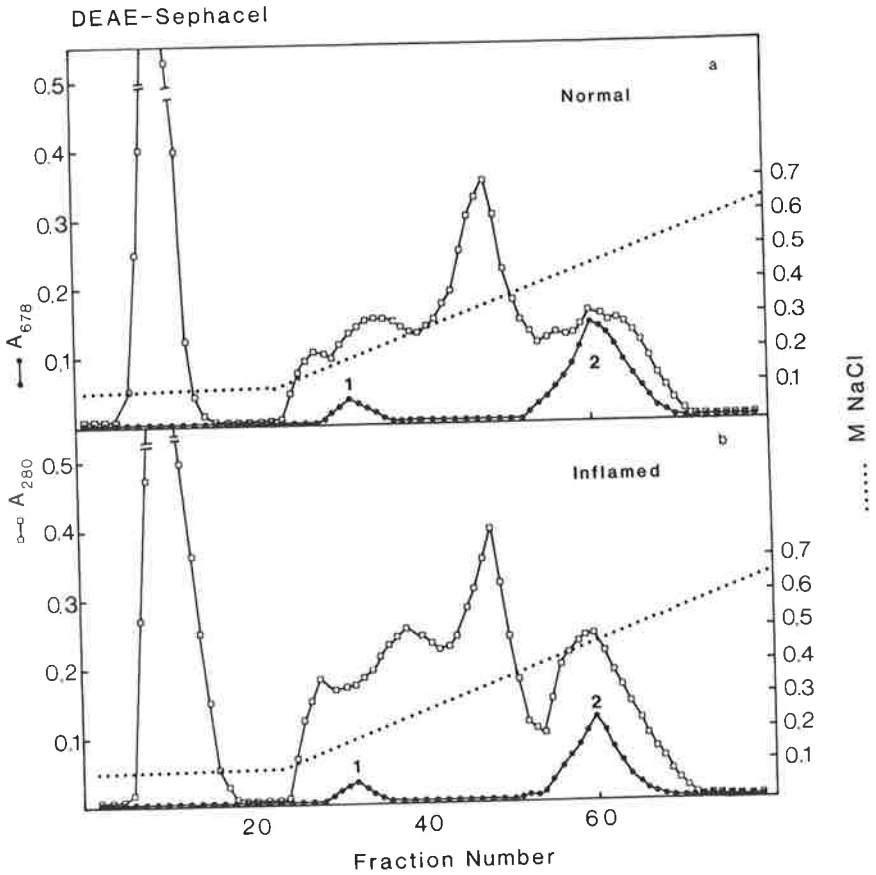

Fig. 4. DEAE-Sephacel ion-exchange chromatography of normal and inflamed gingival extracts. 4 M guanidine HCl extracts of (a) normal and (b) inflamed gingiva were dialyzed against 7 M urea/0.05 M Tris HCl, pH 7.0, containing 0.1 M NaCl and applied to a DEAE-Sephacel column. The various fractions were eluted with a linear 0.1 M to 0.8 M NaCl gradient in the same buffer as described above. Fractions of 2.0 ml were collected at a flow rate of 3 ml/hour. Glycosaminoglycans were identified by the absorbance of Alcian blue complexes at A_{678} and protein was monitored at A_{280}. □——□ protein; ■——■ Glycosaminoglycan.

into 5 peaks. Only 2 peaks positive for glycosaminoglycans, as determined by Alcian blue complex formation, were identified. A relatively symmetrical peak which eluted at a salt concentration of 0.45 M NaCl formed the bulk of the Alcian blue-positive material. A quantitatively smaller Alcian blue-positive peak eluted earlier at a salt concentration of approximately 0.15 M NaCl. Each of the fractions in the Alcian blue-positive peaks isolated by DEAE-Sephacel ion-exchange chromatography were pooled and papain digested, and subjected to cellulose acetate electrophoresis (Fig. 5). Peak 1 from both the normal and inflamed gingival extracts, contained one Alcian blue-positive band which was determined to be hyaluronic acid; this was confirmed by its complete digestion with *Streptomyces* hyaluronidase (results not shown). Peak 2 was found to contain heparan sulfate, dermatan sulfate and chondroitin sulfate for both the normal and inflamed tissue extracts (Fig. 5).

Of peripheral interest to this study was the increased amount of material eluting with an A_{280} which co-eluted with the proteoglycans. A large proportion of this material could be dissociated from the proteoglycans under dissociative molecular sieve chromatography on Sepharose CL-4B (see Fig. 6), and since it did not contain glycosaminoglycans it was not further considered in this study.

The proteoglycans which eluted in Peak 2 from DEAE-Sephacel ion-exchange chromatography were further characterized on columns of Sepharose CL-4B (Fig. 6). Normal gingival proteoglycans have been well characterized previously (6) and, therefore, only one gel filtration profile typical of normal tissue proteoglycans is shown (Fig. 6a). Proteoglycan preparations from 5 separate specimens of inflamed gingiva were analyzed and 3 of the profiles are presented (Fig. 6 b–d). In no instances were identical profiles obtained from proteoglycans isolated from inflamed gingiva. The profiles were similar to the normal gingival proteoglycan profile in that 2 principal proteoglycan peaks (A_{678}) were evident; one being excluded from the gel, and the other eluting with a Kav of 0.45. However, inflamed tissue proteoglycan peak IPG2 was often more heterodisperse than the corresponding peak NPG2 of normal tissue proteoglycans. In addi-

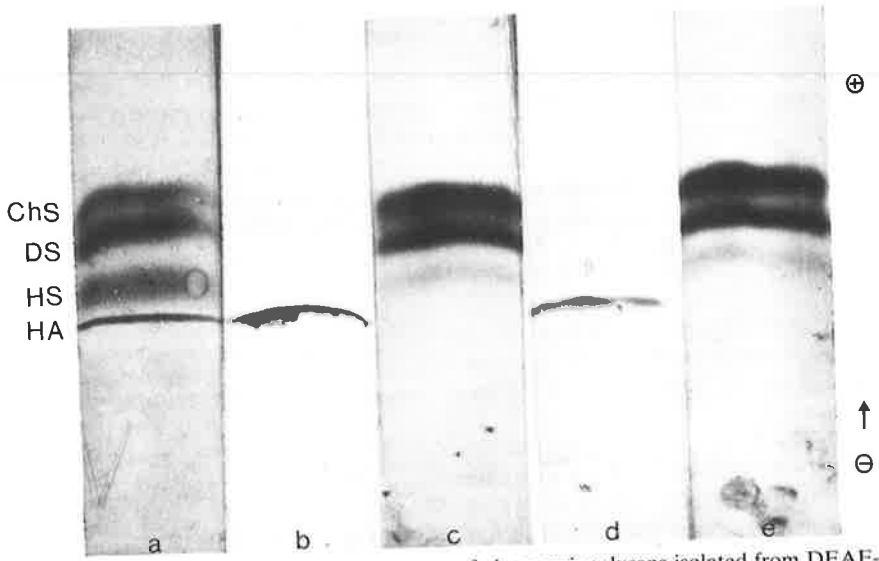

Fig. 5. Cellulose acetate electrophoretograms of glycosaminoglycans isolated from DEAE-Sephacel. Alcian blue positive fractions from DEAE-Seahacel chromatography were pooled and digested with papain, and the released glycosaminoglycans were identified by cellulose acetate electrophoresis under the same conditions as described in Fig. 1. (a) standard glycosaminoglycans; (b) Peak 1 from normal tissue extract; (c) Peak 2 from normal tissue extract; (d) Peak 1 from inflamed tissue extract; (e) Peak 2 from inflamed tissue extract. Abbreviations are the same as in Fig. 1.

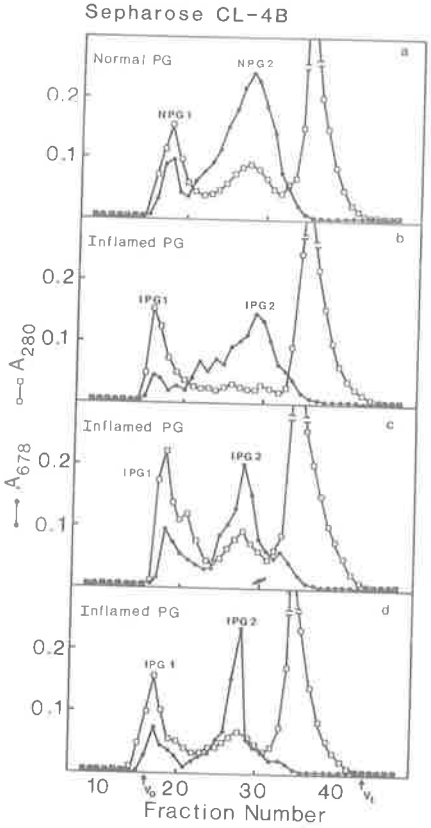

Fig. 6. Gel filtration of normal and inflamed gingival proteoglycans isolated from DEAE-Sephacel. Proteoglycans from (a) normal and (b, c, d) inflamed gingiva were eluted from Sepharose CL-4B in the presence of 4 M guanidine HCl/0.05 M sodium acetate, pH 5.8. Fractions of 0.5 ml were collected at a flow rate of 3 ml/h and proteoglycans were assessed by their Alcian blue complexes (A_{678}); proteins were monitored at A_{280}. The V_o and V_t were determined as described in Fig. 2.

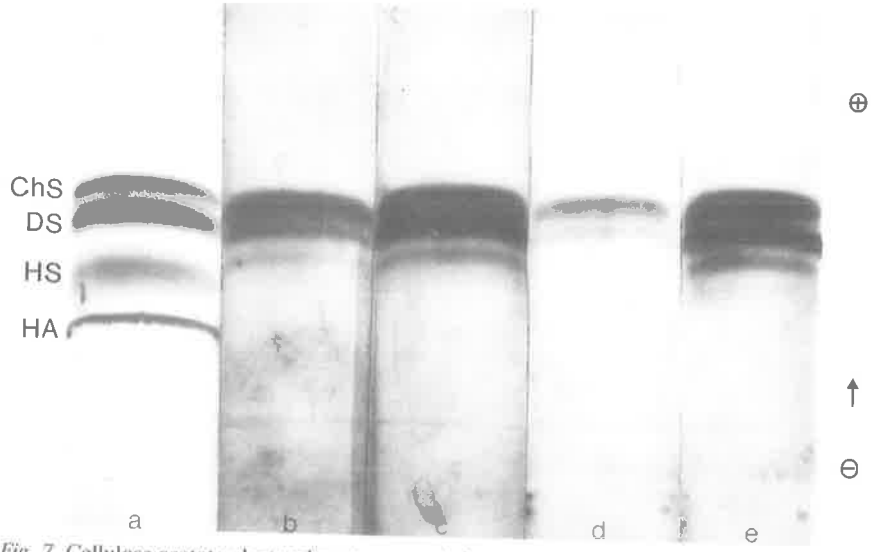

Fig. 7. Cellulose acetate electrophoretograms of glycosaminoglycans eluted from Sepharose CL-4B. Fractions containing proteoglycans from Sepharose CL-4B chromatography were pooled, papain digested and the released glycosaminoglycans electrophoresed under similar conditions to those described in Fig. 1. (a) standard glycosaminoglycans (b) normal proteoglycan Peak 1; (c) normal proteoglycan Peak 2; (d) inflamed proteoglycan Peak 1; (e) inflamed proteoglycan Peak 2. Abbreviations are the same as in Fig. 1.

tion, the proportion of peak IPG1 contributing to the total inflamed tissue proteoglycans was unpredictable ranging from 3%–15% of the total whereas the contribution of NPG1 to the total normal tissue proteoglycans was consistently in the range of 5%–10%. Of particular interest in the inflamed tissue proteoglycan profiles was the clear lack of evidence of small degradation products which would have been expected to elute near the V_t of the column.

Each of the peaks obtained by Sepharose CL-4B chromatography (NPG1 and 2; IPG1 and 2) was digested with papain and the released glycosaminoglycans indentified and quantitated by cellulose acetate electrophoresis (Fig. 7, Table 2). In both cases Peak 1 (NPG1 and IPG1) was comprised principally of chondroitin sulfate (70%–75%) with some dermatan sulfate present (25%–30%). Peak 2 (NPG2 and IPG2) was found to contain variable amounts of heparan sulfate with approximately equal proportions of chondroitin sulfate and dermatan sulfate (50%) as the principal components. There were no statistically significant differences between the relative distributions of the glycosaminoglycans in each of the peaks derived from the normal or inflamed gingival tissue proteoglycans.

Discussion

The effect of inflammation on soft connective tissue proteoglycans has been studied using gingival tissue classified as either normal or inflamed according to its clinical and histological appearance. The differences noted between proteoglycans from these types of tissue have provided a useful insight into the quantitative and qualitative properties of the proteoglycans in the extracellular matrix of inflamed soft connective tissue.

The observations that uronic acid content and individual glycosaminoglycan species present in inflamed tissue are not different from normal glycosaminoglycan species present in inflamed tissue support those of Embery, Oliver and Stanbury (2). However, our studies show that the uronic acid content of the inflamed gingiva varies over a broader range than normal gingiva (Table 1). Such variation could reflect the state of inflammation within the tissue. For example, in any one sample, active inflammatory destruction may have been occurring at the time of sur-

Table 2. Glycosaminoglycan composition of proteoglycans separated on Sepharose CL-4B. Glycosaminoglycans were identified by cellulose acetate electrophoresis and Alcian blue staining. Quantitation was achieved by integration of the densitometric scans of the electropherotograms. Values are expressed as means of three individual determinations for each sample of normal and inflamed gingival proteoglycans. Sata represent scans of glycosaminoglycans derived from the proteoglycans shown in Figure 7. In some cases, trace amounts of a glycosaminoglycan were identified visually, but could not be reliably quantitated densitometrically.

Specimen	Hyaluronic Acid	Heparan Sulfate	Dermatan Sulfate	Chondroitin Sulfate
Normal				
Peak 1	trace	trace	30	70
Peak 2	trace	5	50	45
Inflamed				
Peak 1	trace	trace	25	75
Peak 2	5	trace	50	45

gery, and, therefore, that tissue may have had depleted levels of uronic acid. On the other hand, a similarly classified piece of tissue which was undergoing a phase of remission with a concomitant burst of tissue reparative activity could have an elevated level of uronic acid. Unfortunately, at this stage we are unable to assess whether or not inflamed tissues are undergoing active or remissive phases of inflammatory destruction.

The observation that molecular size of hyaluronic acid is disrupted in inflamed tissue is new. Factors such as hyaluronidase (31, 32) and oxygen-derived free radicals (18, 19) are likely to play important roles in such depolymerization in inflamed tissue. Nonetheless, one cannot rule out the possibility of altered hyaluronic acid synthesis by the fibroblasts residing in inflamed tissue, and studies exploring this possibility are currently underway in our laboratory. Since hyaluronic acid has been implicated in a wide variety of tissue functions (33–36), any damage to this molecule, such as has been seen in the present study, could be expected to have functional repercussions for the whole tissue.

Inflammation did not appear to have any bearing on the net charge of normal and inflamed tissue proteoglycans since both eluted from DEAE-Sephacel at identical salt concentrations as single symmetrical peaks well separated from hyaluronic acid. However, evidence of catabolism of the inflamed tissue proteoglycans was apparent by the heterogeneity of their Sepharose CL-4B profiles. The inflamed gingival proteoglycan profiles were similar to those reported for proteoglycans extracted from inflamed cartilage (37, 38). Nonetheless, despite extreme caution taken to retain all small molecular weight material by using low molecular weight cut-off dialysis tubing (3,500), we were unable to detect any small sized glycosaminoglycan material eluting at, or near, the V_t of the column to indicate extensive degradation of proteoglycans during inflammation. The failure to detect any degradation products may be a result of extremely rapid clearance from the inflammatory site. Indeed, the size of the initially degraded proteoglycans in the extracellular matrix may be important for their subsequent fate; small fragments (either peptides or glycosaminoglycan chain fragments) may enter the circulation and be excreted or further catabolized in the liver (39, 40). Larger fragments (perhaps similar to those seen in the present study) may be more likely to be endocytosed and degraded by the cells in the connective tissue (41–43).

Further assessment of the molecular integrity of the proteoglycans from normal and inflamed tissue, revealed very little alteration occurs to the molecular size of the sulfated glycosaminoglycan components. These moieties from normal and inflamed tissue proteoglycans eluted from Sepharose CL-6B with similar Kav values (0.45) indicative of an approximate molecular weight of 25,000. This molecular weight is in good agreement with previously published values for the sulfated glycosaminoglycans of normal gingival tissue (5). Taken together, these data suggest that the subtle alterations seen in the molecular sieve elution profiles of inflamed tissue proteoglycans most likely arise from disruption to the protein core rather than the carbohydrate side chains.

Indeed, the likelihood of protein cleavage of proteoglycans in inflamed gingiva is high. Recently a neutral metalloproteinase has been identified within inflamed gingival tissue (44), and proteolytic degradation of gingival proteoglycans by a neutral proteinase isolated from human polymorphonuclear leukocytes has also been demonstrated (23, 45). In addition, confirmation that gingival cells produce a proteoglycan-degrading enzyme has also been reported (46).

The roles of tissue hydrolytic enzymes in degrading proteoglycans are confusing. Many hydrolytic enzymes have been identified in inflamed tissues (especially gingiva) and various claims made regarding their role in tissue degradation (31, 47–50). However, in the light of current concepts of proteoglycan degradation (14, 15) as well as the data presented in this study, it is difficult to see how any of these hydrolytic enzymes are involved in the initiation of proteoglycan degradation. They may, however, be involved in subsequent breakdown of glycosaminoglycans after proteoglycan core protein degradation by proteinases.

Thus, the most likely sequence of events in the degradation of soft connective tissue proteoglycans would appear to be initial disruption to the protein core, followed by removal of the glycosaminoglycan/peptide fragments from the inflammatory site to another location where they may be further degraded or excreted. The role of hydrolytic enzymes within these tissues, except for hyaluronidase, which may be instrumental in depolymerizing hyaluronic acid, remains to be established.

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Paper 10.

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The effect of oxygen-derived free radicals on gingival proteoglycans and hyaluronic acid.
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P.M. Bartold's role in this study was:
Provision of research funds
Design of the experiments
Execution of the experiments
Writing of the manuscript

O.W. Wiebkin's role in this study was:
Advisory
Writing of the manuscript

J.C. Thonard's role in this study was:
Advisory
Writing of the manuscript

Journal of Periodontal Research 1984: **19**: 390–400

The effect of oxygen-derived free radicals on gingival proteoglycans and hyaluronic acid

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The effect of a chemically induced oxygen-derived free radical flux has been studied on extracted porcine gingival hyaluronic acid and proteoglycans as well as on cryostat sections of porcine gingivae. The result of the free radical producing system on hyaluronic acid and the proteoglycans was one of reduction of specific viscosity and molecular size. When frozen sections were subjected to the same oxygen-derived free radical flux and subsequently stained with Alcian blue, a noticeable decrease in staining intensity was observed when compared to control sections. These results are considered to reflect the *in vitro* capacity of oxygen-derived free radicals to depolymerize the two major non-fibrous extracellular macromolecules of gingivae. It is postulated that such a mechanism may be responsible, at least in part, for the destruction of gingival proteoglycans and hyaluronic acid observed in inflammatory periodontal disease.

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Introduction

Oxygen-derived free radicals (ODFR), such as the superoxide radical ($O_2^{\cdot -}$) and the hydroxyl radical ($OH^{\cdot}$) are integral reaction products of normal cellular metabolism. One of the most common sources of $O_2^{\cdot -}$ is the reduction of NADPH to $NADP^+$ during electron transport in cellular metabolism (equation 1).

$$2O_2 + NADPH \rightarrow 2O_2^{\cdot -} + NADP^+ + H^+$$

Once in solution the superoxide radical is rapidly involved in the production of hydrogen peroxide by dismutation (reaction 2).

$$O_2^{\cdot -} + O_2 + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$$

At the same time as reaction 2 is occurring, the superoxide radical may also interact with hydrogen peroxide to form an hydroxyl radical (equation 3)

$$H_2O_2 + O_2^{\cdot -} \rightarrow OH^{\cdot} + OH^- + O_2$$

Despite being part of regular aerobic metabolism, ODFR are highly reactive and, if present in abundance within tissues, can cause extensive damage (Freeman & Crapo 1982). For example, free radicals are active in disrupting cellular proteins, nucleic acids, and membrane lipids, as well as effecting depolymerization of extracellular matrix components such as collagen, hyaluronic acid, and possibly proteoglycans (Greenwald, Moy & Lazarus 1976, Greenwald & Moy 1979, Greenwald & Moy 1980).

One way in which tissues may be exposed to an excess of ODFR is during the inflammatory response, because polmorphonuclear leukocytes are particularly active

in reducing molecular oxygen to the superoxide radical when exposed to stimuli which induce phagocytosis (Johnston et al. 1975, Goldstein et al. 1975, Bragt, Bansbury & Banta 1980).

Since extensive degradation of gingival extracellular matrix is observed during the progression of periodontal disease (Page & Schroeder 1976), the question of whether ODFR could be involved in either the early stages or the propagation of the disease process is raised. This is a novel approach since hydrolytic enzymes have historically been considered to be primarily responsible for tissue catabolism in periodontal disease. Indeed, the role of ODFR, generated by inflammatory cells in periodontal disease, has hitherto been ignored. In order to begin to answer this question, tests were made for the susceptibility of gingival proteoglycans and hyaluronic acid to an *in vitro*, chemically induced, flux of ODFR.

Materials and Methods

Gingival tissue

Buccal gingivae were dissected from young adult pigs (1–2 years) which had been freshly slaughtered at the local abattoirs. The excised gingivae were immediately placed into Hanks' Balanced Salt Solution (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) and transported to the laboratory where they were finely chopped prior to proteoglycan or hyaluronic acid extractions.

Gingival proteoglycan extraction and purification

Proteoglycans were extracted from porcine gingivae (20 gm wet weight) in 200 ml 4 M guanidinium chloride/0.05 M sodium acetate, pH 7.4, containing proteinase inhibitors at 4°C for 24 h (Wiebkin, Bartold & Thonard 1979, Oegema, Hascall & Dziewiatkowski 1975). The tissue was then separated from the extractant and subjected to another 24 h extraction in fresh 4 M guanidinium chloride/0.05 M sodium acetate, pH 7.4 at 4°C. Both extractants were pooled, concentrated, exhaustively dialysed against 7 M urea/0.05 M Tris HCl, pH 6.5 at 4°C, and then chromatographed on DEAE Sephadex A-50 ion-exchange resin (Pharmacia, South Seas, Sydney, Australia) (Bartold, Wiebkin & Thonard 1983a). The eluate was assayed for uronic acid (Blumenkrantz & Asboe-Hansen 1973) and demonstrated the bulk of the uronate to be eluted with 2.0 M NaCl. This fraction was pooled, dialysed against 0.5 M sodium acetate, pH 7.4, containing proteinase inhibitors and then against deionized water and finally freeze dried. This crude proteoglycan preparation was then redissolved in 4 M guanidinium chloride/0.05 M sodium acetate and adjusted to a density of 1.35 g/ml with solid cesium chloride. Preparative ultracentrifugation was performed in a Beckman Model L ultracentrifuge at 40,000 rev/min (approx. 100,000 g) at 15°C for 48 h. The centrifuge tubes were then rapidly frozen, and the proteoglycan rich portion (bottom 3/5) was collected for further experimentation (Bartold, Wiebkin & Thonard 1983a).

Gingival hyaluronic acid extraction and purification

Hyaluronic acid was extracted from porcine gingivae essentially as described previously for human gingivae (Bartold, Wiebkin & Thonard 1983b). Acetone dried and defatted gingivae were digested by pronase B (Calbiochem-Behring Corp., La Jolla, California) to release all the glycosaminoglycans. The digestion was performed in 0.5 M sodium acetate, pH 7.5 (50/mg tissue/ml buffer), at 65°C for 24 h (Svejcar & Robertson 1967).

Following digestion, protein was removed by adding 80% trichloroacetic acid to give a final concentration of 10% and left

at 4°C for 1 h. The precipitate was centrifuged down and the supernatant then dialysed against deionized water. The retentate was concentrated by rotary evaporation, and the glycosaminoglycans precipitated by the addition of 4 volumes of 1% sodium acetate in ethanol.

The precipitated glycosaminoglycans were redissolved in 0.15 M Na_2SO_4 and hyaluronic acid fractionated from the sulfated glycosaminoglycans by adding cetylpyridinium chloride to give 1% final concentration and thereby precipitating the sulfated glycosaminoglycans (Scott 1960). Following centrifugation, the hyaluronate in the supernatant was precipitated by adding an equal volume of water to give 60 mM Na_2SO_4 and was recovered by centrifugation. The cetylpyridinium complexes were converted into sodium salts by precipitation with ethanolic sodium acetate via propan-1-ol.

Confirmation of hyaluronic acid fractionation was achieved by electrophoresis on Cellogel (Chemetron, Milan, Italy) in 0.2 M calcium acetate, pH 7.2, and specified by *Steptomyces* hyaluronidase digestion (Bartold, Wiebkin & Thonard 1981).

Oxygen-derived free radical production and depolymerization

Oxygen-derived free radicals were induced by autoxidation of ferrous EDTA chelates (Betts & Cleland 1982). Hyaluronate (standard [Sigma Chemicals] and gingival) and gingival proteoglycans were solubilized in 50 mM K_2HPO_4 containing 0.1 mM EDTA, pH 7.4, to give a concentration of 1 mg uronic acid/ml. To initiate the ODFR flux, 10 μl of a stock solution (made fresh daily) of 50 mM ferrous sulphate and 50 mM EDTA in distilled water was added to 1.7 ml of the hyaluronate or proteoglycan solutions, thus giving a final concentration of $FeSO_4$ of about 300 μM. The effect of the ODFR flux was then observed by viscometry and subsequent column chromatography of the reaction products.

Viscosity measurements

The specific viscosity of hyaluronate and proteoglycan solutions was measured before and after initiation of the ODFR flux by a highly reproducible system using 1 ml plastic tuberculin syringes fitted with a 19 gauge needle (Betts & Cleland 1982). The test solutions were drawn up into the syringe and the time for 0.8 ml to drain was measured. The relative viscosity (η_R) of these solutions was calculated by the equation:

$$\eta_R = \frac{\text{time for test solution}}{\text{time for buffer solution}} \quad (4)$$

The specific viscosity (η_{sp}), which is a measure of the contribution of the solute (hyaluronate or proteoglycan) to the solution viscosity was calculated by the equation:

$$\eta_{sp} = \eta_R^{-1} \quad (5)$$

Column chromatography

Chromatography of the hyaluronate and proteoglycan specimens, both before and after ODFR fluxes, was performed on columns (0.9 cm × 32 cm) of Sepharose 4B-CL (Pharmacia, South Seas, Sydney, Australia). The columns were eluted with 0.5 M sodium acetate, pH 7.4 at a constant flow rate of 3 ml/hour. The void volume (V_0) was determined using Blue Dextran 2000 (Pharmacia, South Seas, Sydney, Australia) whilst the total volume was measured using [^{35}S]-sulfate.

Histochemical observations

Freshly dissected porcine gingivae from slaughtered animals were frozen at −70°C prior to 10 μm cryostat sections being cut. The dried sections were randomly placed into three groups and then immersed in one of the following solutions: (a) 20 ml of 0.1 mM EDTA in 50 mM K_2HPO_4, pH 7.4, (b) 20 ml of 0.1 mM EDTA in 50 mM K_2HPO_4,

pH 7.4, containing 120 μl of 50 mM $FeSO_4$ in 50 mM EDTA, pH 7.4, and (c) 20 ml of the same solution as used for (b) but also containing 100 μl propanol as a free-radical scavenger. The sections were left in these solutions for 1 h at room temperature and then stained overnight by 0.05% Alcian blue in 0.1 M $MgCl_2$ in 0.02 M sodium acetate, pH 5.8 (Scott & Dorling 1965). The slides were then rinsed in distilled water and dehydrated in 70%, 90%, and 100% alcohol, cleared in xylene and mounted.

Results

Hyaluronic acid extraction and purification

Hyaluronic acid was extracted and purified from porcine gingivae following pronase digestion and selective CPC precipitation. The results of electrophoresis of the products of such procedures can be seen in Fig. 1. The band which corresponded to standard hyaluronic acid on Fig. 1b was confirmed by complete elimination following *Steptomyces* hyaluronidase digestion.

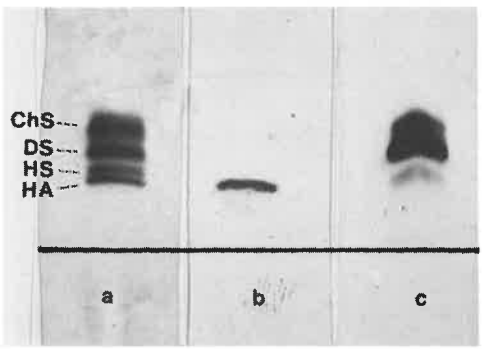

Fig. 1. Electrophoretograms of glycosaminoglycans (buffer: 0.2 M calcium acetate, pH 7.2; 30 volts/cm for 30 min). a) Standard glycosaminoglycans (1 mg/ml), HA (hyaluronic acid), HS (heparan sulphate), DS (dermatan sulphate), ChS (chondroitin sulphate-4). b) Hyaluronic acid isolated from porcine gingivae. c) Sulphated glycosaminoglycans isolated from porcine gingivae.

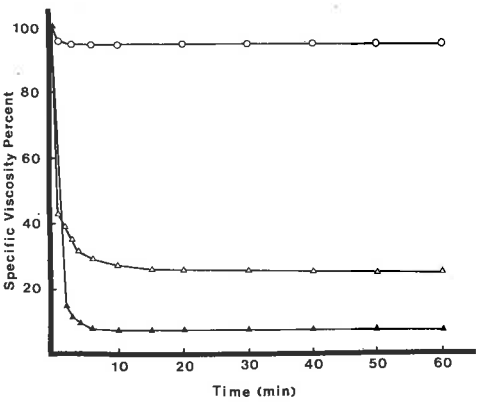

Fig. 2. Effect of an ODFR flux on hyaluronic acid viscosity. ▲—▲ standard hyaluronic acid. △—△ gingival hyaluronic acid. ○—○ standard hyaluronic acid in the presence of propanol. Viscosity measurements were made before and immediately after induction of the ODFR flux, and specific viscosity values are expressed as percentage of control values measured before the ODFR flux.

Hyaluronic acid viscosity measurements

In order to validate the ferrous EDTA system as a satisfactory means of causing hyaluronate depolymerization by ODFR, initial studies using a standard commercial hyaluronic acid preparation (Sigma Chemicals) were performed. The effect of an ODFR flux on the specific viscosity of this material as well as the effect of a free-radical scavenger on the reaction can be seen in Fig. 2. Within 6 minutes the rate of reaction appeared complete, causing a 93% reduction in specific viscosity of the standard hyaluronate preparation. The effect of propanol on this reaction was one of almost total inhibition. Indeed, propanol was added to the test system as a control, in which the active free radicals produced by the ferrous EDTA reaction would be scavanged and rendered inactive.

A similar result was observed for gingival hyaluronic acid in which ODFR caused a reduction in specific viscosity (Fig. 2). The effect could be abolished by the presence of

propanol. The observed change in specific viscosity and the reaction rate was slightly different from the standard hyaluronic acid/ODFR reaction. Gingival hyaluronic acid specific viscosity was reduced by 75% and reached this level within 15 minutes.

Hyaluronic acid column chromatography

The effect of ODFR flux on gingival hyaluronic acid molecular size can be seen in Fig. 3. Before exposure to the ODFR the hyaluronic acid eluted close to the V_0 of the column ($K_{av}=0.07$); following the ODFR flux, the majority of the hyaluronic acid eluted as an included peak ($K_{av}=0.34$) and indicated a decrease in overall molecular size.

Proteoglycan viscosity

Viscosity measurements were made on proteoglycans extracted from porcine gingivae both before and after an ODFR flux as well as an ODFR flux in the presence of propanol as can be seen in Fig. 4. The specific viscosity of the proteoglycan solution decreased by 50% over a 20 min period after which no further decrease was noted. Again, the effect of propanol in scavenging the free radicals was evident.

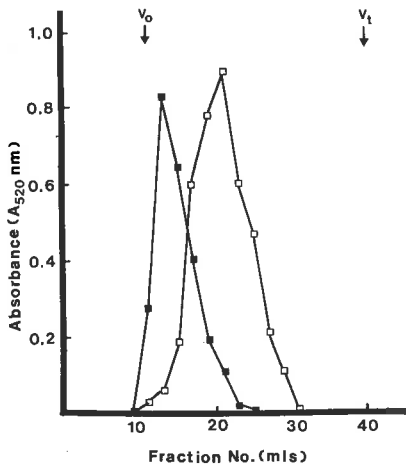

Fig. 3. Column chromatographs of gingival hyaluronic acid before and after an ODFR flux on Sepharose 4B-CL. (Buffer: 0.5 M sodium acetate, pH 7.4; flow rate of 3 ml/hr.). ■—■ before ODFR flux. □—□ after ODFR flux.

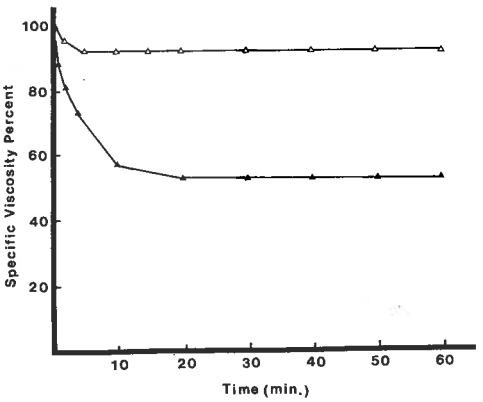

Fig. 4. Effect of an ODFR flux on porcine gingival proteoglycan viscosity. △—△ gingival proteoglycans. ▲—▲ gingival proteoglycans in the presence of propanol. Viscosity measurements were made before and immediately after induction of the ODFR flux, and viscosity values are expressed as percentage values measured before the ODFR flux.

Proteoglycan column chromatography

Gingival proteoglycan purified by ion-exchange chromatography and density-gradient centrifugation chromatographed on Sepharose 4B-CL as a single peak with a K_{av} of 0.14 (Fig. 5). Following treatment with an ODFR flux, the proteoglycan population fractionated into two separate peaks. One peak (which contained the bulk of the proteoglycan) was polydisperse in molecular size with an estimated K_{av} of 0.34. The other peak was quantitatively the minor component and eluted with a K_{av} of 0.93.

Histochemistry

The susceptibility of intercellular proteoglycans and hyaluronic acid within gingival tissue to ODFR was tested on cryostat histological sections. The staining affinity of Alcian blue, a specific dye for polyanionic macromolecules, was used to visualize the

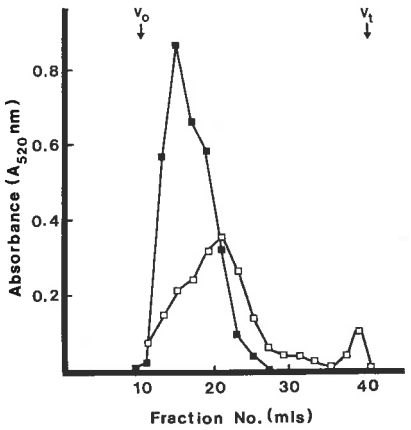

Fig. 5. Column chromatographs of porcine gingival proteoglycans before and after an ODFR flux on Sepharose 4B-CL. (Buffer: 0.5 M sodium acetate pH 7.4; flow rate of 3 ml/hr). ■—■ before. □—□ after.

degree of loss of depolymerized proteoglycans and hyaluronic acid following exposure to free radicals (Fig. 6).

The sections which were subjected to ODFR flux demonstrated a noticeable diminution in Alcian blue staining when compared to either controls or to the ODFR generating system which contained a radical scavenger. This visual assessment was confirmed following densitometric scanning of the whole sections and indeed revealed a 20% reduction in staining intensity following the prior exposure of sections to ODFR.

Discussion

Despite no precise reports of *in vivo* radical concentrations being currently available, ODFR are presumed to be present in elevated amounts in inflammation. This assumption is based upon observations such as those reported by Greenwald (1980) who reported that leukocytes are capable of producing superoxide at a rate of 5.8 nM/10^6 cells/15 min. In addition, he reported that whilst the superoxide production by 5×10^6 leukocytes caused some depolymerization of exogenous hyaluronic acid in the culture medium, the superoxide production by 13×10^6 cells resulted in a substantially greater degree of hyaluronic acid depolymerization. Furthermore, it has been suggested that by calculating the number of leukocytes commonly found at diseased sites as well as the amount of superoxide such cells can produce *in vitro*, superoxide mediated depolymerization of the extracellular matrix components is quantitatively feasible *in vivo* (McCord 1974).

The studies reported herein have utilized the production of ODFR by the autoxidation of ferrous sulfate in the presence of a chelator (EDTA). The following reaction sequence has been presented as the most likely course by which radicals are produced in such a system (Halliwell 1978).

$$Fe^{+++} + O_2^{\cdot -} \rightarrow Fe^{++} + O_2 \qquad (6)$$

$$Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + OH^{\cdot} + OH^{-} \qquad (7)$$

The net reaction is therefore the same as reaction (3) outlined in the introduction.

$$O_2^{\cdot -} + H_2O_2 \quad OH^{\cdot} + OH^{-} + O_2 \qquad (3)$$

The concentration of ferrous sulfate used in these experiments (approximately 300 μM) was chosen because it produced maximum proteoglycan and hyaluronic acid depolymerization. The amount of ODFR produced may, therefore, have been greater than one would expect to find in either inflamed or normal tissue but nonetheless provided a very satisfactory method of *in vitro* production of free radicals. Therefore, by subjecting hyaluronate and proteoglycans extracted from porcine gingivae to a system which generated ODFR, we have demonstrated the *in vitro* capacity of these highly reactive radicals to affect molecular size and specific viscosity of the two major non-fibrous macromolecules of gingival extracellular matrices. Molecular size and con-

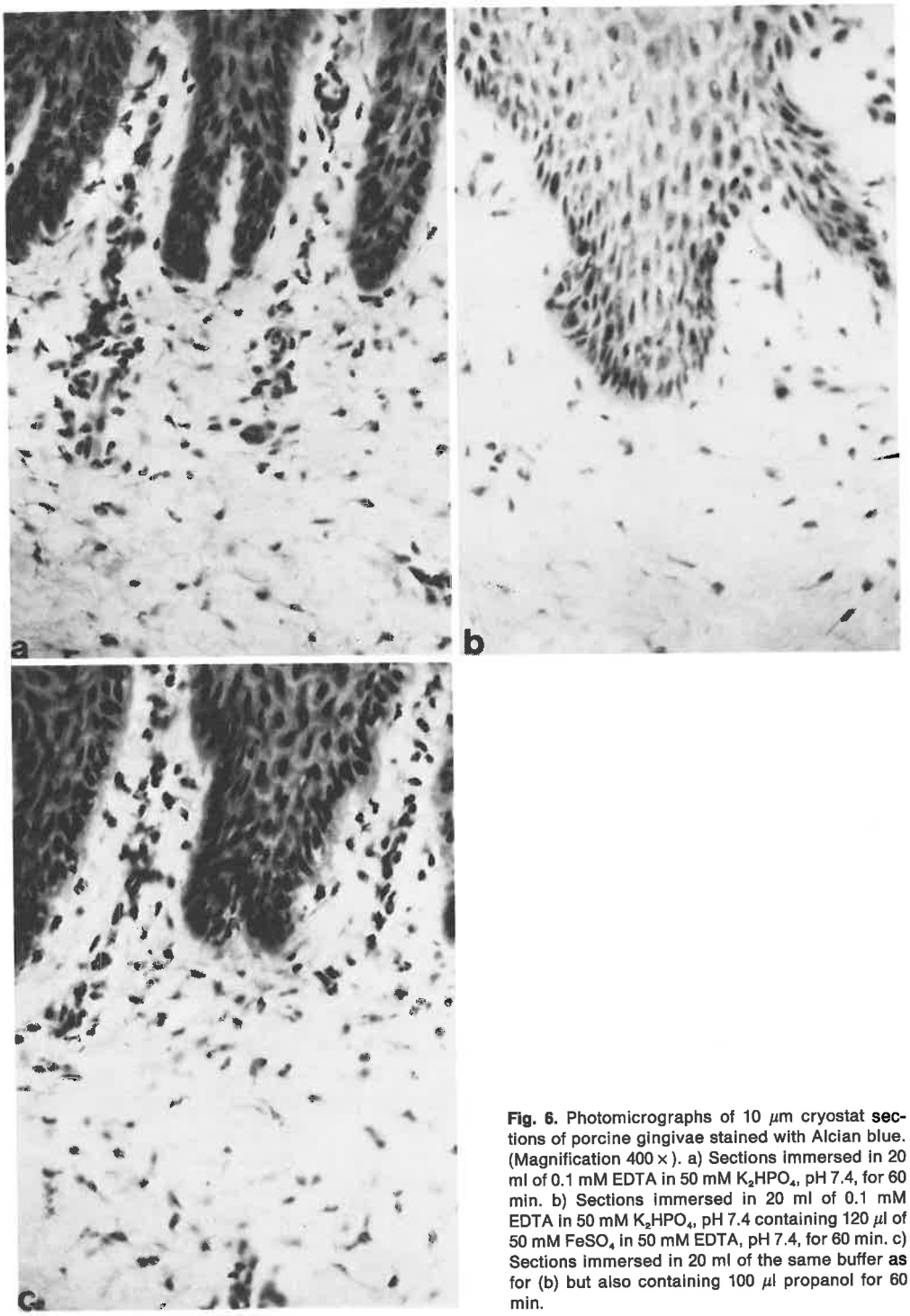

Fig. 6. Photomicrographs of 10 μm cryostat sections of porcine gingivae stained with Alcian blue. (Magnification 400 ×). a) Sections immersed in 20 ml of 0.1 mM EDTA in 50 mM K_2HPO_4, pH 7.4, for 60 min. b) Sections immersed in 20 ml of 0.1 mM EDTA in 50 mM K_2HPO_4, pH 7.4 containing 120 μl of 50 mM $FeSO_4$ in 50 mM EDTA, pH 7.4, for 60 min. c) Sections immersed in 20 ml of the same buffer as for (b) but also containing 100 μl propanol for 60 min.

sequently specific viscosity are likely to be affected during inflammatory degradation of the extracellular matrix components *in vivo*. Indeed, such alterations would be expected to influence many of the mechanical and biophysical properties of tissues (Comper & Laurent 1978) and could manifest ultimately as tissue breakdown similar to the tissue response seen during the progression of inflammatory periodontal disease.

The specific viscosity of gingival hyaluronic acid was more affected by the ODFR flux (75% reduction) than was the proteoglycan viscosity (50% reduction). Whilst specific viscosity is primarily dependent upon concentration, these differences in specific viscosity may also reflect the different chemical structure of hyaluronic acid and proteoglycans. Indeed, hyaluronic acid is a single chain polymer of D-glucuronic acid and D-glucosamine whilst proteoglycans are far more complex comprising a single protein core to which many sulfated glycosaminoglycan chains are covalently bound. Therefore, the complex nature of proteoglycans may be more protective (resistant) to ODFR depolymerization.

The molecular sizes of hyaluronic acid and proteoglycan, as judged by column chromatography were decreased following an ODFR flux. However, these changes were not large enough to indicate a breakdown to very small oligosaccharide fragments or large scale protein degradation. Nonetheless, in the case of the proteoglycans treated with ODFR, a small proportion of uronic acid containing material was observed near the V_t of the column. This material most likely represented cleaved glycosaminoglycan chains whilst the large proteoglycan peak seen following the ODFR flux is probably comprised of protein cores with some residual glycosaminoglycan chains attached.

The proteoglycans and hyaluronic acid which had been depolymerized were subjected to a further ODFR flux after the viscosity decrease had plateaued (i.e. after 20 min). There were no observable changes in viscosity (results not shown) which indicated that depolymerization had progressed to a state where further degradation by free radicals did not occur. This observation is in contrast to that of Wong et al. (1981) who reported additional depolymerization upon supplementary ODFR fluxes. These workers did, however, use an initial lower concentration of ferrous sulfate as well as a higher original hyaluronic acid concentration than that in the present investigation, and accordingly depolymerization was not as complete as reported in the present study.

Additional evidence that free radicals are capable of disrupting the molecular arrangement of gingival hyaluronic acid and proteoglycans has been provided by subjecting gingival cryostat sections to a flux of ODFR. Alcian blue was chosen as the histochemical dye to observe the effects of ODFR on gingival extracellular matrix components because of its specificity for polyanions (Scott & Dorling 1965). In particular, it was anticipated that this dye would demonstrate degradation or stoichiometric alteration to the hyaluronic acid and the proteoglycans of the extracellular matrices of gingival sections. Indeed, this was the case. Sections which were subjected to an ODFR flux demonstrated a decrease in Alcian blue staining intensity within the extracellular regions of both epithelium and connective tissue when compared with the controls or sections subjected to an ODFR flux in the presence of the free-radical scavenger, propanol. Whilst the results do not demonstrate complete loss of all Alcian blue positive material, which might imply massive degradation of the extracellular macromolecules by free radicals, they do support our *in vitro* findings of the effect of ODFR

on hyaluronic acid and proteoglycans described in this study. That is, ODFR cause sufficient depolymerization of these macromolecules to effect changes in both molecular size and specific viscosity. Such alterations may be great enough to permit either solubilization (extraction) of hyaluronic acid and proteoglycan from the sections during processing or cause blockage of the Alcian blue binding sites. Either way, these changes would manifest as a decrease in Alcian blue staining intensity. Nonetheless, on the basis of current work in our laboratories using cartilage sections, a measurable loss of proteoglycan and hyaluronic acid in the test solutions during processing was found. These findings would support the former suggestion above rather than the latter one of Alcian blue binding site alteration.

The data reported herein are particularly interesting in light of the current concepts of the pathogenesis of periodontal disease (Bartold, Wiebkin & Thonard 1983c). At present, enzymes such as hyaluronidase (Gaffer, Coleman & Marcussen 1981), collagenase (Uitto, Appelgren & Robinson 1981, Woolley & Davies 1981, Yanagimura, Hara & Nohara 1983), and proteases released by leukocytes (Cergneaux, Anderson & Cimansoni 1982) are considered to be responsible for much of the disruption to the gingival connective tissues affected by periodontal disease. However, whilst these enzymes can undoubtedly degrade hyaluronic acid, proteoglycans, and collagen *in vitro*, proponents of this philosophy have had difficulty in isolating these enzymes from gingivae as well as difficulties in demonstrating *in vivo* enzymatic activity. Furthermore, since collagenase is secreted by gingival fibroblasts in an inactive form (Wilhelm, Javed & Miller 1983) and many proteolytic enzyme inhibitors have been reported in extracts of gingivae (Pettigrew et al. 1981, Heath et al. 1982), specific enzymatic degradation of gingival extracellular matrix is difficult to explain. Therefore, in the light of such data as well as evidence which suggests that carbohydrases such as hyaluronidase are unlikely to play a major role in the intitial degradation of gingival hyaluronic acid and proteoglycans (Embery, Oliver & Stanbury 1979), the data concerning ODFR depolymerization of these macromolecules adds a new perspective. Since a major source of ODFR is phagocytes and these cells are present in abundance in inflammatory periodontal disease, these highly reactive radicals may indeed be responsible for the initial degradation (depolymerization) of hyaluronic acid and proteoglycans as well as collagen seen in periodontal disease. This initial depolymerization could then be further augmented by lyzosomal enzymes from the phagocytes destroyed by ODFR.

Nonetheless, in making such a novel proposal, one must presuppose that the balance between ODFR released into the extracellular matrices of tissues and the presence of the body's inherent superoxide scavenger, superoxide dismutase, is disturbed. Indeed, superoxide production by stimulated leukocytes has been measured *in vitro* to last for as long as 20 minutes (Salin & McCord 1975) during which time the protection to the tissue components afforded by superoxide dismutase is crucial. However, superoxide dismutase is present in tissue fluids only in limited amounts (McCord & Salin 1975) and therefore superoxide damage to tissue components and cells may occur before the scavengers render the free radicals inactive.

In conclusion, the results presented in this paper have addressed themselves to a model of the simpler systems of extracellular macromolecular depolymerization by ODFR, and the interpretations presented herein warrant further investigation.

Acknowledgements

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Candidate's Contribution to this paper: 95%

P.M. Bartold's role in this study was:
Design of the experiments
Execution of all experimental work
Writing of the paper

R.C. Page's role in this study was:
Provision of research funds
Writing of the paper

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PROTEOGLYCANS SYNTHESIZED BY CULTURED FIBROBLASTS DERIVED FROM NORMAL AND INFLAMED HUMAN GINGIVA

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SUMMARY

The in vitro proliferations rates and ptoteoglycans synthesized by adult human gingival fibroblasts derived from six age- and sex-matched donors of healthy and chronically inflamed gingiva were analyzed. Fibroblasts from inflamed gingiva demonstrated a slower growth rate than cells from healthy tissue. The rate of incorporation of [^{35}S]sulfate into cell layer-associated proteoglycans and the release of these macromolecules into the culture medium did not differ appreciably between the two groups of cells. Similarly, no detectable differences in the overall charge of the proteoglycans synthesized by normal and inflamed gingival fibroblasts, as assessed by their elution from DEAE-Sephacel, were noted. However, Sepharose CL-4B chromatography revealed that the medium-associated proteoglycans made by the inflamed tissue fibroblasts were depleted in one species of chondroitin sulfate proteoglycans and contained more dermatan sulfate than did control cells. In addition, the intracellular proteoglycan pool was found to be greatly diminished in the inflamed tissue fibroblast cell layers. Glycosaminoglycan analysis of the proteoglycans confirmed these observations. Compared to normal gingival fibroblasts, the inflamed tissue fibroblasts released less heparan sulfate into the medium. Additionally, increased levels of dermatan sulfate and depleted amounts of chondroitin sulfate in the medium of inflamed gingival cells were noted. The observed changes were stable through several transfers in culture and indicate that chronically inflamed tissue may contain fibroblasts manifesting a heritable phenotype differing from fibroblasts in normal connective tissue.

Key words: proteoglycans; inflammation; fibroblasts; gingiva; periodontal disease.

INTRODUCTION

Proteoglycans are a complex group of highly anionic macromolecules that have ubiquitous distributions throughout the extracellular matrices of soft connective tissues (1). As a result of their charged nature, proteoglycans are highly hydrated and capable of interacting with a wide variety of matrix and cell surface components (7,11,16). Indeed, such interactions are considered vital to the maintenance of normal tissue function. Consequently, any damage to, or altered synthesis of, these matrix macromolecules could be expected to significantly affect the physiologic well being of tissues.

During soft tissue pathological changes, such as is seen in inflammation, there is a complex interplay between the inflammatory cells and the resident fibroblasts accompanied by degradation of the extracellular matrix. If the causative factor is removed, then under normal circumstances the tissue eventually heals and a healthy state is restored. Gingiva has served as a model for studying the events occurring in the extracellular matrix of soft connective tissues associated with inflammation because it is both readily available and manifests a naturally occurring, progressive condition termed periodontitis (19).

The collagens of gingiva in both health and disease have been studied extensively (19). Histological and biochemical studies have shown that at a very early stage of inflammation, collagen destruction and loss occur (22). Additional in vitro studies have revealed that various chemical agents found at sites of inflammation exert profound effects on fibroblast growth and synthetic activity (12,13,18,21). Furthermore, fibroblasts from inflamed tissue may be phenotypically different from those present in normal tissue. These cells produce a unique collagen with a structure of $\alpha 1[I]_3$, a type not found in cultures of normal fibroblasts and represents a feature that persists throught their cultured life span.

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Much less is known about gingival proteoglycans. To date, the proteoglycan composition of normal gingival tissue has been analyzed (2,9,36), and some studies have considered the in vitro synthesis of proteoglycans and glycosaminoglycans by gingival fibroblasts (3) (Bartold, P. M.; Page, R. C., submitted for publication). In the present study proteoglycans synthesized by cultured fibroblasts isolated from normal and inflamed human gingiva have been analyzed to determine the effect of chronic inflammation on the types of proteoglycans synthesized by these cells. The results obtained indicated that although proteoglycan synthetic rates seemed unaltered, there were differences noted in the quantities and types of proteoglycans made by fibroblasts for normal and diseased tissues, and these differences persisted through numerous transfers in culture.

Materials and Methods

Materials. Guanidine HCl, 6-aminohexanoic acid, benzamidine HCl, *N*-ethylmaleimide, phenylmethylsulfonyl flouride, cysteine HCl, cetylpyridinium chloride, and papain (E. C. 3.4.22.2) were all purchased from Sigma Chemical Co., St. Louis, MO; sodium dodecyl sulfate (SDS) from BioRad Laboratories, Richmond, CA; chondroitinase AC (*Arthrobacter aurescens*) and chondroitinase ABC (*Proteus vulgaris*) from Seikagaku Kogyo, Tokyo, Japan, through Miles Laboratories, Inc., Elkhart, IN; Sephadex G-50, Sepharose CL-4B, Sepharose CL-6B, and DEAE-Sephacel from Pharmacia, Inc., Piscataway, NJ; Dulbecco-Vogt medium, fetal bovine serum (FBS), phosphate buffered saline (PBS), and penicillin-streptomycin from Grand Island Biological Co., Grand Island, NY. Sulfate-depleted medium was made from amino acids and vitamins purchased from Grand Island Biological Co.; 75-cm^2 tissue culture flasks were from Falcon Plastics, Oxnard, CA; 24-well plates from Flow Laboratories; $Na_2[^{35}S]O_4$ (2 mCi/ml; 424.15 mCi/mmol) from New England Nuclear, Boston, MA; Aquamix from WestChem, San Diego, CA; and Aquacide from Calbiochem, La Jolla, CA.

Fibroblast culture. Fibroblasts were obtained from biopsies from human volunteers with healthy gingivae and from age- and sex-matched patients undergoing treatment for chronic periodontitis as previously described (21). The normal tissue fibroblasts (henceforth termed "normal fibroblasts") were designated by the terminology HGF_{24}, HGF_{25}, and HGF_{27}; the inflamed tissue fibroblasts (henceforth termed "inflamed fibroblasts") were designated by the terminology HGF_{10}, HGF_{11}, and HGF_{13}. These are cells from the same parent populations described previously for the study of collagen synthesis by normal and inflamed fibroblasts (21). No test for mycoplasmal contamination was carried out in the present study. The cells were maintained in Dulbecco-Vogt medium supplemented with 10% heat inactivated FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 m*M* glutamine and studied between the 8th and 13th transfer in culture. The fibroblasts were maintained in 75-cm^2 tissue culture flasks in Dulbecco-Vogt medium. For each experiment the cells were released from the tissue culture plates by treatment with 0.05% trypsin in PBS for 5 min at 37° C.

Measurement of cell proliferation. Cells were plated into 24-well tissue culture plates in triplicate at an initial density of 50 000 cells/well. The number of cells in each culture was determined at daily intervals over a 7-d period. Briefly, at each time point the medium was removed and the cells washed twice with 0.5 ml PBS. The cells were then released by trypsinization with 200 μl of 0.05% trypsin at 37° C for 15 min. Cells were pipetted from the wells and each well washed twice with 200 μl PBS. The trypsinized cells and the washes were pooled and cell numbers determined using a Coulter Counter.

[^{35}S]Sulfate incorporation and pulse chase. For the radiolabel incorporation studies, trypsinized cells were seeded in 2 ml medium at a density of 50 000/well into 24-well plates. The cells were allowed to grow to confluence with medium replenishment daily. The culture medium was then removed and replaced with 500 μl of sulfate-depleted medium ($MgSO_4$ replaced with $MgCl_2$) and the cells incubated for 1 h at 37° C. The sulfate-depleted medium was then removed and replaced with sulfate-depleted medium containing [^{35}S]sulfate (75 μCi/ml) and incubated at 37° C for up to 48 h. Incorporation of [^{35}S]sulfate into proteoglycans by normal and inflamed fibroblasts was monitored over 48 h at 2, 4, 8, 24, 30, and 48 h intervals. At each time interval,

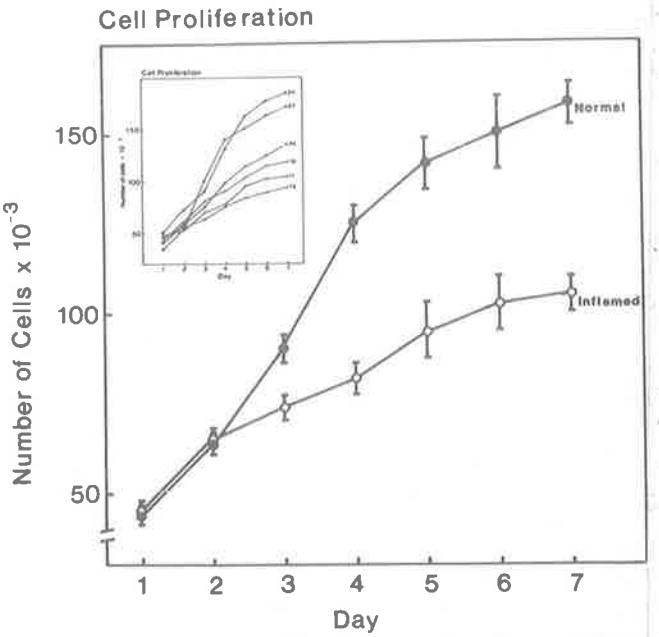

FIG. 1. Fibroblast proliferation rates. Normal and inflamed gingival tissue fibroblasts were seeded into 24-well plates at the same initial plating densities (50 000 cells/well). The cultures were incubated for 7 d and cell numbers were determined daily by counting in a Coulter Counter as described in Materials and Methods. The experiments were repeated three times and representative data from one of these experiments are shown. The mean and SEM of triplicate cultures for each time point for three strains normal and three strains of inflamed fibroblasts are shown. The *inset* shows mean proliferation rates of the individual cell strains: normal fibroblasts are designated 24, 25, and 27 and inflamed fibroblasts designated 10, 11, and 13.

the medium was removed from each culture and the cell layers washed twice with 200 μl PBS. The medium and washes for each cell strain were pooled. The cell layers were then treated with 200 μl of 0.05% trypsin in PBS for 15 min at 37° C and washed twice with 200 μl PBS. The trypsin digest and washes for each cell strain were also pooled. The medium and cell layer extracts were then frozen at −70° C until all of the time points had been completed. Aliquots (300 μl) of the medium and cell layer extracts of each time point were then spotted, in duplicate, onto Whatman 3MM filter paper. The filter paper was dried and then immersed through five changes of 1% cetylpyridinium chloride (CPC) in 0.05 *M* NaCl each of 1 h duration (35). The paper strips were then dried overnight, cut into small pieces and placed into 10 ml of scintillation fluid and assessed for [^{35}S]-activity in a Packard Tri Carb 3255 liquid scintillation counter.

For the pulse-chase experiments, triplicate cultures of cells were plated into 24-well plates, as for the [^{35}S]sulfate incorporation studies, and allowed to reach confluence. Radiolabeling of the cultures with [^{35}S] sulfate was commenced as described above and allowed to proceed for 36 h, by which time a steady state of [^{35}S]sulfate incorporation is reached (3). The medium was then removed and the cells washed three times with PBS to remove residual-free [^{35}S]sulfate and medium-associated [^{35}S]-labeled macromolecules and replaced with 500 μl Dulbecco-Vogt medium. The release of radiolabeled macromolecules into the medium was followed over 48 h at 2, 4, 8, 24, 30, and 48-h intervals. At each time point, the medium and cell layers were harvested and the amount of [^{35}S]sulfate-labeled proteoglycans determined using CPC precipitation onto filter paper and liquid scintillation counting as described above.

Metabolic labeling and proteoglycan extraction. For quantitative and qualitative biochemical analyses of the proteoglycans synthesized by fibroblast cultures, cells wre seeded into 75-cm^2 flasks at a density of 750 000/ flask. The cells were grown to confluence in 75-cm^2

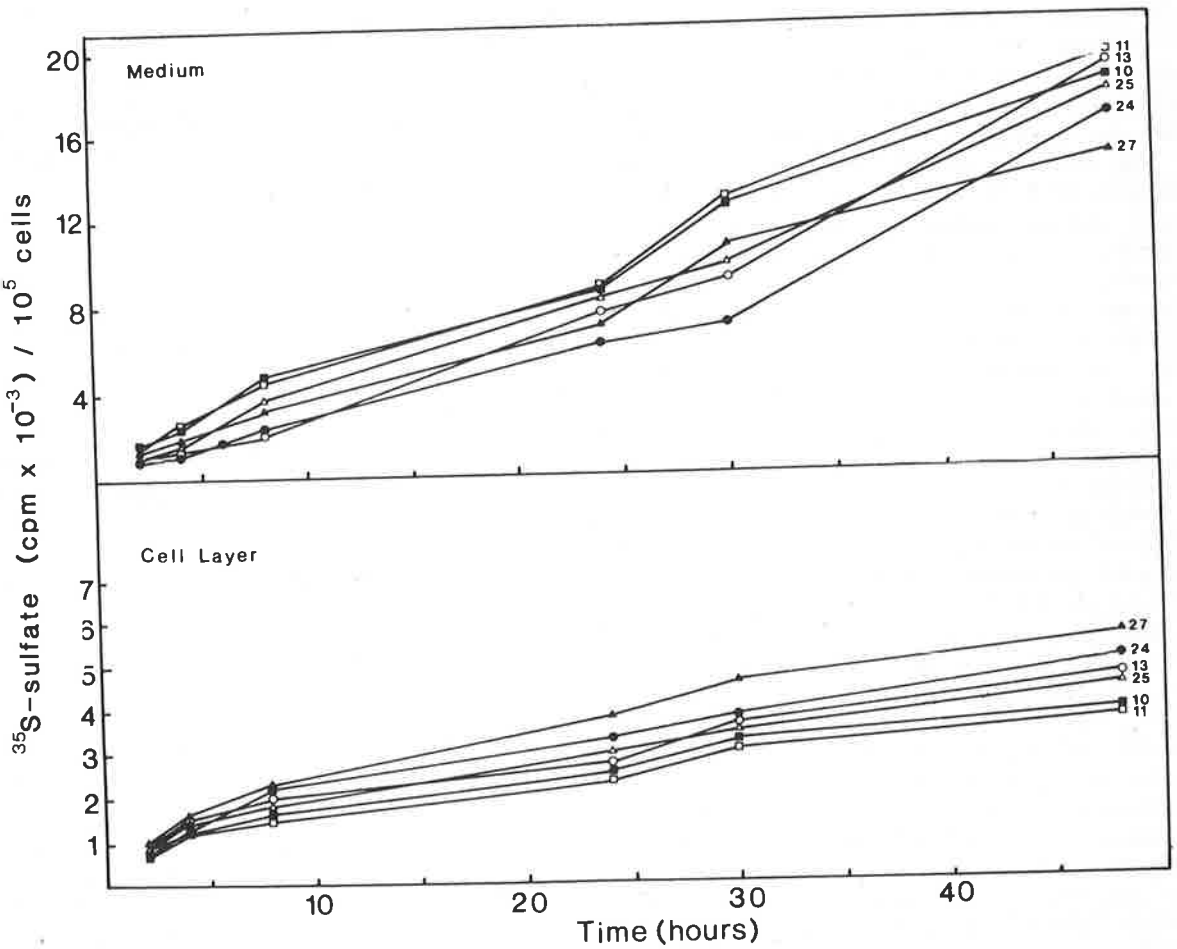

FIG. 2. [^{35}S]Sulfate incorporation into proteoglycans with time. Normal and inflamed gingival tissue fibroblasts were plated into 24-well plates in triplicate and were radiolabeled with [^{35}S]sulfate for various lengths of time between 0 and 48 h. At each time point, the medium and cell layer were extracted with 4 *M* guanidine HCl as described in Materials and Methods. The amount of radioactivity precipitable with cetylpyridinium chloride for each time point is expressed relative to the number of cells present at that time. Data indicate the mean of experiments carried out on each cell strain in triplicate for each time point. The experiments were repeated three times and representative data for one of these experiments are presented. Normal gingival fibroblasts: HGF_{24} ●—●; HGF_{25} △—△; HGF_{27} ▲—▲. Inflamed gingival fibroblasts: HGF_{10} ■—■; HGF_{11} □—□; HGF_{13} ○—○.

flasks with medium replenishment every 2 d. Metabolic labeling of the cells was carried out in 5 ml Dulbecco-Vogt medium containing 75 μCi/ml [^{35}S]sulfate for 48 h at 37° C. For these experiments, sulfate-depleted medium was not used.

[^{35}S]Sulfate-labeled proteoglycans were isolated from the medium and cell layers of normal and inflamed fibroblasts. Briefly, the medium was removed and the cell layers washed twice with 5 ml PBS and pooled. The pooled material was then made approximately 3 *M* in quanidine HCl by adding solid guanidine HCl (0.38 g/ml). Unincorporated radiolabel was then separated from labeled macromolecules by elution from Sephadex G-50 in 4 *M* guanidine HCl 0.05 *M* sodium acetate containing the following protease inhibitors: 0.1 *M* 6-aminohexanoic acid, 5 m*M* benzamidine HCl, 50 m*M* EDTA, 0.1 m*M* phenylmethylsulfonyl flouride, and 10 m*M* *N*-ethylmaleimide, pH 5.8. The cell layers were extracted overnight at 4° C in 4 *M* guanidine HCl 0.05 *M* sodium acetate plus protease inhibitors. The cell layers were then scraped from the culture plates and rinsed once with 4 *M* guanidine HCl-0.05 *M* sodium acetate, plus protease inhibitors. The extract and wash were pooled and centrifuged to remove any insoluble cell debris. The material remaining in this residue represented less than 3% of the total radioactivity associated with the cell layer extracts. The cell layer extracts were also eluted from Sephadex G-50 in 4 *M* guanidine HCl-0.05 *M* sodium acetate plus protease inhibitors to separate the labeled macromolecules that eluted in the void volume from the free radiolabel.

Ion-exchange chromatography. The overall charge of the proteoglycans synthesized by normal and inflamed fibroblasts was assessed by ion-exchange chromatography. Aliquots (5 ml) of the medium and cell layer proteoglycans, obtained from Sephadex G-50 chromatography, were dialyzed against several changes of 0.1 *M* sodium chloride in 7 *M* urea, 0.05 *M* Tris-HCl, and protease inhibitors, pH 7.0, and then applied to a DEAE-Sephacel column (0.7 × 3 cm) equilibrated with the same bufer. After elution of the unbound material, a continuous 0.1 to 0.8 *M* sodium chloride gradient was applied using a total of 30 ml. Fractions of 1.0 ml were collected and 0.5 ml aliquots of these fractions were assessed for [^{35}S]sulfate activity.

Analytical column chromatography. The molecular size distribution of [^{35}S]-labeled proteoglycans was analyzed by gel filtration on a column of Sepharose CL-4B. Aliquots of the [^{35}S]-labeled material excluded from Sephadex G-50 (1.0 ml) were concentrated to 200 μl in dialysis tubing against Aquacide. The concentrate was applied to and eluted from columns of Sepharose CL-4B (0.7 × 100 cm) with 4 *M* guanidine HCl in 0.05 *M* sodium acetate containing the above-mentioned protease inhibitors, pH 5.8 (dissociative conditions). To determine whether any of the proteoglycans were detergent sensitive, aliquots of the excluded material from Sephadex G-50 chromatography (1.0 ml) were dialyzed against 0.15 *M* sodium acetate, 5 m*M* $MgCl_2$, and 1 m*M* $CaCl_2$, pH 5.8. The retentate was then concentrated to 200 μl inside dialysis tubing against Aquacide and then made 0.2% SDS by adding 10× stock SDS in sodium acetate buffer. The concentrates were applied to Sepharose CL-4B columns (0.7 × 100 cm) and eluted with 0.2% SDS in the same sodium acetate buffer as described above (detergent conditions). Fractions of 0.5 ml were collected at a flow rate of 3.0 ml/h and all of the fractions were assayed for [^{35}S]-activity.

Glycosaminoglycan identification. [^{35}S]-Labeled proteoglycans isolated in the excluded peak from Sephadex G-50 chromatography, as well as those in all of the peaks obtained from Sepharose CL-4B chromatography under

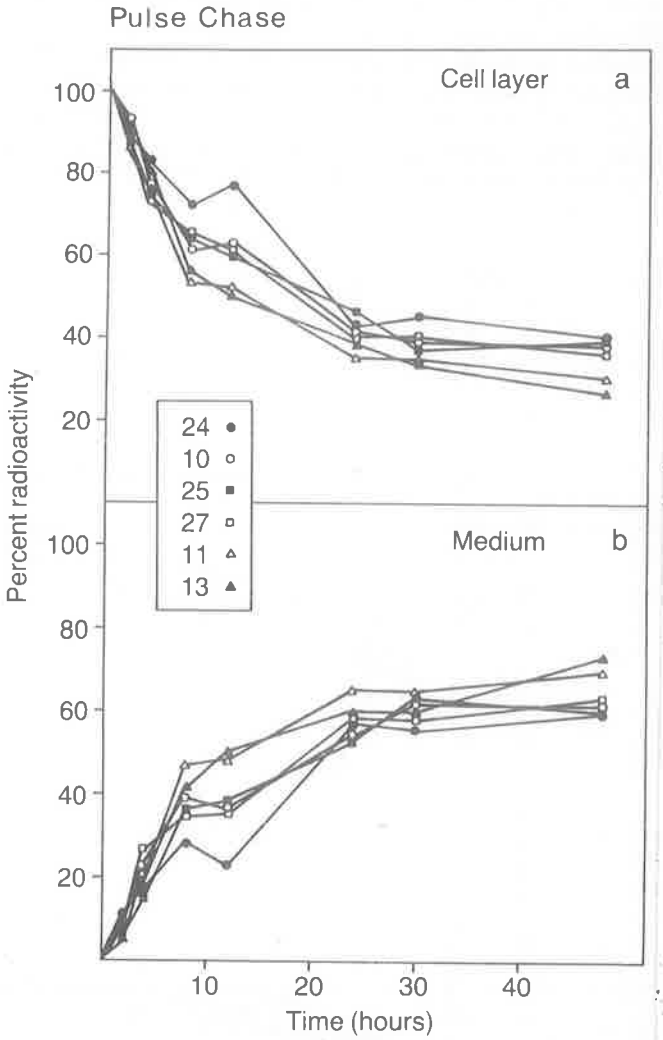

FIG. 3. Pulse chase of [^{35}S]labeled proteoglycans. Normal and inflamed gingival fibroblasts were pulsed for 36 h with [^{35}S]sulfate. The amount of [^{35}S]-labeled proteoglycans associated with the medium and cell layer at various time points over 48 h was then assessed after guanidine HCl extraction and cetylpyridinium chloride precipitation as described in Materials and Methods. Data presented are the mean of triplicate cultures for each time point of each of the six cell strains studied. The experiments were repeated three times and data representative of one of these experiments are shown. Normal gingival fibroblasts: HGF_{24} ●—●; HGF_{25} ■—■; HGF_{27} □—□. Inflamed gingival fibroblasts: HGF_{10} ○—○; HGF_{11} △—△; HGF_{13} ▲—▲.

dissociative conditions, were digested with papain and the released glycosaminoglycans were identified. Briefly, 1.0-ml aliquots of the material that excluded from Sephadex G-50 were dialyzed against 0.2 *M* sodium acetate, 4 m*M* EDTA, and 20 m*M* cysteine HCl, pH 5.7, and then concentrated against Aquacide to 200 μl. The concentrates were then digested with papain (1 mg/ml buffer) overnight and loaded onto Sepharose CL-6B columns (0.7 × 30 cm) and eluted with 0.2% SDS in 0.15 *M* sodium acetate, 5 m*M* $MgCl_2$, and 1 m*M* $CaCl_2$. Fractions of 0.5 ml were collected at a flow rate of 3 ml/h and all of the fractions were assayed for radioactivity. The types of sulfated glycosaminoglycans present were determined after selective enzyme digestion (chondroitinase AC and ABC) and chemical (nitrous acid) degradation (26, 28). Aliquots (1.0 ml) from the material excluded from Sephadex G-50 chromatography were dialyzed against 50 m*M* Tris-HCl, 60 m*M* sodium chloride, and 40 m*M* sodium acetate, pH 8.0, concentrated to 200 μl against Aquacide and then subjected to one of the above-mentioned degradative treatments. The reaction products were then chromatographed on Sephadex G-50 columns (0.7 × 30 cm) using the same SDS-acetate buffer as described above. The relative proportions of each glycosaminoglycan species were determined by calculating the amount of [^{35}S]-labeled material resistant to (void volume) or degraded by (included volume) each treatment.

Results

Cell growth. The proliferative rates of the normal and inflamed fibroblasts were assessed over a 7-d period to determine the time span required for each of the cell strains to reach confluence and thereby to determine the time intervals, at which time metabolic labeling could be commenced. Variability in growth rates among all the strains studied was noted. In general, the fibroblasts obtained from inflamed tissue were slower growing, taking longer to reach confluence than similar age-, sex-, and transfer number-matched cells derived from normal tissue (Fig. 1). Indeed, by Day 3 the differences in the cell numbers between normal and inflamed fibroblast cultures were calculated to be statistically different at the $P<0.05$ level by using analysis of variance.

[^{35}S]Sulfate incorporation. The incorporation of [^{35}S]sulfate into macromolecular material associated with the cell layer and medium compartments was determined over a 48-h period. Despite obvious differences in growth rates of the normal and inflamed fibroblasts seen in Fig. 1, the rate of [^{35}S]sulfate incorporation into proteoglycans did not differ significantly (Fig. 2). The bulk of the [^{35}S]-activity was found in the medium where the amount increased steadily over 48 h. The incorporation of [^{35}S]sulfate into cell layer associated proteoglycans was rapid during the first 8 h and continued to increase up to 30 h; a relatively steady state was reached by 48 h. In all cases, by 48 h approximately 70% of the total [^{35}S]-activity was found in the medium, with the remaining 30% being associated with the cell layer.

Pulse chase experiments. Pulse chase experiments were carried out to determine if there were any detectable differences in the rate of release of proteoglycans into the medium by normal and inflamed fibroblasts. As observed in the above experiments, there seemed to be no remarkable differences in [^{35}S]sulfate incorporation among the cell strains (Fig. 3). The release of [^{35}S]-labeled proteoglycan from the cell layers into the medium was rapid during the first 12 h of the chase, and then seemed to level off at around 50% of the total activity in the medium and cell layer by 24 h. This increase of [^{35}S]-labeled proteoglycans in the medium was accompanied by a concomitant decrease of [^{35}S]-labeled proteoglycans associated with the cells.

Quantitation of glycosaminoglycans. The glycosaminoglycan composition of the newly synthesized labeled macromolecules, as determined by selective enzymatic and chemical degradation, is shown in Table 1. Heparan sulfate was not only the predominant sulfated glycosaminoglycan associated with the cell layers of normal fibroblasts, where it comprised 56% of the total [^{35}S]-activity, but it was also predominant in the cell layers of the inflamed fibroblasts (53%). Overall, no

TABLE 1

PROPORTIONS OF SULFATED GLYCOSAMINOGLYCANS SYNTHESIZED BY NORMAL AND INFLAMED GINGIVAL TISSUE FIBROBLASTS[a]

	Heparan Sulfate	Dermatan Sulfate	Chondroitin Sulfate
Medium			
Normal HGF 24	20 ± 2	50 ± 2	30 ± 2
Normal HGF 25	30 ± 1	44 ± 4	27 ± 2
Normal HGF 27	33 ± 6	46 ± 4	22 ± 5
Mean	27 ± 7[b]	47 ± 4[b]	27 ± 5[b]
Inflamed HGF 10	12 ± 2	70 ± 2	19 ± 2
Inflamed HGF 11	11 ± 2	64 ± 4	25 ± 2
Inflamed HGF 13	13 ± 2	66 ± 4	21 ± 4
Mean	12 ± 2[b]	67 ± 4[b]	21 ± 4[b]
Cell Layer			
Normal HGF 24	61 ± 3	22 ± 5	18 ± 2
Normal HGF 25	56 ± 1	29 ± 4	14 ± 2
Normal HGF 27	48 ± 7	30 ± 5	21 ± 6
Mean	56 ± 7	26 ± 6	18 ± 4
Inflamed HGF 10	57 ± 3	25 ± 5	18 ± 2
Inflamed HGF 11	52 ± 6	29 ± 9	19 ± 3
Inflamed HGF 13	51 ± 3	28 ± 4	21 ± 4
Mean	53 ± 5	30 ± 6	19 ± 3

[a]The data are expressed as the percentage of the total radioactive material loaded onto Sephadex G-50 columns which was sensitive to specific enzymatic and chemical degradation procedures. All experiments were performed in triplicate; mean values and SD of the mean are presented.

[b]Represent data that are statistically different ($P<0.05$) from each other within corresponding groups. Data points determined for each glycosaminoglycan species associated with each fibroblast strain were pooled together as either normal or inflamed. Analysis of variance was used to determine if the differences between the normal and inflamed fibroblast strains were significant.

significant differences were noted between the cell layer associated glycosaminoglycans of normal and inflamed fibroblasts. Dermatan sulfate was the predominant sulfated glycosaminoglycan associated with the medium of both normal and inflamed fibroblasts, but the proportion was elevated in the medium of inflamed fibroblasts where it accounted for between 15 and 20% more than was seen in the medium of the normal strains. This increase was accompanied by a concomitant decreased proportion of chondroitin sulfate and heparan sulfate. All of the differences noted between the media associated glycosaminoglycans of normal and inflamed fibroblasts were significant ($P < 0.05$) as assessed by analysis of variance.

Ion-exchange chromatography. The isolated proteoglycans from the medium and cell layer of normal and inflamed fibroblasts were initially characterized by ion-exchange chromatography to assess their charge (Fig. 4). No differences in the elution position of the medium and cell layer proteoglycans of normal and inflamed fibroblasts were detected. In all cases, the major [^{35}S]-labeled peak was relatively symmetrical and eluted from the column at a salt concentration of approximately 0.4 *M*.

Molecular Sieve Chromatography

Dissociative conditions. More detailed information regarding the properties of the proteoglycans was obtained by assessing their molecular size distribution after elution from columns of Sepharose CL-4B (Fig. 5). Under dissociative conditions (4 *M* guanidine HCl), the proteoglycans of normal fibroblasts eluted as three discrete populations. These corresponded to K_{av} values of 0, 0.3, and 0.4 and were termed NM1, NM2, and NM3, respectively (Fig. 5 *a*). When the proteoglycans associated with the medium of the inflamed fibroblasts were subjected to similar preparative steps and analyzed by elution from Sepharose CL-4B under dissociative conditions, the peak corresponding to K_{av} 0.3 was either absent or severely depleted (Fig. 5 *b*). The peaks corresponding to K_{av} of 0 and 0.4 were termed IM1 and IM2.

Some of the differences in the dissociative Sepharose CL-4B elution profiles of the cell layer proteoglycans were also noted (Fig. 5). These were, however, more subtle than the differences seen for the medium proteoglycans. In all cases (both normal and inflamed fibroblasts), four peaks could be clearly identified eluting with K^{av} values of 0, 0.1, 0.4, and 0.55 (Fig. 5 *c* and *d*). These peaks were termed NC1 through NC4 and IC1 through IC4 for the normal and inflamed cell layer proteoglycans, respectively. The relative proportions of total radioactivity in each of these peaks varied somewhat (Table 2). The quantitative major peaks were those corresponding to K_{av} of 0.1 and 0.55 and accounted for approximately 60 to 70% of the total [^{35}S]-labeled material associated with the cell layers of normal and inflamed fibroblasts. Recoveries of [^{35}S]-labeled material from these columns eluted with 4 *M* guanidine HCl were approximately 80 to 85% of the total radioactivity applied.

Detergent conditions. Inasmuch as proteoglycans in both the medium and cell layer preparations of normal and inflamed fibroblasts were excluded from Sepharose CL-4B under dissociative conditions, and because

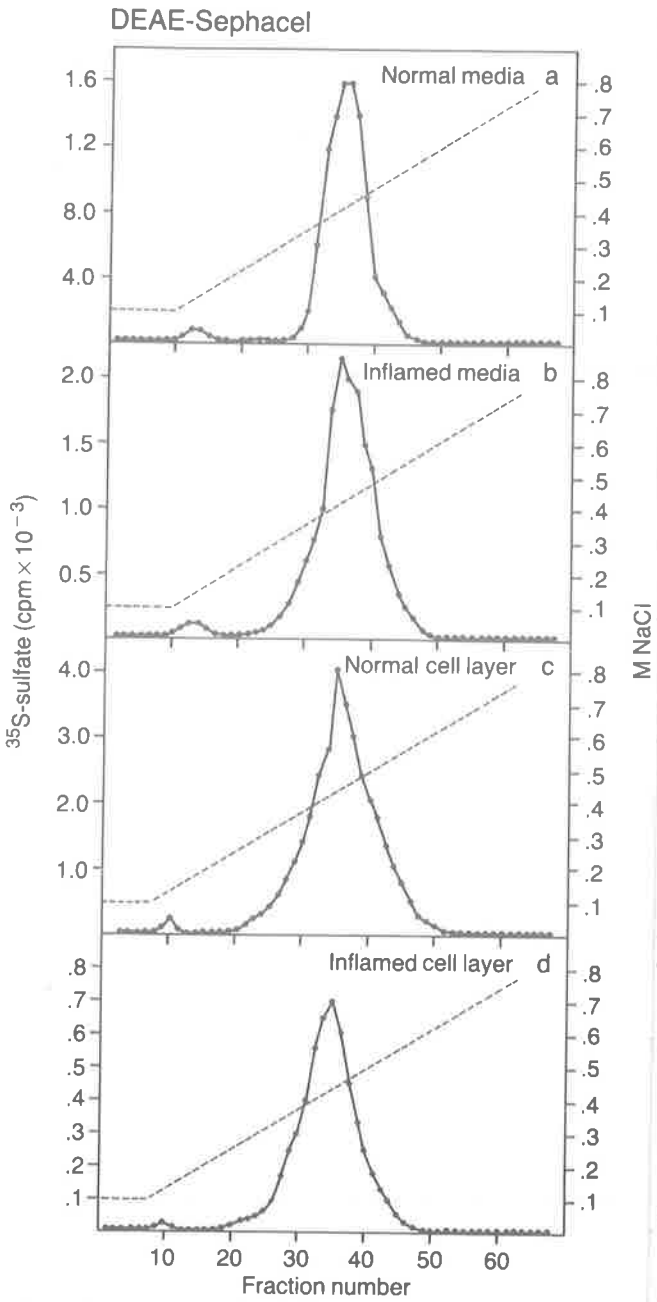

FIG. 4. DEAE-Sephacel ion-exchange chromatography of [^{35}S]-labeled macromolecules. Fibroblasts from normal and inflamed gingiva were incubated for 48 h in the presence of [^{35}S]sulfate and the macromolecular-labeled material in the media (*a* and *b*) and cell layer (*c* and *d*) extracts was isolated in the void volume after elution from Sephadex G-50 in 7 *M* urea. The excluded peaks for the medium and cell layer extracts were applied to DEAE-Sephacel and eluted with a linear NaCl gradient from 0.1 to 0.8 *M*. Representative data for one each of the normal (HGF_{25}) and inflamed (HGF_{11}) gingival fibroblast cell strains are presented.

previous reports have suggested that the cell layer excluded material is lipid associated (Bartold, P. M.; Page, R. C., submitted for publication) we also analyzed the proteoglycans on Sepharose CL-4B columns eluted under detergent conditions (0.2% SDS in acetate buffer) (Fig. 6). When compared to the dissociative conditions (Fig. 5 *a* and *b*), the medium proteoglycans were not affected by treatment with detergent (Fig. 6 *a* and *b*). The proteoglycans of the medium of normal fibroblasts separated into three populations corresponding to K_{av} values of 0, 0.3, and 0.4, whereas only two peaks corresponding to K_{av} values of 0. and 0.4 were observed for samples from cultures of inflamed fibroblasts. The peak of K_{av} 0.3 was absent.

When cell layer extracts were chromatographed under detergent conditions, elution profiles different from those obtained under dissociative conditions were seen (Fig. 6 *c* and *d*). Three peaks were identified corresponding to K_{av} values of 0.1, 0.4, and 0.55. The excluded volume peak eluted under dissociative conditions was absent and suggests that cultures of both normal and inflamed fibroblasts have lipid associated proteoglycans in their cell layers. The relative proportion of radioactivity in each of the peaks obtained under detergent conditions varied between the normal and inflamed cell layer proteoglycans. The [^{35}S]-label was more evenly distributed throughout the profiles of each of the three peaks of the normal fibroblasts, accounting for 28, 25, and 46%, respectively, for the K_{av} values of 0.1, 0.4, and 0.55. However, the inflamed cell layer proteoglycans showed a noticeable difference in distribution. The peak of K_{av} 0.1 was the quantitative predominant peak (45% of the total radioactivity), and the peak corresponding to K_{av} of 0.55 was the quantitative minor component accounting for 25% of the total radioactivity. The recoveries of [^{35}S]-activity from the columns run under detergent conditions were greater (90%) than those for columns eluted with guanidine HCl.

Chemical analysis of dissociatively prepared proteoglycans. The proteoglycan nature of each of the peaks isolated after preparative Sepharose CL-4B chromatography under dissociative conditions (NM1-3; IM1 and

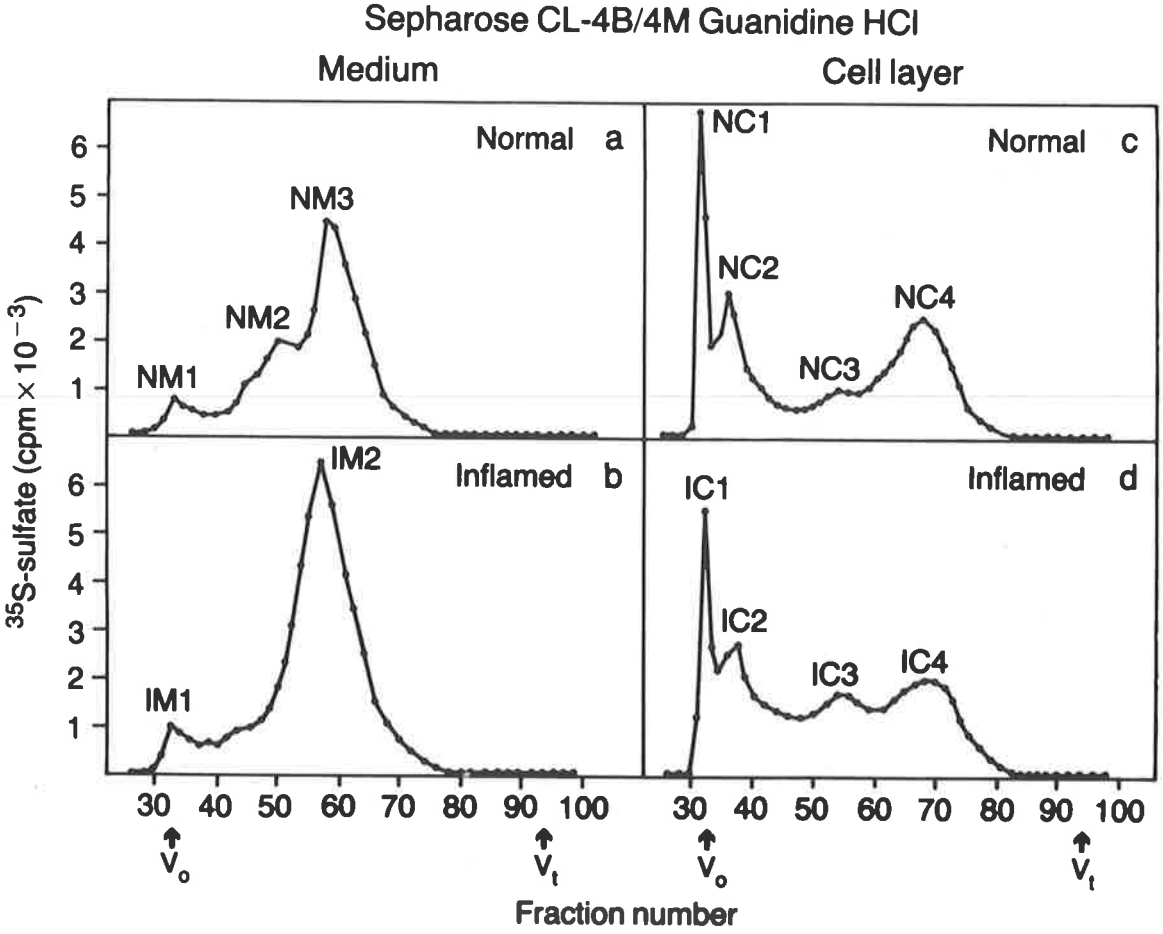

FIG. 5. Gel filtration chromatography of media and cell layer extracts: dissociative conditions. [^{35}S]-Labeled culture medium and cell layers were extracted with 4 *M* guanidine HCl and eluted from Sephadex G-50 in the presence of guanidine HCl as described in the Materials and Methods. The macromolecular radioactivity eluting at the void volume was applied to a Sepharose CL-4B column (0.7 × 100 cm) and eluted with the same buffer used for Sephadex G-50 chromatography. The void volume (*Vo*) and total volume (*Vt*) were identified with [^{3}H]DNA and Na_2 [^{35}S] O_4, respectively. Representative data for the normal (HGF_{2S}) and inflamed (HGF_{11}) gingival fibroblasts are shown.

IM2; NC1-4 and IC1-4) was determined by treatment with papain and subsequent Sepharose CL-6B chromatography. [^{35}S]-Labeled material in all of the peaks except NC4 and IC4 contained proteoglycans as demonstrated by susceptibility to papain digestion (Fig. 7). The [^{35}S]-labeled molecules in peaks NC4 and IC4 were considered to be free glycosaminoglycan chains because their elution position was not altered by papain treatment. The [^{35}S]-labeled glycosaminoglycan chains released from the proteoglycans eluted from Sepharose CL-6B with K_{av} values of 0.45 and therefore most likely had an average molecular weight in the range of 25 000 (34).

The glycosaminoglycan composition of each [^{35}S]-labeled peak identified by dissociative Sepharose CL-4B chromatography (Fig. 5) was determined by treating aliquots from each peak with chondroitinase AC, chondroitinase ABC, or nitrous acid, followed by chromatography on Sephadex G-50 columns. Qualitative and quantitative differences between the glycosaminoglycan composition of the proteoglycan peaks from normal and inflamed fibroblasts were observed (Table 2). For the medium proteoglycans, peak IM1 contained more heparan sulfate and less chondroitin sulfate than the corresponding normal fibroblast proteoglycan peak NM1. In addition, a small amount (17%) of dermatan sulfate was identified in peak IM1 but was absent in peak NM1. The proportions of glycosaminoglycans constituting the proteoglycans of peaks NM3 IM2 were found to be similar, with dermatan sulfate predominating in both. The cell layer proteoglycans also demonstrated some qualitative glycosaminoglycan differences. Chondroitin sulfate was present in greater proportions in peak IC4 than compared to peak NC4, whereas heparan sulfate, which was the principal glycosaminoglycan identified in peak NC4, contributed less to the total proteoglycans in the corresponding normal fibroblast peak NC4. In addition, peak NC3 of the normal fibroblasts contained more dermatan sulfate but less chondroitin sulfate than the corresponding inflamed peak of IC3.

Discussion

Fibroblasts from normal and chronically inflamed human gingiva have been cultured and their growth and proteoglycan production assessed. Several differences were noted between normal and inflamed tissue fibroblasts; these were persistent during several transfers in vitro and therefore presumed to be heritable. For example, all three strains of inflamed fibroblasts grew at a rate significantly less ($P < 0.05$) than similar age- sex- and transfer-matched normal strains. Moreover, several differences were observed in the constituent sulfated glycosaminoglycans of the proteoglycans synthesized by these cells.

The incorporation of [^{35}S]sulfate into proteoglycans over a 48-h period, and pulse chase experiments over a similar time course, failed to highlight any noticeable differences in the capacity of normal and inflamed fibroblasts to synthesize and release [^{35}S]-labeled proteoglycans. Furthermore, ion-exchange chromatography on DEAE-Sephacel of the newly synthesized macromolecules also failed to demonstrate any significant charge differences between the proteoglycans made by normal and inflamed fibroblasts. However, more detailed analysis revealed structural and qualitative differences in the proteoglycans. Heparan sulfate, the major sulfated glycosaminoglycan associated with the cell layers of both normal and inflamed fibroblasts, was depleted in the medium of inflamed tissue fibroblast cultures. Additionally, dermatan sulfate, the principal sulfated glycosaminoglycan in the medium of both normal and inflamed tissue fibroblast cultures, was significantly elevated in the inflamed fibroblast medium. This increase in dermatan sulfate was accompanied by a decrease in the relative proportion of chondroitin sulfate in the medium of inflamed fibroblasts. These differences were reproducible and they were observed in all three strains of inflamed fibroblasts studied.

At first we suspected that the decrease in amounts of chondroitin sulfate and heparan sulfate in the medium of inflamed fibroblasts could reflect their different growth rates, because actively dividing cells are reported to shed heparan sulfate from their cell surface into the medium (14). In addition, an increase in the release of chondroitin sulfate into the medium of cultures stimulated to divide

TABLE 2

GLYCOSAMINOGLYCAN COMPOSITION OF PROTEOGLYCAN FRACTIONS[a]

Peak	Percent Fraction	Heparan Sulfate	Dermatan Sulfate	Chondroitin Sulfate
Medium				
NM1	5	18 ± 7	—[b]	82 ± 5[b]
NM2	20	30 ± 4	—	70 ± 4
NM3	75	23 ± 5	66 ± 4	12 ± 3
IM1	6	31 ± 5[b]	17 ± 1[b]	52 ± 6[b]
IM2	94	24 ± 6	66 ± 4	11 ± 2
Cells				
NC1	20	60 ± 8	10 ± 1[b]	30 ± 8
NC2	30	64 ± 5	—	30 ± 5
NC3	15	45 ± 3	44 ± 4[b]	12 ± 1
NC4	35	69 ± 7[b]	31 ± 8	—
IC1	15	58 ± 3	—[b]	42 ± 3
IC2	35	54 ± 8	—	46 ± 7
IC3	20	50 ± 7	26 ± 9[b]	25 ± 4[b]
IC4	30	40 ± 5[b]	42 ± 3	18 ± 5[b]

[a] Glycosaminoglycan content was determined on each peak obtained from Sepharose CL-4B chromatography by sequential digestion with chondroitinase AC, chondroitinase ABC, and nitrous acid. Data presented (mean values of three normal and three inflamed fibroblast proteoglycan preparations and SD of the mean) are expressed as percentage of the total ^{35}S-activity remaining after degradation. Fractions refer to the peaks obtained by Sepharose CL-4B chromatography as seen in Fig. 5.

[b] Represents data within corresponding groups (i.e. NM1 and IM1) which are statistically different ($P < 0.05$). Triplicate determinations for each glycosaminoglycan species in each of the peaks of normal and inflamed fibrobalsts were assessed statistically using analysis of variance to determine significant differences between the two groups of fibroblasts.

by exposure to 10% serum relative to nondividing cells in medium with 0.5% serum has been reported by Vogel and Sapien (31). They have suggested that such differences in proteoglycan production and release may be a response to some external signal provided by the serum. Our Sepharose CL-4B profiles for proteoglycans from inflamed and normal fibroblasts are remarkably similar to profiles of proteoglycans from fibroblasts cultured in 0.5 and 10% serum, respectively (31). However, it is notable that the behavior of our inflamed fibroblasts in this regard does not seem to be dependent on serum concentration.

Increased levels of dermatan sulfate in the medium of inflamed fibroblasts may have significant biologic implications if the cells behave similarly in vivo. During chronic inflammation there is a rapid loss of collagen at the inflammatory site (22), after which the fibroblasts are required to try to repair this damage by depositing new collagen and other essential matrix components. Dermatan sulfate proteoglycan is closely associated with mature collagen (27) and it may play an important role in collagen fibrillogenesis (8,30,32). Therefore, elevated amounts of dermatan sulfate at sites of inflammation could enhance collagen deposition and reconstitution of the extracellular matrix.

The decreased amount of [^{35}S]-labeled material in the cell layer Sepharose CL-4B peak of K_{av} 0.55 (peak IC4) of inflamed fibroblasts also invites speculation. This peak contains free glycosaminoglycan chains of intracellular origin and most likely represents the degradative or turnover pool of the synthesized proteoglycans (Bartold, P. M.; Page, R. C., submitted for publication). Depletion of radioactive material in this peak from inflamed fibroblasts may be a consequence of an accelerated rate of degradation of newly synthesized proteoglycans by these cells. This seems unlikely, however, because the pulse chase experiments failed to reveal differences between the normal and inflamed fibroblasts with regard to synthesis and release. On the other hand, the endocytotic process (15,37) by which proteoglycans from the medium are transported to the internal compartments of the cell for subsequent degradation may be impaired in inflamed fibroblasts. This is an attractive possibility because suppression of degradative activity during a period of tissue repair and reconstitution of the matrix could be biologically advantageous.

Taken together, our data demonstrate the existence of clear-cut reproducible differences between strains of fibroblasts obtained from chronically inflamed and

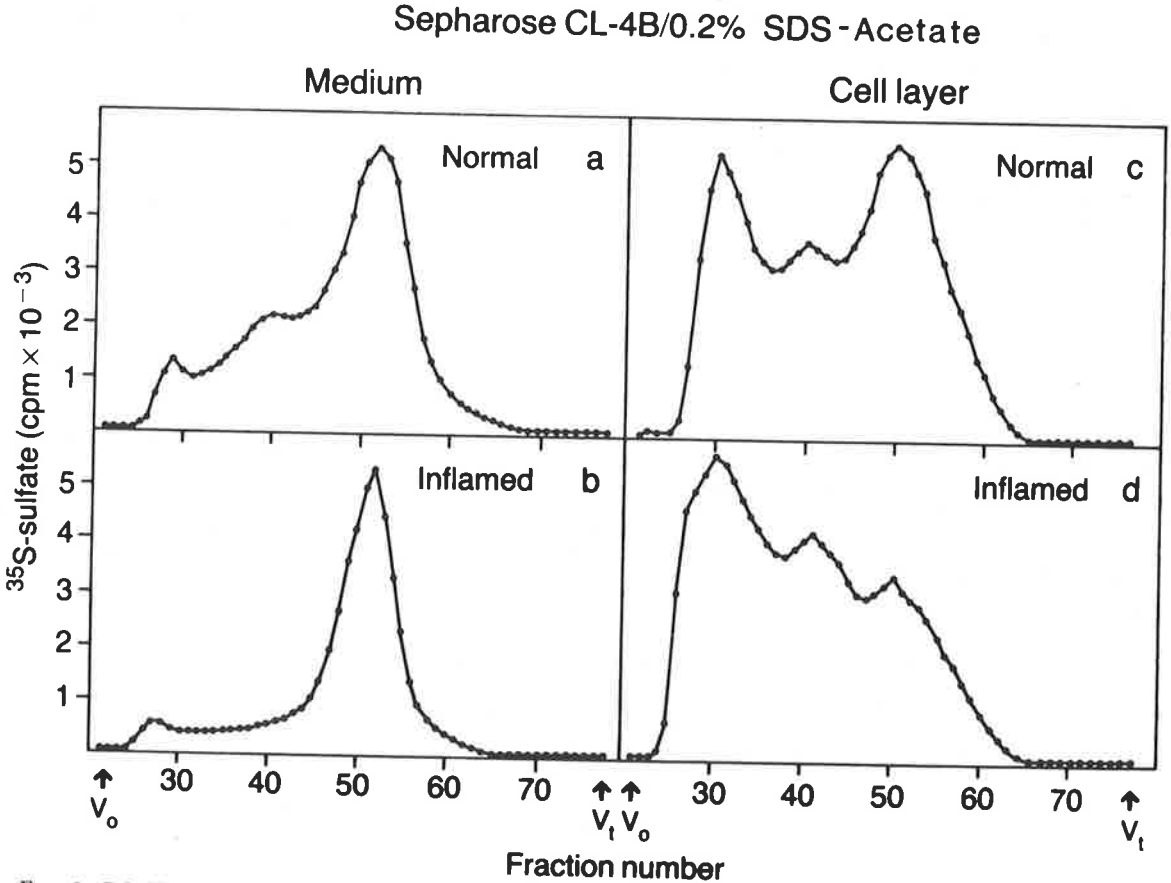

FIG. 6. Gel filtration chromatography of media and cell layer extracts: detergent conditions. [^{35}S]-labeled culture medium and cell layers were extracted with 4 *M* guanidine HCl as described in Materials and Methods. The extracts were dialyzed against 0.15 *M* sodium acetate, 0.001 *M* magnesium chloride, 0.001 *M* calcium chloride, pH 5.8, and then made 0.2% in SDS before elution on a Sepharose CL-4B column (0.7 × 100 cm) eluted with the same buffer used to dialyze the samples but also containing 0.2% SDS. The *Vo* and *Vt* were determined as described in Fig. 5. Representative data for the normal (HGF_{25}) and inflamed (HGF_{11}) gingival fibroblasts are shown.

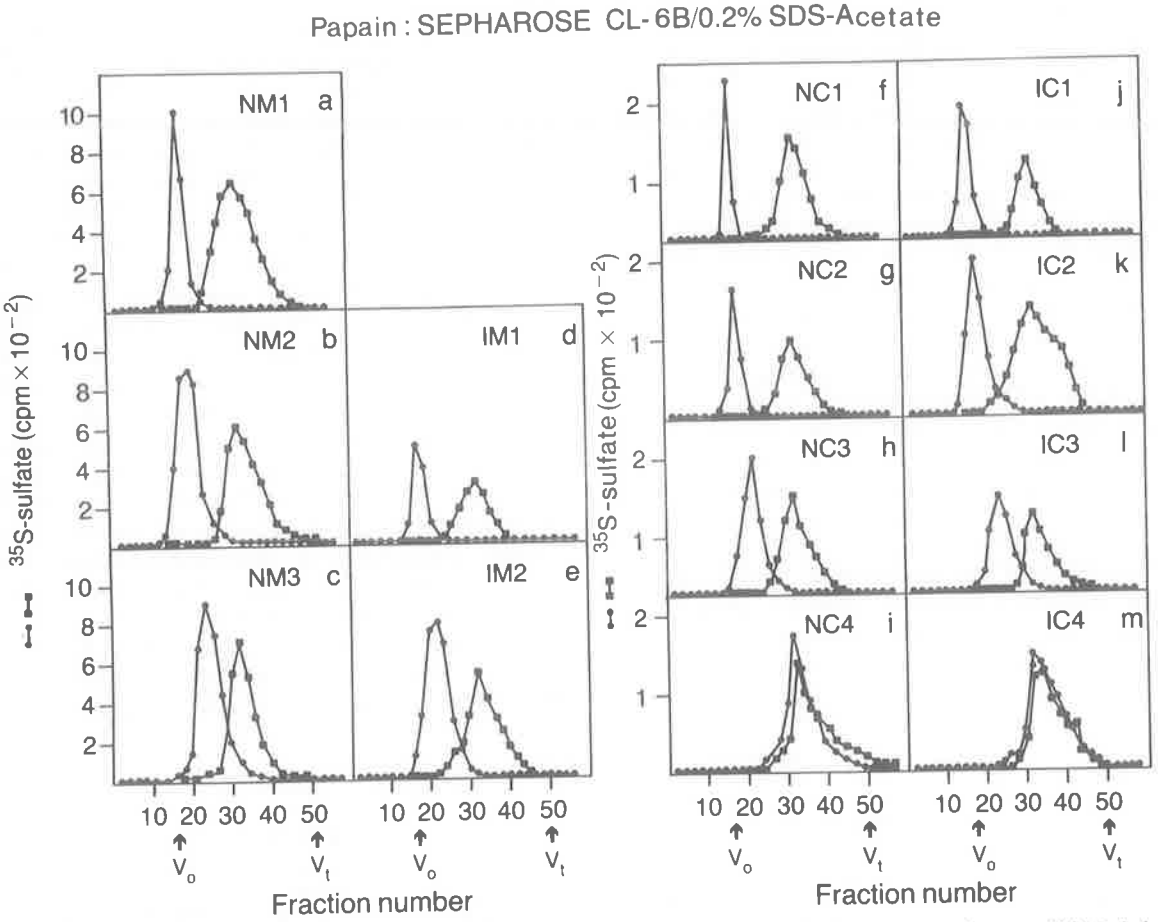

FIG. 7. Gel filtration chromatography of papain digested medium and cell layer proteoglycans. [^{35}S]-Labeled proteoglycans were isolated by elution from Sepharose CL-4B (*see* Fig. 5). Each of the peaks was pooled, dialyzed against papain digest buffer and aliquots were incubated at 60° C for 12 h in the presence or absence of papain. After incubation, each sample was applied to a Sepharose CL-6B column and eluted with the same buffer as described in Fig. 6. Both the *Vo* and *Vt* were identified as described in Fig. 5. Representative data for the normal (HGF_{25}) and inflamed (HGF_{11}) gingival fibroblasts are shown.

normal connective tissues. These differences persist in culture and are therefore heritable. In this regard, they are comparable to differences seen in collagen production by cells from chronically inflamed, fibrotic, and normal connective tissue (10,31,34) and the differences in strains of cells from arthritic tissue (5,6). Such differences have been taken to imply that the fibroblasts obtained from pathological sites are genetically different from those from normal healthy sites. Whether the differences we report are due to selection of preexisting subsets of resident fibroblasts within the tissue (4,17,29), or to persistent phenotypic alterations caused by an altered environment (20,23–25,33), remain to be established.

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Paper 12

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P.M. Bartold's role in this study was:
Design of the experiments
Execution of all experimental work
Writing of the paper

R.C. Page's role in this study was:
Provision of research funds
Writing of the paper

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Hyaluronic Acid Synthesized by Fibroblasts Cultured from Normal and Chronically Inflamed Human Gingivae

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Abstract

Hyaluronic acid is an important component of the extracellular matrix of gingivae and its quantity and molecular size appear to be altered under inflammatory conditions. Whether gingival fibroblasts from inflamed tissues synthesize quantities and molecular sizes of hyaluronic acid that differ from normal gingival fibroblasts is not known. To determine this, we isolated fibroblasts from three biopsies each of healthy and chronically inflamed human gingiva and incubated them in the presence of [^{3}H]-glucosamine. The release of labeled macromolecules into the medium was approximately 50% greater for the inflamed tissue fibroblasts than for the normal tissue fibroblasts. Of this labeled material, 35% was identified as hyaluronic acid in the medium of inflamed cell cultures, compared to only 25% in normal fibroblast cultures. Sepharose CL-4B chromatography of the labeled material revealed that most of the newly synthesized hyaluronic acid was of large molecular size in inflamed fibroblast cultures. The proportions of [^{3}H]-labeled hyaluronic acid in these peaks varied and indicated that an increase in the amount of large molecular size hyaluronic acid was responsible for the increase in labeled hyaluronic acid noted in the medium of the inflamed tissue fibroblasts. Thus, the decrease in molecular size of hyaluronic acid previously noted in inflamed tissue most likely arises from extracellular factors rather than synthesis of smaller molecular weight species by the fibroblasts. More importantly, however, the differences noted between normal and inflamed gingival fibroblasts persisted over time in culture. This indicates that such differences between the cells may be of a stable and heritable nature.

Key words: fibroblasts, gingiva, hyaluronic acid, inflammation.

Introduction

The role of proteoglycans and hyaluronic acid in the extracellular matrix of soft connective tissues is now recognized as being important in maintaining normal tissue function (Hascall and Hascall, 1981; Toole, 1981). During chronic inflammation, changes in the structure of these molecules occur, and these may be partially responsible for the observed pathophysiology.

Gingival tissue is a particularly useful model for studying the effects of chronic inflammation on extracellular components (Narayanan and Page, 1983). Using this model, we have shown that chronic inflammation results in marked depolymerization of hyaluronic acid and breakdown of proteoglycans (Bartold and Page, 1986 a). In addition, we have demonstrated that fibroblasts from chronically inflamed gingivae differ phenotypically from those from normal tissue with regard to proteoglycan synthesis (Bartold and Page, 1986 b). Others have demonstrated that extracts of bacteria associated with gingival inflammation can induce increased hyaluronic acid synthesis by gingival fibroblasts in culture (Larjava et al., 1983; Larjava, 1984). In order to determine the role fibroblasts play in the alterations of the extracellular nonfibrous macromolecules during inflammation, we have cultured these cells from normal and chronically inflamed human gingivae and studied their newly synthesized hyaluronic acid.

Materials and Methods

Materials

Guanidine HCl was purchased from Sigma Chemical Co., St. Louis, MO; *Streptomyces* hyaluronidase and chondroitinase ABC *(Proteus vulgaris)* from Seikagaku Kogyo Ltd., Tokyo Japan through Miles Laboratories Inc., Elkhart, IN; sodium dodecylsulfate (SDS)' from BioRad Laboratories, Richmond, CA, Sephadex G-50, Sepharose CL-4B and DEAE-Sephacel from Pharmacia Inc., Piscataway, NJ; Dulbecco-Vogt medium, fetal calf serum (FCS), phosphate-buffered saline (PBS), trypsin, penicillin and streptomycin from Grand Island Biological Co., Grand Island, NY; all tissue culture plastic ware was from Falcon Plastics, Oxnard, CA:D-[6-^{3}H]-glucosamine and [^{35}S]-sulfate from New England Nuclear, Boston, MA; Aquacide from Calbiochem, LaJolla, CA and Aquamix from WestChem, San Diego, CA.

Fibroblast Cultures

Fibroblasts were obtained from biopsies from human volunteers with healthy gingivae and from age- and sex-matched patients undergoing treatment for chronic periodontitis, as described previously (Narayanan et al., 1978). The normal gingival fibroblasts (henceforth termed "normal fibroblasts") were designated HGF_{24}, HGF_{25} and HGF_{27}; the inflamed tissue fibroblasts (henceforth termed "inflamed fibroblasts") were designated HGF_{10}, HGF_{11} and HGF_{13}. These cells were from the same parent populations used previously for the study of collagens and proteoglycans synthesized by normal and inflamed fibroblasts (Narayanan et al., 1978; Bartold and Page, 1986 c). The cells were maintained in 75 cm^2 tissue culture flasks by incubation in Dulbecco-Vogt medium supplemented with 10% heat-inactivated, fetal calf serum, 100 units/ml of penicillin, 100 µg/ml of streptomycin and 2 mM glutamine at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells between the 8th and 12th passage in culture were used.

[^{3}H]-Glucosamine Labeling

Cells were released from the 75 cm^2 flasks by treatment with 0.05% trypsin in phosphate-buffered saline (PBS) for 5 min at 37 °C. The cells were then plated in triplicate at an initial density of 500,000 cells into 35 mm petri dishes in 5 ml of

medium. The cells were allowed to attach, spread and reach confluence with daily replenishment of medium. The time taken to reach confluence varied among the cell strains studied, with the inflamed fibroblasts having a slower proliferation rate (Bartold and Page, 1986 b). Once the cells were confluent, the medium was removed and replaced with 3 ml of medium containing 17.5 µCi/ml D-[6-^{3}H]-glucosamine (specific activity 34.6 Ci/mmol) for 48 h, by which time radiolabel incorporation into cell-layer glycosaminoglycans has been shown to reach a steady state (Bartold and Page 1985, 1986 c). The medium was then removed and the cell layers washed with 5 ml PBS, which was pooled with the medium to make up the medium fraction for subsequent analyses. The cells were released from the culture flasks by trypsinization and recovered by centrifugation. The supernatant was removed, the cells were washed once with 2 ml PBS and harvested by centrifugation. The supernatants were pooled to form the cell layer fraction for future analyses. The cells were resuspended in 500 µl PBS and aliquots were taken to determine cell numbers using a Coulter counter (Coulter Electronics, Hialeah, FL).

Analysis of [^{3}H]-Labeled Material

The medium and cell layer material thus obtained was dialyzed exhaustively against 0.1 M sodium acetate, 0.15 M sodium chloride, 1 mM magnesium chloride and 1 mM calcium chloride, pH 5.8, to remove all unincorporated [^{3}H]-glucosamine. After exhaustive dialysis, the final volumes of each sample were recorded. Aliquots (100 µl) were taken from each sample and added to 6 ml scintillation fluid (Aquamix) and counted for tritium activity in a liquid scintillation counter (Packard Tri Carb 3255).

To determine the contribution of hyaluronic acid to the total [^{3}H]-glucosamine-labeled material, aliquots (200 µl) from each sample were digested with 6 TRU *Streptomyces* hyaluronidase for 3 h at 60 °C (Ohya and Kaneko, 1970; Bartold et al., 1979). The digestion products were then eluted from a Sephadex G-50 column (0.7 × 30 cm) with 0.2% sodium dodecylsulfate (SDS) in 0.15 M sodium acetate, 1 mM magnesium chloride and 1 mM calcium chloride, pH 5.8. Fractions of 0.25 ml were collected at a flow rate of 3 ml/hour, and the whole fractions were assessed for [^{3}H]-activity in a liquid scintillation counter. The amount of material digested by *Streptomyces* hyaluronidase was determined as that which eluted in the included volume; undigested (resistant) material was detected in the void volume. The recoveries of labeled material from these columns were always greater than 90%.

Since differences in specific activity of sugar nucleotide precursors may vary between cell types when using labeled glucosamine, we assessed the specific activity of radiolabeled products by comparing the [^{35}S]/[^{3}H] ratios using a dual label protocol. Briefly, the cells were incubated for 48 hours in the presence of [^{35}S]-sulfate (50 µCi/ml) and [^{3}H]-glucosamine (20 µCi/ml). The medium and PBS wash of the cell layers were pooled and dialysed against 7 M urea in 50 mM Tris HCl containing 0.1 M sodium chloride. After exhaustive dialysis the samples were loaded onto a 5 ml DEAE-Sephacel column which had been equilibrated in the same buffer as described above. After washing the column with 5 volumes of buffer a linear 0.1–0.8 M sodium chloride gradient was applied to the column. A major peak which contained both [^{35}S] and [^{3}H] activity eluted last and was pooled. This peak has been shown previously to contain proteoglycans (Bartold and Page 1986 c). An aliquot from this pooled material was analysed for radioactivity by liquid scintillation counting using a dual label programme and the ratio of [^{35}S]/[^{3}H] was thus determined.

In addition to the above, a portion of the media obtained incubating the cells in the presence of [^{35}S]-sulfate and [^{3}H]-glucosamine was dialysed exhaustively against 50 mM Tris-HCl, 60 mM sodium chloride and 40 mM sodium acetate, pH 8.0. This was then digested with chondroitinase ABC as previously described (Saito et al., 1968). The digestion products were eluted from a Sephadex G-50 column using the same conditions as described above. The fractions were collected and assessed for radioactivity. The liberated disaccharides from this treatment eluted near the total volume of the column and thus the ratio of [^{35}S]/[^{3}H] in this peak could be readily determined.

Since non-enzymatic glycosylation of serum proteins may account for the presence of labeled macromolecules eluting at the void volume of the Sephadex G-50 columns together with metabolically labeled components (Thonar et al., 1983), we incubated 5 ml of medium for 48 h in the absence of cells but with [^{3}H]-glucosamine (20 μCi/ml) added. The medium was then collected and a small portion was eluted from the same Sephadex G-50 columns as used for the previously described experiments. The proportion of labeled material eluting at the void volume and total volume was thus determined and used as a measure of the degree of non-enzymatic glycosylation. This was found to account for 0.05% of the total label and thus was not further considered as a significant contributor to the appearance of labeled material eluting within the void volume of Sephadex G-50.

In order to confirm that *Streptomyces* hyaluronidase was responsible for digestion of labeled hyaluronic acid alone and not other labeled components such as proteoglycans, an aliquot of the [^{35}S]/[^{3}H]-labeled medium was treated with *Streptomyces* hyaluronidase as described above. The digestion products were then eluted from Sephadex G-50. We found that only [^{3}H]-labeled material eluted in the included volume of the column. No [^{35}S]-labeled material was found to be retarded by this gel. This confirms our earlier observation that *Streptomyces* hyaluronidase does not appear to affect the elution position of [^{35}S]-labeled proteoglycans on Sephadex G-50 (Bartold and Page 1985). Thus, this method appears to represent a valid way of assessing the presence of hyaluronic acid.

Molecular Size Distribution of Hyaluronic Acid

Since previous studies have shown disruption to the molecular size of hyaluronic acid within inflamed gingiva (Bartold and Page, 1985 a), we studied the molecular size distribution of hyaluronic acid synthesized by fibroblasts from normal and inflamed gingiva. Because we were interested in obtaining a rough estimate of the relative proportions of "large" and "small" sized hyaluronic acid species on the basis of exclusion and inclusion within a gel chromatographic system, we chose to use Sepharose CL-4B. Using this gel, we took the material which eluted in the void volume to represent "large" molecular weight species and pooled the included peaks and took these to be representative of "small" molecular weight species. It was not the purpose of these studies to obtain accurate estimates of the various hyaluronic acid populations present. Thus, 1 ml aliquots of medium and cell layer preparations were concentrated to 200 μl in dialysis tubing against Aquacide. The concentrates were eluted from columns of Sepharose CL-4B (0.7 cm × 100 cm) in 4 M guanidine HCl/50 mM sodium acetate, pH 5.8. Fractions of 0.5 ml were collected at a flow rate of 3 ml/h and 50 μl aliquots counted for [^{3}H]-activity in a liquid scintillation counter. The material which eluted in the void volume was pooled separately from that eluting in the included volume. The recovery of labeled material from these columns was in the range of

80–85%. These fractions were then dialyzed against 0.15 M sodium acetate, 1 mM magnesium chloride and 1 mM calcium chloride, pH 5.8, and 1 ml aliquots concentrated to 100 µl against Aquacide. The concentrates were digested with 6 TRU *Streptomyces* hyaluronidase as described earlier, and then eluted from Sephadex G-50 columns (0.7 cm × 30 cm) with 0.2% SDS in the same buffer described above. This allowed quantitation of hyaluronic acid in each of the samples. Thus, the contribution of the different sized hyaluronic acid populations to the total hyaluronic acid content of the medium and cell layer was determined.

Statistical Analyses

All data were subjected to statistical analysis using either the methods of analysis of variance or student's T-test.

Results

The amount of [^{3}H]-glucosamine incorporated into macromolecules isolated from cultures of normal and inflamed gingival fibroblasts is shown in Table I. Label incorporated for each of the three strains of inflamed cells was significantly greater than that incorporated by any of the strains of normal fibroblasts ($P < 0.05$). About two-thirds of the total label was found in the medium of cultures of cell strains of both types, and the amount of label found in the medium of strains of inflamed fibroblasts was on average, 44% greater than that in medium of normal cells ($P < 0.05$). Label in the cell layer fraction of cultures of inflamed cells was also greater (39%) than that found in normal cells but the difference was not statistically significant because of the large variance ($P < 0.2$).

Table I. [^{3}H]-Glucosamine Incorporated into Macromolecules[a].

Cell Strains		Medium[b]	Cell Layer	Total CPM
Normal	HGF_{24}	80743 ± 7025	42423 ± 1547	123,166
	HGF_{25}	79477 ± 1578	37285 ± 6385	116,762
	HGF_{27}	85232 ± 827	45176 ± 3035	130,408
			Mean	123,445
Inflamed	HGF_{10}	108353 ± 8947	47154 ± 2201	155,507
	HGF_{11}	149793 ± 11693	91129 ± 3895	240,922
	HGF_{13}	115068 ± 9867	66102 ± 1499	181,170
			Mean	192,533

[a] Normal and inflamed gingival fibroblasts were incubated for 48 h in the presence of [^{3}H]-glucosamine. The medium and cell-layer fractions were dialyzed exhaustively to remove unincorporated radiolabel, and aliquots of the retentate were assayed for radioactivity. Data are presented as mean values and standard deviation of the mean of triplicate cultures, and expressed as cpm/10^5 cells.

[b] The data for medium of normal fibroblasts are statistically different from those for medium of inflamed fibroblasts ($p < 0.05$).

Table II. Proportion of [^{3}H]-Glucosamine Present as Hyaluronic Acid[a].

Cell Strains		Medium		Cell Layer	
		% Total ^{3}H Activity[c]	cpm/10^5 Cells[d]	% Total ^{3}H Activity	cpm/10^5 Cells
Normal	HGF_{24}	21 ± 2	15621 ± 1887	10 ± 3	5020 ± 110
	HGF_{25}	24 ± 4	20331 ± 1805	11 ± 1	4829 ± 1029
	HGF_{27}	25 ± 3	22750 ± 2039	17 ± 2	7723 ± 662
Inflamed	HGF_{10}	38 ± 2	38772 ± 3466	15 ± 2	7809 ± 1040
	HGF_{11}	35 ± 3	35519 ± 2727	16 ± 3	15623 ± 1192
	HGF_{13}	35 ± 2	31800 ± 6987	23 ± 4	19388 ± 1919

[a] Macromolecular material labeled with [^{3}H]-glucosamine was digested with *Streptomyces* hyaluronidase to determine the amount of label in hyaluronic acid. Data are presented as mean values and standard deviation of the mean of triplicate determinations on each of the cell strains. Values are expressed as percentage of the total [^{3}H]-glucosamine material susceptible to the enzyme digestion, and as cpm/10^5 cells.

[b] The data for medium of normal gingival fibroblasts are statistically different from those for the medium of inflamed gingival fibroblasts.

[c] ($p < 0.05$).

[d] ($p < 0.01$).

The specific activity of sugar nucleotide precursors may vary between different cell strains. Therefore, we compared the [^{35}S]/[^{3}H] ratio in the proteoglycans isolated by ion-exchange chromatography. In addition, the [^{35}S]/[^{3}H] ratio of the disaccharides released by digestion of proteoglycans with chondroitinase ABC was also determined. Using ion-exchange chromatography we determined normal fibroblast proteoglycans to have a ratio of 0.8 (± 0.07) while the proteoglycans synthesized by fibroblasts from inflamed tissues had a value of 0.74 (± 0.1). The [^{35}S]/[^{3}H] ratio of the disaccharides released by chondroitinase ABC was found to be 0.2 (± 0.05) for normal and 0.16 (± 0.08) for inflamed fibroblast proteoglycans. Thus, the differences noted are minimal and indicate that large differences in specific activity of sugar nucleotide precursors between normal and inflamed tissue fibroblasts are unlikely to be responsible for the differences in the amounts of [^{3}H]-labeled material associated with either of these cell populations.

The medium and cell layer fractions of all the cultures were subjected to digestion with *Streptomyces* hyaluronidase to assess the amount of label in hyaluronic acid. The digestion products were well separated from the enzyme-resistant material following elution from Sephadex G-50 and allowed accurate quantitation of label. As seen in Table II, the medium from the inflamed fibroblasts had significantly more labeled material present than did the medium from cultures of normal fibroblasts. When these data were normalized on the basis of cell number, the amount of hyaluronic acid present in the inflamed fibroblast medium fraction was 45% greater than that present in medium from cultures of normal fibroblasts. Values for hyaluronic acid content were also elevated in the cell layer fraction from inflamed fibroblasts but the difference was not statistically significant.

Because about 50% of the total hyaluronic acid in extracts of inflamed gingival tissue is of low molecular weight (Bartold and Page, 1985 a), the size distribution of hyaluronic acid synthesized by normal and inflamed gingival fibroblasts was assessed.

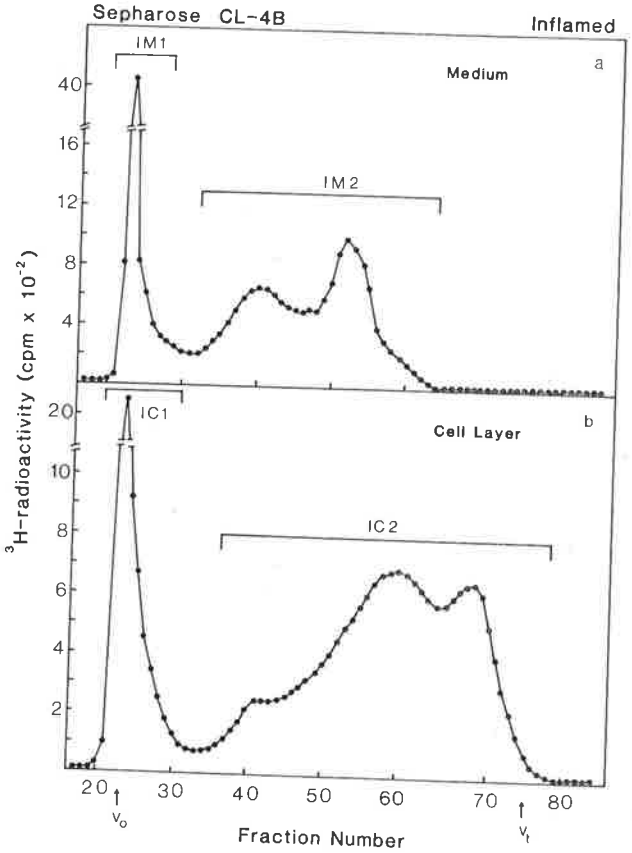

Fig. 1. Sepharose CL-4B gel-filtration chromatograms of medium and cell-layer macromolecules isolated from cultures of normal gingival fibroblasts (HGF_{24}) incubated in the presence of [^{3}H]-glucosamine. Material was eluted from columns of Sepharose CL-4B (0.7 cm × 100 cm) with 4 M guanidine HCl in 0.05 M sodium acetate, pH 5.8. Fractions of 0.5 ml were collected at a flow rate of 3 ml/h. The void volume (V_o) and total volume (V_t) were determined with [^{3}H]-DNA and phenol red respectively. The fractions within the regions marked by the bars were pooled for subsequent analysis.

[^{3}H]-labeled material from the medium or cell layer preparations eluted from Sepharose CL-4B as either excluded or included peaks which varied in their quantitative distribution (Figures 1 and 2). The excluded peak from the inflamed fibroblast medium (IM1), representing large macromolecules, contained 28% of the labeled material; while the corresponding peak from normal fibroblast medium (NM1) contained only 18% of the total. The large molecular weight peak for the cell layer fractions of normal (NC1) and inflamed (IC1) fibroblasts contained 23% of the total eluted radioactivity.

The high and low molecular weight peaks from all samples were pooled separately as shown in Figures 1 and 2 and aliquots were digested with *Streptomyces* hyaluronidase, and then eluted on Sephadex G-50 to determine the hyaluronic acid content

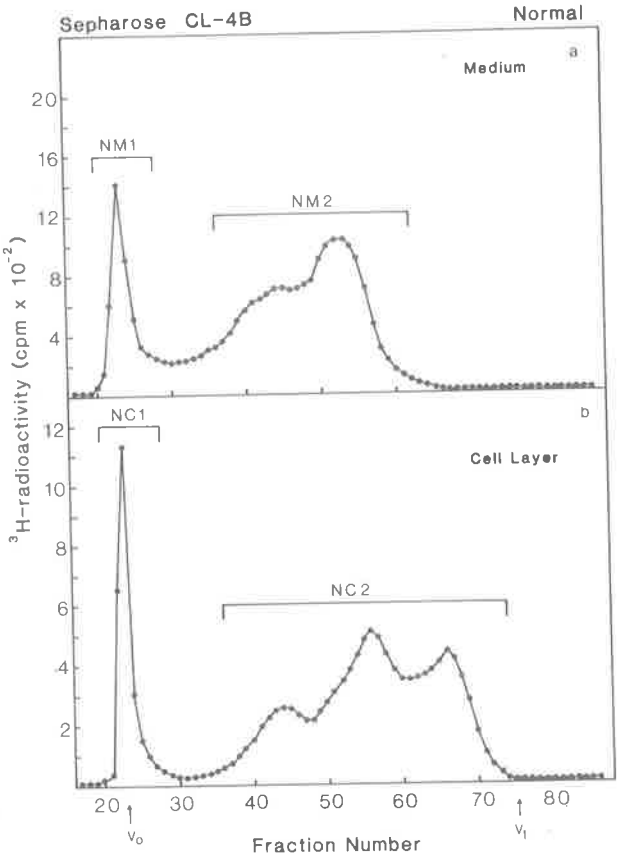

Fig. 2. Sepharose CL-4B gel-filtration chromatograms of medium and cell-layer macromolecules isolated from inflamed gingival fibroblasts (HGF_{10}). The column elution conditions and characterizations were the same as described in Figure 1. Fractions within the regions marked by the bars were pooled for subsequent analysis.

(Figs. 3 and 4). Most of the labeled material sensitive to hyaluronidase digestion was located in the high molecular weight fractions from the medium (NM1 and IM1) with only 7% and 10% of the total found in the low molecular weight fractions of normal (NM2) and inflamed (IM2) fibroblast preparations (Fig. 3 and Table III). Of the total labeled material in peaks NM1 and IM1, 85% was digestable (Table III). However, since the high molecular weight fraction of the medium of inflamed fibroblasts contained 28% of the total label in contrast to 18% for this fraction from normal fibroblasts, the inflamed fibroblasts produced a significantly enhanced amount of hyaluronic acid (Table III). Consideration of the amount of digestable material in both high and low molecular weight fractions revealed that hyaluronic acid accounted for 21% of the total labeled material in the medium from normal fibroblasts and 31% of that from the inflamed fibroblast cultures. These values are in close agreement with those in Table II

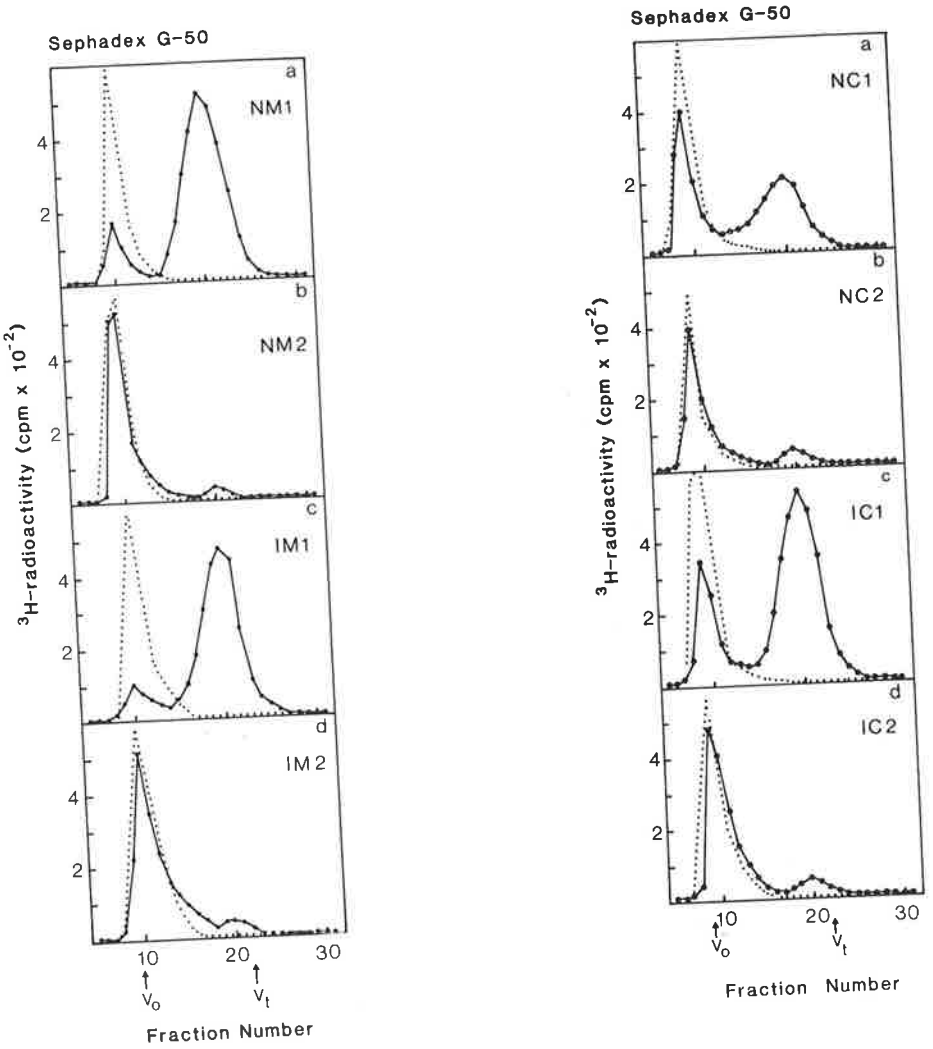

Fig. 3. *Streptomyces* hyaluronidase digestion of normal and inflamed fibroblast culture medium (left). Samples were either digested with *Streptomyces* hyaluronidase (●——●) or treated as controls (....). They were then brought to 0.2% SDS and eluted from Sephadex G-50 columns (0.7 cm × 30 cm) with 0.2% SDS in 0.15 M sodium acetate, 1 mM magnesium chloride. 1 mM calcium chloride, pH 5.8. Fractions were collected at flow rate of 3 ml/h. Both V_o and V_t were determined as described in Figure 1.

Fig. 4. *Streptomyces* hyaluronidase digestion of normal and inflamed fibroblast cell-layer material (right). Samples were either digested with *Streptomyces* hyaluronidase (●——●) or treated as controls (....) and then eluted from Sephadex G-50 under the same conditions as described in Figure 3. Both the V_o and V_t were determined as described in Figure 1.

Table III. Distribution of Hyaluronic Acid in Sepharose CL-4B Peaks[a]

Peak	Total [^{3}H]-activity in Peak[b]	% Peak Digested by Hyaluronidase[c]	% Total [^{3}H]-activity[d]	[^{3}H]-activity as Hyaluronic acid[e]
NM1	18	85	15.3	
NM2	82	7	5.7	21.0
IM1	28	85	23.8	
IM2	72	10	7.7	31.5
NC1	23	53	12.1	
NC2	77	7	5.4	17.8
IC1	23	70	16.1	
IC2	77	8	6.1	22.2

[a] Peaks from Sepharose were pooled as shown in Figures 1 and 2 and treated with *Streptomyces* hyaluronidase, then eluted from columns of Sephadex G-50. The proportion of hyaluronic acid present was determined by calculating the amounts of [^{3}H]-activity susceptible to the enzyme treatment.

[b] [^{3}H]-activity in each peak is expressed as a percentage of the total [^{3}H]-activity eluted from Sepharose CL-4B.

[c] Values represent the percentage of material in the Sepharose CL-4B peaks which was sensitive to *Streptomyces* hyaluronidase digestion.

[d] The percentage of total [^{3}H]-activity represented by hyaluronic acid was determined by multiplying the percent of [^{3}H]-activity in the Sepharose peaks by the percentage of that material identified as hyaluronic acid.

[e] The total [^{3}H]-activity in hyaluronic acid associated with either the medium or cell layer material was calculated by adding together the values representing the percentage of hyaluronic acid in each peak (see footnote d). Data represent percentage of total [^{3}H]-activity in either the medium or cell layer present as hyaluronic acid.

and indicate good recoveries from the Sepharose CL-4B columns during the preparative stages. Chromatograms and quantitations of the large and small molecular weight cell-layer material which was eluted from Sephadex G-50 after hyaluronidase digestion are shown in Figure 4 and Table III. 53% of the high molecular weight material from normal fibroblasts and 70% of that from inflamed fibroblasts was digestible. Since the high molecular weight fractions accounted for 23% of the total label incorporated for both types of cells, the amount of hyaluronic acid present was 17% and 22% for normal and inflamed fibroblasts respectively. These values, too, are in very close agreement with those reported in Table II.

Discussion

The effect of chronic gingival inflammation on hyaluronic acid has only recently been considered an important avenue of investigation to better understand the pathogenesis of inflammatory periodontal disease. From these studies, it is apparent that although the quantity of hyaluronic acid in inflamed tissues remains relatively unaltered, changes in its molecular weight are evident (Embery et al., 1979; Bartold and Page, 1986 a). *In vitro* studies using gingival fibroblasts, aimed at determining the effect of extracts of periodontal bacteria on hyaluronic acid synthesis have also demonstrated

that modulation of production of this macromolecule by agents that induce inflammation is possible (Larjava et al., 1983; Larjava, 1984).

In the present study we demonstrate that fibroblasts obtained from inflamed tissue are phenotypically different from fibroblasts obtained from normal tissue with respect to hyaluronic acid production. The inflamed tissue fibroblasts incorporate significantly more glucosamine than normal fibroblasts and secrete approximately 50% more macromolecules of a significantly higher molecular weight into the medium. These differences persisted through many transfers in culture. In addition fibroblasts from normal and inflamed tissues also differ with regard to the amounts and types of collagen produced (Narayanan and Page, 1983) and their rates of proliferation and growth (Bartold and Page, 1986 b).

Therefore, our observations provide additional support for the hypothesis that human connective tissue fibroblasts are heterogeneous (Hassell et al., 1976; Bordin et al., 1984).

Although the medium of inflamed fibroblasts had significantly more [^{3}H]-labeled material compared to the medium of normal fibroblasts, there was not a significant difference between the cell-layer associated material. Indeed, the amount of hyaluronic acid associated with the cell layer would not be expected to differ greatly unless each cell strain contained variable intracellular [^{3}H]-glucosamine pools available for cellular transport or retained variable amounts of hyaluronic acid within their pericellular coats (Toole, 1981). However, the cells were labeled over a time period which allowed for a steady state of incorporation of radiolabel to be reached (Bartold and Page, 1986 c) and the cell number at the end of the labeling period were found to be very similar. Therefore, the absence of significant differences in radiolabel uptake between the six cell strains studied, indicated that differences in intracellular pool sizes of [^{3}H]-glucosamine or cell surface associated hyaluronic acid were not significant.

Since hyaluronic acid synthesis can be affected by cell proliferation and cell density (Hopwood and Dorfman, 1977; Toole, 1981), care must be taken in planning experiments of the type described in this report. For this reason, we have previously defined the proliferation rates of the normal and inflamed fibroblasts used in the present study, and found that the inflamed fibroblasts have a slower proliferation rate (Narayaynan and Page, 1976; Bartold and Page, 1986 b). Thus, in the present study, the cultured fibroblasts were monitored daily using phasecontrast microscopy and radiolabeling with [^{3}H]-glucosamine was not commenced until the cells were visually assessed to have just become confluent. In addition, the data presented in Table I, which demonstrate the total [^{3}H]-glucosamine activity associated with the medium and cell-layer, have been normalized on the basis of cell numbers present in each culture. By the end of the labeling period, approximately the same number of cells was present in each of the cultures (620,000 $\pm$ 17,000; data not shown in results). Therefore it appears that the differences we report are unlikely to be directly related to cell density or proliferation rates, but rather represent inherent differences between the normal and inflamed cell strains.

When extracts were assayed for hyaluronic acid content, we found an increased proportion of [^{3}H]-labeled hyaluronic acid in the medium of inflamed fibroblasts compared to the medium of the normal fibroblasts. Analysis of the molecular size of hyaluronic acid on Sepharose CL-4B revealed two principal populations. The major component was of large molecular size and excluded from the gel. The other was of smaller size, eluted within the included volume of the column and was quantitatively a minor component. These studies confirmed the association of a greater proportion of

[^{3}H]-labeled hyaluronic acid of a large molecular size with the inflamed fibroblasts relative to the normal fibroblasts. Thus, the previously reported decrease in molecular size of hyaluronic acid in inflamed gingivae (Bartold and Page, 1985 a) is most likely due to factors other than selective synthesis of small molecular weight hyaluronic acid by inflamed gingival fibroblasts. Factors such as enzymatic or oxygen-derived free radical depolymerization of hyaluronic acid may play important roles in the observed alterations (Goggins, et al., 1969; Orkin and Toole, 1980; Bartold, et al., 1984). The biological significance of our findings is not yet clear, but it is interesting to speculate that since the amount of hyaluronic acid associated with the inflamed fibroblasts appears to be increased, then this may play a role in host defence and wound healing. For example, high molecular weight hyaluronic acid can inhibit phagocytosis by macrophages and may play a role in inhibiting inflammatory cell function during the later stages of wound healing (Forrester and Balazs, 1980). In addition, hyaluronic acid can inhibit the migration of mononuclear cells (Balazs and Darzynkiewicz, 1973), inhibit lymphocyte transformation by mitogens (Darzynkiewicz and Balazs, 1971), as well as regulate the synthesis of extracellular matrix macromolecules (Wiebkin and Muir, 1975). Therefore, the stimulation of high molecular weight hyaluronic acid production by fibroblasts in inflamed gingiva may be an important factor in the process of inflammation and tissue repair and thus invites further investigation.

Acknowledgements

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Paper 13.

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Distribution of chondroitin sulfate and dermatan sulfate in normal and inflamed human gingiva.
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Distribution of Chondroitin Sulfate and Dermatan Sulfate in Normal and Inflamed Human Gingivae

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The effect of inflammation on the distribution of chondroitin sulfate and dermatan sulfate proteoglycans was assessed after normal and inflamed human gingivae were stained with monoclonal antibodies against these extracellular matrix macromolecules. The tissues were obtained following periodontal surgery and reacted with specific antibodies after pre-treatment with chondroitinase ACII or chondroitinase ABC, and staining was visualized by the immunoperoxidase technique. The results indicated that these two proteoglycans were present in both the 4-sulfated and 6-sulfated isomeric forms. While chondroitin sulfate appeared to be uniformly distributed throughout the connective tissue, dermatan sulfate showed greater intensity of staining in the areas immediately subjacent to the epithelium. Positive staining for chondroitin sulfate was noted in the intercellular spaces of the epithelium. In inflamed tissues, there was significant staining associated with 4-sulfated dermatan sulfate and chondroitin sulfate, but this had lost the structured pattern of staining noted in normal sections. The 6-sulfated isomeric forms were greatly reduced in inflamed tissues and tended to show a predilection to be localized within the perivascular tissues. In the inflamed tissues, there was intense staining for chondroitin sulfate associated with the infiltrating inflammatory cells. These findings corroborate earlier biochemical studies on normal and inflamed gingival tissues. The specific tissue localization of dermatan sulfate and chondroitin sulfate in tissues damaged by inflammation indicates that, as opposed to the large loss of collagenous material noted during inflammation, there is not a corresponding large loss of proteoglycan. Indeed, at specific inflammatory foci, the intensity of staining for these macromolecules may intensify.

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Introduction.

Following recognition that the gingival tissues provide the milieu for the first line of defense against bacterial initiation of the periodontal diseases, both the fibrous and non-fibrous components of these tissues have received considerable attention (Narayanan and Page, 1983; Bartold, 1987). The major extracellular non-fibrous macromolecules of human gingivae are proteoglycans and hyaluronate. By virtue of their highly anionic charge, these components have been implicated in numerous cell-cell, cell-matrix, and matrix-matrix interactions (Ruoslahti, 1988).

The proteoglycans of human gingival connective tissue and epithelium have been studied biochemically and the principal components identified as dermatan sulfate, chondroitin sulfate, and heparan sulfate (Embery *et al.*, 1979; Bartold *et al.*, 1981). Although not strictly considered a proteoglycan, due to the absence of a protein core, hyaluronate is the other major uronate-containing macromolecule identified in gingival tissues (Embery *et al.*,1979; Bartold *et al.*, 1981). Interestingly, although significant changes in the amounts and types of collagens present in inflamed gingivae compared with healthy gingivae have been noted (Payne *et al.*, 1975), very few changes in the total proteoglycan and hyaluronate content of normal and inflamed gingivae have been found (Bartold and Page, 1986). Despite this, some evidence of structural changes has been noted for proteoglycans extracted from inflamed tissues (Purvis *et al.*, 1984; Bartold and Page, 1986). These findings led to a proposal that the rates of proteoglycan synthesis may be altered in inflamed tissues and that there was probably rapid clearance of degraded proteoglycans from the site of inflammation (Bartold and Page, 1986).

Although the previous studies have been useful in determining the gross biochemical events associated with inflammatory-mediated changes in gingival tissues, they have not been able to provide any information regarding the focal nature of periodontal inflammation. Early histochemical studies indicated a loss of polyanionic material at the site of inflammation but an increase at the periphery (Melcher, 1967). However, due to the lack of specificity of the stains used, very little could be said of the molecular species being identified. With the development of antibodies to specific epitopes of extracellular matrix components, it is now possible to identify these components positively in tissues with a degree of accuracy not possible previously.

Therefore, it was the aim of the study to use well-characterized monoclonal antibodies capable of detecting specific carbohydrate epitopes on proteoglycans to determine the distribution of chondroitin sulfate and dermatan sulfate within both normal and inflamed human gingival tissues.

Materials and methods.

Materials.—Biotinylated species-specific anti-mouse immunoglobulin and biotinylated horseradish peroxidase-Streptavidin complex were purchased from Amersham, North Ryde, NSW, Australia. Monoclonal antibodies 9-A-2 and 3-B-3 were purchased from ICN Biochemicals, Lisle, IL. Chondroitinase ACII and chondroitinase ABC were purchased from Seikagaku Kogyo Pty. Ltd., Tokyo, Japan. All chemicals used, unless otherwise stated, were of analytical grade.

Gingival tissues.—Biopsies of gingival tissues were obtained from six patients (ranging in age from 28 to 47 years) who were undergoing surgical treatment for crown-lengthening procedures (normal tissue) or management of adult-type periodontitis (inflamed tissue). The tissues came from the buccal, lingual, and interproximal surfaces of the maxillary molar teeth. Following surgical removal, the gingivae were placed immediately into 2% paraformaldehyde, 2% glutaraldehyde, 0.3% cetylpyridinium chloride, and 30 mmol/L NaCl in 0.1 mol/L phosphate buffer, pH 7.4, and fixed for two h at 4°C (Ripellino *et al.*, 1985). After fixation, the tissues were embedded in paraffin, and 5-µm sections were cut. Although the surgical specimens were initially judged, on clinical criteria, as being derived from normal or inflamed sites, routine staining with hematoxylin and eosin was carried out to confirm the inflammatory status of the tissues.

Primary antibodies.—The primary antibodies used were 9-A-

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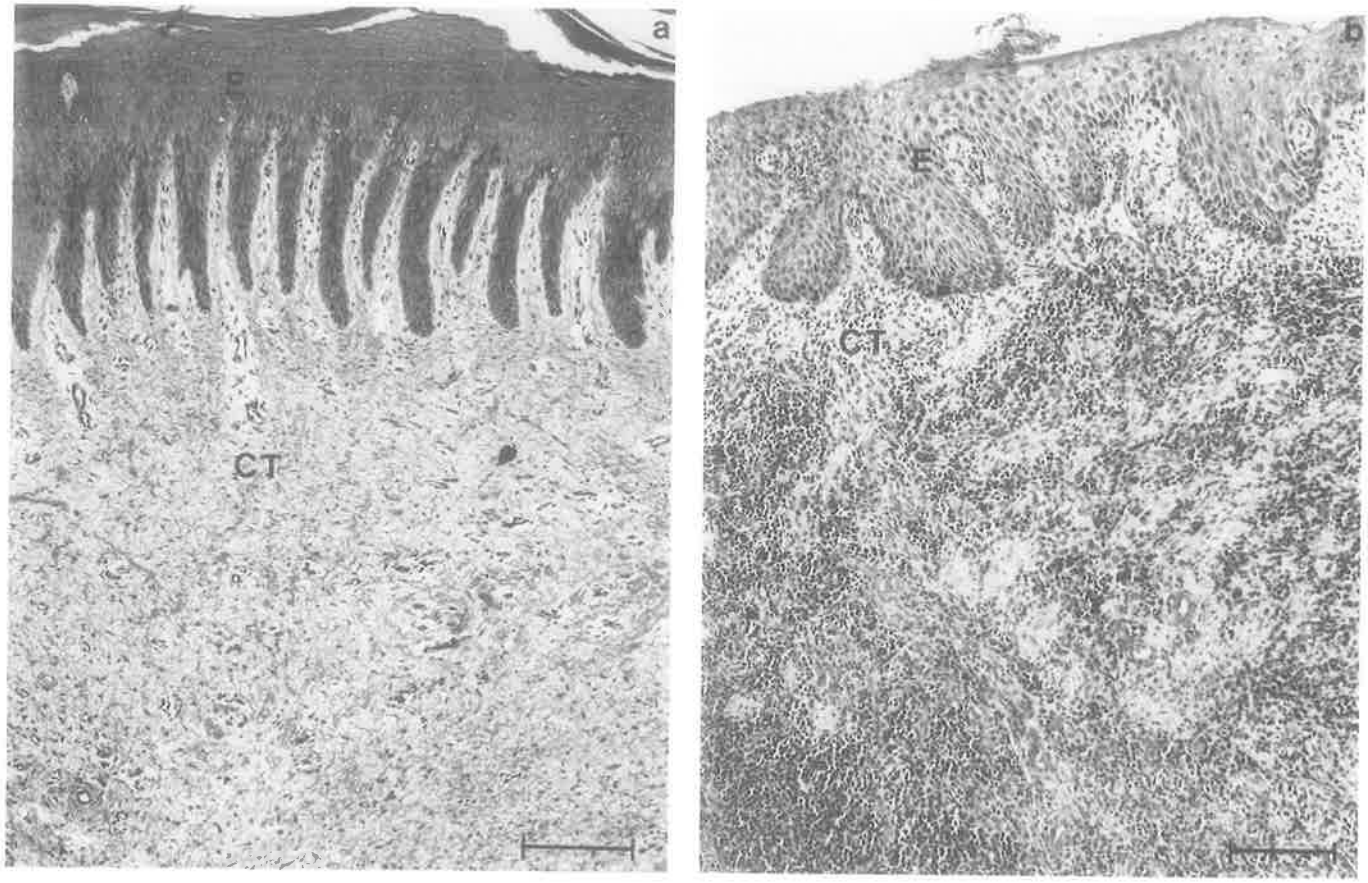

Fig. 1—Histological appearance of representative hematoxylin- and eosin-stained sections of tissues assessed clinically as normal or inflamed. The normal tissue (a) shows a dense collagenous connective tissue with only a few inflammatory cells present. The inflamed tissue (b) shows a very dense infiltration of inflammatory cells with little collagenous connective tissue evident. Bar = 100 µm. Abbreviations: E, epithelium; CT, connective tissue.

2 and 3-B-3, and these have been well-characterized and used extensively for proteoglycan localization within a variety of tissues (Couchman *et al.*, 1984). They were originally raised against chondroitinase-ABC-digested cartilage proteoglycans and recognize the oligosaccharide stubs of degraded glycosaminoglycan chains of dermatan sulfate and chondroitin sulfate. These epitopes contain delta-unsaturated hexuronic acid residues adjacent to N-acetyl galactosamine residues in either the 4- (antibody 9-A-2) or 6- (antibody 3-B-3) isomeric form. Because these disaccharide epitopes are ubiquitous to all chondroitin sulfates and dermatan sulfates, these antibodies are not tissue- or species-specific. Thus, they represent a very useful tool for the general screening of any tissue for the presence of dermatan sulfate and chondroitin sulfate.

Immunohistochemical staining.—Sections were de-paraffinized, re-hydrated through graded alcohol solutions, and then exposed to methanol containing 2.5% hydrogen peroxide for 20 min to block any endogenous peroxidase activity in the sections. After the sections were washed with phosphate-buffered saline (PBS), the epitopes for antibody recognition were generated by digestion with chondroitinase ACII or chondroitinase ABC in 70 mmol/L sodium acetate, pH 6.0, or 50 mmol/L Tris HCl, 60 mmol/L sodium acetate, and 50 mmol/L sodium chloride, pH 8.0, respectively, for 30-60 min at 37°C. Control sections were incubated in the respective buffers but in the absence of any enzyme. The primary antibody was then applied at a 1:32,000 dilution to the sections and allowed to incubate at 4°C overnight. After removal of the unbound antibody by being washed in PBS, the sections were incubated with biotinylated species-specific sheep anti-mouse antibody for 60 min at room temperature. Finally, the sections were reacted with Streptavidin-biotinylated horseradish peroxidase complex for 30 min at room temperature prior to color development with 3,3'-diaminobenzidine (120 mg in 200 mL of tris-buffered saline) containing 0.08% nickel chloride to enhance the staining intensity (Gown and Vogel, 1984).

Results.

Control sections.—The appearance of representative sections from normal and inflamed human gingival specimens stained with hematoxylin and eosin is shown in Fig. 1. The healthy tissues (Fig. 1a) demonstrated moderately fibrous connective tissues with a mild inflammatory cell infiltrate. The epithelium showed no overt degenerative changes. The inflamed tissues (Fig. 1b) were characterized by dense inflammatory cell infiltration, loss of collagenous architecture, and some early degenerative changes in the epithelial tissues. The results of reaction of slides with either the 9-A-2 or 3-B-3 antibody in the absence of pre-treatment with an appropriate enzyme are shown in Fig. 2. The degree of nonspecific staining by the two antibodies was negligible.

Epithelium.—In all sections reacted with antibody 9-A-2 following chondroitinase ACII digestion, some degree of staining of the intercellular spaces was evident (Fig. 3). The level of staining was not uniform throughout all layers of the epithelium, with the middle third characteristically being most heavily stained. Regardless of the degree of inflammation present in the underlying connective tissues, the staining pattern remained relatively unchanged with respect to that of healthy tissues. Similar

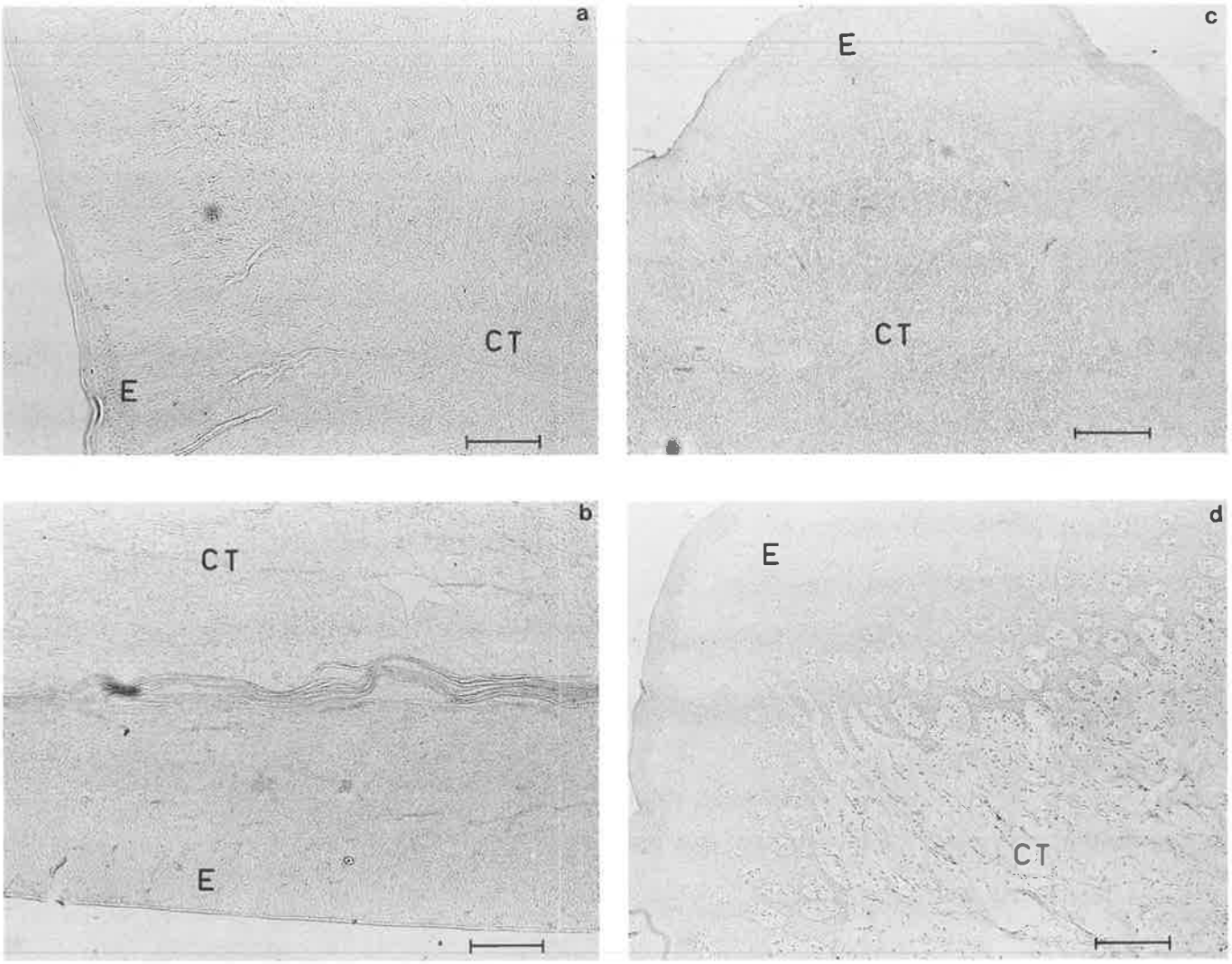

Fig. 2—Control immunoperoxidase staining of normal (a,b) and inflamed (c,d) gingiva. Sections were incubated in the absence of either chondroitinase ACII or chondroitinase ABC and were then reacted with monoclonal antibody 9-A-2 (a,c) or 3-B-3 (b,d). Bar = 100 μm. Abbreviations: E, epithelium; CT, connective tissue.

patterns of staining were achieved following chondroitinase ABC digestion (results not shown).

Connective tissue: distribution of 4-sulfated isomers.—Pretreatment of tissue sections with chondroitinase ACII or chondroitinase ABC followed by reaction with antibody 9-A-2 permitted the detection of chondroitin- or dermatan-4-sulfate, respectively (Fig. 4). While the distribution of chondroitin-4-sulfate was relatively uniform throughout the connective tissue, 4-sulfated dermatan sulfate demonstrated a stronger reaction and in particular localized in the tissues immediately subjacent to the epithelium. In healthy tissues, these components showed a strong localization to the collagenous elements demonstrating a densely-packed fiber arrangement. In inflamed tissues, much of this uniform architecture was lost, with obvious disruption to the fibrous appearance and generalized evidence of tissue disruption. The sections stained for chondroitin-4-sulfate also showed strong positive reaction at sites of inflammatory cell infiltration localizing to the cell membranes and the immediate pericellular environment.

Connective tissue: distribution of 6-sulfated isomers.—Chondroitin sulfate and dermatan sulfate in the 6-sulfated isomeric form were detected following reaction of sections with antibody 3-B-3 after chondroitinase ACII and chondroitinase ABC, respectively (Fig. 5). The pattern of staining was slightly different from that noted for the 4-sulfated isomers. In normal tissues, dermatan-6-sulfate was present in reduced amounts compared with the 4-sulfated isomer and showed little specificity for the immediate areas subjacent to the epithelium. Although the 6-sulfated isomer of chondroitin sulfate continued to show a uniform distribution throughout the connective tissue of the normal gingival specimens, it also showed a degree of localization around blood vessels which was not evident for the 4-sulfated isomer. In inflamed tissues, the distribution of the 6-isomeric form of chondroitin sulfate indicated an obvious loss of tissue integrity, with an increase in staining localized to the cell surfaces of infiltrating inflammatory cells. The pattern of staining for dermatan sulfate in inflamed tissues became very sparse, with small areas of dense staining associated with residual collagenous structures.

Discussion.

By use of the antibodies 9-A-2 and 3-B-3 in conjunction with

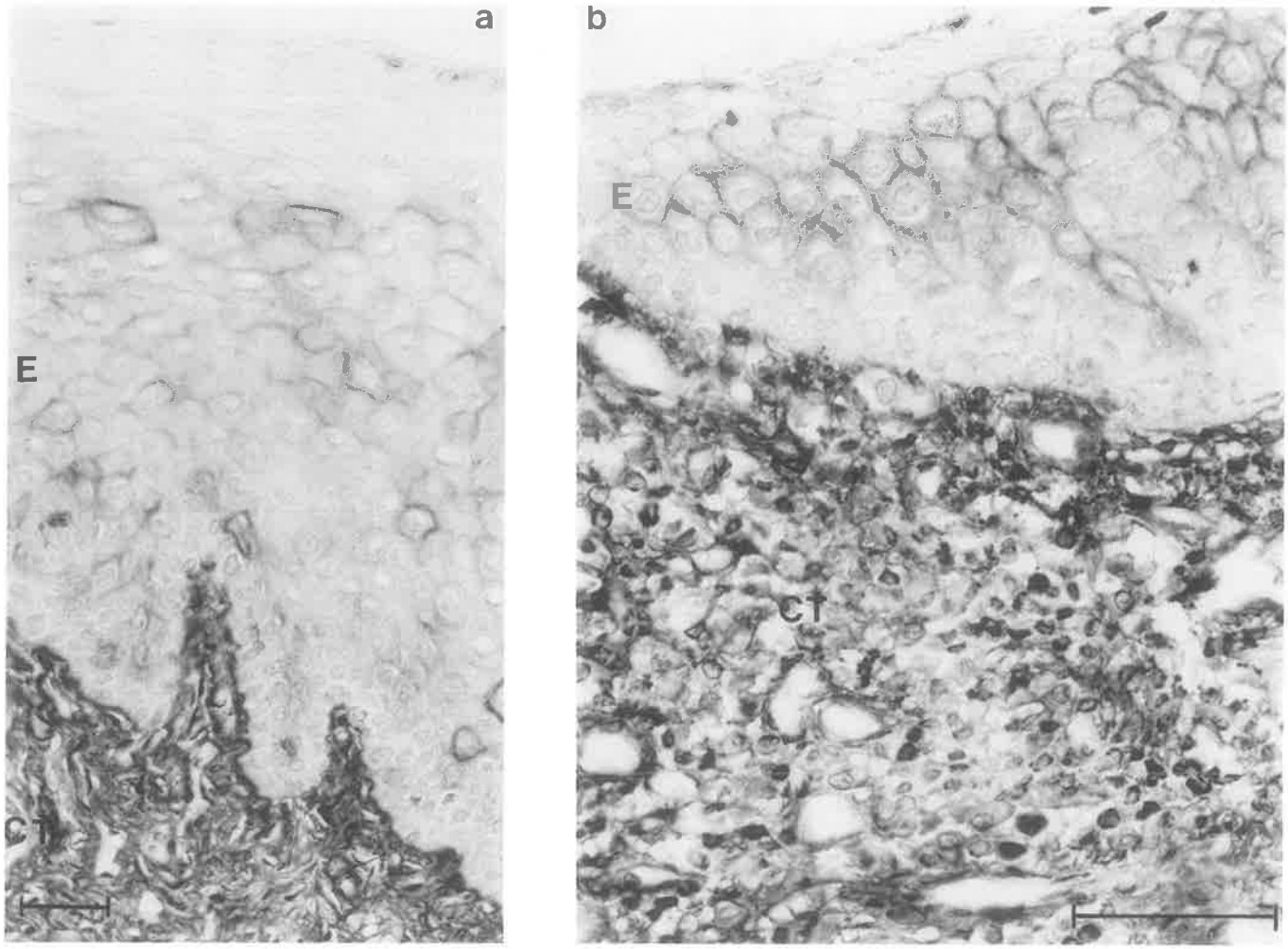

Fig. 3—Immunoperoxidase staining of normal (a) and inflamed (b) gingiva with monoclonal antibody 9-A-2 after chondroitinase ACII digestion. Note the presence of reactive material within the intercellular spaces of the epithelial tissues. Bar = 50 μm. Abbreviations: E, epithelium; CT, connective tissue.

specific glycosaminoglycan lyase elimination, it has been possible to demonstrate the presence of the 4- and 6-sulfated isomers of chondroitin sulfate and dermatan sulfate in normal and inflamed human gingivae. Such specific histochemical identification and localization has hitherto been unattainable due to the poor specificity of various histochemical dyes and methodologies. Although other glycosaminoglycans such as hyaluronate and heparan sulfate are present in gingival tissues, this study focused upon the quantitatively major sulfated glycosaminoglycans of human gingivae, chondroitin sulfate, and dermatan sulfate. This is not intended to downplay the importance of heparan sulfate and hyaluronate but merely reflects the ready availability of specific probes and represents a logical reference point from which to begin localization studies on other uronate-containing macromolecules of normal and inflamed human gingivae.

The demonstration of reactive material in the intercellular spaces of gingival epithelium is particularly noteworthy. Despite reasonable histochemical, biochemical, and autoradiographic evidence indicating the probable presence of sulfated glycosaminoglycans in gingival epithelium (Thonard and Scherp, 1962; Embery *et al.*, 1979; Bartold *et al.*, 1981; Wiebkin and Thonard, 1981; Takata *et al.*, 1990), there have been reports casting doubt on such a presence (Pedlar, 1979). Moreover, immunohistochemical studies have noted an absence of staining for glycosaminoglycans in skin epidermis when the 9-A-2 and 3-B-3 antibodies are used (Couchman *et al.*, 1984; Sorrell *et al.*, 1990). Indeed, during the initial stages of this study, we encountered similar problems in which no staining of the epithelial intercellular spaces was noted (results not shown). However, upon addition of cetylpyridinium chloride to the fixative, it was possible for the localization of proteoglycans in the gingival epithelium to be significantly improved. Such results are presumed to arise from the precipitation of glycosaminoglycans in the tissue spaces by the CPC (Kupchella *et al.*, 1984). At present, it is unclear why there should be selective loss of staining material from the epithelium and not from connective tissue. Nonetheless, these results highlight the need for particular care in attempts to localize epithelial proteoglycans. From the evidence presented in this study, it seems clear that at least chondroitin sulfate (and possibly a minor amount of dermatan sulfate) is present in the intercellular spaces of human gingival epithelium. From previous autoradiographic studies (Wiebkin and Thonard, 1981), the source of this material is presumed to be the epithelial cells, and with the development of molecular probes, it is anticipated that the epithelial cells specifically responsible for proteoglycan synthesis will soon be identified. Indeed, although cell-surface- and epithelial-tissue-specific proteoglycans such as Syndecan have been identified in other ectodermal tissues and cells, precise identification of the particular proteoglycans in gingival epithelium must await further use of specific antibodies and cDNA probes.

The distribution of chondroitin sulfate and dermatan sulfate

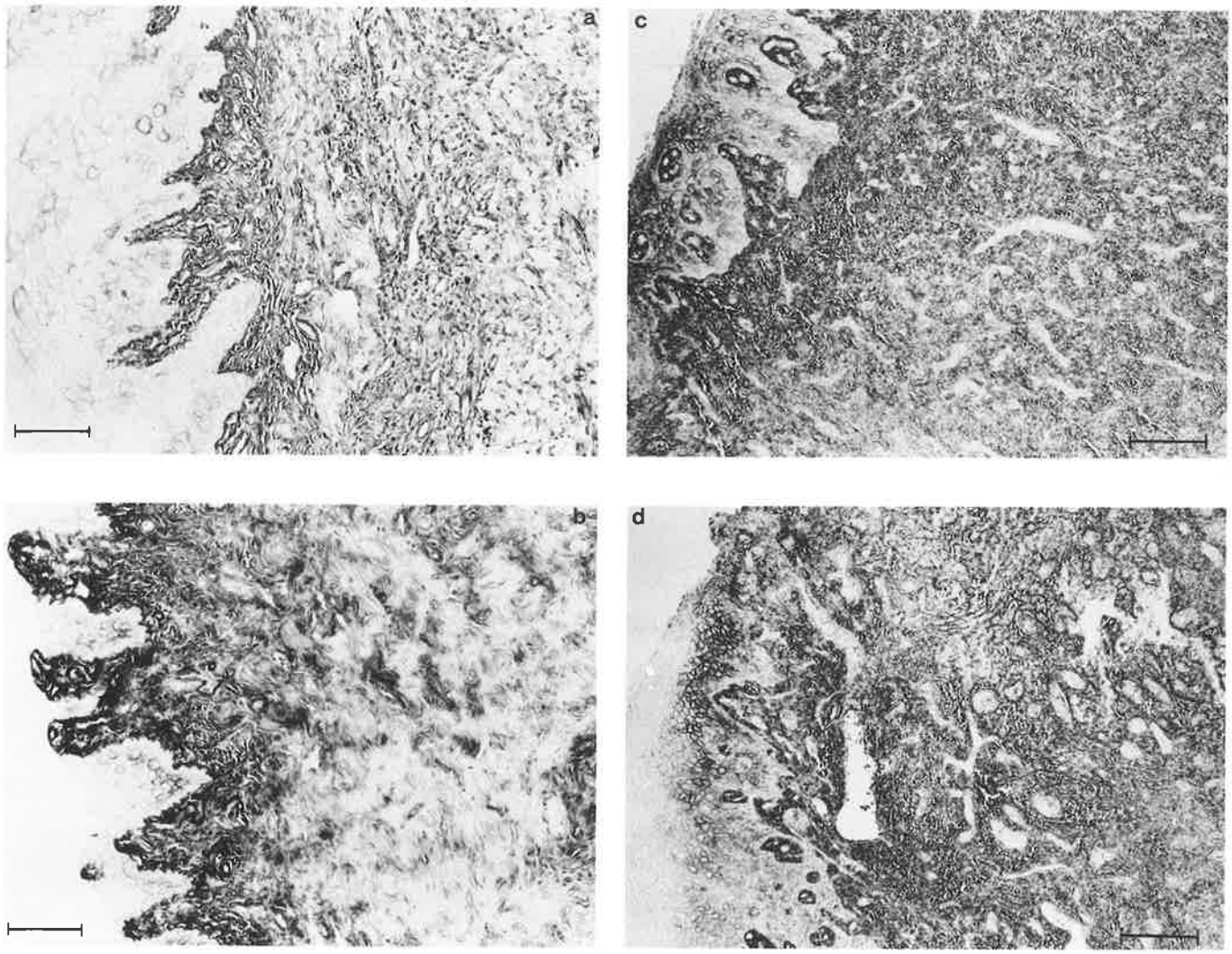

Fig. 4—Immunoperoxidase staining for chondroitin-4-sulfate in normal (a,b) and inflamed (c,d) gingiva with monoclonal antibody 9-A-2 after digestion with chondroitinase ACII (a,c) and chondroitinase ABC (b,d). Bar = 50 μm.

observed in the present study is in agreement with that reported by Shibutani *et al.* (1989). Dermatan-4-sulfate is the most strongly staining glycosaminoglycan and tends to localize to the immediate subepithelial regions. Chondroitin-4-sulfate tends to be more evenly distributed throughout the connective tissue. The 6-sulfated isomers of dermatan sulfate and chondroitin sulfate show some differences in distribution, with dermatan-6-sulfate being uniformly distributed throughout the connective tissues and chondroitin-6-sulfate being specifically located in the perivascular regions as well as more generally throughout the connective tissue. All of the isomers studied appeared to be closely associated with the fibrous elements of the gingival connective tissue, confirming previous reports on the association between various glycosaminoglycans and collagens (Scott and Orford, 1981).

An additional anomaly to arise from this study was the identification of chondroitin-6-sulfate. This was unexpected, since previous biochemical studies on human, canine, and bovine gingiva had failed to identify this isomer (Tawa *et al.*, 1976; Sakamoto *et al.*, 1978; Embery *et al.*, 1979; Bartold *et al.*, 1981). Nonetheless, recent immunohistochemical studies (Shibutani *et al.*, 1989) have indicated the probable presence of trace amounts of chondroitin-6-sulfate in gingival tissues. Such findings highlight the extreme sensitivity of antibodies for detecting specific connective components.

The distribution of the various glycosaminoglycans under consideration in inflamed tissues showed some specific differences with respect to the healthy tissues. Of particular note was the obvious loss of normal tissue architecture. However, despite the reported massive loss of collagen in inflamed tissues (up to 70% within seven days of initiation of inflammation) (Payne *et al.*, 1975), it is interesting that, even with the obvious inflammatory-induced changes, there were still significant deposits of chondroitin sulfate and dermatan sulfate in these tissues. In particular, the 4-sulfated isomeric forms appeared to remain quite prevalent in inflamed tissues. Such a finding helps to explain the earlier biochemical findings of few quantitative changes in the levels of glycosaminoglycans between normal and inflamed gingival tissues (Bartold and Page, 1986). It was also interesting to note the intense staining of chondroitin sulfate in association with the infiltrating inflammatory cells. Such an observation lends additional support to observations that both polymorphonuclear leukocytes and lymphocytes synthesize and secrete chondroitin sulfate and that for lymphocytes this synthesis can be dramatically increased by mitogen stimulation (Bartold *et al.*, 1989a,b). Consequently, it now remains to be established

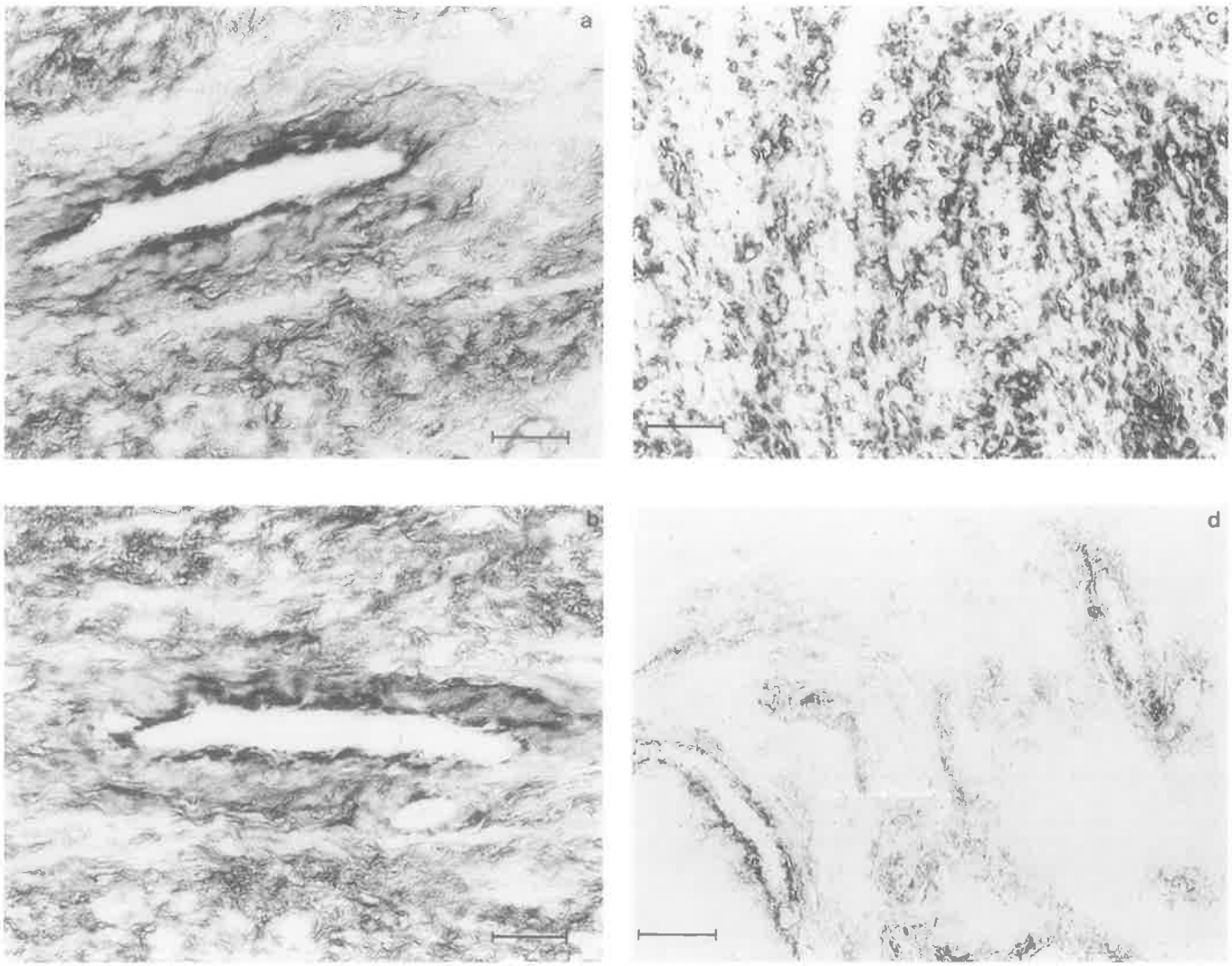

Fig. 5—Immunoperoxidase staining for chondroitin-4-sulfate in normal (a,b) and inflamed (c,d) gingiva with monoclonal antibody 3-B-3 after digestion with chondroitinase ACII (a,c) and chondroitinase ABC (b,d). Bar = 50 μm.

whether the production of proteoglycans by inflammatory cells is sufficient to replace the native extracellular matrix proteoglycans destroyed by the inflammatory process and thus explain the enigmatic presence of significant quantities of proteoglycans in inflamed gingival tissues.

In conclusion, the present data serve to confirm that the gingival tissues undergo significant structural alterations during inflammation. Furthermore, the presence of discernible quantities of proteoglycans localizing in the immediate vicinity of inflammatory foci indicates a possible role for these macromolecules in inflammatory cell function and wound repair.

Acknowledgments.

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Paper 14.

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Biochemical and immunohistochemical studies on overgrown gingival tissues associated with mannosidosis.
Virchows Archiv B **62:** 391-399; 1992.

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Biochemical and immunohistochemical studies on overgrown gingival tissues associated with mannosidosis

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Summary. The gingival tissues of a male patient suffering from mannosidosis and presenting with gingival overgrowth have been studied. Routine histological assessment highlighted the presence of highly enlarged and vacuolated lymphocytes. The morphology of the connective tissues, fibroblasts and epithelium appeared normal. Immunohistochemical staining of the tissues for chondroitin sulfate proteoglycan demonstrated a normal distribution of this component throughout the connective tissues and intense staining associated with the vacuolated lymphocytes. In vitro studies indicated that fibroblasts isolated from the overgrown tissue did not differ from age and sex matched control fibroblasts with respect to proliferation, protein and proteoglycan synthesis. Taken together, these findings imply that the gingvial overgrowth noted in this patient was not due to a defect in the resident fibroblasts but rather reflected a secondary response of the tissues to impaired host defence mechanisms.

Key words: Mannosidosis – Fibroblasts – Proteoglycans – Gingiva

Introduction

Mannosidosis is a rare lysosomal enzyme storage disease of which approximately 100 patients have been reported (Beaudet and Thomas 1989). Originally described by Ökerman in 1967, this autosomal recessive disorder manifests as an accumulation of mannose-containing oligosaccharides due to a lack of activity of either α-mannosidase or β-mannosidase. Of the reported cases to date, α-mannosidosis is more common than β-mannosidosis (Cooper et al. 1986; Wenger et al. 1986). Although only seven cases of β-mannosidosis have been reported (van Pelt et al. 1990), this condition is very common in some animals, especially the goat in which the phenotypic expression of β-mannosidosis is more severe than in man (Jones and Dawson 1981; Hancock et al. 1986). In addition to the two different enzymes which may be affected, mannosidosis, in humans, may be further classified according to its onset and severity (Desnick et al. 1976). For example, type I mannosidosis is a severe and often fatal infantile form while type II mannosidosis is a milder form affecting juveniles and adults (Beaudet and Thomas 1989). Although mannosidosis demonstrates a degree of clinical heterogeneity, some general features may be noted. Of these, mental retardation, coarse facies, deafness, hepatomegaly, hernias and ocular opacities are common findings (Spranger et al. 1976; Yunis et al. 1976; Gordon et al. 1980; Mitchell et al. 1981; Patton et al. 1982). Nonetheless, the ultimate diagnosis is derived from laboratory tests which demonstrate absence of either α-mannosidase or β-mannosidase in fibroblasts, lymphocytes and serum. In addition, vacuolated lymphocytes together with the absence of mucopolysaccharidurie but presence of elevated levels of mannose-containing oligosaccharides in the urine are diagnostic features of mannosidosis (Loeb et al. 1969; Autro et al. 1973; Mónus et al. 1977).

Most of the lysosomal enzyme disorders in humans are related to incomplete catabolism of glycoproteins, glycolipids or glycosaminoglycans. As a consequence, there is an accumulation of partly degraded metabolic products which leads to compromised cellular and tissue function. Because glycolipids, glycoproteins and glycosaminoglycans are fundamental metabolic components of all cells, the end result of lysosomal disorders is multifaceted. Therefore, it is not surprising that these disorders demonstrate a wide range of connective tissue alterations, many of which have been well documented. Nonetheless, the effects of mannosidosis on the oral tissues (both hard and soft) have been poorly defined.

Materials and methods

Materials. Dulbecco's Modification of Eagle's Medium (DMEM), fetal calf serum, non-essential amino acids, penicillin and strepto-

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mycin were all purchased from Flow Laboratories Australasia, North Ryde, NSW; all tissue culture ware was purchased from Nunc, Roskilde, Denmark; guanidine HCl, L-glutamine and diaminobenzidine were purchased from Sigma Chemical Co., St. Louis, MO; monoclonal antibody 9-A-2 against the unsatturated disaccaride (ΔDi-4-sulfate) of chondroitin sulfate was purchased from ICN Biochemicals, Lisle, IL; biotinylated species specific anti-mouse immunoglobulin and biotinylated horseradish peroxidase-Streptavidin complex were purchased from Amersham, North Ryde, N.S.W. Australia; chondroitinase ACII (*Athrobacter aurescens*) was from Seikagaku Kogyo Pty. Ltd., Tokyo, Japan; [6-^{3}H]-thymidine (22 µCi/mMol), L-[5–3H]-proline (15–40 Ci/mMol) and [^{35}S]-sulfate (25–40 Ci/mg) were from Amersham (Australia), Surrey Hills, NSW, Australia; Sephadex PD-10 columns from Pharmacia Fine Chemicals, North Ryde, NSW, Australia; Ready Solv liquid scintillation fluid from Beckman (Australia), Gladesville, NSW, Australia. Zeta probe membranes and Bio-dot microfiltration apparatus were from Bio-Rad Laboratories, North Ryde, NSW Australia.

Source of tissue. A 40 year old white male was referred to the Periodontics Unit of the Westmead Hospital Clinical Dental School (Sydney, Australia) for management of gingival overgrowth. A comprehensive medical history indicated that the patient had been previously diagnosed as suffering from α-mannosidosis (based on an assessment of mannosidase levels in skin fibroblasts, oligosaccharide scans and morphology of peripheral blood lymphocytes carried out at the Center for Lysosomal Storage disorders, Adelaide Children's Hospital). Of particular dental note was the presentation of excessive gingival overgrowth which covered over two-thirds of the teeth present. Since there was no known history of ingestion of medications which might induce gingival overgrowth, a preliminary diagnosis of gingival overgrowth associated with the condition of mannosidosis was made. A comprehensive case report of the clinical features is currently being compiled for publication by the clinicians dealing with this case.

Histology. Portions of overgrown gingival tissues from around the labial surface of the upper anterior teeth obtained during gingivectomy were fixed in 10% formal-saline, embedded in paraffin nd 5 µm sections were cut. Routine staining with either Haematoxylin and Eosin or Periodic Acid Schiff was carried out prior to histological assessment for general cell and tissue morphology.

Immunohistochemistry. Sections (5 µm) were cut from the paraffin blocks and, following removal of the paraffin and rehydration, the sections were pre-treated with chondroitinase ACII (0.5 Units/ml) for 3 h, washed with phosphate buffered saline, and incubated with the monoclonal antibody 9-A-2 for 2 h (Couchman et al. 1984). The sections were then reacted with biotinylated peroxidase-conjugated streptavidin prior to color development with diaminobenzidine and nickel chloride enhancement. Control sections were incubated in the absence of chondroitinase ACII.

Fibroblast cultures. Human gingival fibroblasts were obtained by explant culture as described previously (Narayanan and Page 1976) from overgrown gingival tissue biopsies from the mannosidosis patient. Age- and sex-matched fibroblasts derived from healthy human gingival biopsies served as a reference source. The cultures thus obtained were routinely tested for mycoplasma infection and found to be negative for this organism. The cells were maintained in Dulbecco's Modification of Eagles Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 Units penicillin, 100 µg/ml streptomycin and non-essential amino acids in a humidified atmosphere of air/CO_2 (9:1) at 37° C. Cells between the 4th and 10th transfer in culture were used.

Fibroblast DNA synthesis and proliferation assays. To determine the level of DNA synthesis in cells derived from both normal and overgrown tissues, human gingival fibroblasts were seeded, in triplicate, into 24-well plates (which hold approximately 10^5 cells at confluence) at an initial density of 20000 cells per well and allowed to attach and spread overnight in DMEM containing 10% FCS. The medium was then replaced with 500 µl/well of DMEM alone and incubated for a further 48 hour. This medium was then removed and replaced with medium containing 10% FCS. After 20 h incubation [^{3}H]-thymidine was added to the medium to give a final concentration of 1 µCi/ml. The cells were incubated for a further 4 h after which the medium was removed and the cells washed three times with 500 µl PBS. The cells were then washed twice with PBS and DNA then precipitated with 600 µl TCA at at 4° C for 2 h. The cell layers were then lysed with an equal volume of 0.1 M sodium hydroxide for 60 min at 50° C. The radioactivity in the extracted material was determined in a Beckman LS-2800 Liquid Scintillation Counter. This assay has been shown to represent accurately in vitro division of fibroblasts (Wahl et al. 1979).

Cell growth was also assessed over a 5 day period using a colorimetric assay (Matthews and Neale 1987). Cells were plated at an initial density of 10000 cells per well into 4-well plates and incubated in DMEM supplemented with 10% FCS. At daily intervals the medium was removed and the cells fixed in 2.5% (v/v) gluteraldehyde). At the end of 5 days all fixed cells were washed with distilled water and then exposed to 400 µl 1% aqueous crystal violet. After staining for 5 min the plates were washed exhaustively with water and the cells solubilized with 1 ml of 33% (v/v) glacial acetic acid and the absorbance read at 580 nm after 5 min.

Protein synthesis. Protein synthesis by gingival fibroblasts was assessed by determination of the incorporation of [^{3}H]-proline into macromolecular material over a 48 h period. Briefly, triplicate cultures of confluent cells (1×10^5 cells per well) from either normal or overgrown gingiva in 24-well plates were incubated in the presence of 10 µCi/ml [^{3}H]-proline. At 2, 4, 8, 24 and 48 h intervals the medium was removed and the cells washed once with 250 µl PBS. The wash and medium were pooled and the cell layers extracted with 500 µl guanidine HCl at 5° C for 24 h. Aliquots from both the medium and cell layer extracts (500 µl) were chromatographed on Sephadex PD-10 columns in the presence of 4 M guanidine HCl/0.05 M sodium acetate pH 5.8. Radioactivity in 0.4 ml effluent fractions was determined by liquid scintillation counting. The amount recovered in the void volume provided a measure of incorporation of radiolabel into newly synthesized macromolecules. Recovery from these columns was approximately 85%.

Proteoglycan synthesis. Proteoglycan synthesis by fibroblasts derived from normal and overgrown gingiva was assessed using a solid-phase assay (Rapraeger and Yeaman 1989). Briefly, triplicate cultures of confluent cells from either normal or overgrown gingiva in 24-well plates were incubated in the presence of 20 µCi/ml [^{35}S]-sulfate for 48 h. At various time intervals (2, 4, 8, 24 and 48 h) the medium wash removed and the cell layers washed with 500 µl PBS. The medium and was were pooled to make up the medium fraction. The cell layers were then extracted overnight at 4° C in 8 M urea containing 10 mM Tris HCl, 1 mM sodium sulfate and 0.1% Triton X-100. Aliquots (200 µl) from both the medium and cell layer fractions were applied onto Zeta probe membranes assembled in a Bio-Dot apparatus and filtered through under vacuum. The membranes were removed from the apparatus, washed twice each in Tris-buffered saline, deionized water and 95% ethanol. After drying, the membrane was cut into small pieces and added to 5 ml scintillation fluid and the amount of radioactivity present determined by liquid scintillation counting.

Statistical analyses. All data were subjected to statistical analysis using the method of analysis of variance.

Results

Routine histological assessment of the excised overgrown gingival tissue revealed a relatively normal width

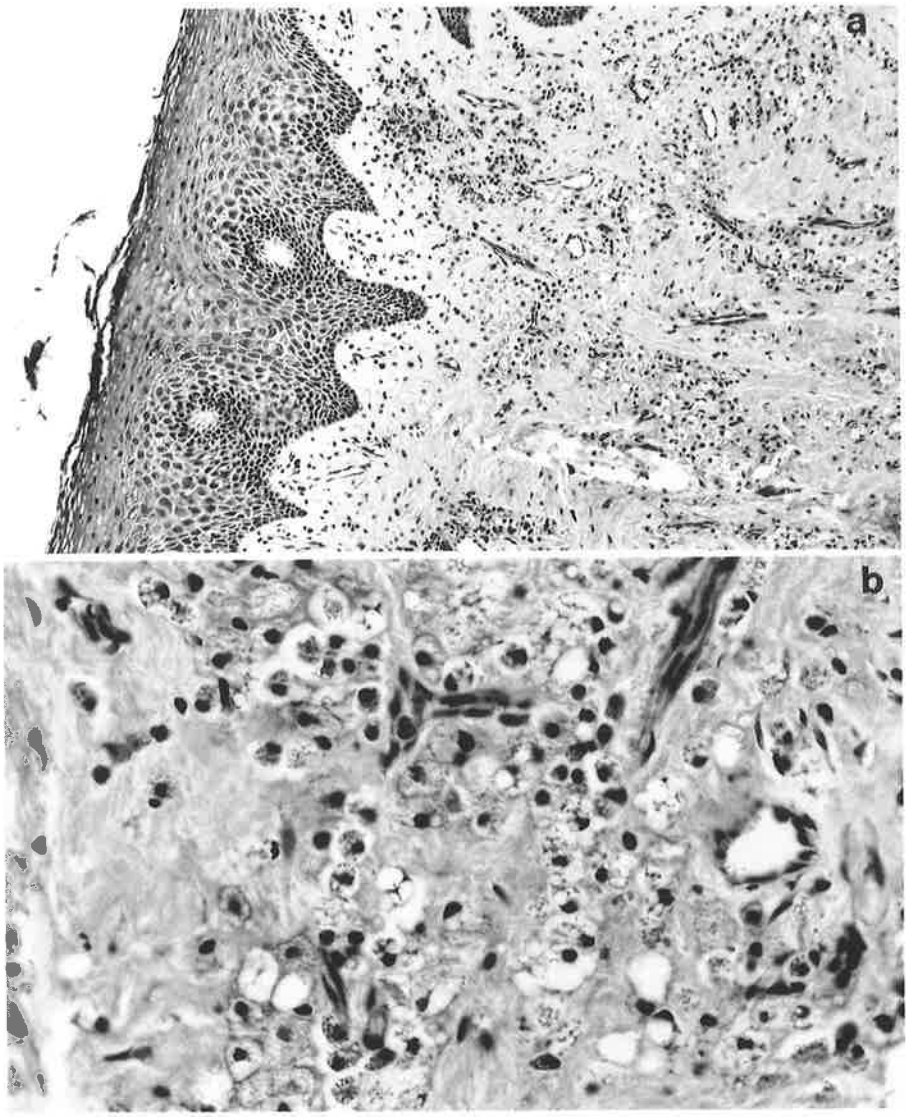

Fig. 1a, b. Histological appearance of gingival biopsy sections. **a** Sections stained with Haematoxylin and Eosin showing relatively normal epithelium with a moderately inflamed underlying connective tissue. Magnification 25×. **b** Higher power view showing enlarged and vacuolized infiltrating lymphocytes. Note the normal appearance of resident fibroblasts. Magnification 100×

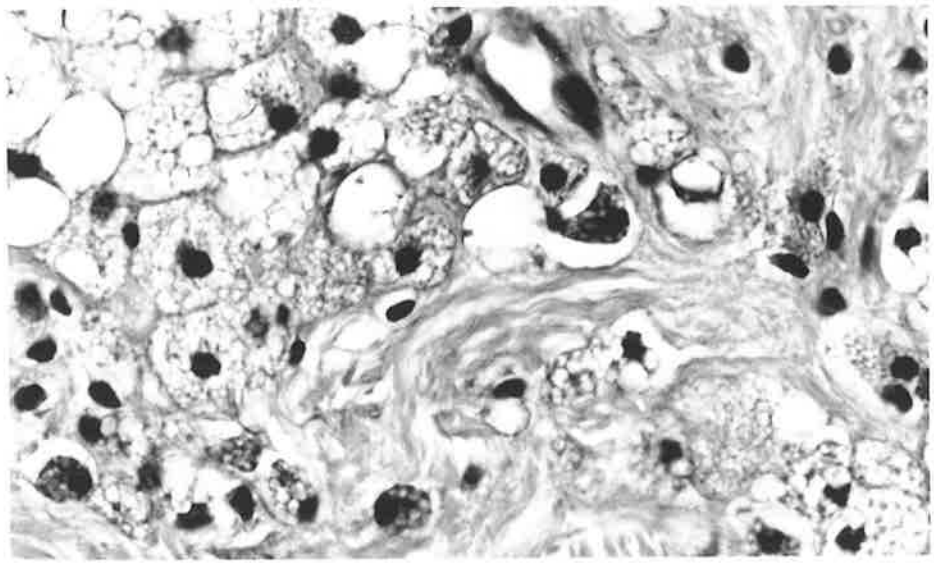

Fig. 2. Gingival biopsy sections stained with PAS showing abundant positive material for glycoproteins within the vacuoles of the enlarged lymphocytes. Magnification 200×

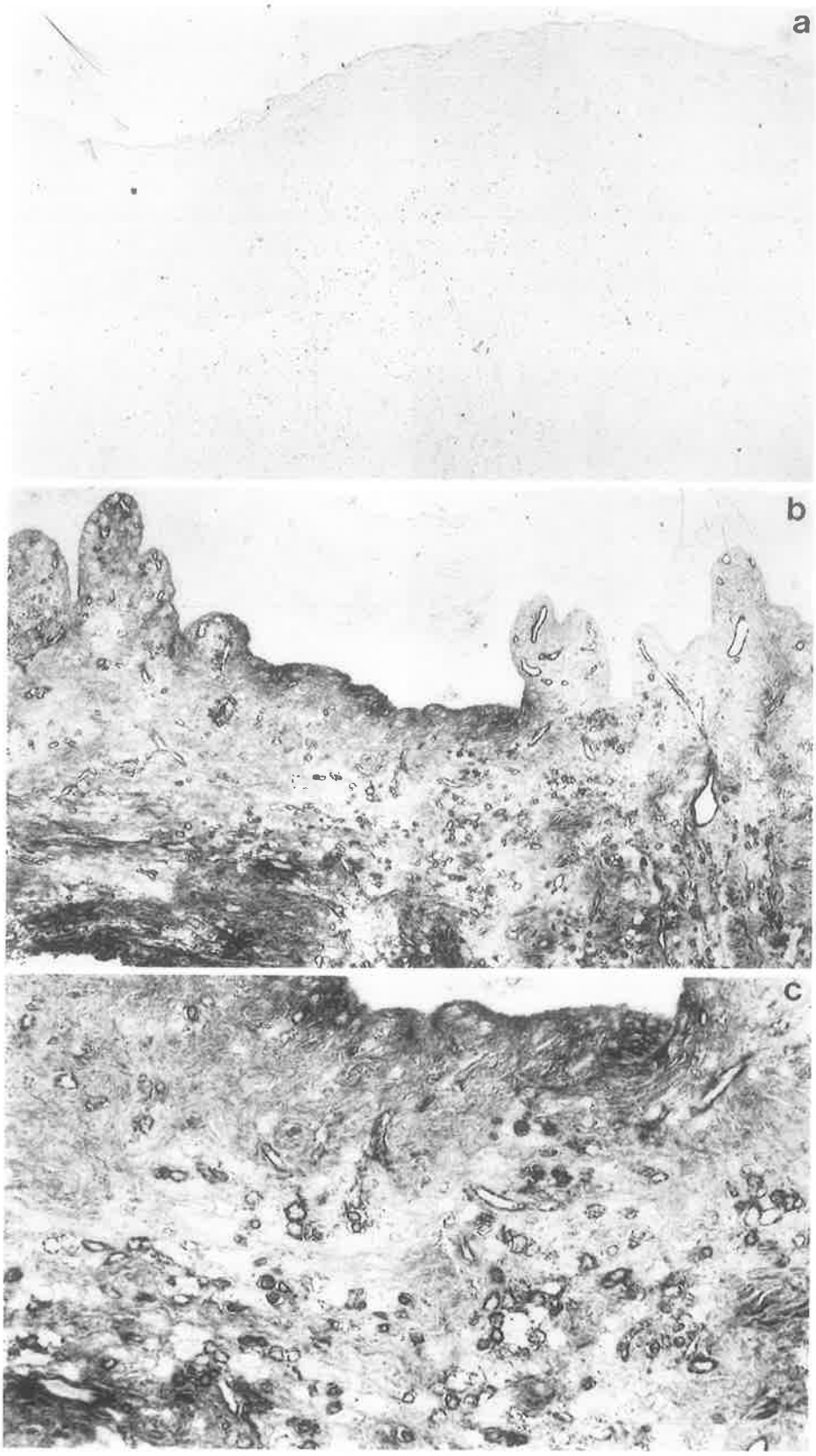

Fig. 3a–c. Immunohistochemical localization of chondroitin sulfate in sections of the gingival biopsy. **a** Control section incubated with monoclonal antibody 9-A-2 in the absence of chondroitinase ACII digestion. Magnification 25×. **b** Section incubated with monoclonal antibody 9-A-2 after digestion with chondroitinase ACII. Magnification 25×. **c** Section incubated with monoclonal antibody 9-A-2 after digestion with chondroitinase ACII. Magnification 50×

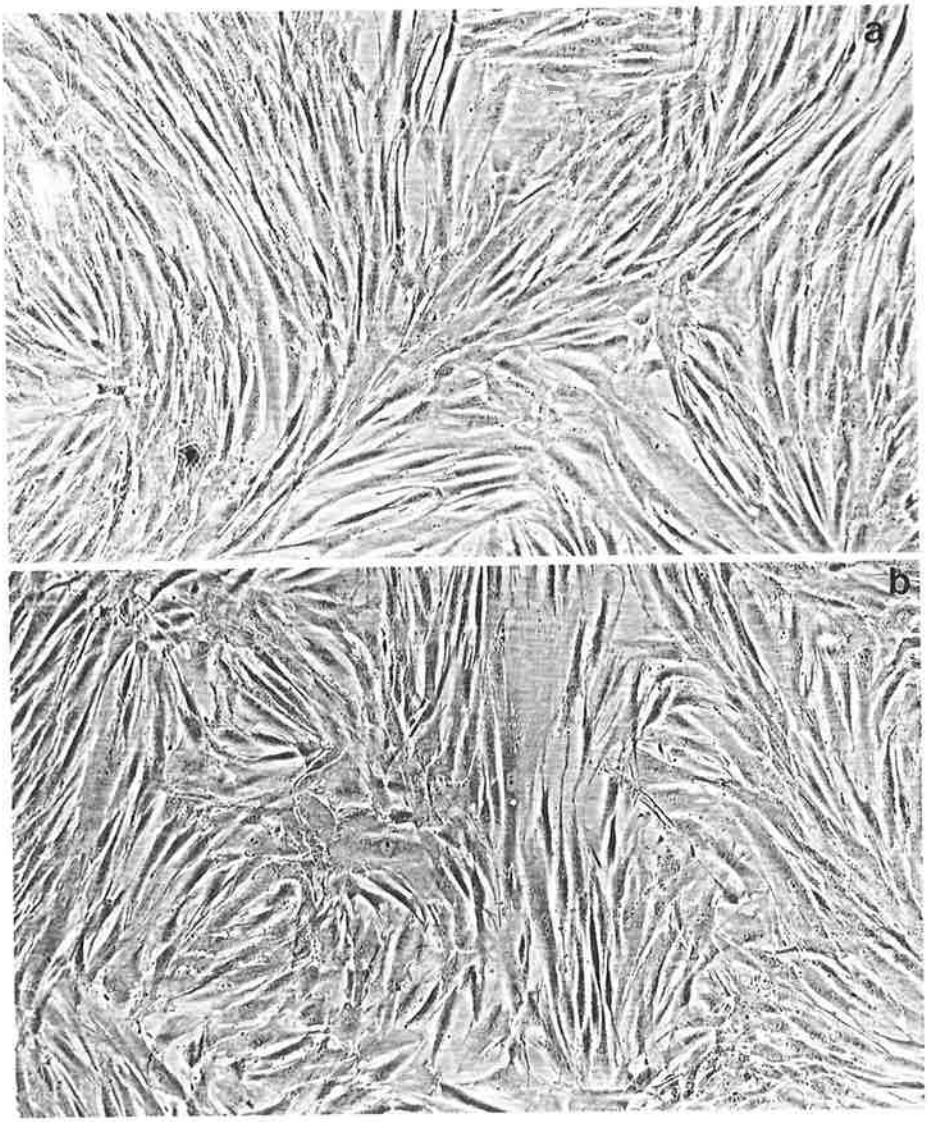

Fig. 4. Microscopic appearance of gingival fibroblasts from (**a**) normal and (**b**) mannosidosis patient. Magnification 230×

of epithelium overlying a moderately fibrotic connective tissue (Fig. 1). Although not specifically quantitated, there did not appear to be an excessive number of fibroblasts present. The morphology of both the epithelial cells and fibroblasts appeared normal. Small foci of inflammatory cell infiltrates were noted. These comprised principally of lymphocytes and macrophages with very few polymorphonuclear leukocytes being noted. One very striking feature was the presence of large numbers of engorged, vacuolated lymphocytes. At the sites of heavy lymphocyte infiltration there was an obvious loss of normal collagenous architecture.

Reaction of the sections with PAS revealed a typical staining pattern in the epithelial tissues. The connective tissue elements were well defined in areas not infiltrated by inflammatory cells. Of particular interest was the positive staining for glycoproteins (possibly mannose-rich storage products) in the vacuoles of the infiltrating lymphocytes (Fig. 2).

Immunohistochemical investigations using the monoclonal antibody 9-A-2 which recognizes the unsatturated disaccharide (ΔDi-4S) of chondroitinase ACII digested chondroitin sulfates, revealed relatively uniform staining throughout the connective tissue (Fig. 3). Virtually no staining was evident in the epithelium. At the sites of heavy lymphocyte infiltration, there was a noticeable loss of normal connective tissue architecture and staining for chondroitin sulfate. The enlarged, vacuolated lymphocytes showed intense staining with this antibody on their cell surface and, to some extent, within the engorged vacuoles.

The appearance of fibroblasts isolated from gingival tissues of the mannosidosis patient as well as an age and sex matched control subject is shown in Fig. 4. At the light microscopic level, there appeared to be no evidenced of intracellular vacuolization or other morphological changes in the mannosidosis cells.

The incorporation of [^{3}H]-thymidine into DNA by the cells from both normal and overgrown gingivae is shown in Fig. 5. Control cultures of cells incubated in

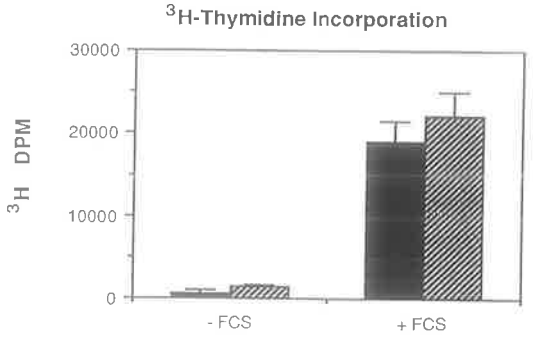

Fig. 5. ^{3}H-thymidine incorporation into DNA by fibroblasts isolated from normal and overgrown gingival tissues. Data represent the mean and standard deviation of the mean of triplicate cultures. ■ Normal; ▨ mannosidosis

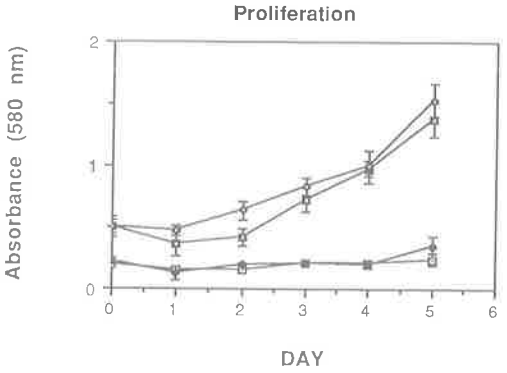

Fig. 6. Proliferative response of fibroblasts isolated from (**a**) normal and (**b**) overgrown gingival tissues. The cell were cultured in medium either with or without 10% FCS. Data represent the mean and standard deviation of the mean of triplicate cultures. —□— Normal-FCS; —◇— mannosidosis-FCS; —■— normal + FVCS; —◆— mannosidosis + FCS

the absence of FCS demonstrated the expected lack of [^{3}H]-thymidine uptake. Between 20–24 h following stimulation of the cells with 10% FCS, there was a large increase in [^{3}H]-thymidine incorporation into DNA. No statistical difference was found between this activity for cells from either normal or overgrown tissues.

Although the incorporation of [^{3}H]-thymidine into DNA is good indicator of cell proliferation, it is not a definitive measure since variability in the cell cycle between different cells may be sufficient to produce aberrent results. Therefore, to confirm the above results, the growth rates of cells derived form normal and overgrown gingivae were assessed over a 5 day period (Fig. 6). As would be expected, the cells cultured in the absence of FCS failed to show any significant growth. On the other hand, cells grown in the presence of 10% FCS showed a steady increase in numbers after a brief lag period during the first 48 h. No significant difference between cells derived from normal of overgrown gingival tissues were noted.

In addition to proliferation, analyses of the cells' biosynthetic activity was also assessed by monitoring the total protein synthesis over a 48 h period (Fig. 7). The

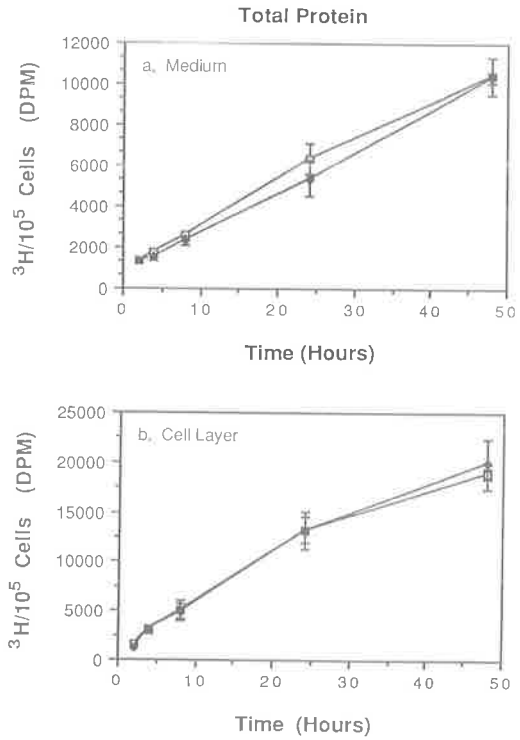

Fig. 7a, b. Total protein synthesis by fibroblasts isolated from normal and overgrown gingival tissues. The protein associated with (**a**) the medium or, (**b**) the cell layer was determined. Data represent the mean and standard deviation of the mean of triplicate cultures. —□— Normal; —◆— mannosidosis

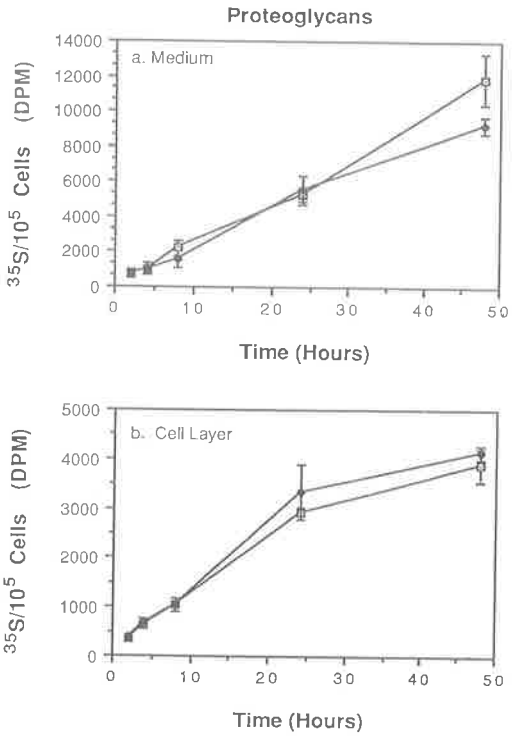

Fig. 8a, b. Proteoglycan synthesis by fibroblasts isolated from normal and overgrown gingival tissues. The amount of proteoglycan in (**a**) medium and (**b**) cell layer was determined and the data represent the mean and standard deviation of the mean of triplicate cultures. —□— Normal; —◆— mannosidosis

release of proteins into the culture medium did not differ significantly between the different cells studied. Similarly, analysis of the newly synthesized protein remaining associated with the cells revealed no significant differences. Analysis of proteoglycans synthesis also failed to show any significant differences between the medium and cell layer fractions of cells derived from normal and overgrown gingivae (Fig. 8).

Discussion

Gingival overgrowth has been observed in a variety of situations. The best recognized are those induced by various medications such as phenytoin, cyclosporine and nifedipine (Hassell et al. 1980; Bartold 1987; Lederman et al. 1984). Nonetheless, heavy deposits of dental plaque, inherited familial as well as unidentified fibromatoses, and metabolic disorders have also been reported to demonstrate various degrees of gingival overgrowth (Fletcher 1966; Johnson et al. 1986; Goodman and Gorlin 1977). Of these, the metabolic defects are of direct interest to the present study. Although many of the drug-induced gingival overgrowth conditions may be associated with an ultimate alteration in fibroblast function, their manifestations are generally regarded as reversible since remission of the lesions commonly occurs after withdrawal of the prescribed medication (Little et al. 1975; Daly et al. 1986). However, this is not the case for the disorders in which there is an existing underlying metabolic defect in normal cellular metabolism (Emerson 1965).

To date few reports concerning the metabolic disorders have made note of effects on the gingival tissues. Those which have made such a note include reports on gangliosidosis and mucolipidosis (Goodman and Gorlin 1977; Nolan and Sly 1989). Both of these conditions have a common feature in that they are lysosomal enzyme disorders and thus have a large potential to affect the normal metabolism and structure of all connective tissues. In this respect it is not surprising that, in the present report, gingival overgrowth has been found in a patient suffering from mannosidosis which is also a lysosomal enzyme defect affecting the complete degradation of mannose-containing glycoproteins.

Whether the gingival response in mannosidosis represents a direct manifestation of the disorder or is a secondary response is not entirely clear. For example, only two reports dealing with mannosidosis have indicated the manifestation of gingival overgrowth in this condition (Kistler et al. 1977; Daniel et al. 1981). The low prevalence of this feature could be due to many factors. For example it could be because it is not a normal feature of mannosidosis, because it has been overlooked due to more serious medical problems, or because few patients have been studied into their mid-adult life and thus time for milder complications to develop has not been available.

Regardless of the underlying metabolic problems, another important aetiologic feature in gingival overgrowth is dental plaque. An abundance of dental plaque appears to be common to all but the hereditary familial gingival fibromatoses. For most of the drug-induced gingival overgrowth conditions, these responses may not be a primary response to the medication but rather a reversible secondary response to the presence of large quantities of irritating sub-gingival plaque (Hassell et al. 1976; Bartold 1989; Friskopp and Klintman 1986). Such is likely to also be the situation in the presently reported case. The patient was moderately retarded, and self care was not optimal. As a result, oral hygiene was poor. Due to the likely compromised connective tissue metabolism associated with mannosidosis, the protective mechanisms normally present in healthy individuals may not operate at satisfactory levels. Indeed, due to the compromised lysosomal enzyme function in the fibroblasts and lymphocytes (Taylor et al. 1975), together with a possible defect in neutrophil function (Quie and Cates 1977), one would anticipate the potential for uncontrolled host response to bacterial plaque irritation to be high.

Several investigations have studied fibroblasts from patients with mannosidosis with the principal area of concern being the synthesis of α-mannosidase. To date several forms of this enzyme have been identified which differ not only in their pH optima but also in their subcellular and extracellular localization (Carrol et al. 1972; Marsh and Goulay 1971; Tabas and Kornfeld 1979). While there is normal excretion of α-mannosidase into the culture medium of mannosidosis fibroblasts (Hultberg and Masson 1977) a defect lies in the enzymes associated with the cell surface and intracellular compartments (Taylor et al. 1975; Ben Yoseph et al. 1982; Halley et al. 1980). Regardless of these findings, there does appear to be some variability to the degree in which the enzyme activity is affected (Tsvetkova et al. 1980) and this may account for the observed clinical heterogeneity of mannosidosis (Beaudet and Thomas 1989).

Although the manifestation of gingival overgrowth in this mannosidosis patient implies a modification of normal connective tissue tun rover or metabolism, no significant differences were found between fibroblasts from normal and mannosidosis-associated gingival tissues with respect to cell morphology at the light microscopic level, proliferative responses or synthetic activity. These findings imply that the gingival response is not related to an altered phenotypic expression of the mannosidosis fibroblasts with respect to proliferation and matrix synthesis. The absence of proliferative changes is consistent with most of the reported lysosomal storage disorders except for mucolipidosis III (pseudo-Hurler polydystrophy) in which fibroblasts with this disorder show some loss of contact-inhibited growth regulation (Oohira et al. 1987). In addition, our findings are consistent with other lysosomal enzyme storage disorders in that, although glycoprotein degradation is severely affected in these disorders, the problem appears to reside solely with a defect in the degradative enzymes and not in synthesis of the glycoproteins (Fratantoni et al. 1968).

In conclusion, the gingival tissues have been studied of a patient suffering from the lysosomal enzyme defect

mannosidosis. Since no reliable defect in the isolated fibroblasts could be found, yet significant morphological changes were noted in the infiltrating lymphocytes, the noted gingival overgrowth is considered to be a secondary response to external factors as a result of compromised host defense mechanisms.

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VIII. FACTORS INFLUENCING PROTEOGLYCAN SYNTHESIS

Paper 15. Bartold, P.M., Boyd, R.R. and Page, R.C.
Proteoglycans synthesized by fibroblasts derived from donors of different ages.
Journal of Cellular Physiology **126:** 37-46, 1986.

Paper 16. Bartold, P.M.
The effect of interleukin 1β on proteoglycans synthesized by human gingival fibroblasts *in vitro.*
Connective Tissue Research **17:** 287-304, 1988.

Paper 17. Bartold, P.M.
The effect of interleukin 1β on hyaluronic acid synthesized by adult human gingival fibroblasts *in vitro.*
Journal of Periodontal Research **23:** 139-147, 1988.

Paper 18. Bartold, P.M. and Millar, S.J.
Effect of lipopolysaccharide on proteoglycan synthesis by adult human gingival fibroblasts *in vitro.*
Infection and Immunity **56:** 2149-2155, 1988.

Paper 19. Bartold, P.M.
Lipopolysaccharide stimulation of hyaluronate synthesis by human gingival fibroblasts *in vitro.*
Archives of Oral Biology **36:** 791-797, 1991.

Paper 20. Bartold, P.M., Gully, N.J., Zilm, P.S. and Rogers, A.H.
Identification of components in Fusobacterium nucleatum chemostat-culture supernatants that are potent inhibitors of human gingival fibroblast proliferation.
Journal of Periodontal Research **26:** 314-322, 1991.

Paper 21. Bartold, P.M.
Regulation of human gingival fibroblast growth and synthetic activity by Cyclosporine-A *in vitro.*
Journal of Periodontal Research **24:** 314-321, 1989.

Paper 22. Bartold, P.M., Kylstra, A. and Lawson, R.
Substance P: An immunohistochemical and biochemical study in human gingival tissues. A role for neurogenic inflammation?
Journal of Periodontology **65:** 1113-1121; 1994

Paper 23 Bartold, P.M.
Platelet-derived growth factor stimulates hyaluronate but not proteoglycan synthesis by human gingival fibroblasts *in vitro.*
Journal of Dental Research **72:** 1473-1480; 1993

Paper 24. Bartold, P.M. and Raben, A.
Growth factor modulation of fibroblasts in simulated wound healing
Journal of Periodontal Research In Press 1995.

Papers 15 - 24 present the results of studies designed to investigate a variety of factors which might influence proteoglycan and hyaluronan synthesis in the human periodontal tissues. These studies compliment the earlier studies which had considered the normal and inflamed tissues, but did not consider individual parameters which might affect cell metabolism in the context of periodontal inflammation.

Paper 15 provides a description of the differences in proteoglycans synthesized by fibroblasts derived from the gingivae of donors of different ages. These studies were important in that they departed from the classical approach for aging studies which used *in vitro* senescence. Rather, cells of low passage number from patients of varying ages were used. The major findings of this study were that with increasing donor age, the cells proliferated at a slower rate and that proteoglycan synthesis decreased. In addition, with increasing donor age, the proteoglycans synthesized by the fibroblasts became smaller and contained an increased amount of heparan sulfate which became progressively richer in N-sulfate with age. These various changes were considered to reflect the general metabolic changes which occur with aging. In particular, the relationship between a decrease in proliferative activity and an increase in heparan sulfate synthesis were considered to be directly related.

Papers 16 and 17 describe the effect of the cytokine interleukin-1β (IL-1β) on proteoglycan and hyaluronate synthesis by human gingival fibroblasts. At the time of doing these studies, considerable attention was focused on IL-1β as being a prime player in matrix degradation during inflammation. In addition, recombinant forms of IL-1β had just become available. Thus, it became possible to address the effects of IL-1β using recombinant forms of this molecule which were free from the confusing issue of possible contaminants often found in purified tissue extracts of IL-1β. These studies were amongst the first to consider the effects of IL-1β on human fibroblasts, and demonstrated that IL-1β was mitogenic for fibroblasts, as well as caused a dose-dependent increase in both proteoglycan and hyaluronan synthesis. Although other studies had implicated IL-1β in increased matrix degradation due to stimulation of the release of matrix metalloproteinases, the results of the studies described in papers 16 & 17 were thought to reflect some of the early changes which occur in inflamed tissues, namely an increase in matrix synthesis to compensate for loss due to enzymatic degradation.

Papers 18, 19 and 20 consider the effects of bacterial products on proteoglycan and hyaluronan synthesis by human gingival fibroblasts. Lipopolysaccharide from a variety of oral and non-oral sources was found to modulate both cell proliferation and matrix synthesis. In culture supernatants from *F. nucleatum*, ammonia and butyrate were identified as the principal components which significantly influenced cell proliferation and matrix synthesis. These studies served to highlight the importance of both metabolic and structural components of oral bacteria in influencing fibroblast function.

During the late 1980's, numerous medications, apart from phenytoin, were noted to be associated with gingival overgrowth. Of these, cyclosporine was particularly interesting, in that its principle mode of action was via immunosuppression, yet notable connective tissue changes were observed in

the gingival tissues of patients manifesting cyclosporine-induced gingival overgrowth. In paper 21, several aspects of the direct effects of cyclosporine on human gingival fibroblasts were explored. Cyclosporine was found to stimulate cell proliferation in a dose dependent manner but did not significantly alter protein or proteoglycan synthesis. This was considered to be consistent with the histological picture of a generalized overgrowth of the tissues with a proportional increase in both cell numbers and matrix. A particularly interesting finding from this study was that cyclosporine appeared to negate the inhibitory effects of lipopolysaccharide on fibroblast proliferation which could explain, in part, why the gingival overgrowth is often most prominent in areas of heavy dental plaque accumulation.

Paper 22 addresses the novel hypothesis that neurogenic inflammation may be an important component in matrix modification during the establishment of periodontitis. The tissue distribution of the neuropeptide substance P was studied by immunohistochemistry, and found to be increased in inflamed tissues where it was found to localize strongly around blood vessels and in close proximity to the inflammatory cell infiltrates. Upon exposure to this neuropeptide *in vitro*, gingival fibroblasts showed a dose dependent mitogenic response. In addition, exposure of gingiva fibroblasts to substance P led to an increased accumulation of protein and proteoglycans within these cells. Such findings suggested a potential role for substance P in gingival pathology, and that there is a need for ongoing investigations into the largely unexplored area of neurogenic inflammation and periodontitis.

In recent years considerable attention has focused on means of stimulating and inducing regeneration of damaged periodontal structures. Papers 23 and 24 consider the effects that platelet derived growth factor might have in the regenerative process. Paper 23 describes a series of experiments investigating the ways in which this growth factor modulates cell proliferation and proteoglycan synthesis. The findings indicated that platelet derived growth factor is capable of stimulating responses in fibroblasts which would be considered to be important functions during the very early phases of wound repair and tissue regeneration. Paper 24 used a novel *in vitro* model of wounding and wound healing to investigate the effect of platelet derived growth factor in modulating wound repair. This study found that platelet derived growth factor stimulated cell proliferation and cell migration as well as proteoglycan synthesis in a manner consistent with the events associated with accelerated wound repair.

Paper 15.

Bartold, P.M., Boyd, R.R. and Page, R.C.
Proteoglycans synthesized by fibroblasts derived from donors of different ages.
Journal of Cellular Physiology **126:** 37-46, 1986.

Candidate's Contribution to this paper: 70%

P.M. Bartold's role in this study was:
Design of the experiments
Execution of experimental work
Writing of the paper

R.R. Boyd's role in this study was:
Summer Vacation Student Scholar
Assistance with experimental work

Dr Page's role was:
Provision of research funds
Writing of the paper

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PROTEOGLYCANS SYNTHESIZED BY CULTURED FIBROBLASTS DERIVED FROM NORMAL AND INFLAMED HUMAN GINGIVA

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(Received 18 November 1985; accepted 21 January 1986)

SUMMARY

The in vitro proliferations rates and ptoteoglycans synthesized by adult human gingival fibroblasts derived from six age- and sex-matched donors of healthy and chronically inflamed gingiva were analyzed. Fibroblasts from inflamed gingiva demonstrated a slower growth rate than cells from healthy tissue. The rate of incorporation of [^{35}S]sulfate into cell layer-associated proteoglycans and the release of these macromolecules into the culture medium did not differ appreciably between the two groups of cells. Similarly, no detectable differences in the overall charge of the proteoglycans synthesized by normal and inflamed gingival fibroblasts, as assessed by their elution from DEAE-Sephacel, were noted. However, Sepharose CL-4B chromatography revealed that the medium-associated proteoglycans made by the inflamed tissue fibroblasts were depleted in one species of chondroitin sulfate proteoglycans and contained more dermatan sulfate than did control cells. In addition, the intracellular proteoglycan pool was found to be greatly diminished in the inflamed tissue fibroblast cell layers. Glycosaminoglycan analysis of the proteoglycans confirmed these observations. Compared to normal gingival fibroblasts, the inflamed tissue fibroblasts released less heparan sulfate into the medium. Additionally, increased levels of dermatan sulfate and depleted amounts of chondroitin sulfate in the medium of inflamed gingival cells were noted. The observed changes were stable through several transfers in culture and indicate that chronically inflamed tissue may contain fibroblasts manifesting a heritable phenotype differing from fibroblasts in normal connective tissue.

Key words: proteoglycans; inflammation; fibroblasts; gingiva; periodontal disease.

INTRODUCTION

Proteoglycans are a complex group of highly anionic macromolecules that have ubiquitous distributions throughout the extracellular matrices of soft connective tissues (1). As a result of their charged nature, proteoglycans are highly hydrated and capable of interacting with a wide variety of matrix and cell surface components (7,11,16). Indeed, such interactions are considered vital to the maintenance of normal tissue function. Consequently, any damage to, or altered synthesis of, these matrix macromolecules could be expected to significantly affect the physiologic well being of tissues.

During soft tissue pathological changes, such as is seen in inflammation, there is a complex interplay between the inflammatory cells and the resident fibroblasts accompanied by degradation of the extracellular matrix. If the causative factor is removed, then under normal circumstances the tissue eventually heals and a healthy state is restored. Gingiva has served as a model for studying the events occurring in the extracellular matrix of soft connective tissues associated with inflammation because it is both readily available and manifests a naturally occurring, progressive condition termed periodontitis (19).

The collagens of gingiva in both health and disease have been studied extensively (19). Histological and biochemical studies have shown that at a very early stage of inflammation, collagen destruction and loss occur (22). Additional in vitro studies have revealed that various chemical agents found at sites of inflammation exert profound effects on fibroblast growth and synthetic activity (12,13,18,21). Furthermore, fibroblasts from inflamed tissue may be phenotypically different from those present in normal tissue. These cells produce a unique collagen with a structure of $\alpha 1[I]_3$, a type not found in cultures of normal fibroblasts and represents a feature that persists throught their cultured life span.

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Much less is known about gingival proteoglycans. To date, the proteoglycan composition of normal gingival tissue has been analyzed (2,9,36), and some studies have considered the in vitro synthesis of proteoglycans and glycosaminoglycans by gingival fibroblasts (3) (Bartold, P. M.; Page, R. C., submitted for publication). In the present study proteoglycans synthesized by cultured fibroblasts isolated from normal and inflamed human gingiva have been analyzed to determine the effect of chronic inflammation on the types of proteoglycans synthesized by these cells. The results obtained indicated that although proteoglycan synthetic rates seemed unaltered, there were differences noted in the quantities and types of proteoglycans made by fibroblasts for normal and diseased tissues, and these differences persisted through numerous transfers in culture.

Materials and Methods

Materials. Guanidine HCl, 6-aminohexanoic acid, benzamidine HCl, *N*-ethylmaleimide, phenylmethylsulfonyl flouride, cysteine HCl, cetylpyridinium chloride, and papain (E. C. 3.4.22.2) were all purchased from Sigma Chemical Co., St. Louis, MO; sodium dodecyl sulfate (SDS) from BioRad Laboratories, Richmond, CA; chondroitinase AC (*Arthrobacter aurescens*) and chondroitinase ABC (*Proteus vulgaris*) from Seikagaku Kogyo, Tokyo, Japan, through Miles Laboratories, Inc., Elkhart, IN; Sephadex G-50, Sepharose CL-4B, Sepharose CL-6B, and DEAE-Sephacel from Pharmacia, Inc., Piscataway, NJ; Dulbecco-Vogt medium, fetal bovine serum (FBS), phosphate buffered saline (PBS), and penicillin-streptomycin from Grand Island Biological Co., Grand Island, NY. Sulfate-depleted medium was made from amino acids and vitamins purchased from Grand Island Biological Co.; 75-cm² tissue culture flasks were from Falcon Plastics, Oxnard, CA; 24-well plates from Flow Laboratories; $Na_2[^{35}S]O_4$ (2 mCi/ml; 424.15 mCi/mmol) from New England Nuclear, Boston, MA; Aquamix from WestChem, San Diego, CA; and Aquacide from Calbiochem, La Jolla, CA.

Fibroblast culture. Fibroblasts were obtained from biopsies from human volunteers with healthy gingivae and from age- and sex-matched patients undergoing treatment for chronic periodontitis as previously described (21). The normal tissue fibroblasts (henceforth termed "normal fibroblasts") were designated by the terminology HGF_{24}, HGF_{25}, and HGF_{27}; the inflamed tissue fibroblasts (henceforth termed "inflamed fibroblasts") were designated by the terminology HGF_{10}, HGF_{11}, and HGF_{13}. These are cells from the same parent populations described previously for the study of collagen synthesis by normal and inflamed fibroblasts (21). No test for mycoplasmal contamination was carried out in the present study. The cells were maintained in Dulbecco-Vogt medium supplemented with 10% heat inactivated FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 m*M* glutamine and studied between the 8th and 13th transfer in culture. The fibroblasts were maintained in 75-cm² tissue culture flasks in Dulbecco-Vogt medium. For each experiment the cells were released from the tissue culture plates by treatment with 0.05% trypsin in PBS for 5 min at 37° C.

Measurement of cell proliferation. Cells were plated into 24-well tissue culture plates in triplicate at an initial density of 50 000 cells/well. The number of cells in each culture was determined at daily intervals over a 7-d period. Briefly, at each time point the medium was removed and the cells washed twice with 0.5 ml PBS. The cells were then released by trypsinization with 200 μl of 0.05% trypsin at 37° C for 15 min. Cells were pipetted from the wells and each well washed twice with 200 μl PBS. The trypsinized cells and the washes were pooled and cell numbers determined using a Coulter Counter.

[^{35}S]Sulfate incorporation and pulse chase. For the radiolabel incorporation studies, trypsinized cells were seeded in 2 ml medium at a density of 50 000/well into 24-well plates. The cells were allowed to grow to confluence with medium replenishment daily. The culture medium was then removed and replaced with 500 μl of sulfate-depleted medium ($MgSO_4$ replaced with $MgCl_2$) and the cells incubated for 1 h at 37° C. The sulfate-depleted medium was then removed and replaced with sulfate-depleted medium containing [^{35}S]sulfate (75 μCi/ml) and incubated at 37° C for up to 48 h. Incorporation of [^{35}S]sulfate into proteoglycans by normal and inflamed fibroblasts was monitored over 48 h at 2, 4, 8, 24, 30, and 48 h intervals. At each time interval

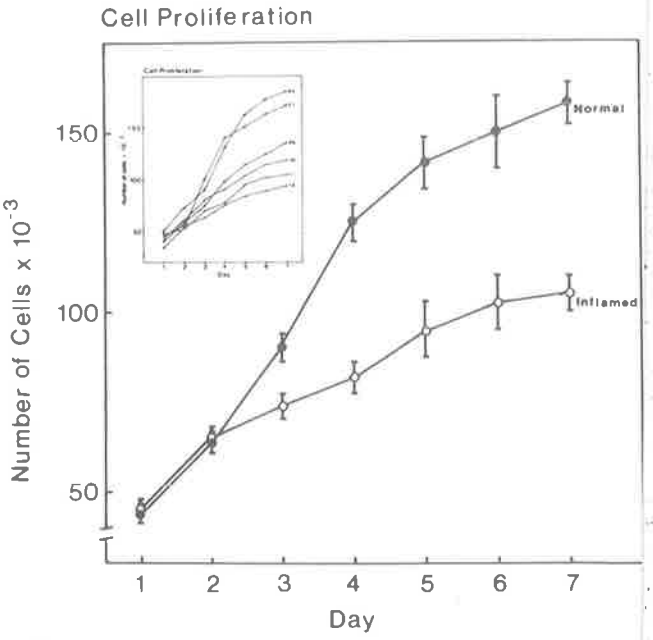

FIG. 1. Fibroblast proliferation rates. Normal and inflamed gingival tissue fibroblasts were seeded into 24-well plates at the same initial plating densities (50 000 cells/well). The cultures were incubated for 7 d and cell numbers were determined daily by counting in a Coulter Counter as described in Materials and Methods. The experiments were repeated three times and representative data from one of these experiments are shown. The mean and SEM of triplicate cultures for each time point for three strains normal and three strains of inflamed fibroblasts are shown. The *inset* shows mean proliferation rates of the individual cell strains; normal fibroblasts are designated 24, 25, and 27 and inflamed fibroblasts designated 10, 11, and 13.

the medium was removed from each culture and the cell layers washed twice with 200 μl PBS. The medium and washes for each cell strain were pooled. The cell layers were then treated with 200 μl of 0.05% trypsin in PBS for 15 min at 37° C and washed twice with 200 μl PBS. The trypsin digest and washes for each cell strain were also pooled. The medium and cell layer extracts were then frozen at −70° C until all of the time points had been completed. Aliquots (300 μl) of the medium and cell layer extracts of each time point were then spotted, in duplicate, onto Whatman 3MM filter paper. The filter paper was dried and then immersed through five changes of 1% cetylpyridinium chloride (CPC) in 0.05 *M* NaCl each of 1 h duration (35). The paper strips were then dried overnight, cut into small pieces and placed into 10 ml of scintillation fluid and assessed for [^{35}S]-activity in a Packard Tri Carb 3255 liquid scintillation counter.

For the pulse-chase experiments, triplicate cultures of cells were plated into 24-well plates, as for the [^{35}S]sulfate incorporation studies, and allowed to reach confluence. Radiolabeling of the cultures with [^{35}S] sulfate was commenced as described above and allowed to proceed for 36 h, by which time a steady state of [^{35}S]sulfate incorporation is reached (3). The medium was then removed and the cells washed three times with PBS to remove residual-free [^{35}S]sulfate and medium-associated [^{35}S]-labeled macromolecules and replaced with 500 μl Dulbecco-Vogt medium. The release of radiolabeled macromolecules into the medium was followed over 48 h at 2, 4, 8, 24, 30, and 48-h intervals. At each time point, the medium and cell layers were harvested and the amount of [^{35}S]sulfate-labeled proteoglycans determined using CPC precipitation onto filter paper and liquid scintillation counting as described above.

Metabolic labeling and proteoglycan extraction. For quantitative and qualitative biochemical analyses of the proteoglycans synthesized by fibroblast cultures, cells wre seeded into 75-cm² flasks at a density of 750 000/ flask. The cells were grown to confluence in 75-cm²

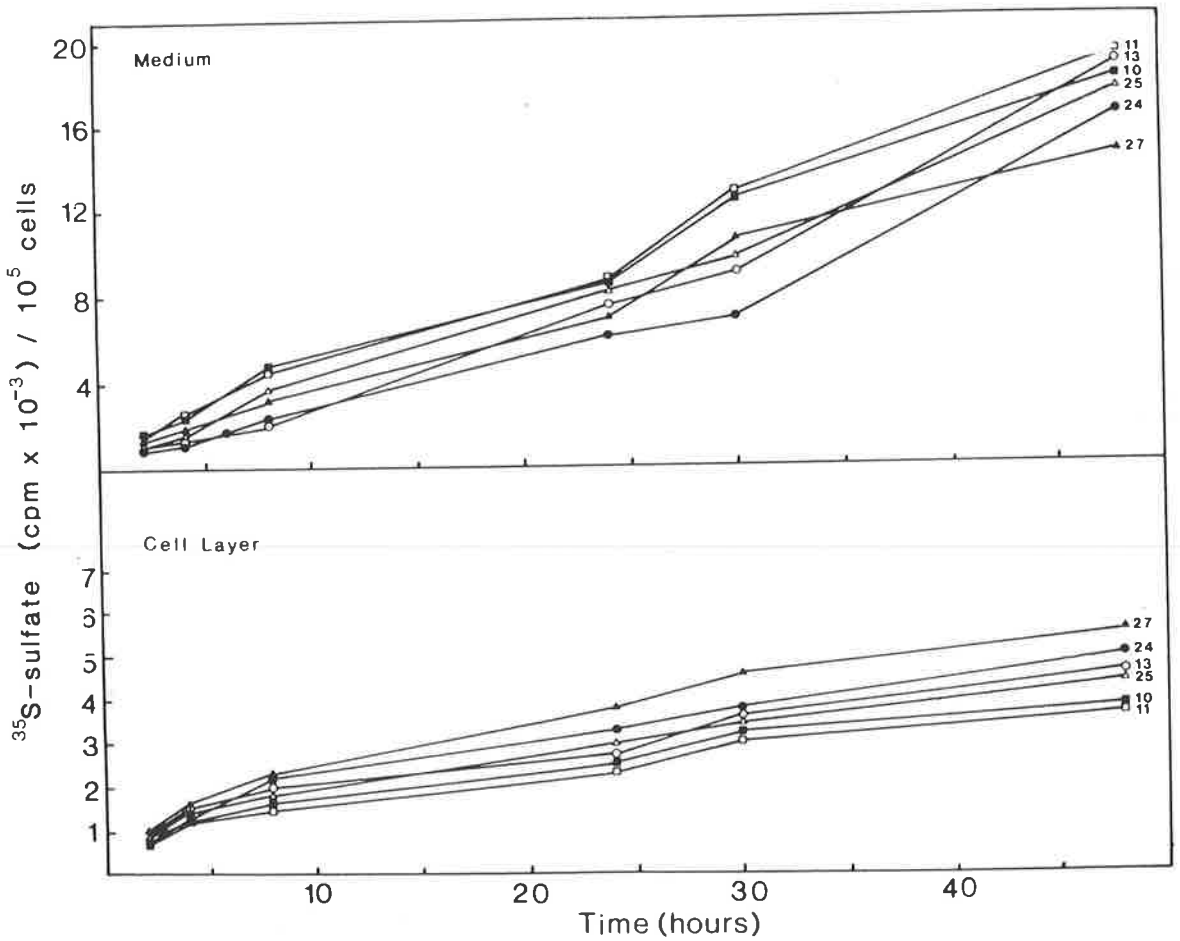

FIG. 2. [^{35}S]Sulfate incorporation into proteoglycans with time. Normal and inflamed gingival tissue fibroblasts were plated into 24-well plates in triplicate and were radiolabeled with [^{35}S]sulfate for various lengths of time between 0 and 48 h. At each time point, the medium and cell layer were extracted with 4 *M* guanidine HCl as described in Materials and Methods. The amount of radioactivity precipitable with cetylpyridinium chloride for each time point is expressed relative to the number of cells present at that time. Data indicate the mean of experiments carried out on each cell strain in triplicate for each time point. The experiments were repeated three times and representative data for one of these experiments are presented. Normal gingival fibroblasts: HGF_{24} ●—●; HGF_{25} △—△; HGF_{27} ▲—▲. Inflamed gingival fibroblasts: HGF_{10} ■—■; HGF_{11} □—□; HGF_{13} ○—○.

flasks with medium replenishment every 2 d. Metabolic labeling of the cells was carried out in 5 ml Dulbecco-Vogt medium containing 75 μCi/ml [^{35}S]sulfate for 48 h at 37° C. For these experiments, sulfate-depleted medium was not used.

[^{35}S]Sulfate-labeled proteoglycans were isolated from the medium and cell layers of normal and inflamed fibroblasts. Briefly, the medium was removed and the cell layers washed twice with 5 ml PBS and pooled. The pooled material was then made approximately 3 *M* in quanidine HCl by adding solid guanidine HCl (0.38 g/ml). Unincorporated radiolabel was then separated from labeled macromolecules by elution from Sephadex G-50 in 4 *M* guanidine HCl 0.05 *M* sodium acetate containing the following protease inhibitors: 0.1 *M* 6-aminohexanoic acid, 5 m*M* benzamidine HCl, 50 m*M* EDTA, 0.1 m*M* phenylmethylsulfonyl flouride, and 10 m*M* *N*-ethylmaleimide, pH 5.8. The cell layers were extracted overnight at 4° C in 4 *M* guanidine HCl 0.05 *M* sodium acetate plus protease inhibitors. The cell layers were then scraped from the culture plates and rinsed once with 4 *M* guanidine HCl-0.05 *M* sodium acetate, plus protease inhibitors. The extract and wash were pooled and centrifuged to remove any insoluble cell debris. The material remaining in this residue represented less than 3% of the total radioactivity associated with the cell layer extracts. The cell layer extracts were also eluted from Sephadex G-50 in 4 *M* guanidine HCl-0.05 *M* sodium acetate plus protease inhibitors to separate the labeled macromolecules that eluted in the void volume from the free radiolabel.

Ion-exchange chromatography. The overall charge of the proteoglycans synthesized by normal and inflamed fibroblasts was assessed by ion-exchange chromatography. Aliquots (5 ml) of the medium and cell layer proteoglycans, obtained from Sephadex G-50 chromatography, were dialyzed against several changes of 0.1 *M* sodium chloride in 7 *M* urea, 0.05 *M* Tris-HCl, and protease inhibitors, pH 7.0, and then applied to a DEAE-Sephacel column (0.7 × 3 cm) equilibrated with the same bufer. After elution of the unbound material, a continuous 0.1 to 0.8 *M* sodium chloride gradient was applied using a total of 30 ml. Fractions of 1.0 ml were collected and 0.5 ml aliquots of these fractions were assessed for [^{35}S]sulfate activity.

Analytical column chromatography. The molecular size distribution of [^{35}S]-labeled proteoglycans was analyzed by gel filtration on a column of Sepharose CL-4B. Aliquots of the [^{35}S]-labeled material excluded from Sephadex G-50 (1.0 ml) were concentrated to 200 μl in dialysis tubing against Aquacide. The concentrate was applied to and eluted from columns of Sepharose CL-4B (0.7 × 100 cm) with 4 *M* guanidine HCl in 0.05 *M* sodium acetate containing the above-mentioned protease inhibitors, pH 5.8 (dissociative conditions). To determine whether any of the proteoglycans were detergent sensitive, aliquots of the excluded material from Sephadex G-50 chromatography (1.0 ml) were dialyzed against 0.15 *M* sodium acetate, 5 m*M* $MgCl_2$, and 1 m*M* $CaCl_2$, pH 5.8. The retentate was then concentrated to 200 μl inside dialysis tubing against Aquacide and then made 0.2% SDS by adding 10× stock SDS in sodium acetate buffer. The concentrates were applied to Sepharose CL-4B columns (0.7 × 100 cm) and eluted with 0.2% SDS in the same sodium acetate buffer as described above (detergent conditions). Fractions of 0.5 ml were collected at a flow rate of 3.0 ml/h and all of the fractions were assayed for [^{35}S]-activity.

Glycosaminoglycan identification. [^{35}S]-Labeled proteoglycans isolated in the excluded peak from Sephadex G-50 chromatography, as well as those in all of the peaks obtained from Sepharose CL-4B chromatography under

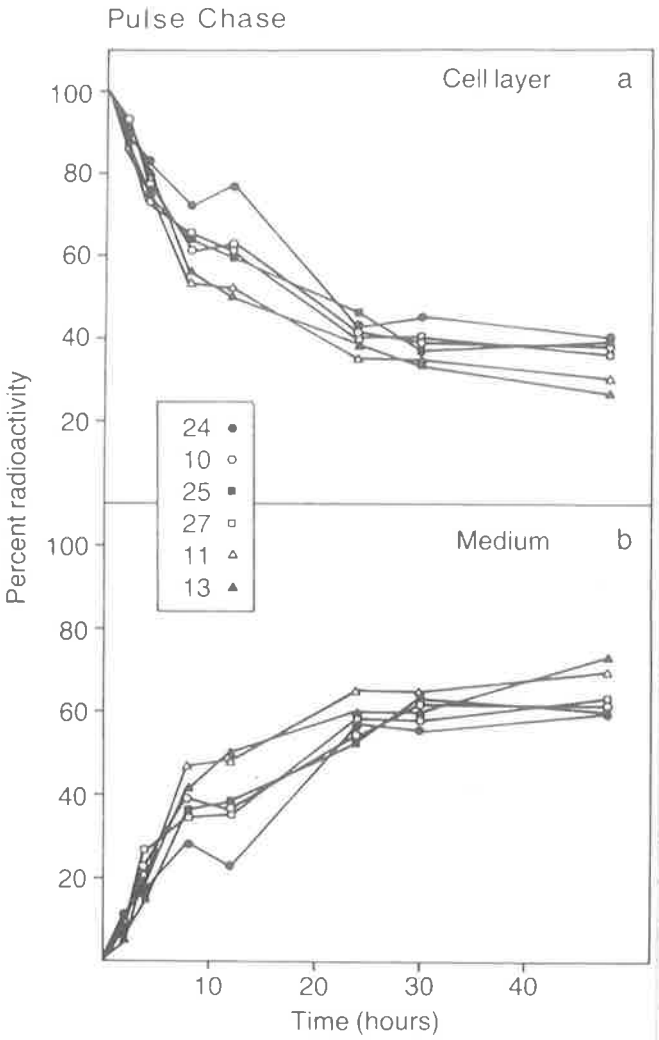

FIG. 3. Pulse chase of [^{35}S]labeled proteoglycans. Normal and inflamed gingival fibroblasts were pulsed for 36 h with [^{35}S]sulfate. The amount of [^{35}S]-labeled proteoglycans associated with the medium and cell layer at various time points over 48 h was then assessed after guanidine HCl extraction and cetylpyridinium chloride precipitation as described in Materials and Methods. Data presented are the mean of triplicate cultures for each time point of each of the six cell strains studied. The experiments were repeated three times and data representative of one of these experiments are shown. Normal gingival fibroblasts: HGF_{24} ●—●; HGF_{25} ■—■; HGF_{27} □—□. Inflamed gingival fibroblasts: HGF_{10} ○—○; HGF_{11} △—△; HGF_{13} ▲—▲.

dissociative conditions, were digested with papain and the released glycosaminoglycans were identified. Briefly, 1.0-ml aliquots of the material that excluded from Sephadex G-50 were dialyzed against 0.2 *M* sodium acetate, 4 m*M* EDTA, and 20 m*M* cysteine HCl, pH 5.7, and then concentrated against Aquacide to 200 μl. The concentrates were then digested with papain (1 mg/ml buffer) overnight and loaded onto Sepharose CL-6B columns (0.7 × 30 cm) and eluted with 0.2% SDS in 0.15 *M* sodium acetate, 5 m*M* $MgCl_2$, and 1 m*M* $CaCl_2$. Fractions of 0.5 ml were collected at a flow rate of 3 ml/h and all of the fractions were assayed for radioactivity. The types of sulfated glycosaminoglycans present were determined after selective enzyme digestion (chondroitinase AC and ABC) and chemical (nitrous acid) degradation (26, 28). Aliquots (1.0 ml) from the material excluded from Sephadex G-50 chromatography were dialyzed against 50 m*M* Tris-HCl, 60 m*M* sodium chloride, and 40 m*M* sodium acetate, pH 8.0, concentrated to 200 μl against Aquacide and then subjected to one of the above-mentioned degradative treatments. The reaction products were then chromatographed on Sephadex G-50 columns (0.7 × 30 cm) using the same SDS-acetate buffer as described above. The relative proportions of each glycosaminoglycan species were determined by calculating the amount of [^{35}S]-labeled material resistant to (void volume) or degraded by (included volume) each treatment.

Results

Cell growth. The proliferative rates of the normal and inflamed fibroblasts were assessed over a 7-d period to determine the time span required for each of the cell strains to reach confluence and thereby to determine the time intervals, at which time metabolic labeling could be commenced. Variability in growth rates among all the strains studied was noted. In general, the fibroblasts obtained from inflamed tissue were slower growing, taking longer to reach confluence than similar age-, sex-, and transfer number-matched cells derived from normal tissue (Fig. 1). Indeed, by Day 3 the differences in the cell numbers between normal and inflamed fibroblast cultures were calculated to be statistically different at the $P<0.05$ level by using analysis of variance.

[^{35}S]Sulfate incorporation. The incorporation of [^{35}S]sulfate into macromolecular material associated with the cell layer and medium compartments was determined over a 48-h period. Despite obvious differences in growth rates of the normal and inflamed fibroblasts seen in Fig. 1, the rate of [^{35}S]sulfate incorporation into proteoglycans did not differ significantly (Fig. 2). The bulk of the [^{35}S]-activity was found in the medium where the amount increased steadily over 48 h. The incorporation of [^{35}S]sulfate into cell layer associated proteoglycans was rapid during the first 8 h and continued to increase up to 30 h; a relatively steady state was reached by 48 h. In all cases, by 48 h approximately 70% of the total [^{35}S]-activity was found in the medium, with the remaining 30% being associated with the cell layer.

Pulse chase experiments. Pulse chase experiments were carried out to determine if there were any detectable differences in the rate of release of proteoglycans into the medium by normal and inflamed fibroblasts. As observed in the above experiments, there seemed to be no remarkable differences in [^{35}S]sulfate incorporation among the cell strains (Fig. 3). The release of [^{35}S]-labeled proteoglycan from the cell layers into the medium was rapid during the first 12 h of the chase, and then seemed to level off at around 50% of the total activity in the medium and cell layer by 24 h. This increase of [^{35}S]-labeled proteoglycans in the medium was accompanied by a concomitant decrease of [^{35}S]-labeled proteoglycans associated with the cells.

Quantitation of glycosaminoglycans. The glycosaminoglycan composition of the newly synthesized labeled macromolecules, as determined by selective enzymatic and chemical degradation, is shown in Table 1. Heparan sulfate was not only the predominant sulfated glycosaminoglycan associated with the cell layers of normal fibroblasts, where it comprised 56% of the total [^{35}S]-activity, but it was also predominant in the cell layers of the inflamed fibroblasts (53%). Overall, no

TABLE I

PROPORTIONS OF SULFATED GLYCOSAMINOGLYCANS SYNTHESIZED BY NORMAL AND INFLAMED GINGIVAL TISSUE FIBROBLASTS[a]

	Heparan Sulfate	Dermatan Sulfate	Chondroitin Sulfate
Medium			
Normal HGF 24	20 ± 2	50 ± 2	30 ± 2
Normal HGF 25	30 ± 1	44 ± 4	27 ± 2
Normal HGF 27	33 ± 6	46 ± 4	22 ± 5
Mean	27 ± 7[b]	47 ± 4[b]	27 ± 5[b]
Inflamed HGF 10	12 ± 2	70 ± 2	19 ± 2
Inflamed HGF 11	11 ± 2	64 ± 4	25 ± 2
Inflamed HGF 13	13 ± 2	66 ± 4	21 ± 4
Mean	12 ± 2[b]	67 ± 4[b]	21 ± 4[b]
Cell Layer			
Normal HGF 24	61 ± 3	22 ± 5	18 ± 2
Normal HGF 25	56 ± 1	29 ± 4	14 ± 2
Normal HGF 27	48 ± 7	30 ± 5	21 ± 6
Mean	56 ± 7	26 ± 6	18 ± 4
Inflamed HGF 10	57 ± 3	25 ± 5	18 ± 2
Inflamed HGF 11	52 ± 6	29 ± 9	19 ± 3
Inflamed HGF 13	51 ± 3	28 ± 4	21 ± 4
Mean	53 ± 5	30 ± 6	19 ± 3

[a]The data are expressed as the percentage of the total radioactive material loaded onto Sephadex G-50 columns which was sensitive to specific enzymatic and chemical degradation procedures. All experiments were performed in triplicate; mean values and SD of the mean are presented.

[b]Represent data that are statistically different ($P<0.05$) from each other within corresponding groups. Data points determined for each glycosaminoglycan species associated with each fibroblast strain were pooled together as either normal or inflamed. Analysis of variance was used to determine if the differences between the normal and inflamed fibroblast strains were significant.

significant differences were noted between the cell layer associated glycosaminoglycans of normal and inflamed fibroblasts. Dermatan sulfate was the predominant sulfated glycosaminoglycan associated with the medium of both normal and inflamed fibroblasts, but the proportion was elevated in the medium of inflamed fibroblasts where it accounted for between 15 and 20% more than was seen in the medium of the normal strains. This increase was accompanied by a concomitant decreased proportion of chondroitin sulfate and heparan sulfate. All of the differences noted between the media associated glycosaminoglycans of normal and inflamed fibroblasts were significant ($P < 0.05$) as assessed by analysis of variance.

Ion-exchange chromatography. The isolated proteoglycans from the medium and cell layer of normal and inflamed fibroblasts were initially characterized by ion-exchange chromatography to assess their charge (Fig. 4). No differences in the elution position of the medium and cell layer proteoglycans of normal and inflamed fibroblasts were detected. In all cases, the major [^{35}S]-labeled peak was relatively symmetrical and eluted from the column at a salt concentration of approximately 0.4 *M*.

Molecular Sieve Chromatography

Dissociative conditions. More detailed information regarding the properties of the proteoglycans was obtained by assessing their molecular size distribution after elution from columns of Sepharose CL-4B (Fig. 5). Under dissociative conditions (4 *M* guanidine HCl), the proteoglycans of normal fibroblasts eluted as three discrete populations. These corresponded to K_{av} values of 0, 0.3, and 0.4 and were termed NM1, NM2, and NM3, respectively (Fig. 5 *a*). When the proteoglycans associated with the medium of the inflamed fibroblasts were subjected to similar preparative steps and analyzed by elution from Sepharose CL-4B under dissociative conditions, the peak corresponding to K_{av} 0.3 was either absent or severely depleted (Fig. 5 *b*). The peaks corresponding to K_{av} of 0 and 0.4 were termed IM1 and IM2.

Some of the differences in the dissociative Sepharose CL-4B elution profiles of the cell layer proteoglycans were also noted (Fig. 5). These were, however, more subtle than the differences seen for the medium proteoglycans. In all cases (both normal and inflamed fibroblasts), four peaks could be clearly identified eluting with K^{av} values of 0, 0.1, 0.4, and 0.55 (Fig. 5 *c* and *d*). These peaks were termed NC1 through NC4 and IC1 through IC4 for the normal and inflamed cell layer proteoglycans, respectively. The relative proportions of total radioactivity in each of these peaks varied somewhat (Table 2). The quantitative major peaks were those corresponding to K_{av} of 0.1 and 0.55 and accounted for approximately 60 to 70% of the total [^{35}S]-labeled material associated with the cell layers of normal and inflamed fibroblasts. Recoveries of [^{35}S]-labeled material from these columns eluted with 4 *M* guanidine HCl were approximately 80 to 85% of the total radioactivity applied.

Detergent conditions. Inasmuch as proteoglycans in both the medium and cell layer preparations of normal and inflamed fibroblasts were excluded from Sepharose CL-4B under dissociative conditions, and because

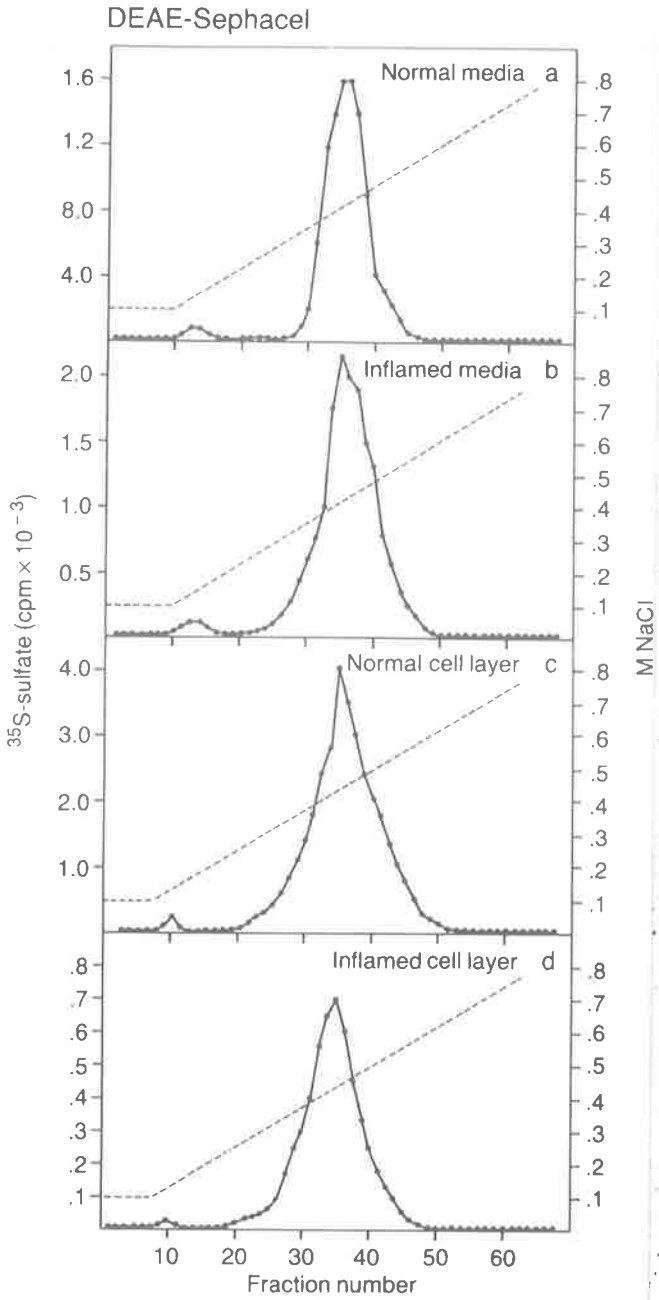

FIG. 4. DEAE-Sephacel ion-exchange chromatography of [^{35}S]-labeled macromolecules. Fibroblasts from normal and inflamed gingiva were incubated for 48 h in the presence of [^{35}S]sulfate and the macromolecular-labeled material in the media (*a* and *b*) and cell layer (*c* and *d*) extracts was isolated in the void volume after elution from Sephadex G-50 in 7 *M* urea. The excluded peaks for the medium and cell layer extracts were applied to DEAE-Sephacel and eluted with a linear NaCl gradient from 0.1 to 0.8 *M*. Representative data for one each of the normal (HGF_{25}) and inflamed (HGF_{11}) gingival fibroblast cell strains are presented.

previous reports have suggested that the cell layer excluded material is lipid associated (Bartold, P. M.; Page, R. C., submitted for publication) we also analyzed the proteoglycans on Sepharose CL-4B columns eluted under detergent conditions (0.2% SDS in acetate buffer) (Fig. 6). When compared to the dissociative conditions (Fig. 5 *a* and *b*), the medium proteoglycans were not affected by treatment with detergent (Fig. 6 *a* and *b*). The proteoglycans of the medium of normal fibroblasts separated into three populations corresponding to K_{av} values of 0, 0.3, and 0.4, whereas only two peaks corresponding to K_{av} values of 0. and 0.4 were observed for samples from cultures of inflamed fibroblasts. The peak of K_{av} 0.3 was absent.

When cell layer extracts were chromatographed under detergent conditions, elution profiles different from those obtained under dissociative conditions were seen (Fig. 6 *c* and *d*). Three peaks were identified corresponding to K_{av} values of 0.1, 0.4, and 0.55. The excluded volume peak eluted under dissociative conditions was absent and suggests that cultures of both normal and inflamed fibroblasts have lipid associated proteoglycans in their cell layers. The relative proportion of radioactivity in each of the peaks obtained under detergent conditions varied between the normal and inflamed cell layer proteoglycans. The [^{35}S]-label was more evenly distributed throughout the profiles of each of the three peaks of the normal fibroblasts, accounting for 28, 25, and 46%, respectively, for the K_{av} values of 0.1, 0.4, and 0.55. However, the inflamed cell layer proteoglycans showed a noticeable difference in distribution. The peak of K_{av} 0.1 was the quantitative predominant peak (45% of the total radioactivity), and the peak corresponding to K_{av} of 0.55 was the quantitative minor component accounting for 25% of the total radioactivity. The recoveries of [^{35}S]-activity from the columns run under detergent conditions were greater (90%) than those for columns eluted with guanidine HCl.

Chemical analysis of dissociatively prepared proteoglycans. The proteoglycan nature of each of the peaks isolated after preparative Sepharose CL-4B chromatography under dissociative conditions (NM1-3; IM1 and

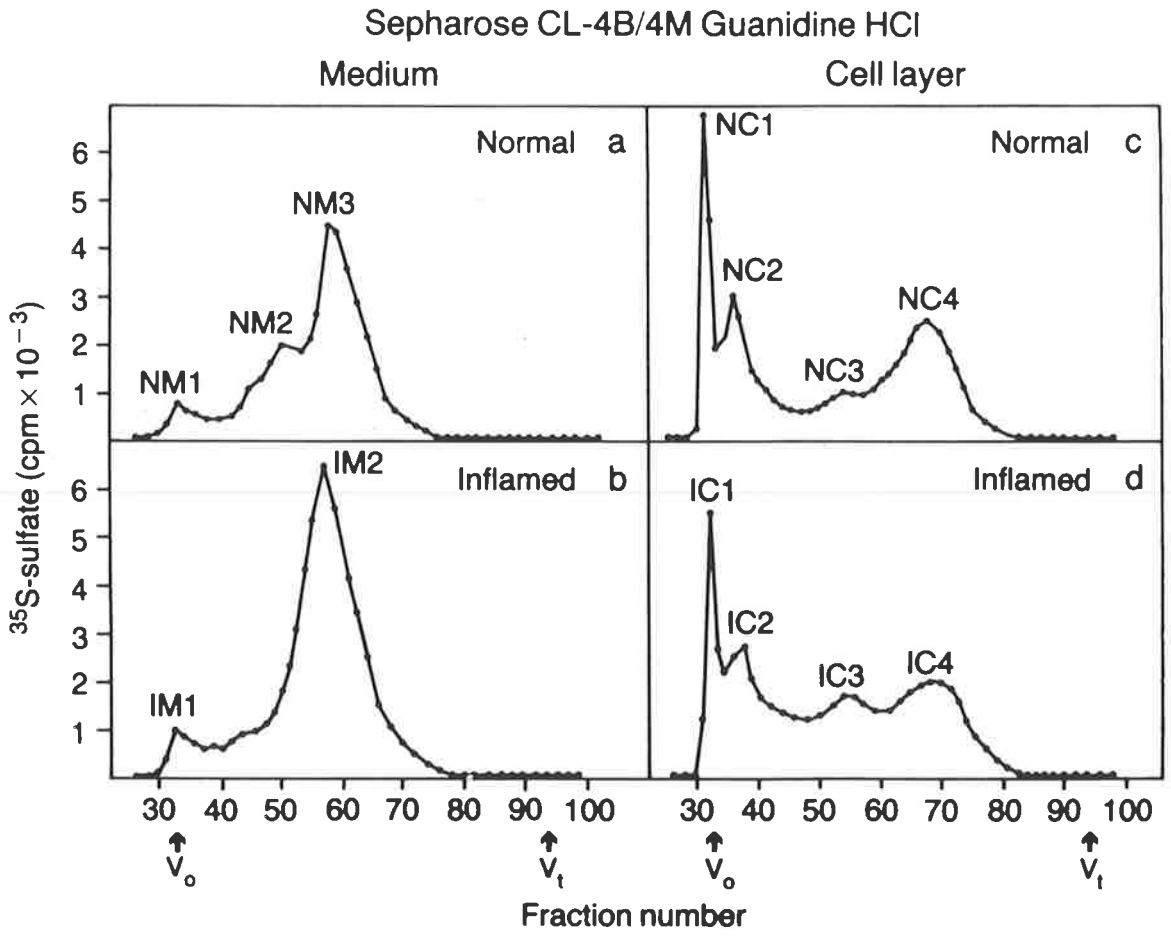

FIG. 5. Gel filtration chromatography of media and cell layer extracts: dissociative conditions. [^{35}S]-Labeled culture medium and cell layers were extracted with 4 *M* guanidine HCl and eluted from Sephadex G-50 in the presence of guanidine HCl as described in the Materials and Methods. The macromolecular radioactivity eluting at the void volume was applied to a Sepharose CL-4B column (0.7 × 100 cm) and eluted with the same buffer used for Sephadex G-50 chromatography. The void volume (*Vo*) and total volume (*Vt*) were identified with [^{3}H]DNA and Na_2 [^{35}S] O_4, respectively. Representative data for the normal (HGF_{2S}) and inflamed (HGF_{1I}) gingival fibroblasts are shown.

IM2; NC1-4 and IC1-4) was determined by treatment with papain and subsequent Sepharose CL-6B chromatography. [^{35}S]-Labeled material in all of the peaks except NC4 and IC4 contained proteoglycans as demonstrated by susceptibility to papain digestion (Fig. 7). The [^{35}S]-labeled molecules in peaks NC4 and IC4 were considered to be free glycosaminoglycan chains because their elution position was not altered by papain treatment. The [^{35}S]-labeled glycosaminoglycan chains released from the proteoglycans eluted from Sepharose CL-6B with K_{av} values of 0.45 and therefore most likely had an average molecular weight in the range of 25 000 (34).

The glycosaminoglycan composition of each [^{35}S]-labeled peak identified by dissociative Sepharose CL-4B chromatography (Fig. 5) was determined by treating aliquots from each peak with chondroitinase AC, chondroitinase ABC, or nitrous acid, followed by chromatography on Sephadex G-50 columns. Qualitative and quantitative differences between the glycosaminoglycan composition of the proteoglycan peaks from normal and inflamed fibroblasts were observed (Table 2). For the medium proteoglycans, peak IM1 contained more heparan sulfate and less chondroitin sulfate than the corresponding normal fibroblast proteoglycan peak NM1. In addition, a small amount (17%) of dermatan sulfate was identified in peak IM1 but was absent in peak NM1. The proportions of glycosaminoglycans constituting the proteoglycans of peaks NM3 IM2 were found to be similar, with dermatan sulfate predominating in both. The cell layer proteoglycans also demonstrated some qualitative glycosaminoglycan differences. Chondroitin sulfate was present in greater proportions in peak IC4 than compared to peak NC4, whereas heparan sulfate, which was the principal glycosaminoglycan identified in peak NC4, contributed less to the total proteoglycans in the corresponding normal fibroblast peak NC4. In addition, peak NC3 of the normal fibroblasts contained more dermatan sulfate but less chondroitin sulfate than the corresponding inflamed peak of IC3.

Discussion

Fibroblasts from normal and chronically inflamed human gingiva have been cultured and their growth and proteoglycan production assessed. Several differences were noted between normal and inflamed tissue fibroblasts; these were persistent during several transfers in vitro and therefore presumed to be heritable. For example, all three strains of inflamed fibroblasts grew at a rate significantly less ($P < 0.05$) than similar age- sex- and transfer-matched normal strains. Moreover, several differences were observed in the constituent sulfated glycosaminoglycans of the proteoglycans synthesized by these cells.

The incorporation of [^{35}S]sulfate into proteoglycans over a 48-h period, and pulse chase experiments over a similar time course, failed to highlight any noticeable differences in the capacity of normal and inflamed fibroblasts to synthesize and release [^{35}S]-labeled proteoglycans. Furthermore, ion-exchange chromatography on DEAE-Sephacel of the newly synthesized macromolecules also failed to demonstrate any significant charge differences between the proteoglycans made by normal and inflamed fibroblasts. However, more detailed analysis revealed structural and qualitative differences in the proteoglycans. Heparan sulfate, the major sulfated glycosaminoglycan associated with the cell layers of both normal and inflamed fibroblasts, was depleted in the medium of inflamed tissue fibroblast cultures. Additionally, dermatan sulfate, the principal sulfated glycosaminoglycan in the medium of both normal and inflamed tissue fibroblast cultures, was significantly elevated in the inflamed fibroblast medium. This increase in dermatan sulfate was accompanied by a decrease in the relative proportion of chondroitin sulfate in the medium of inflamed fibroblasts. These differences were reproducible and they were observed in all three strains of inflamed fibroblasts studied.

At first we suspected that the decrease in amounts of chondroitin sulfate and heparan sulfate in the medium of inflamed fibroblasts could reflect their different growth rates, because actively dividing cells are reported to shed heparan sulfate from their cell surface into the medium (14). In addition, an increase in the release of chondroitin sulfate into the medium of cultures stimulated to divide

TABLE 2

GLYCOSAMINOGLYCAN COMPOSITION OF PROTEOGLYCAN FRACTIONS[a]

Peak	Percent Fraction	Heparan Sulfate	Dermatan Sulfate	Chondroitin Sulfate
Medium				
NM1	5	18 ± 7	—[b]	82 ± 5[b]
NM2	20	30 ± 4	—	70 ± 4
NM3	75	23 ± 5	66 ± 4	12 ± 3
IM1	6	31 ± 5[b]	17 ± 1[b]	52 ± 6[b]
IM2	94	24 ± 6	66 ± 4	11 ± 2
Cells				
NC1	20	60 ± 8	10 ± 1[b]	30 ± 8
NC2	30	64 ± 5	—	30 ± 5
NC3	15	45 ± 3	44 ± 4[b]	12 ± 1
NC4	35	69 ± 7[b]	31 ± 8	—
IC1	15	58 ± 3	—[b]	42 ± 3
IC2	35	54 ± 8	—	46 ± 7
IC3	20	50 ± 7	26 ± 9[b]	25 ± 4[b]
IC4	30	40 ± 5[b]	42 ± 3	18 ± 5[b]

[a]Glycosaminoglycan content was determined on each peak obtained from Sepharose CL-4B chromatography by sequential digestion with chondroitinase AC, chondroitinase ABC, and nitrous acid. Data presented (mean values of three normal and three inflamed fibroblast proteoglycan preparations and SD of the mean) are expressed as percentage of the total ^{35}S-activity remaining after degradation. Fractions refer to the peaks obtained by Sepharose CL-4B chromatography as seen in Fig. 5.

[b]Represents data within corresponding groups (i.e. NMI and IMI) which are statistically different ($P < 0.05$). Triplicate determinations for each glycosaminoglycan species in each of the peaks of normal and inflamed fibrobalsts were assessed statistically using analysis of variance to determine significant differences between the two groups of fibroblasts.

by exposure to 10% serum relative to nondividing cells in medium with 0.5% serum has been reported by Vogel and Sapien (31). They have suggested that such differences in proteoglycan production and release may be a response to some external signal provided by the serum. Our Sepharose CL-4B profiles for proteoglycans from inflamed and normal fibroblasts are remarkably similar to profiles of proteoglycans from fibroblasts cultured in 0.5 and 10% serum, respectively (31). However, it is notable that the behavior of our inflamed fibroblasts in this regard does not seem to be dependent on serum concentration.

Increased levels of dermatan sulfate in the medium of inflamed fibroblasts may have significant biologic implications if the cells behave similarly in vivo. During chronic inflammation there is a rapid loss of collagen at the inflammatory site (22), after which the fibroblasts are required to try to repair this damage by depositing new collagen and other essential matrix components. Dermatan sulfate proteoglycan is closely associated with mature collagen (27) and it may play an important role in collagen fibrillogenesis (8,30,32). Therefore, elevated amounts of dermatan sulfate at sites of inflammation could enhance collagen deposition and reconstitution of the extracellular matrix.

The decreased amount of [^{35}S]-labeled material in the cell layer Sepharose CL-4B peak of K_{av} 0.55 (peak IC4) of inflamed fibroblasts also invites speculation. This peak contains free glycosaminoglycan chains of intracellular origin and most likely represents the degradative or turnover pool of the synthesized proteoglycans (Bartold, P. M.; Page, R. C., submitted for publication). Depletion of radioactive material in this peak from inflamed fibroblasts may be a consequence of an accelerated rate of degradation of newly synthesized proteoglycans by these cells. This seems unlikely, however, because the pulse chase experiments failed to reveal differences between the normal and inflamed fibroblasts with regard to synthesis and release. On the other hand, the endocytotic process (15,37) by which proteoglycans from the medium are transported to the internal compartments of the cell for subsequent degradation may be impaired in inflamed fibroblasts. This is an attractive possibility because suppression of degradative activity during a period of tissue repair and reconstitution of the matrix could be biologically advantageous.

Taken together, our data demonstrate the existence of clear-cut reproducible differences between strains of fibroblasts obtained from chronically inflamed and

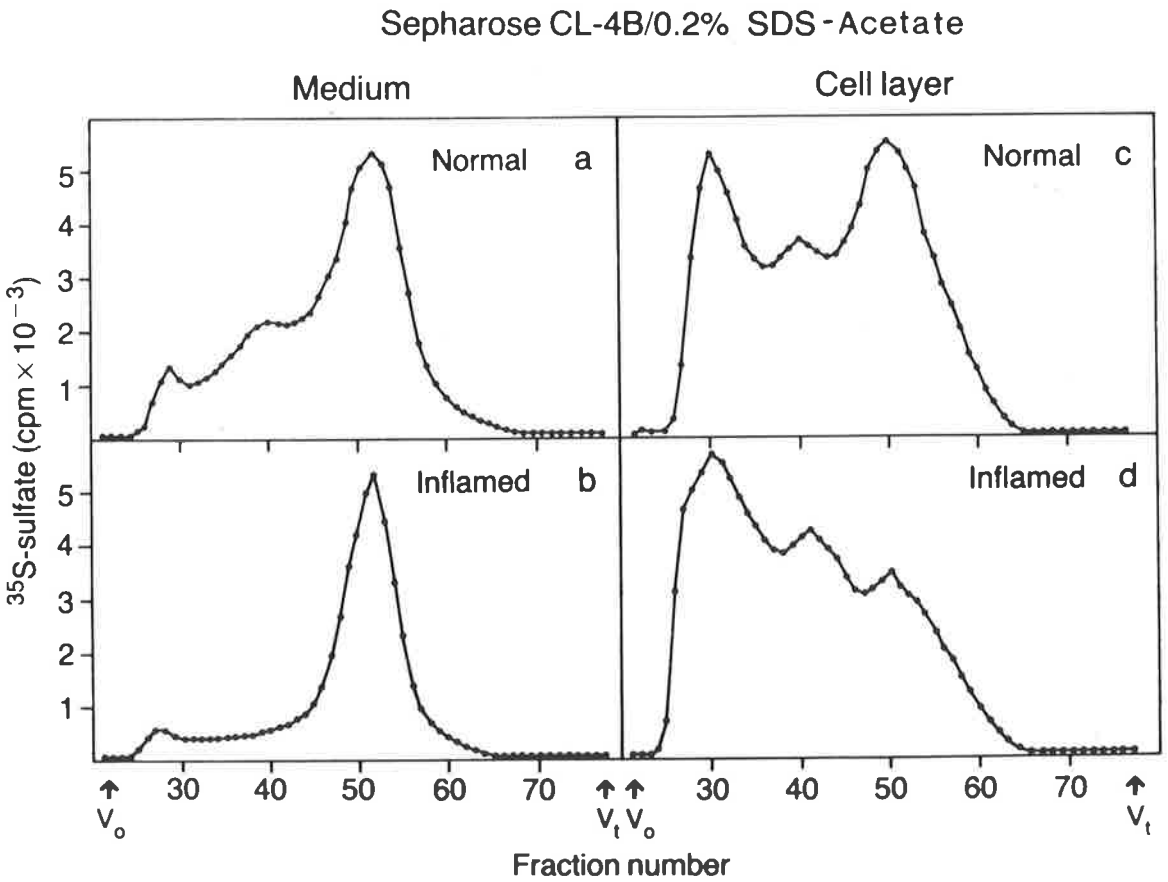

FIG. 6. Gel filtration chromatography of media and cell layer extracts: detergent conditions. [^{35}S]-labeled culture medium and cell layers were extracted with 4 *M* guanidine HCl as described in Materials and Methods. The extracts were dialyzed against 0.15 *M* sodium acetate, 0.001 *M* magnesium chloride, 0.001 *M* calcium chloride, pH 5.8, and then made 0.2% in SDS before elution on a Sepharose CL-4B column (0.7 × 100 cm) eluted with the same buffer used to dialyze the samples but also containing 0.2% SDS. The *Vo* and *Vt* were determined as described in Fig. 5. Representative data for the normal (HGF_{2s}) and inflamed (HGF_{1i}) gingival fibroblasts are shown.

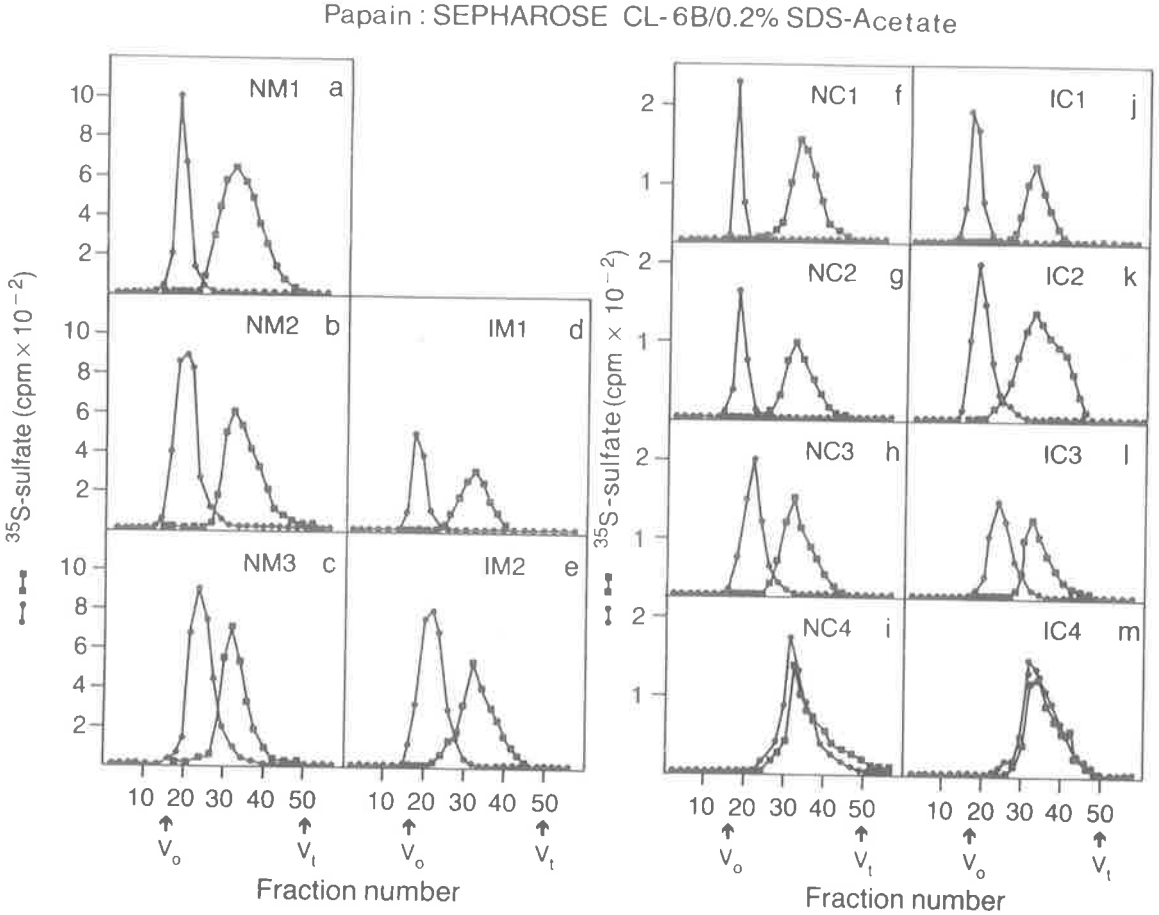

FIG. 7. Gel filtration chromatography of papain digested medium and cell layer proteoglycans. [^{35}S]-Labeled proteoglycans were isolated by elution from Sepharose CL-4B (*see* Fig. 5). Each of the peaks was pooled, dialyzed against papain digest buffer and aliquots were incubated at 60° C for 12 h in the presence or absence of papain. After incubation, each sample was applied to a Sepharose CL-6B column and eluted with the same buffer as described in Fig. 6. Both the *Vo* and *Vt* were identified as described in Fig. 5. Representative data for the normal (HGF_N) and inflamed (HGF_H) gingival fibroblasts are shown.

normal connective tissues. These differences persist in culture and are therefore heritable. In this regard, they are comparable to differences seen in collagen production by cells from chronically inflamed, fibrotic, and normal connective tissue (10,31,34) and the differences in strains of cells from arthritic tissue (5,6). Such differences have been taken to imply that the fibroblasts obtained from pathological sites are genetically different from those from normal healthy sites. Whether the differences we report are due to selection of preexisting subsets of resident fibroblasts within the tissue (4,17,29), or to persistent phenotypic alterations caused by an altered environment (20,23–25,33), remain to be established.

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Paper 16.

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THE EFFECT OF INTERLEUKIN 1β ON PROTEOGLYCANS SYNTHESIZED BY HUMAN GINGIVAL FIBROBLASTS *IN VITRO*.

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The effect of recombinant interleukin-1β (IL-1β) on proteoglycan synthesis by human gingival fibroblasts was investigated. IL-1β stimulated the gingival fibroblasts to proliferate. When compared to human foreskin fibroblasts, the gingival fibroblasts demonstrated a greater proliferative response at higher concentrations of IL-1β. The midpoint of the proliferation response for both cell types was in the 10^{-11} M IL-1β range. The rate of [^{35}S]-sulfate incorporation into proteoglycans by human gingival fibroblasts was enhanced by 40% at 10^{-9} M IL-1β. This stimulatory effect appeared to be independent of cell proliferation and prostaglandin synthesis since blocking of these functions with hydroxyurea and indometacin respectively, resulted in similar dose responses to IL-1β. Pulse chase experiments indicated the kinetics of degradation in the presence or absence of IL-1β were essentially identical. Therefore, the turnover rate of proteoglcyans was not altered by IL-1β. no significant differences between molecular species, size or glycosaminoglycan composition of the proteoglycans synthesized in the presence or absence of IL-1β was noted. Thus, IL-1β can modulate extracellular matrix synthesis by human gingival fibroblasts and may therefore be partially responsible for the early events of healing following inflammatory episodes.

INTRODUCTION

Macrophage-derived secretion produces are important mediators of inflammatory reactions.[1] Of these factors, interleukin-1 (IL-1) has been the focus of much attention, since it has many biological properties.[2] For example, this polypeptide hormone is able to stimulate T-lymphocyte proliferation,[3] increase fibroblast proliferation,[4,5] stimulate prostaglandin E_2 synthesis by fibroblasts,[6,7] increase the release of proteolytic enzymes by synovial cells,[8,9] decrease collagen synthesis by fibroblasts,[10] cause loss of proteoglycans from cartilage matrix,[1,12] increase hyaluronic acid synthesis by fibroblasts[13,14] and increase proteoglycan synthesis by fibroblasts.[15]

Two major forms of human IL-1 have been identified which differ in their isoelectric points[16] and are termed interleukin-1α (IL-1α) and interleukin-1β (IL-1β). These have only 26% homology in amino acid sequence and are probably separate gene products.[16] Nonetheless, their biological effects appear to be similar and are presumed to be effected via specific membrane receptors. Of the many cells investigated, fibroblasts bind IL-1 most avidly.[17] Such an affinity for IL-1 may be important in the regulatory effect of IL-1 on extracellular matrix synthesis and metabolism during inflammation.

At present the literature contains many references to the effect of IL-1 on fibroblasts. However most of these studies have used cell lines,[4,17] cells from foetal or newborn skin,[5,9,10,18] or cells from synovium.[6,8,13,14] Apart from the synovial cells, the others are not representative of cells present in adult tissues affected by chronic inflammatory lesions. Therefore, gingiva, a tissue which has been used as a model for studying the biochemical and cellular events of inflammation,[19] may be a more stuitable source of fibroblasts for investigating inflammatory mediated changes in extracellular matrix synthesis. In addition, the large number of membrane receptors for IL-1 on gingival fibroblasts[17] lends further support to their suitability for such studies.

Amongst the extracellular macromolecules, proteoglycans can regulate many physiological properties of tissues.[20] Such functions are related not only to total quantity of proteoglycans but also to molecular composition and size. Thus factors such as IL-1 which can influence connective tissue metabolism are important. This study not only considers the kinetics of proteoglycan synthesis, but also describes the effect of IL-1 on molecular size and composition of the proteoglycans synthesized by gingival fibroblasts.

MATERIALS AND METHODS

Materials

Guanidine HCl, 6-aminohexanoic acid, benzamidine HCl, N-ethymaleimide, phenylmethylsulfonyl fluoride, indomethacin, hydroxyurea, Tris HCl, sodium pyruvate, glutamine and trypsin were all purchased from Sigma Chemical Co., St. Louis, MO; Dulbecco-Vogt medium (DVM), fetal calf serum (FCS), penicillin, streptomycin, nonessential amino acids were from Flow Laboratories, Irvine, Ayrshire, Scotland; Tissue culture plastic ware was from Nunc, Roskilde, Denmark; [6-^{3}H]-thymidine (22 Ci/mMol), $Na_2(^{35}S)O_4$ (1042 mCi/mMol), D-[6-^{3}H]-glucosamine HCl (33 Ci/mMol) were from Amersham (Australia) Sydney, Australia; Ready Solv EP scintillation fluid from Beckman (Australia), Adelaide, Australia; Chondroitinase AC II (*Arthrobacter aurescens*) and chondroitinase ABC (*Proteus vulgaris*) from Seikagaku Kogyo Co., Ltd., Tokyo, Japan; Sepharose CL-4B, Sephadex G-50, Sephadex G-25 (PD-10) were from Pharmacia Fine Chemicals, North Ryde, Sydney, Australia; Aquacide from Calbiochem, La Jolla, CA; Recombinant interleukin-1β was generously donated by Immunex Corporation, Seattle, WA.

Fibroblast Cultures

Gingival fibroblasts were obtained from explant culture of healthy gingiva from adult human volunteers as described previously.[21] The cells were maintained in Dulbecco-Vogt Medium (DVM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 Units/ml of penicillin, 100 µg/ml of streptomycin, 2 mM glutamine, 10 mM sodium pyruvate and non-essential amino acids. Unless otherwise stated this was the medium used for all experiments. The fibroblasts

were maintained in 75 cm^2 tissue culture flasks in DVM at 37°C with CO_2/air (1:9). Cells between 4th and 8th transfer in culture were used. For each experiment, the cells were released from the culture plates by treatment with 0.05% (w/v) trypsin in phosphate buffered saline (PBS) for 5 min at 37°C.

Human foreskin fibroblast cultures were a generous gift from S. Hay (Department of Pathology, University of Adelaide) and were maintained in the same medium and conditions as for the gingival fibroblasts.

Measurement of Cell Proliferation

Human gingival fibroblasts and foreskin fibroblast were seeded in triplicate into 24-well plates (which hold approximately 10^5 cells at confluence) at an initial density of 20,000 cells per well and allowed to attach and spread overnight. The medium was then replaced with 500 µl/well of medium containing increasing concentrations of IL-1β from 10^{-13} to 10^{-9} M as well as controls which contained no IL-1β. After 72 hr incubation the medium was replaced with 500 µl of medium containing IL-1β as well as 1 µCi/ml [^{3}H]-thymidine. The cells were incubated for a further 3 hr after which the medium was removed and the cell layers washed 3 times with 500 µl PBS. The cells were released from the wells by trypsinization for 15 min at 37°C and recovered by centrifugation. The cells were lysed with 100 µl 0.1 M sodium hydroxide for 60 min at 60°C and the the radioactivity was determined in a Beckman LS-2800 Liquid Scintillation Counter.

Dose Response Experiments

The effect of increasing concentrations of IL-1β on [^{35}S]-sulfate incorporation into macromolecules was studied. Briefly, 75,000 cells were seeded, in quadruplicate, into 24-well culture plates and allowed to attain confluence over 2 days. The medium was then removed and replaced with 500 µl/well medium containing a range of concentrations of IL-1β from 10^{-13} to 10^{-9} M. After incubation at 37°C for 48 hr, the medium was removed and replaced with fresh medium containing IL-1β and [^{35}S]-sulfate (20 µCi/ml) and incubated for a further 48 hr. The cells in one well at each concentration were released by trypsinization and cell numbers were determined by counting in a haemocytometer. The medium was then removed from the remaining three wells for each concentration of IL-1β and the cell layers washed once with 500 µl PBS and the medium and the wash were pooled. The cell layers were extracted overnight at 4°C in 500 µl 4 M guanidine HCl/0.05 M sodium acetate containing the following as protease inhibitors: 0.1 M 6-aminohexanoic acid, 5 mM benzamidine HCl, 50 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and 10 mM N-ethylmaleimide, pH 5.8. The extract was removed and the plates washed once with 500 µl 4 M guanidine HCl/0.05 M sodium acetate containing protease inhibitors. The cell layer extract and washes were pooled. Aliquots of 0.4 ml from the medium and cell layer were applied to separate pre-packed Sephadex G-25 (PD-10) columns and eluted with 4 M guanidine HCl/0.05 M sodium acetate plus protease inhibitors, pH 5.8. Radioactivity in 0.4 ml effluent fractions was determined by liquid scintillation counting and the amount recovered in the void volume provided a measure of incorporation of

[^{35}S]-sulfate into newly synthesized macromolecules.[22] Recovery of radioactivity from these columns was approximately 90%.

[^{35}S]Sulfate Incorporation with Time

To determine the effect of IL-1β on the kinetics of proteoglycan synthesis, incorporation of [^{35}S]-sulfate was followed over time. Cells were seeded in quadruplicate at an initial density of 75,000 cell per well and allowed to attach, spread and reach confluence as described above. The medium was then removed and replaced with medium containing 10^{-11} M IL-1β and [^{35}S]-sulfate (20 μCi/ml). Incorporation of [^{35}S]-sulfate into macromolecules was determined at 12, 24, 48 and 72 hr after introduction of the radiolabel and IL-1β. The amount of radiolabelled macromolecules present at each time point was determined by liquid scintillation counting of the effluents from PD-10 columns. Cell numbers were determined at each time point following trypsiniztion and counting in a haemocytometer.

Pulse Chase Experiments

To determine whether or not the effect of IL-1β on proteoglycan synthesis was due to an alteration in intracellular metabolism, pulse chase experiments were carried out. Fibroblasts were seeded in quadruplicate at an initial density of 75,000 cells per well and allowed to reach confluence. The confluent cells were then incubated in the presence of 20 μCi/ml [^{35}S]-sulfate and 10^{-11} M IL-1β for 24 hr, washed 4 times with PBS and then chased in 500 μl isotope free medium containing 10^{-11} M-IL-1β for various periods of time. At each time point, macromolecular radioactivity in either the cell layer or the medium was determined by PD-10 chromatography and liquid scintillation counting. Cell numbers were determined at each time point following trypsinization and counting in a haemocytometer.

Blocking Cell Proliferation and Prostaglandin Synthesis

Since IL-1 may influence fibroblast proliferation and prostaglandin E_2 (PGE_2) synthesis,[4–7] the possibility that the effects of IL-1β may have been a secondary response to such alterations was addressed. Therefore, cell proliferation and PGE_2 were inhibited by addition of 10^{-3} M hydroxyurea[23] 10^{-6} M indomethacin[24] respectively to the cells. Dose response experiments as described above were then carried out with increasing concentrations of IL-1β either in the presence or absence of hydroxyurea or indomethacin.

Proteoglycan Extration

For metabolic labelling of the cells prior to proteoglycan extraction, the fibroblasts were maintained in 75 cm^2 flasks. The cells were allowed to grow to confluence with medium replenishment every second day. Upon reaching confluence (approximately 1.6×10^6 cells), the medium was removed and replaced with 5 ml of

medium with or without 10^{-11} M IL-1β. The cells were then incubated for 48 hr after which the medium was removed from each flask and replaced with fresh medium either with or without IL-1β but containing 20 μCi/ml [^{45}S]-sulfate. Metabolic labelling was allowed to proceed for 48 hr.[25]

The [^{35}S]-sulfate-labelled macromolecules were isolated from the medium and cell layer as described previously.[25] Briefly, the medium was removed and the cell layers were washed with 5 ml PBS and pooled. The pooled medium fraction was adjusted to approximately 3 M in guanidine HCl by adding solid guanidine HCl (0.38 g/l), and then eluted from a Sephadex G-50 column (2.5 cm × 30 cm) in 4 M guanidine HCl/0.05 M sodium acetate containing protease inhibitors, pH 5.8. The cell layers were extracted overnight at 4°C in 4 M guanidine HCl/0.05 M sodium acetate containing protease inhibtors. The cell layers were scraped from the flasks and the flasks were then rinsed with 5 ml 4 M guanidine HCl/0.05 M sodium acetate containing protease inhibitors. Extracts from the cell layers were also pooled and then eluted from Sephadex G-50, as for the medium fraction, to separate [^{45}S]-sulfate-labelled macromolecules which eluted in the void volume from free radiolabel. Recoveries from these columns were between 85–90%. Approximately 5% of the total radioactivity remained with the culture flasks but was not further assessed.

Analytical Column Chromatography

The molecular size distribution of [^{35}S]-sulfate-labelled proteoglycans was analyzed by gel filtration from Sepharose CL-4B. Aliquots (1 ml) of the [^{35}S]-sulfate-labelled material which was excluded from Sephadex G-50 were concentrated in dialysis tubing against Aquacide to 200 μl. The concentrate was then eluted from columns of Sepharose CL-4B (0.7 cm × 100 cm) with 4 M guanidine HCl/0.05 M sodium acetate containing protease inhibitors, pH 5.8 Fractions of 0.5 ml were collected at a flow rate of 3 ml/hr and all of the fractions were assayed for radioactivity. Recovery of radiolabelled macromolecules from these columns was in the range of 80–90%.

Glycosaminoglycan Analyses

The types of glycosaminoglycans associated with the medium and cell layer proteoglycans of IL-1β treated and untreated cultures were determined following selective enzyme digestion (chondroitinase AC II and chondroitinase ABC) and chemical degradation (nitrous acid).[26,27] Aliquots (1.0 ml) of the material excluded from Sephadex G-50 were dialysed against 0.05 M Tris HCl, 0.06 M sodium chloride and 0.04 M sodium acetate, pH 8.0, concentrated to 200 μl against Aquacide and then subjected to one of the above degradative procedures. The reaction products were then eluted from Sephadex G-50 columns (0.7 cm × 30 cm) with 0.2% SDS in 0.15 M sodium acetate, 1 mM magnesium chloride, 1 mM calcium chloride, pH 5.8. Fractions of 0.5 ml were collected at a flow rate of 3 ml/h and all of the fractions were assayed for radioactivity. The relative proportion of each glycosaminoglycan species was determined by calculation of the amount of radiolabelled material resistant to (void volume) or degraded by

(included volume) each treatment. For specific activity determinations, cells were incubated in the presence or absence of IL-1β as described above and labelled in the presence of both [^{35}S]-sulfate (20 μCi/ml) and [^{3}H]-glucosamine (17.5 μCi/ml) for 24 hr. The disaccharides released by chondroitinase ABC digestion were assessed for their $^{35}S/^{3}H$ ratio as described previously.[28].

Statistical Analyses

All data were subjected to statistical analysis using the methods of analysis of variance or the Student's T-test.

RESULTS

Cell Proliferation

The proliferation rate of human gingival and foreskin fibroblasts in the presence of increasing concentrations of IL-1β was assessed by [^{3}H]-thymidine uptake (Fig. 1). Human foreskin fibroblasts were used as a positive control, since these cells have been shown previously to be stimulated to proliferate in the presence of

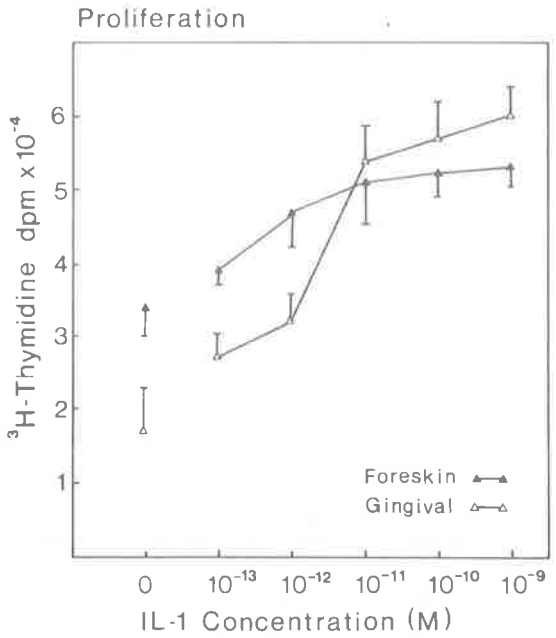

FIGURE 1 *Stimulation of fibroblast proliferation by IL-1β*
5×10^{4} fibroblasts were cultured in the presence of varying concentrations of IL-1β for 72 hr. The cultures were then pulsed with 1 μCi/ml [^{3}H]-thymidine for 3 h and processed for determination of [^{3}H]-thymidine incorporation. Data represent the mean and standard deviation of the mean of triplicate cultures from a representative experiment.

IL-1.[5] In the present study, increasing concentrations of IL-1β stimulated [^{3}H]-thymidine uptake by both cell types. In the absence of IL-1β the gingival fibroblasts had a slower basal proliferation rate compared to the foreskin fibroblasts. However, with increasing concentrations of IL-1β the gingival fibroblasts were stimulated to proliferate to a greater extent than the foreskin fibroblasts. For both cell types, the midpoint of the proliferation curve was in the 10^{-11} M range. No stimulation of proliferation was noted if the assay was performed in the absence of serum (results not shown).

Dose Response

The effects of increasing concentrations of IL-1β on incorporation of [^{35}S]-sulfate into macromolecules associated with the medium and cell layers is seen in Fig. 2. IL-1β stimulated the synthesis of [^{35}S]-sulfate-labelled macromolecules. This stimulatory effect was most evident in the medium fraction (Fig. 2a) where IL-1β stimulated macromolecular synthesis in a dose dependent fashion after 96 h in culture. The greatest effect was at a concentration of 10^{-9} M-IL-1β and represented a 30–40% increase in [^{35}S]-sulfate-labelled macromolecules. However, at concentrations of 10^{-12} M and greater the stimulatory effect of IL-1β was determined to be statistically significant ($p < 0.05$). For the cell layer, synthesis of [^{35}S]-sulfate-labelled macromolecules was greatest at 10^{-11} M-IL-1β where it accounted for a 20% increase over control cultures (Fig. 2b). This increase was, however, not statistically significant. Since the increase in ^{35}S-labelled proteoglycan may reflect an increase in sulfation rather than an increase in total amount, the cells were cultured in the presence of both [^{35}S]-sulfate and [^{3}H]-glucosamine. If sulfation was altered by IL-1β, then the ratio of ^{35}S/^{3}H in proteoglycans from IL-1β treated and untreated cultures would change. The ratio in disaccharides released by chondroitinase ABC digestion revealed no significant change at 0.2 (± 0.04) and 0.19 (± 0.01) for cultures treated or untreated with IL-1β respectively.

Inhibition of Cell Proliferation and Prostaglandin Synthesis

Since IL-1β stimulated gingival fibroblast proliferation (Fig. 1) and PGE_2 synthesis by fibroblasts may be elevated by IL-1,[6,7] the possibility was investigated that the increased proteoglycan synthesis noted in Fig. 2 was a secondary effect due to altered proliferation rates or PGE_2 synthesis by the cells.

Cell proliferation can be effectively inhibited by the addition of hydroxyurea to the culture medium.[23] At 10^{-2} M hydroxyurea, human gingival fibroblast proliferation was inhibited 90% (results not shown). The effect of such inhibition of cell proliferation on stimulation of [^{35}S]-sulfate incorporation into macromolecules secreted into the culture medium by IL-1β can be seen in Fig. 3. Regardless of the presence or absence of hydroxyurea, a dose dependent increase in biosynthesis of [^{35}S]-sulphate-labelled macromolecules was noted. These differences became statistically significant ($p < 0.05$) at concentrations of 10^{-11} and greater.

Indomethacin can effectively inhibit PGE_2 synthesis by fibroblasts at concentrations between 10^{-5}–10^{-7} M.[24] The effect of 10^{-6} M indomethacin on

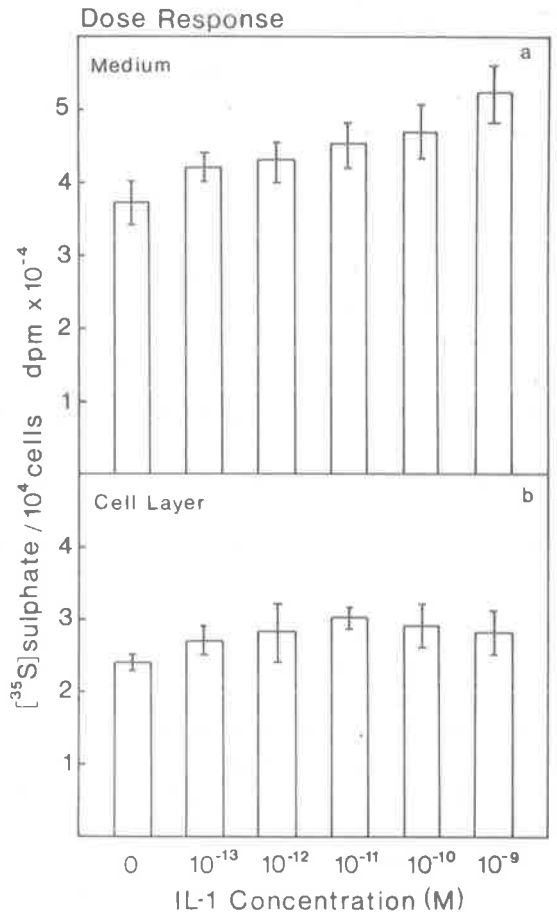

FIGURE 2 *Proteoglycan synthesis by human gingival fibroblasts in the presence of increasing co centrations of IL-1β.*
Human gingival fibroblasts were cultured in the absence or presence of increasing concentrations IL-1β and incorporation of [^{35}S]-sulfate into macromolecules was assessed by Sephadex G-25 (PD-1 chromatography. Data represent mean and standard deviation of the mean of triplicate cultures from representative experiment

IL-1β-induced stimulation of [^{35}S]-sulfate incorporation into macromolecules is shown in Fig. 4. Depsite treatment of the cells with indomethacin, a dose dependent increase in biosynthesis of [^{35}S]-sulfate-labelled macromolecules was noted. The stimulation of proteoglycan synthesis by IL-1β became statistically significant ($p < 0.05$) at concentrations of 10^{-11} and greater for both control and indomethacin-treated cultures.

[^{35}S]-sulfate Incorporation with Time

The rate of incorporation of [^{35}S]-sulfate into medium and cell layer macromolecules in the presence or absence of IL-1β (10^{-11} M) over a 72 h period is shown in

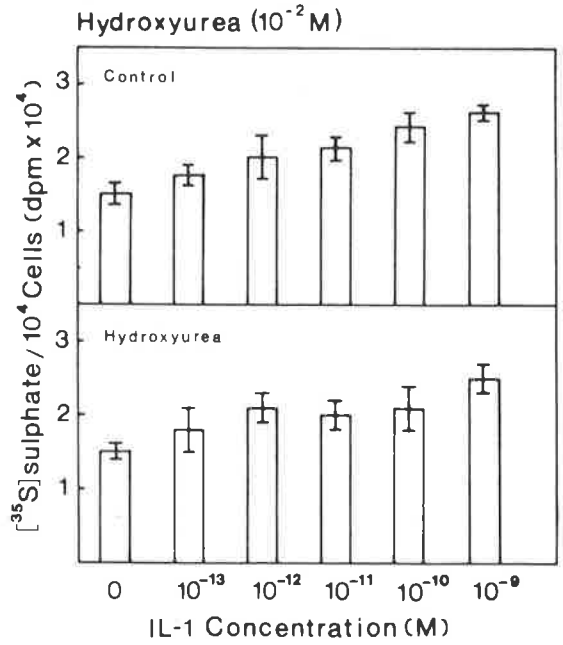

FIGURE 3 *Effect of cell proliferation on synthesis of proteoglycans in the presence of IL-1β* Hydroxyurea at a concentration of 10^{-2}M will inhibit human gingival fibroblast proliferation by 90%. Cells were incubated for 48 h in the presence or absence of 10^{-2}M-hydroxyurea and increasing concentrations of IL-1β and then pulsed for 48 hr in the presence of [^{35}S]-sulfate. Incorporation of [^{35}S]-sulfate into proteoglycans was monitored by PD-10 chromatography. Data are expressed as means and standard deviation of mean of triplicate cultures of a representative experiments.

Fig. 5. A steady increase in release of [^{35}S]-sulfate-labelled proteoglycans into the medium over 72 h for both treated and untreated cultures. At all time points IL-1β treated cells released more [^{35}S]-proteoglycans into the medium. This difference was, however, not statistically significant until 72 h ($p < 0.05$). The radiolabelled material associated with the cell layers of treated and untreated cultures increased rapidly during the first 24 h, after which the incorporation of [^{35}S]-sulfate slowed somewhat increasing only 50% over the next 48 h. Although the IL-1β treated cells appeared to have greater amounts of [^{35}S]-sulfate-labelled proteoglycans associated with their cell layers, no significant difference between IL-1β treated and untreated cultrues was detected.

Pulse Chase Experiments

The effect of IL-1β on the turnover rate of cell associated [^{35}S]-sulfate-labelled material was assessed following chase for various periods of time (Fig. 6). There appeared to be no remarkable differences between the kinetics of release of [^{35}S]-sulfate-labelled macromolecules into the medium by treated or untreated cells. The release of [^{35}S]-sulfate-labelled macromolecules from the cell layer into the medium was rapid during the first 8 hr of the chase, and then seemed to level off

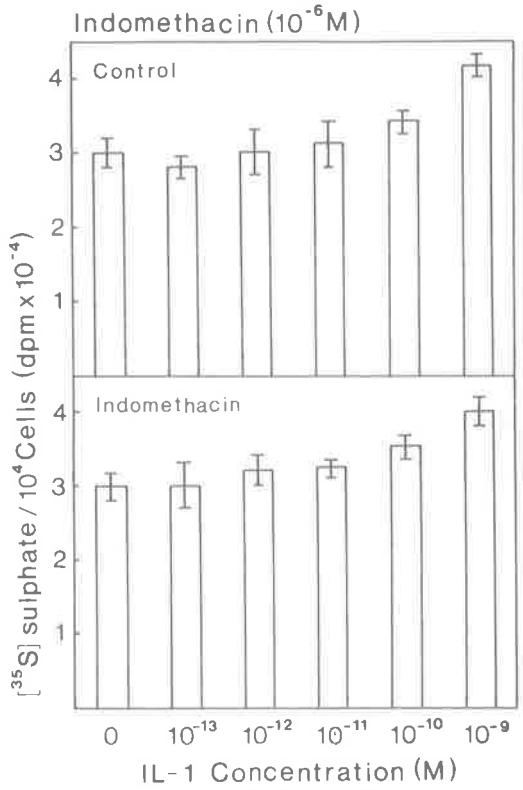

FIGURE 4 *Effect of prostaglandin on the synthesis of proteoglycans in the presence of IL-1β.* Cells were incubated for 48 hr in the presence of indomethacin with increasing concentrations of IL-1β and then pulsed for 48 hr in the presence of [^{35}S]-sulphate. Incorporation of [^{35}S]-sulfate into proteoglycans was monitored by PD10 chromatography. Data are expressed as means and standard deviation of the mean of triplicate cultures of a representative experiment.

at around 30% of the total activity in the medium by 24 h. This increase of [^{35}S]-sulfate-labelled material in the medium was accompanied by a concomitant decrease of [^{35}S]-sulfate-labelled material associated with the cells.

Proteoglycan Characterization

The molecular size distribution of newly synthesized proteoglycans was determined by gel filtration on Sepharose CL-4B (Figs. 7 & 8). Under dissociative conditions (4 M guanidine HCl) the proteoglycans isolated from the medium of IL-1β-treated and untreated cells demonstrated similar elution profiles (Fig. 7). In general, three different size classes could be identified in the medium (M1, M2 and M3) and the relative proportions of these classes of proteoglycan associated with the medium compartment did not vary between treated and untreated cells. Peak M3 was the quantitative major component of both IL-1β treated and untreated cells and represented 73% and 67% respectively. Peaks M2 and M3 constituted the

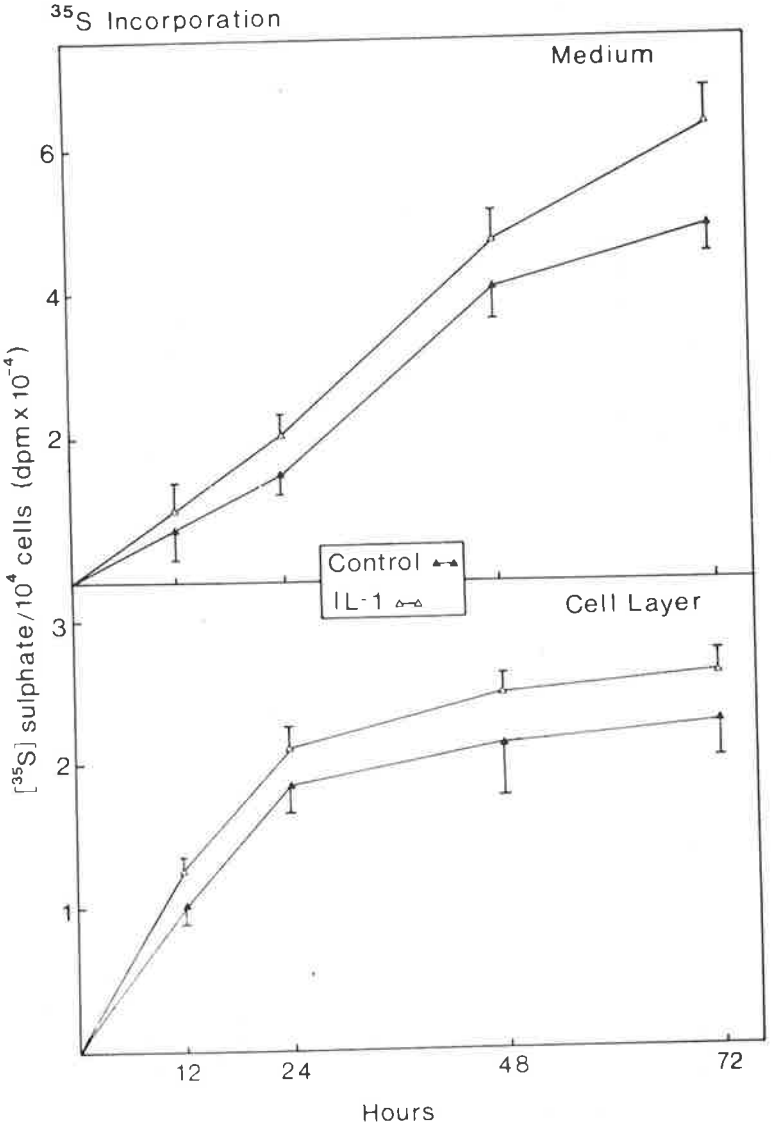

FIGURE 5 *Incorporation of [^{35}S]-sulfate into proteoglycans with time.*
Human gingival fibroblasts were cultured in the presence (10^{-11} M) or absence of IL-1β. The rate of incorporation of [^{35}S]-sulfate into proteoglycans over various time periods was assessed by PD-10 chromatography. Data are expressed as means and standard deviation of the mean of triplicate cultures of a respresentative experiment.

remaining 30% of total [^{35}S]-sulfate-labelled material eluted from the columns and did not differ appreciably between treated and untreated cells.

Similar analyses of cell layer-associated [^{35}S]-sulfate-labelled material on Sepharose Cl-4B revealed four identifiable regions in the elution profiles and were termed C1, C2, C3 and C4 (Fig. 8). As seen for the medium proteoglycans, the

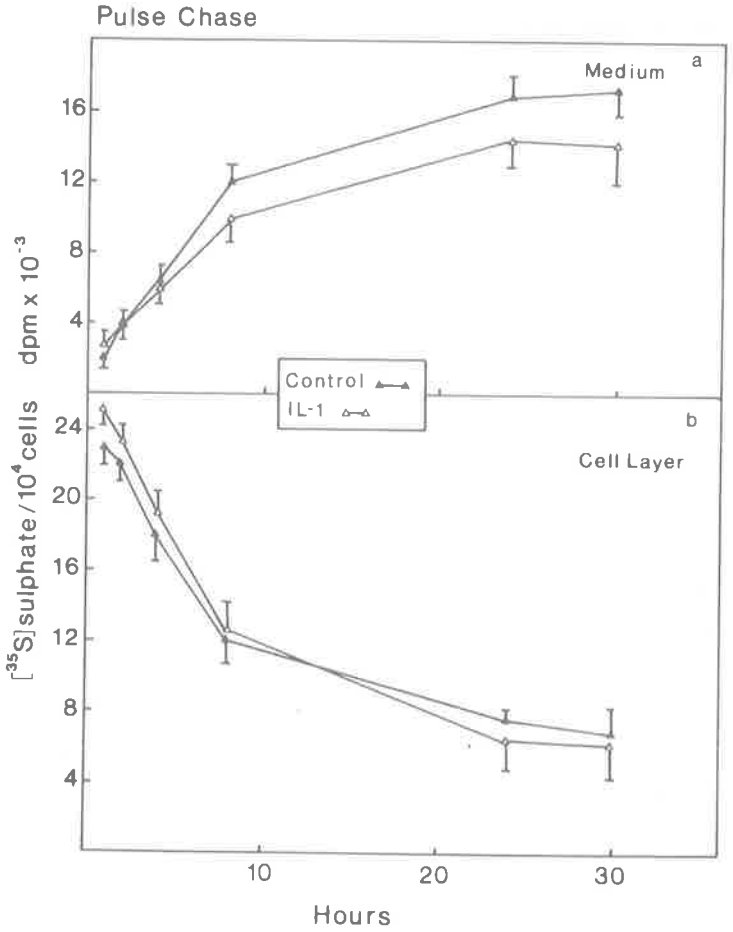

FIGURE 6 *Kinetics of degradation of newly synthesized proteoglycans*
Human gingival fibroblasts were cultured in the presence (10^{-11} M) or absence of IL-1β. After labelling with [^{35}S]-sulfate for 24 h, the medium was removed and the cell layers washed extensively and the cells were then incubated in isotope-free medium for various periods of time. The presence of [^{35}S]-sulfate-labelled proteoglycans in the medium and cell layer after each pulse period was determined by PD-10 chromatography. The data represent means and standard deviation of the mean of triplicate cultures of representative experiments..

relative proportions of the cell layer proteoglycans did not vary noticeably between treated and untreated cells. Peak C4, which accounted for between 45–47% of the total labelled material eluted from the columns, was the quantitative major peak for both the treated and untreated cells. Slight quantitative differences between peaks C1 and C2 were evident but were, however, not statistically significant (Table 1).

The absence of any small molecular weight material eluting at or near the total volume of the column suggests that no degradation of the proteoglycans occured during the incubation period or subsequent analyses.

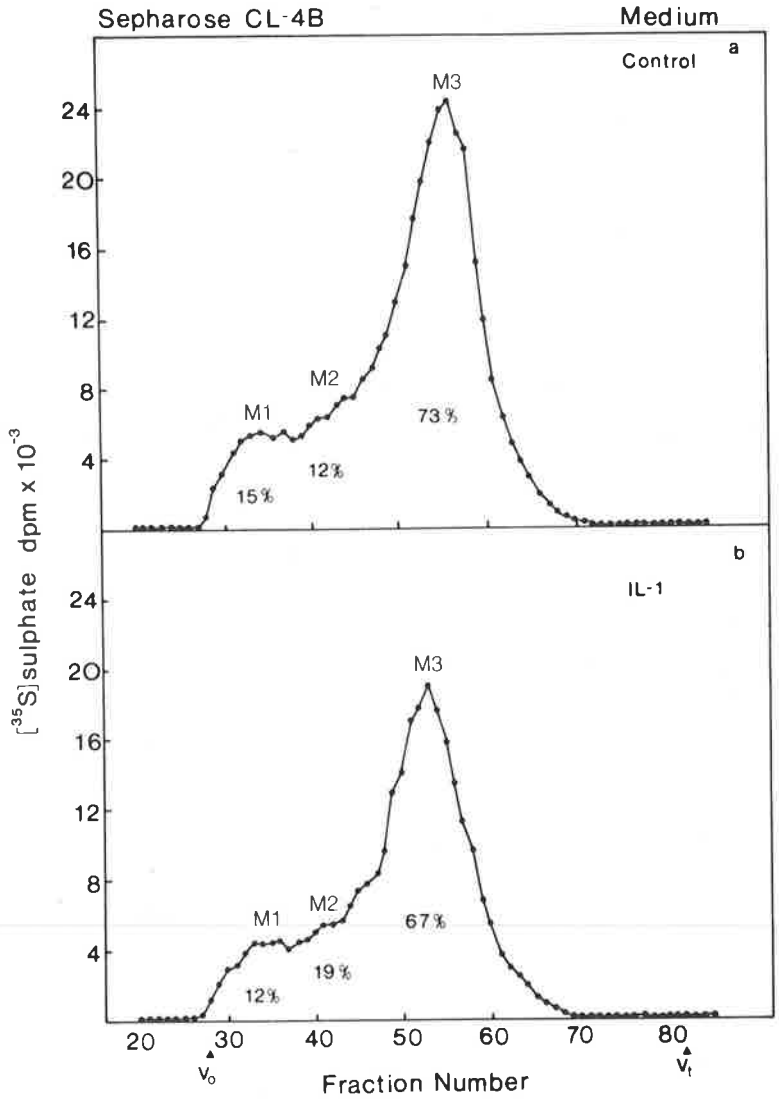

FIGURE 7 *Sepharose CL-4B gel filtration chromatography of medium proteoglycans associated with human gingival fibroblasts cultured in the presence or absence of IL-1β.*
[^{35}S]-sulphate-labelled culture medium from human gingival fibroblasts cultured in the presence (10^{-11} M) or absence of IL-1β was eluted Sephadex G-50 in the presence of 4 M-guanidine HCl and protease inhibitors. An aliquot from the material eluting in the void volume was applied to a Sepharose CL-4B column (0.7 cm × 100 cm) and eluted with 4 M-guanidine HCl/0.05 M-sodium acetate, pH 5.8 containing protease inhibitors. Fractions of 0.5 ml were collected and the radioactivity in each fraction was determined by liquid scintillation counting. The void volume (V_o) and total volume (V_t) were identified with [^{3}H]-DNA and $Na_2[^{35}S]O_4$ respectively. M1–M3 represent the different size classes of proteoglycans identified in these extracts.

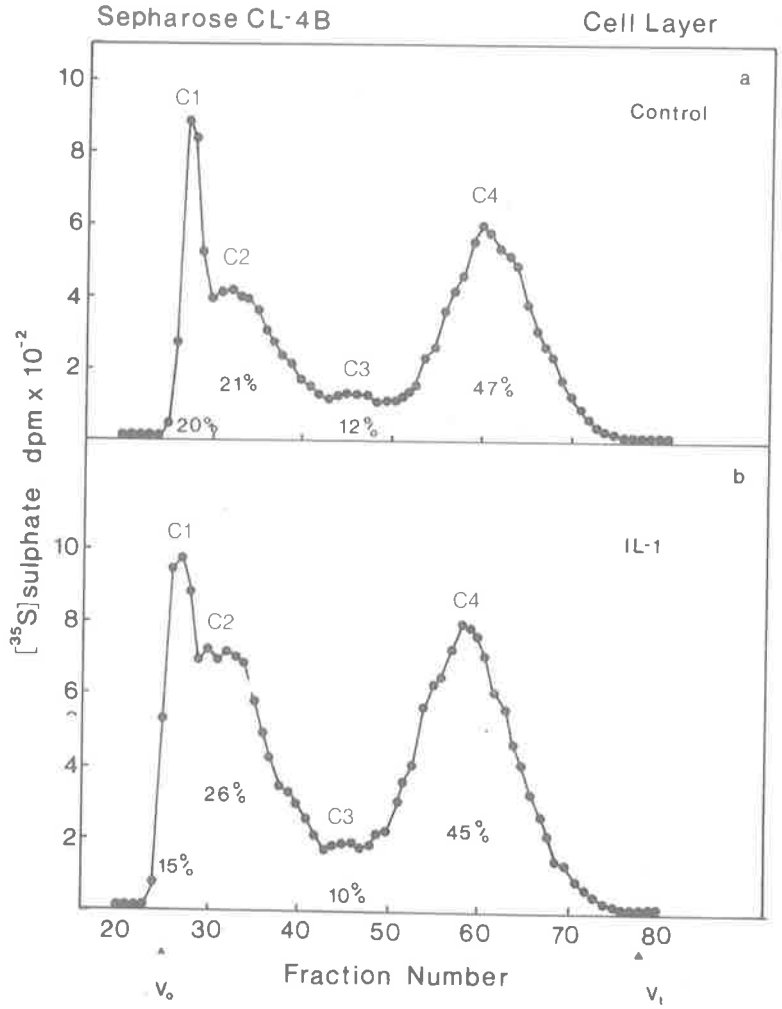

FIGURE 8 *Sepharose CL-4B gel filtration chromatography of cell layer proteoglycans associated with human gingival fibroblasts cultured in the presence or absence of IL-1β.*
[^{35}S]-sulfate-labeled cell layers of human gingival fibroblasts cultured in the presence (10^{-11} M) or absence of IL-1β were extracted with 4 M-guanidine HCl and eluted from Sephadex G-50 in the presence of 4 M-guanidine HCl and protease inhibitors. An aliquot from the material eluting in the void volume was applied to a Sepharose CL-4B column (0.7 cm × 100 cm) and eluted with 4 M-guanidine HCl/0.05 M-sodium acetate, pH 5.8 containing protease inhibitors. Fractions of 0.5 ml were collected and the radioactivity in each fraction was determined by liquid scintillation counting. The void volume (V_o) and total volume (V_t) determinations were the same as described in Figure 7. C1-C4 represent the different size classes of proteoglycans identified in these extracts.

Glycosaminoglycan Analysis

Preliminary analyses of the proteoglycans synthesized by gingival fibroblasts in the presence or absence of IL-1β were directed at determining their glycosaminoglycan composition. After enzymatic and chemical degradation of [^{35}S]-sulfate-labelled proteoglycans, the types of sulfated glycosaminoglycans were identified and quantitated. The effect of IL-1β on the relative proportions of these components

TABLE I

Proportions of [^{35}S]-sulfate-labelled material in peaks eluted from Sepharose CL-4B.

	MEDIUM			CELL LAYER			
	M1	M2	M3	C1	C2	C3	C4
Control	15 ± 2	12 ± 4	72 ± 5	19 ± 1	21 ± 3	11 ± 2	55 ± 10
Treated	12 ± 4	19 ± 3	74 ± 10	14 ± 1	21 ± 6	10 ± 1	57 ± 11

[^{35}S]-sulfate-labelled proteoglycans were isolated in the void volume after elution from Sephadex G-50 and then eluted from Sepharose CL-4B. The various peaks were identified as shown in Figs. 7 & 8, and the proportion of radioactivity in each peak determined and converted to a percentage of the total radioactivity eluted from the column. The data are expressed as mean and standard deviations of the mean of three separate chromatographic analyses.

in the medium and cell layer compartments is shown in Table II. Dermatan sulfate was the predominant glycosaminoglycan identified in the medium and heparan sulfate was the predominant glycosaminoglycan identified in the cell layer preparations. No significant differences were detected between the glycosaminoglycans synthesized by cells treated or untreated with IL-1β.

DISCUSSION

Interleukin-1 is now recognized as being important not only in regulating proliferation and differentiation of lymphoid cells but also as being a key factor in the regulation of connective tissue cells during inflammation.[2] In the present study, human gingival fibroblasts have been studied in relation to their response, *in vitro*, to recombinant IL-1β.

The stimulation of human gingival fibroblast proliferation by IL-1β noted in the

TABLE II

Sulfated glycosaminoglycans in the medium and cell layer.

	Haparan Sulfate	Dermatan Sulfate	Chondroitin Sulfate
MEDIUM			
Control	29 ± 1	43 ± 4	27 ± 3
Treated	27 ± 1	51 ± 7	21 ± 7
CELL LAYER			
Control	60 ± 3	26 ± 3	14 ± 1
Treated	64 ± 1	22 ± 4	17 ± 2

[^{35}S]-sulfate-labelled proteoglycans were isolated from medium and cell layer by elution from Sephadex G-50. The material in the void volume was pooled and the sulfated glycosaminoglycans were identified by digestion with chondroitinase ACII, chondroitinase ABC or nitrous acid followed by elution from Sephadex G-50. The digestion products eluted in the included volume and the undigested material remained in void volume. Thus, the percentage of material susceptible to each degradative treatment was calculated. Data represent mean and standard deviation of the mean of three separate determinations and are expressed as percentages of the total sulfated glycosaminoglycan recovered from the column.

present study is in agreement with other studies.[4,5,29] However, it is of interest to note that, when compared to human foreskin fibroblasts, the human gingival fibroblasts were stimulated to a greater extent even though they had a lower basal rate of proliferation. In addition, it is noteworthy that this effect was not evident in the absence of serum, idicating serum to contain some unidentified factor(s) which augment the biological effect of IL-1β.

The elevated release of proteoglycans into the medium of cultures exposed to IL-1β contrasts the findings of Tyler[30,31] who reported a decrease in proteoglycan synthesis when cartilage slices were incubated in the presence of IL-1. However, other reports have suggested that in cell culture (as opposed to organ culture), proteoglycan synthesis may be elevated by IL-1.[14,32,33,34] Such discrepancies may reflect a singificant difference between organ and cell culture with respect to bio-availability and bilogical activity of various mediators. Whether this increase in proteoglycan synthesis is biologically significant remains to be established. Nonetheless, the fact that IL-1β can induce such a synthetic change in fibroblasts should not be overlooked considering its effect on other parameters such as enzyme release and collagen synthesis.[9,34] Thus, these changes may act in concert to potently influence connective tissue metabolism.

The release of ^{35}S-labelled proteoglycans by cells treated with IL-1β may be related to modulation of intracellular metabolism or sulfation. However, in the present study, the turnover rate of proteoglycans and degree of sulfation of proteoglycans synthesized by cells treated with IL-1β was essentially identical to the untreated cells. Therefore, it is improbable that the increased amount of ^{35}S-labelled proteoglycans noted in the presence of IL-1β is due to alterations in proteoglycan turnover or sulfation. Thus the effect noted probably represents a true stimulation of proteoglycan biosynthesis.

Besides altered intracellular metabolism of proteoglycans, an increase in cell proliferation or PGE_2 synthesis induced by IL-1 may influence proteoglycan synthesis by cells in culture. However, when these two parameters were blocked by specific agents, IL-1β still stimulated proteoglycan synthesis. In this regard, it should be noted that a direct inhibitory effect of indomethacin on PGE_2 synthesis was not investigated in the present study. Therefore, the data relating to the effect of indomethacin on IL-1β-induced proteoglycan synthesis are only circumstantial. Nonetheless, these findings imply that the stimulation of proteoglycan synthesis by IL-1β is independent of both cell proliferation and PGE_2 synthesis.

In addition to altered rates of synthesis, the structure and composition of newly synthesized proteoglycans can have a significant influence on connective tissue function. In this study, IL-1β did not cause a selective increase in synthesis of one proteoglycan at the expense of another. Thus, the immediate effect of Il-1β on fibroblast proteoglycan synthesis appears to be quantitative rather than qualitative.

Since IL-1 has been reported to stimulate the release of proteolytic enzymes from a variety of cells.[9,35–37] it is of interest to note that in the present study, no evidence of proteoglycan degradation was noted. This most likely reflected the presence of FCS in the culture medium which contains several inhibitors of secreted proteinases. This was highlighted when the media preparations were incubated for 24 hr at 37°C prior to molecular size analysis; no changes were noted in elution profiles between treated and untreated cultures (results not shown). Nonetheless

proteoglycanases are released from IL-1-stimulated fibroblast in an inactive form.[9] Thus, analysis of the role that IL-1 plays in proteoglycan degradation requires further investigation.

In conclusion, the observations that IL-1β causes increased synthesis of proteoglycans as well as stimulation of human gingival fibroblast proliferation demonstrates the biological activity of this polypeptide. The events associated with inflammation and repair are exceedingly complex, however, the role of IL-1β in such processes cannot be discounted. Indeed, since proteoglycan synthesis appears to precede collagen synthesis in experimental granuloma formation,[28] it is not inconsistent that IL-1β may mediate such an early reparative response by stimulation of fibroblast proliferation and proteoglycan synthesis.

ACKNOWLEDGMENTS

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Paper 17.

Bartold, P.M.
The effect of interleukin 1β on hyaluronic acid synthesized by adult human gingival fibroblasts i*n vitro*.
Journal of Periodontal Research **23:** 139-147, 1988.

The effect of interleukin-1 β on hyaluronic acid synthesized by adult human gingival fibroblasts *in vitro*

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Bartold PM. The effect of interleukin 1β on hyaluronic acid synthesized by adult human gingival fibroblasts in vitro. *J Periodont Res 1988; 23: 139–147.*

The effect of recombinant interleukin-1β (IL-1β) on hyaluronic acid synthesis by human gingival fibroblasts was studied. IL-1β caused a dose-dependent increase in the incorporation of (^{3}H)-glucosamine into hyaluronic acid. The ^{35}S/^{3}H ratios of labeled macromolecules did not change regardless of the presence or absence of IL-1β and indicates stimulation of hyaluronic acid synthesis. Inhibition of cell proliferation by hydroxyurea caused an increase in hyaluronic acid synthesis. The effect of IL-1β on hyaluronic acid synthesis in the presence of hydroxyurea was increased over untreated and IL-1β-treated controls, but equivalent to the hydroxyurea-treated controls. Thus the effect of IL-1β on hyaluronic acid synthesis may be independent of cell proliferation. Furthermore, inhibition of prostaglandin E_2 synthesis by indomethacin abolished the effect of IL-1β on hyaluronic acid synthesis. Inhibition of new protein synthesis by cycloheximide negated the effect of IL-1β on hyaluronic acid synthesis. This may be related to inhibition of new hyaluronate synthetase synthesis, since IL-1β stimulated the level of hyaluronate synthetase activity. Sepharose CL-2B chromatography revealed that most of the newly synthesized hyaluronic acid was of large molecular size. The cells exposed to IL-1β retained more large molecular size hyaluronic acid in their cell layer environment than did the control cells. These responses by fibroblasts to IL-1β may be indicative of early tissue repair.

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Introduction

Inflammation in soft connective tissues is characterized by early tissue destruction followed by deposition of new extracellular matrix. These events are closely associated with the infiltration of neutrophils and monocytes and the accumulation of fibroblasts. The way in which these cells interact is not yet clear. However, the identification of interleukin 1 (IL-1), a polypeptide released by activated macrophages, which can modulate fibroblast activity has led to interest in this hormone's role in regulating extracellular matrix synthesis.

Currently, two forms of IL-1, interleukin 1α (IL-1α) and interleukin 1β (IL-1β), have been identified (1). Although these differ in their isoelectric points, have only 26% homology in amino acid composition and are most likely separate gene products, they seem to share similar biological properties (2–4). The development of recombinant analogues of IL-1α and IL-1β now permits investigations into their biological activity in the absence of contaminants. Biochemical studies on normal and inflamed human gingiva have revealed alterations to the collagens, proteoglycans and hyaluronic acid under inflammatory conditions (5–7). In addition, fibroblasts derived from inflamed gingiva synthesize different proportions of extracellular matrix components compared to cells from healthy gingiva (8–11). Whether these represent alterations in existing fibroblast populations or selection of predominant subsets of cells within the tissues under inflammatory conditions remains to be established. Nonetheless, it is probable that inflammatory mediators, such as IL-1, will play a prime role in such phenotypic expression by fibroblasts.

The purpose of this study was to investigate the effect of recombinant IL-1β on hyaluronic acid synthesis by fibroblasts derived from healthy human gingiva.

Material and Methods

Materials

Guanidine HCl, indomethacin, hydroxyurea, cycloheximide, sodium pyruvate, glutamine, adenosine 5' phosphate (ATP) and UDP-N-acetylyglucosamine were all purchased from the Sigma Chemical Co., St. Louis, MO; Cetylpyridinium chloride from Ajax Chemicals Australia, Aulam, N. S. W.; sodium dodecyl sulfate (SDS) from Bio Rad Laboratories, Richmond, CA; Cellulose powder from Whatman, Maidstone, U.K.; *Streptomyces* hyaluronidase and chondroitinase ABC (*Proteus vulgaris*) from Seikagaku Kogyo Ltd., Tokyo, Japan; Sephadex G-50 and Sepharose CL-2B from Pharmacia Fine Chemicals, North Ryde, Sydney, Australia; Dul-

becco-Vogt medium (DVM), fetal calf serum (FCS), penicillin, streptomycin and non-essential amino acids were all from Flow Laboratories, Irvine, Ayrshire, Scotland; tissue culture plastic ware from Nunc, Roskilde, Denmark; D-(6-^{3}H)-glucosamine HCl (33 Ci/mMol); UDP-D-(U-^{14}C)-glucuronic acid (225 mCi/mMol) and $Na_2(^{35}S)0_4$ (1042 mCi/mMol were from Amersham (Australia), Sydney, Australia; Ready Solv EP scintillation fluid from Beckman (Australia), Adelaide, Australia; Aquacide from Calbiochem, La Jolla, CA; recombinant interleukin-1β was a generous gift from Immunex Corporation, Seattle, WA.

Fibroblast cultures

Fibroblasts were derived by explant culture from biopsies of healthy human gingiva from human volunteers as described previously (8). The cells were maintained in Dulbecco-Vogt medium (DVM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2 μM glutamine, 10 mM sodium pyruvate and non-essential acids at 37°C in an atmosphere of 5% CO_2 and 95% air. Cells between the 4th and 8th transfer in culture were used. All experiments were done in the presence of FCS unless otherwise stated.

Dose response experiments

Fibroblasts were seeded in triplicate into 24-well plates. After reaching confluence, the cells were preincubated in medium containing IL-1β ranging from 10^{-13} to 10^{-9} M for 48 h, since it is by this time that human gingival fibroblasts become maximally stimulated by IL-1 (22). The medium was removed and replaced with fresh medium containing IL-1β and (^{3}H)-glucosamine (17.5 μCi/ml) and incubated for a further 24 h. The medium was then removed and the cell layers washed once with PBS, which was pooled with the medium to make up the medium fraction for subsequent analyses. The cells were released from the culture plates by trypsinization, and cell numbers determined using a hemocytometer. The culture plates were further extracted with 4 M guanidine HCl/0.05 M sodium acetate, pH 5.8 overnight at 4°C and these were pooled with the trypsin digest to make up the cell layer fraction for subsequent analyses. These experiments, as for all others described, were repeated three times to validate the results.

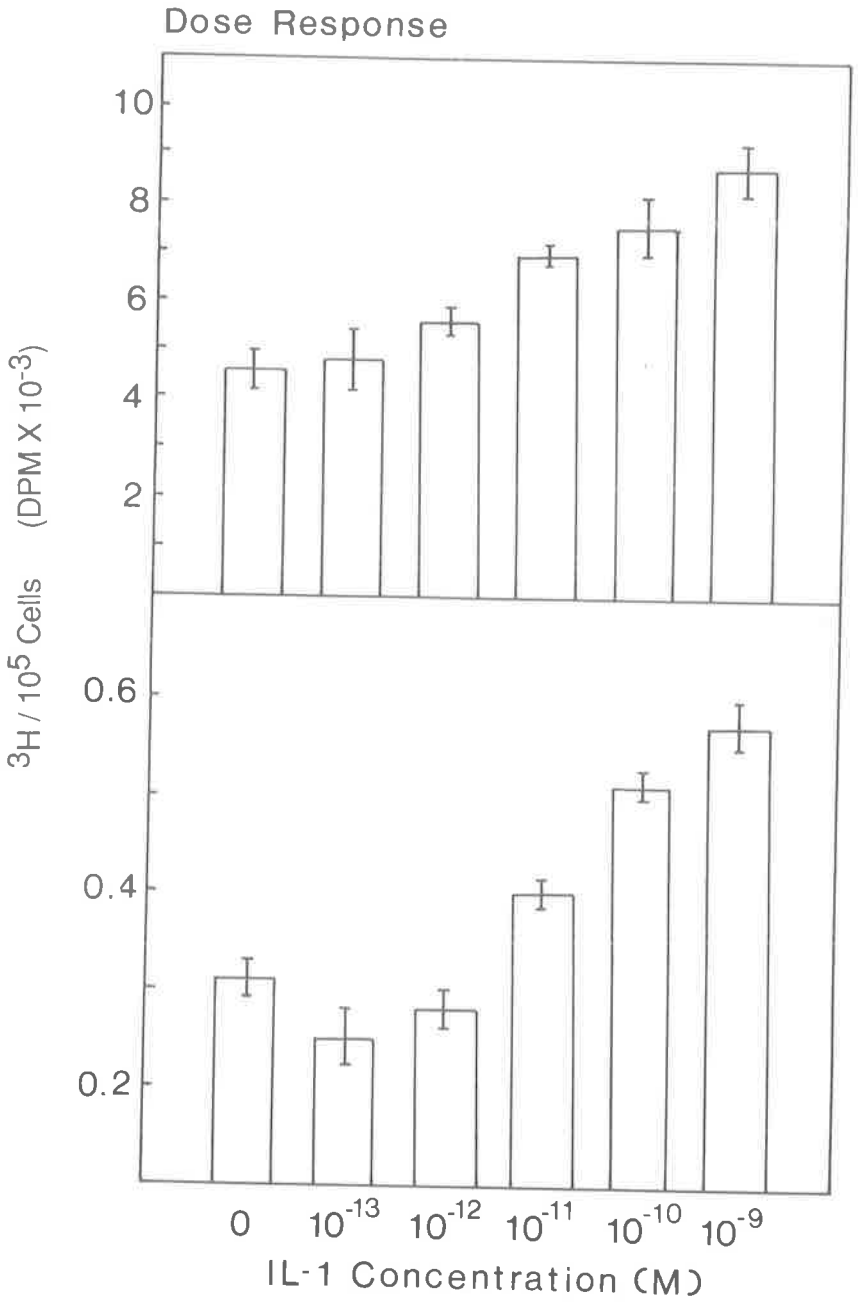

Fig. 1. Effect of increasing concentrations of IL-1β on the incorporation of (^{3}H)-glucosamine into hyaluronic acid by human gingival fibroblasts. Data represent the mean and standard deviation of the mean of triplicate cultures from a representative experiment. (Top) Media; (Bottom) Cell Layer.

(^{3}H)-glucosamine incorporation into hyaluronic acid with time

To determine the effect of IL-1β on the kinetics of hyaluronic acid synthesis, cells were cultured as described above in 24-well plates and incorporation of radiolabel into hyaluronic acid was determined at 4, 8, 24, 30, 48 and 72 h after introduction of the radiolabel and IL-1β. Cell numbers were determined at each time point after trypsinization using a hemocytometer.

Analysis of (^{3}H)-labeled macromolecules

To determine the amount of (^{3}H)-glucosamine incorporated into all macromolecules, the medium and cell layer material was dialysed exhaustively against 0.15 M sodium acetate, 0.15 M

sodium chloride, 1 mM magnesium chloride and 1 mM calcium chloride, pH 5.8. The final volume of each sample was recorded and the ^{3}H-radioactivity was determined for 100 μl aliquots.

Quantitation of hyaluronic acid was achieved using a modification of the procedure described by Saarni & Tammi (13). Aliquots (250 μl) of the medium or cell layer fractions were diluted with an equal volume of 2% hyaluronic acid in distilled water and the glycosaminoglycans precipitated by addition of 50 μl 10% cetylpyridinium chloride (CPC) (15). The samples were then immediately applied onto CPC-cellulose microcolumns (0.5 cm × 6.0 cm) (15) and unincorporated radiolabel and glycoproteins were washed from the columns with 12 ml 1% CPC in 0.05 M sodium chloride. Hyaluronic acid was then eluted with 10 ml of 1% CPC in 0.5 M hydrochloric acid. Using this method, 80% of standard ^{3}H-labeled hyaluronic acid was recovered in the 0.5 M HCl wash; less than 2% eluted in the initial wash and the remaining 15% remained bound to the column and could be eluted with 1.0 M sodium chloride.

^{35}S/^{3}H ratios

The specific activity of specific sugar nucleotide precursors can change with varying culture conditions when (^{3}H)-glucosamine is used as a metabolic precursor (16). Therefore, using a dual label protocol, the ^{35}S/^{3}H ratios of isolated macromolecules and disaccharides liberated from glycosaminoglycan digestion were assessed as described by Bartold & Page (11).

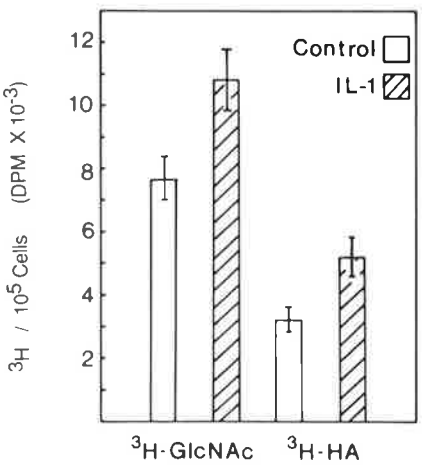

Fig. 2. The effect of 10^{-10} M IL-1β on the incorporation of (^{3}H)-glucosamine into total macromolecules and hyaluronic acid.

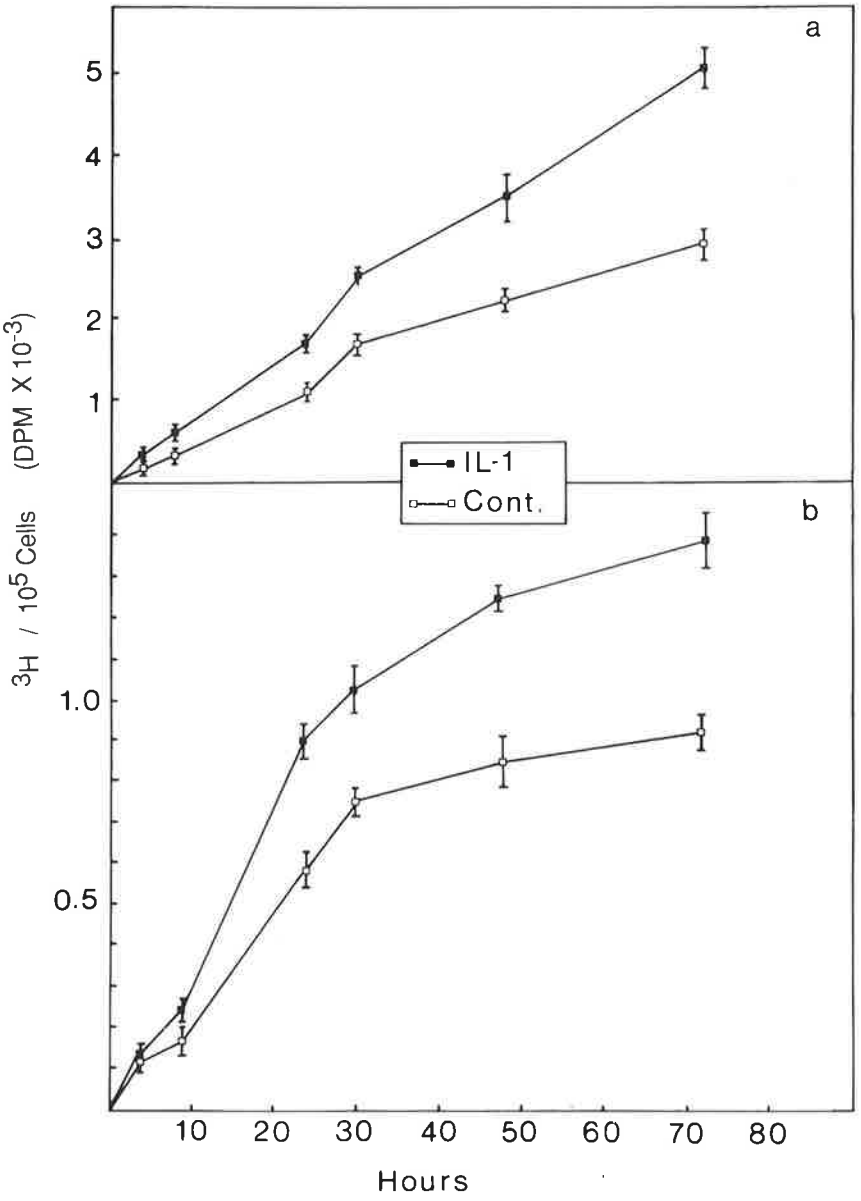

Fig. 3. Effect of IL-1β on the incorporation of (^{3}H)-glucosamine into hyaluronic acid with time. Data are expressed as means and standard deviation of the mean of triplicate cultures of a representative experiment. (a) Medium; (b) Cell Layer.

Inhibition of cell proliferation

Because IL-1 can influence fibroblast proliferation (18, 19) which in turn may influence hyaluronic acid synthesis (20, 21), it was necessary to determine the effect of IL-1β on hyaluronic acid synthesis in the absence of cell proliferation. Hydroxyurea at 10^{-2} M can inhibit gingival fibroblast proliferation up to 90% (22). Therefore, subconfluent cultures of fibroblasts (20 000 cells/well) were cultured in the presence of 10^{-2} M hydroxyurea and hyaluronic acid synthesis was assessed after 48 h incubation in the presence or absence of 10^{-10} M IL-1β.

Inhibition of prostaglandin synthesis

Since PGE_2 can influence hyaluronic acid synthesis (23–28), endogenous PGE_2 synthesis was inhibited by the addition of indomethacin to the culture medium (23). Hyaluronic acid synthesis was thus assessed after 48 h incubation in the presence or absence of 10^{-10} M IL-1β in medium containing indomethacin ranging in concentration from 10^{-6} to 10^{-4} M.

Table 1. Effect of IL-1β on $^{35}S/^{3}H$ ratio in isolated macromolecules[1]

	Total labeled Macromolecules	Chondroitinase ABC Disaccharides
Media Control 1	1:0.074	1:0.24
Media Control 2	1:0.083	1:0.17
Media Control 3	1:0.088	1:0.19
Media IL-1 1	1:0.067	1:0.20
Media IL-1 2	1:0.072	1:0.19
Media IL-1 3	1:0.081	1:0.21

[1] Human gingival fibroblasts were incubated in the presence of (^{3}H)-glucosamine and (^{35}S)-sulfate and the labeled macromolecules were isolated. The $^{35}S/^{3}H$ ratios were determined for both the total isolated macromolecules and chondroitinase ABC digested macromolecules.

Effect of IL-1β on hyaluronic acid synthetase

To determine whether new protein synthesis (and therefore new enzyme synthesis) was a prerequisite for the effect of IL-1β on hyaluronic acid synthesis, the fibroblasts were incubated in the presence or absence of cycloheximide. Cycloheximide at a concentration of 20 μg/ml caused a 80% decrease in total protein synthesis (results not shown). Therefore, gingival fibroblasts were cultured in the presence or absence of 10^{-10} M IL-1β in medium containing either 0 μg/ml or 20 μg/ml cycloheximide and hyaluronic acid synthesis was determined after 48 h.

Hyaluronic acid synthetase activity was assessed by incubating lysed IL-1β-treated and untreated cells in the presence of 0.1 μCi UDP-D-(U-^{14}C)glucuronic acid and 5×10^{-3} μMoles UDP-N-acetylglucosamine (29). The amount of ^{3}H-hyaluronic acid produced was determined by elution from CPC-cellulose microcolumns.

Molecular size distribution of hyaluronic acid

The effect of IL-1β on the molecular size of newly synthesized hyaluronic acid was also studied. Medium and cell layer (^{3}H)-labeled macromolecules were eluted from columns of Sepharose CL-2B (0.7 cm $\times$ 100 cm) with 4 M guanidine HCl/0.05 M sodium acetate, pH 5.8. Fractions of 0.5 ml were collected at a flow rate of 3 ml/h and 50 μl aliquots from each fraction were assessed for (^{3}H)-activity. The material which eluted in the void volume was pooled separately from that which eluted in the included volume. The recoveries of radiolabeled material applied onto these columns were between 80–85%. The hyaluronic acid content of the pooled material was assessed using *Streptomyces* hyaluronidase as described by Bartold & Page (11).

Statistical analysis

All data were subjected to statistical analysis using either the methods of analysis of variance or the Student's t-test.

Results

Dose response

The synthesis of (^{3}H)-labeled hyaluronic acid was stimulated in a dose-dependent fashion by IL-1β (Fig. 1). At a concentration of 10^{-9}M, an 80% and 100% increase in hyaluronic acid synthesis was noted for the medium and cell layer respectively. This stimulatory effect was

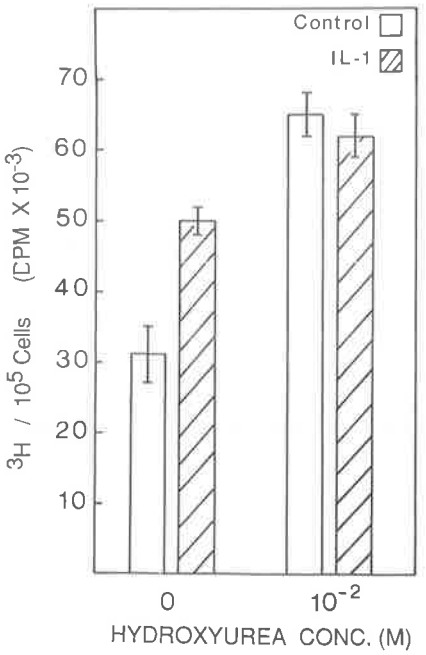

Fig. 4. Effect of inhibition of cell proliferation on hyaluronic acid levels in media. Data represent the means and standard deviation of the mean of triplicate cultures from a representative experiment.

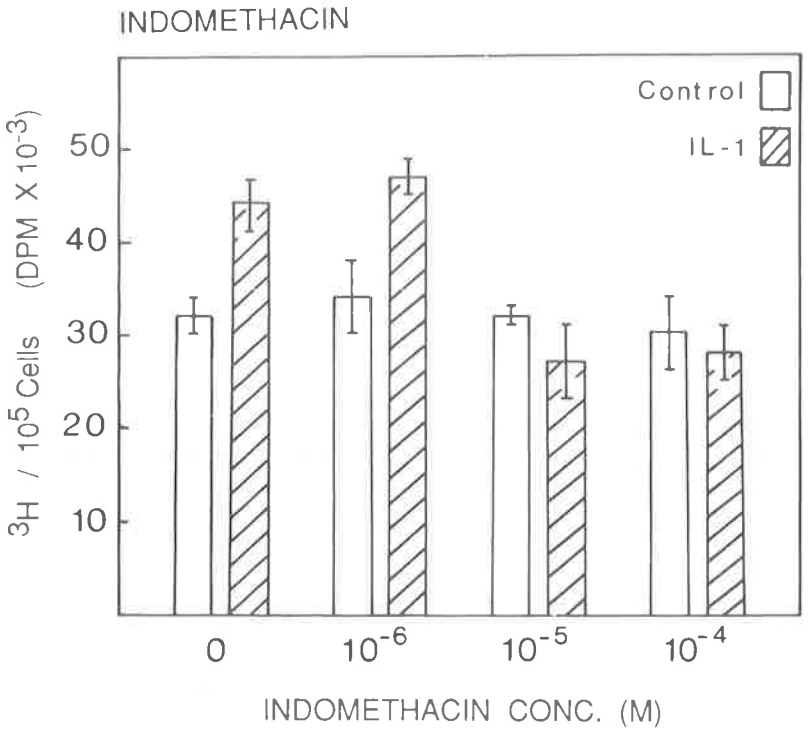

Fig. 5. Effect of indomethacin inhibition of prostaglandin E_2 synthesis on hyaluronic acid levels in media. Data represent the means and standard deviation of the mean of triplicate cultures from a representative experiment.

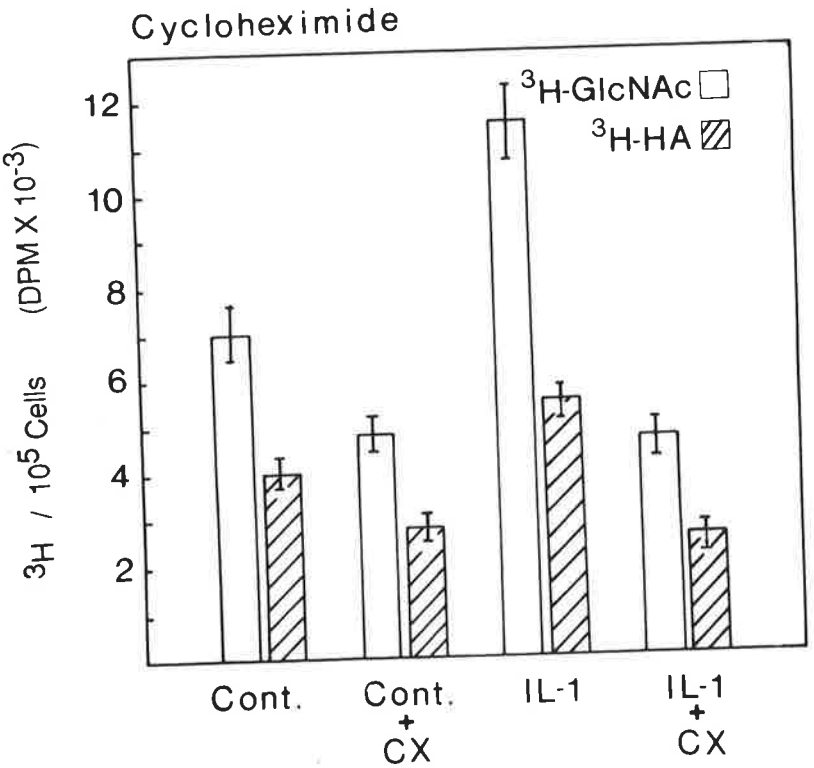

Fig. 6. Effect of cycloheximide (20 μg/ml) on the levels of hyaluronic acid in the medium. Data represent the means and standard deviation of the mean of triplicate cultures from a representative experiment. Abbreviations: cycloheximide (CX).

statistically significant at concentrations of 10^{-11} M and greater ($p < 0.05$). When the effect of IL-1β at 10^{-10} M on total ^{3}H-labeled macromolecules was compared to the effect on total ^{3}H-labeled hyaluronic acid (Fig. 2), the stimulatory effect of IL-1β on hyaluronic acid synthesis was confirmed. However, since the ratio of total labeled macromolecules to total labeled hyaluronic acid changed from 1:2.8 to 1:3.1 it is possible that IL-1β may also stimulate the synthesis of macromolecules other than hyaluronic acid.

(^{3}H)-glucosamine incorporation with time

The amount of (^{3}H)-labeled hyaluronic acid released into the medium increased steadily over a 72-h period (Fig. 3). At all time points, IL-1β treated cells released more (^{3}H)-labeled hyaluronic acid into the medium than did the untreated cells and this difference became statistically significant ($p < 0.05$) after 24 h in culture. For the cell layers, (^{3}H)-labeled hyaluronic acid increased rapidly during the first 24 h, after which the incorporation of (^{3}H)-glucosamine slowed somewhat increasing approximately 40% over the next 48 h. As for the medium, the IL-1β treated cells demonstrated elevated (^{3}H)-labeled hyaluronic acid levels at all time points with the differences becoming statistically significant by 24 h.

^{35}S/^{3}H ratios of labeled macromolecules

The ^{35}S/^{3}H ratio of isolated labeled macromolecules was compared between cultures incubated in the presence or absence of IL-1β (Table 1). In the total pool of macromolecules this ratio was determined to be 0.073 (±0.007) and 0.081 (±0.007) for treated and untreated cells respectively. These differences were not statistically significant.

Further confirmation that IL-1β did not significantly alter the ^{35}S/^{3}H ratio in newly synthesized macromolecules was achieved by analysis of glycosaminoglycan disaccharides. These, which represent the activity in chondroitin sulfate and dermatan sulfate glycosaminoglycans, and were found to be 0.2 (±0.01) and 0.21 (±0.05) respectively for treated and untreated cells.

Inhibition of cell proliferation and prostaglandin synthesis

The effect of inhibition of cell proliferation on hyaluronic acid synthesis by cells exposed to IL-1β is shown in Fig. 4. At 10^{-2} M hydroxyurea, an elevated level of hyaluronic acid production above that of control cultures was noted. In the presence of 10^{-10} M IL-1β this elevated level of hyaluronic acid production persisted at approximately the same level as for hydroxyurea alone.

The effect of blocking prostaglandin E_2 (PGE_2) synthesis with indomethacin on hyaluronic acid synthesis is shown in Fig. 5. Indomethacin in the absence of IL-1β had no marked effect on hyaluronic acid production. At a concentration of 10^{-6} M, indomethacin did not markedly affect the stimulatory effect of IL-1β on hyaluronic acid synthesis. However, at a concentration of 10^{-5} M and greater, indomethacin supressed IL-1β-induced hyaluronic acid synthesis.

Effect of cycloheximide on hyaluronic acid synthesis

The effect of cycloheximide inhibition of protein synthesis on the stimulation of hyaluronic acid by IL-1β is shown in Fig. 6. For comparison, the data for total glucosamine levels in total macromolecules as well as hyaluronic acid are shown. At 20 μg/ml, cycloheximide caused a diminution in hyaluronic acid synthesis. In the presence of IL-1β and cycloheximide, the stimulatory effect of IL-1β on hyaluronic acid synthesis was no longer evident. Hyaluronic acid synthesis was markedly reduced in the presence of cycloheximide and IL-1β compared to treatment of cells with IL-1β alone.

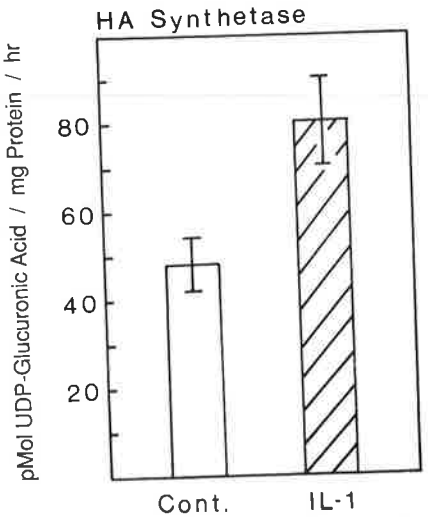

Fig. 7. Effect of IL-1β on hyaluronic acid synthetase activity as assessed by the incorporation of UDP-D-(^{14}C)-glucuronic acid into hyaluronic acid. Data represent the means and standard deviation of the mean of triplicate cultures from a representative experiment.

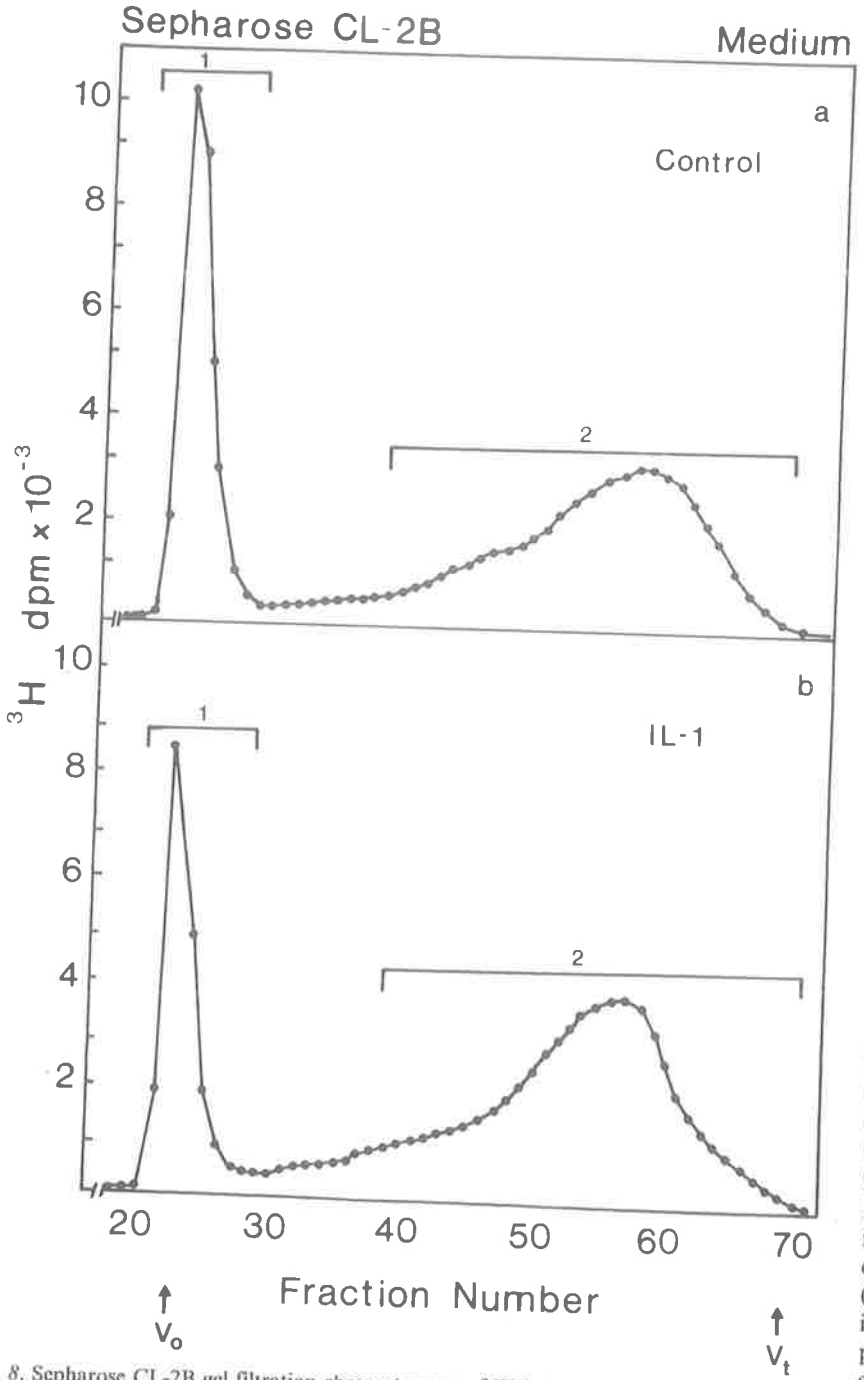

Fig. 8. Sepharose CL-2B gel filtration chromatogram of ^{3}H-labeled medium macromolecules isolated from cultures of human gingival fibroblasts incubated in the presence of 10^{-10} M IL-1β. The void volume (V_o) and total volume (V_t) were determined with (^{3}H)-DNA and (^{35}S)-sulfate respectively. The fractions in the regions marked by the bars were pooled for further analyses.

Effect of IL-1β on hyaluronic acid synthetase activity

Hyaluronic acid synthetase activity was monitored in the presence or absence of IL-1β (Fig. 7). At a concentration of 10^{-10} M, IL-1β caused an increase in hyaluronic acid synthetase activity of 40% compared to cells cultured in the absence of IL-1β.

Effect of IL-1β on hyaluronic acid molecular size

Radiolabeled material from the medium and cell layer of treated and untreated cells eluted from Sepharose CL-2B as either excluded or included peaks (Figs. 8 and 9). The excluded peak from the treated cultures, which represented large macromolecules, accounted for 15% of the total labeled material eluting from the column; while the corresponding peak for the control cultures represented 20% of the total radioactivity (Table 2). The large molecular sized material from the cell layers of treated and untreated cells represented 46% and 40% of the total respectively.

For the medium, most (>90%) of the labeled material in peak 1 was sensitive to hyaluronidase digestion (Table 2). Despite the differences in total (^{3}H)-labeled material in each of the peaks, no significant differences in the distribution of hyaluronic acid on the basis of molecular size could be detected for treated and untreated cultures (Table 2).

Quantitation of large and small molecular size hyaluronic acid associated with the cell layer is also shown in Table 2. Of the labeled material in peak 1 for treated and untreated cells, 64% and 51% respectively was digestable (Table 2). When these values were related to the total radiolabel distribution, it was evident that the cells exposed to IL-1β produced an elevated amount of high molecular weight hyaluronic acid which remained associated with the cell layer (Table 2).

Discussion

Inflammatory lesions in soft connective tissues cause many changes to the composition and structure of the extracellular matrix which may compromise its function (30). In order to understand the basic cellular responses to inflammation, fibroblasts from normal and inflamed tissues have been studied and found to have altered proliferation rates, increased proteoglycan synthesis, increased collagen synthesis and increased release of proteolytic enzymes (6, 7, 10). Many of these features are induced when cells are cultured in the presence of supernatants from stimulated mononuclear cells (24, 31–33). Consequently, with the identification of IL-1 as a potent inflammatory mediator in such supernatants, interest has focused upon this hormone as a likely source of activation of connective tissue cells.

In the presence of increasing concentrations of IL-1β, gingival fibroblasts responded by incorporating elevated amounts of (^{3}H)-glucosamine into macromolecules. Most of this increase could be accounted for by an increase in hyaluronic acid synthesis and is similar to the effect reported for mononuclear supernatants on synovial cells (28,

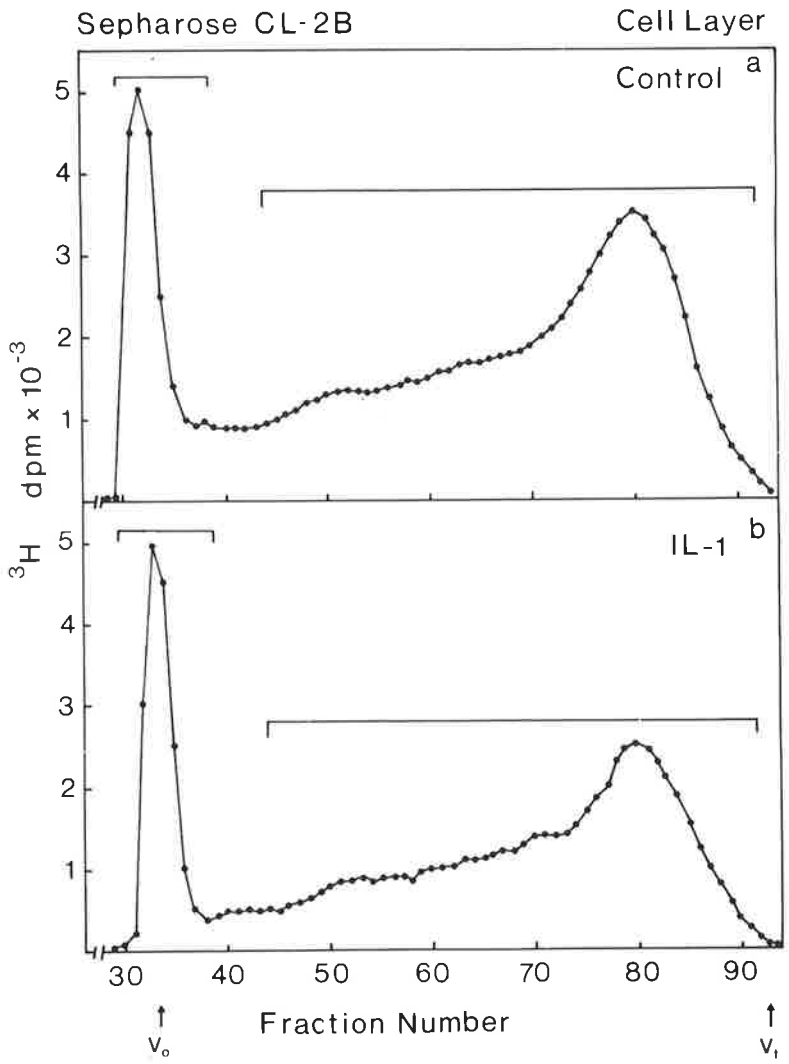

Fig. 9. Sepharose CL-2B gel filtration chromatograms of ^{3}H-labeled cell layer macromolecules isolated from cultures of human gingival fibroblasts incubated in the presence or absence of 10^{-10} M IL-1β. The column elution conditions and characterizations were the same as described in Fig. 7. Fractions within the regions marked by the bars were pooled for subsequent analyses.

34–36). In the present study the ^{35}S/^{3}H ratios of isolated macromolecules did not change appreciably regardless of the presence or absence of IL-1β. This indicates that the specific activity of the sugar nucleotide precursors was not affected by the different culture conditions. Therefore, the increased incorporation of (^{3}H)-glucosamine into hyaluronic acid can be interpreted as representing a true increase in hyaluronic acid synthesis.

Analysis of the rate of (^{3}H)-glucosamine incorporation into hyaluronic acid in the presence or absence of IL-1β revealed that differences could be detected 24 h after the addition of IL-1β. While this may seem to be slow, it is one of the earliest responses that the cells show. Stimulation of proliferation by IL-1β is maximal by d 3 and proteoglycan synthesis only becomes significantly different after 72 h in culture (8, 22). Indeed, this response to IL-1β highlights the observations that increased hyaluronic acid synthesis is a characteristic feature of fibroblast activation (11, 37, 38).

The contribution of cell proliferation to IL-1β-induced hyaluronic acid production was also studied. When treated with hydroxyurea alone, the cells responded by producing more hyaluronic acid relative to the control cells. In the presence of hydroxyurea and IL-1β, elevated hyaluronic acid synthesis was also noted, but it was no greater than that noted when the cells were incubated in hydroxyurea alone. Thus, the influence of IL-1β on cell proliferation may be related to the observed increase in hyaluronic acid production. The effect of hydroxyurea on hyaluronic acid synthesis appears to be a novel finding. However, it is consistent with elevated hyaluronic acid production by cells which are in a subconfluent state, since hyaluronic acid production is related not only to proliferation but also to cell density (20, 21).

To study the role of PGE_2 synthesis on the observed stimulation of hyaluronic acid by IL-1β, the cells were incubated in the presence of indomethacin. At concentrations of 10^{-5} M and greater of indomethacin, the effect of IL-1β on hyaluronic acid synthesis was suppressed. These data are consistent with the findings of Bocquet et al. (35), who reported that inhibition of PGE_2 synthesis negated the effect of mononuclear cell supernatants on hyaluronic acid by synovial cells.

Since increased hyaluronic acid synthesis is associated with altered levels of hyaluronic acid synthetase (29, 39), the effect of blocking new protein synthesis by cycloheximide was analyzed. In the absence of IL-1β, cycloheximide caused a marked decrease in hyaluronic acid synthesis. This is consistent with the effect of cycloheximide on hyaluronic acid synthesis in other systems (40, 41). However, when the cells were cultured in the presence of cycloheximide and IL-1β, no stimulation of hyaluronic acid synthesis was noted. This implies that new protein synthesis is a prerequisite for IL-1β to induce hyaluronic acid synthesis. Indeed, direct assessment of enzyme activity indicated that IL-1β significantly increased hyaluronic acid synthetase activity. Taken together, these data indicate that IL-1β stimulates hyaluronic acid synthesis via stimulation of new hyaluronic acid synthetase.

Although no differences in absolute molecular size distribution of newly synthesized hyaluronic acid were noted, the hyaluronic acid associated with the cell layer was composed of more large molecular weight hyaluronic acid. These data are in partial agreement with others (35, 36) who have reported no changes in molecular size. However, in these studies no quantitation of the relative distribution of large and small molecular sized hyaluronic acid was made.

Hyaluronic acid has been implicated in many biological functions (43, 44). As such, a role for dictating developmental stages has been proposed for this macromolecule (21). In addition, hyal-

Table 2. Effect of IL-1β on the molecular weight distribution of hyaluronic acid[1]

	% total ^{3}H-activity in peak 3[2]	% peak digested by hyaluronidase[3]	% total ^{3}H-activity[4]
Media			
Control Peak 1	20	94	18
Control Peak 2	80	28	22
IL-1 Peak 1	15	93	14
IL-1 Peak 2	85	20	17
Cell layer			
Control Peak 1	40	51	20
Control Peak 2	60	5	3
IL-1 Peak 1	46	64	29
IL-1 Peak 2	54	4	2

[1] ^{3}H-glucosamine labeled macromolecules were pooled as shown in Figs. 7 & 8 after elution from Sepharose CL-2B. The proportion of hyaluronic acid present was determined by calculating the amount of ^{3}H-activity susceptible to *Streptomyces* hyaluronidase digestion.

[2] ^{3}H-activity in each peak is expressed as a percentage of the total ^{3}H-activity eluted from Sepharose CL-2B.

[3] Values represent the percentage of material in the Sepharose CL-2B peaks which was sensitive to *Streptomyces* hyaluronidase digestion.

[4] The percentage of the total ^{3}H-activity represented by hyaluronic acid was determined by multiplying the percentage of ^{3}H-activity in the Sepharose peaks by the percentage of that material identified as hyaluronic acid.

uronic acid production may be enhanced under inflammatory conditions (11, 41, 45) and increased hyaluronic acid is one of the earliest events of tissue remodelling and healing (12). Therefore, stimulation of hyaluronic acid synthesis by a mediator like IL-1β may represent an attempt by the cells to initiate tissue repair.

Acknowledgments

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Effect of Lipopolysaccharide on Proteoglycan Synthesis by Adult Human Gingival Fibroblasts In Vitro

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The effect of lipopolysaccharide preparations from *Salmonella enteritidis*, *Bacteroides gingivalis*, and *Actinobacillus actinomycetemcomitans* on human gingival fibroblasts was studied. Lipopolysaccharide from all sources inhibited fibroblast proliferation in the concentration range of 0.5 to 50 μg/ml, with the lipopolysaccharide from *A. actinomycetemcomitans* having the strongest inhibitory effect. Assessment of the effect of lipopolysaccharide on gingival fibroblast metabolism indicated both total protein and proteoglycan synthesis to be inhibited with increasing concentrations of lipopolysaccharide. As for the antiproliferative effect, lipopolysaccharide from *A. actinomycetemcomitans* had the greatest inhibitory effect on cell synthetic activity. This inhibitory effect was determined by pulse-chase experiments to be a true depression in synthesis. Furthermore, the effect was independent of lipopolysaccharide-induced changes in cell proliferation and prostaglandin synthesis. This study confirmed the toxic effect of lipopolysaccharide on fibroblasts and, in particular, indicated that various lipopolysaccharide preparations vary in their potency to influence cell proliferation and extracellular matrix synthesis.

An interrelationship between bacterial plaque and host defense mechanisms in the development of gingivitis and periodontitis is now well established (15). In particular, organisms such as *Bacteroides gingivalis* and *Actinobacillus actinomycetemcomitans* appear to be associated with the more severe forms of the periodontal diseases (35, 36). Consequently, numerous bacterial surface components and metabolic products have been implicated in the development and propagation of the disease process. Of these components, lipopolysaccharide (LPS) has received considerable attention (1–3, 11). To date, it has been shown that LPS can modulate cell growth (21, 36, 40), synthetic activity (9, 19, 20), and bone metabolism (17, 24) as well as stimulate macrophages to secrete a variety of lymphokines (16). Thus, this cell surface component of gram-negative bacteria has the capacity to significantly alter the physical properties of tissues either directly by influencing the connective tissue cells or indirectly by inducing an inflammatory response.

The extracellular matrix of gingival connective tissue is composed principally of collagens and cells embedded within a complex nonfibrous gel of which proteoglycans are considered to be a major component (7). Thus, any alteration to the metabolism or synthesis of the proteoglycans could be expected to significantly influence the integrity of the tissues.

Therefore, given the well-documented potency of LPS on connective tissue metabolism and the relative importance of proteoglycans in maintaining physiological balance within tissues, this study examined the effect of LPS derived from two periodontal pathogens, as well as from a standard source, on proteoglycan synthesis by human gingival fibroblasts in vitro.

MATERIALS AND METHODS

Materials. LPS from *Salmonella enteritidis* (trichloroacetic acid extract), sodium pyruvate, L-glutamine, guanidine hydrochloride, phenylmethylsulfonyl fluoride, *N*-ethylmaleimide, 6-aminohexanoic acid, benzamidine hydrochloride, hydroxyurea, and indomethacin were all from Sigma Chemical Co., St. Louis, Mo. Dulbecco-Vogt medium, penicillin, streptomycin, fetal calf serum, and nonessential amino acids were from Flow Laboratories, Irvine, Ayreshire, Scotland. Tissue culture plasticware was from Nunc, Roskilde, Denmark. [6-^{3}H]thymidine (22 Ci/mmol) and $Na_2{}^{35}SO_4$ (1,042 mCi/mmol) were from Amersham (Australia), Sydney, Australia. Ready Solv EP scintillation fluid was from Beckman (Australia), Adelaide, Australia. Chondroitinase ACII (*Arthrobacter aurescens*) and chondroitinase ABC (*Proteus vulgaris*) were from Seikagaku Kogyo Ltd., Tokyo, Japan. Sepharose CL-4B, Sephadex G-100, Sephadex G-50, and Sephadex G-25 (PD-10) were from Pharmacia Fine Chemicals, North Ryde, Sydney, Australia.

LPS extraction and purification. LPSs from *B. gingivalis* 381 and *A. actinomycetemcomitans* AA75 were extracted and purified as described previously (24). Briefly, after cell breakage in a Braun homogenizer, the membrane fraction was prepared first by low-speed centrifugation and finally by ultracentrifugation. The LPS was extracted by the phenol-water method (41) and further purified by ultracentrifugation followed by gel filtration chromatography on Sephadex G-100. Fractions were analyzed for neutral sugars (30). The second and third carbohydrate-positive peaks, designated peaks 2 and 3, have previously been shown to contain the LPS components and were used for subsequent experiments (24). The LPS from *A. actinomycetemcomitans* was extracted in a less rigorous manner. The cells were extracted by the phenol-water procedure (41), and the aqueous phase was used as a crude LPS preparation. This crude fraction contains LPS as evidenced by the presence of the fatty acid 3-hydroxytetradecanoic acid (32).

Fibroblast cultures. Gingival fibroblasts were obtained from explant culture of healthy gingiva from adult volunteers as described previously (26). The cells were maintained in Dulbecco-Vogt medium supplemented with 10% heat-inactivated fetal calf serum, 100 units of penicillin per ml, 100 μg of streptomycin per ml, 2 mM glutamine, 10 mM sodium

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pyruvate, and nonessential amino acids (1%, vol/vol). The fibroblasts were maintained in 75-cm^2 tissue culture flasks in Dulbecco-Vogt medium at 37°C with CO_2-air (1:9). Cells between transfers 4 and 8 in culture were used.

Measurement of cell proliferation. Human gingival fibroblasts were seeded into 24-well plates in triplicate at an initial density of 20,000 cells per well and allowed to attach and spread overnight. The medium was then replaced with 500 μl of medium per well containing increasing concentrations of LPS from 0.5 to 50 μg/ml as well as controls which contained no LPS. After 48 h of incubation, the medium was replaced with 500 μl of medium containing LPS as well as 1 μCi of [^{3}H]thymidine per ml. The cells were incubated for a further 3 h, after which the medium was removed and the cells were washed three times with 500 μl of phosphate-buffered saline. The cells were released from the wells by trypsinization for 15 min at 37°C and were recovered by centrifugation. The cells were lysed with 100 μl of 0.1 M sodium hydroxide for 60 min at 60°C, and the radioactivity was determined in a Beckman LS-2800 liquid scintillation counter.

Effect of LPS on total protein synthesis. The effect of increasing concentrations of LPS on protein synthesis by gingival fibroblasts was assessed by incorporation of [^{3}H]proline into proteins by the cells. Cells were plated in triplicate into 24-well plates and allowed to reach confluence. The medium was removed and replaced with medium containing a range of LPS concentrations from 0 to 50 μg/ml. After 48 h in culture, the medium was replaced with medium containing the same concentration ranges of LPS and also 10 μCi of [^{3}H]proline per ml. After incubation for 24 h, the plates were frozen (−70°C) and thawed. The contents of each well were removed, and the wells were washed twice with 20 μl of 0.1 M sodium hydroxide. Protein was then precipitated by the addition of 200 μl of 50% tricholoroacetic acid–5% tannic acid and incubated at 4°C overnight. The precipitate was recovered by centrifugation, washed twice with 1 ml of 10% trichloroacetate acid–1% tannic acid, and finally dissolved in 50 μl of 0.1 M sodium hydroxide. The sample was then assessed for ^{3}H activity in a liquid scintillation counter.

Effect of LPS on proteoglycan synthesis. The effect of increasing concentrations of LPS on [^{35}S]sulfate incorporation into macromolecules was also studied. Cells were seeded in quadruplicate into 24-well culture plates and allowed to attain confluence. The medium was then removed and replaced with 500 μl of medium per well containing a range of concentrations of LPS from 0 to 50 μg/ml. After incubation at 37°C for 48 h, the medium was removed and replaced with fresh medium containing LPS and [^{35}S]sulfate (20 μCi/ml) and incubated for a further 48 h. The medium was then removed, the cell layers were washed once with 500 μl of phosphate buffered saline, and the medium and wash were pooled. The cell numbers in each of the wells were determined by counting released cells in a hemacytometer. Samples of 0.4 ml of the pooled medium were applied to separate prepacked Sephadex G-25 (PD-10) columns and eluted with 4 M guanidine hydrochloride–0.05 M sodium acetate, pH 5.8. Radioactivity in 0.4-ml fractions was determined by liquid scintillation counting, and the amount recovered in the void volume provided a measure of incorporation of [^{35}S]sulfate into newly synthesized macromolecules (43). Recovery of labeled material loaded onto the columns was monitored and found to be between 85 and 90%.

[^{35}S]sulfate incorporation with time. To determine the effect of LPS on the kinetics of proteoglycan synthesis, we followed incorporation of [^{35}S]sulfate over time. Cells were seeded in quadruplicate into 24-well plates and allowed to reach confluence. The medium was replaced with medium containing 50 μg of *S. enteritidis* LPS per ml and [^{35}S]sulfate (20 μCi/ml). Incorporation of [^{35}S]sulfate into macromolecules was determined at 8, 24, 30, 48, and 72 h after introduction of the radiolabel and LPS. The amount of radiolabeled macromolecules present at each time point was determined by liquid scintillation counting of the effluents from PD-10 columns. Cell numbers were determined by each time point after trypsinization and counting in a hemacytometer.

Pulse-chase experiments. To determine whether or not the effect of LPS on proteoglycan synthesis was due to an alteration in intracellular metabolism, we did pulse-chase experiments. Quadruplicate cultures of confluent cells in 24-well plates were incubated in the presence of 20 μCi of [^{35}S]sulfate per ml and 50 μg of LPS per ml for 24 h, washed four times with phosphate-buffered saline, and then chased in 500 μl of isotope-free medium containing 50 μg of LPS per ml for various periods. At each time point, macromolecular radioactivity in either the cell layer or the medium was determined by PD-10 chromatography and liquid scintillation counting. Cell numbers were determined at each time point after trypsinization and counting in a hemacytometer.

Blocking cell proliferation and prostaglandin synthesis. Since LPS can influence both fibroblast proliferation and prostaglandin E_2 (PGE_2) synthesis (10, 12, 37), we investigated the influence of these parameters on proteoglycan synthesis in the presence of LPS. Cell proliferation and PGE_2 were inhibited by the addition of 10^{-2} M hydroxyurea (34) and 10^{-4} to 10^{-6} M indomethacin (12), respectively, to the cells. Dose-response experiments as described above were then done with increasing concentrations of *S. enteritidis* LPS in either the presence or absence of hydroxyurea or indomethacin.

Proteoglycan extraction. For metabolic labeling of the cells prior to proteoglycan extraction, the fibroblasts were allowed to grow to confluence in 75-cm^2 flasks. The medium was then removed, and the cells were incubated for 48 h in 5 ml of medium with or without 50 μg of LPS per ml. The medium was replaced with fresh medium either with or without LPS and containing 20 μCi of [^{35}S]sulfate per ml, and metabolic labeling proceeded for 48 h (6).

The [^{35}S]sulfate-labeled macromolecules were isolated from the medium and cell layer components by Sephadex G-50 gel filtration in 4 M guanidine hydrochloride–0.05 M sodium acetate (pH 5.8) containing protease inhibitors as described previously (6). Recoveries of [^{35}S]sulfate from these columns were between 85 and 90%. Approximately 5% of the total radioactivity remained with the culture flasks but was not further assessed.

Analytical column chromatography. The molecular size distribution of [^{35}S]sulfate-labeled proteoglycans was analyzed by gel filtration from Sepharose CL-4B. Samples (1 ml) of the [^{35}S]sulfate-labeled material which was excluded from Sephadex G-50 were concentrated in dialysis tubing against Aquacide to 200 μl. The concentrate was then eluted from columns of Sepharose CL-4B (0.7 by 100 cm) with 4 M guanidine hydrochloride–0.05 M sodium acetate (pH 5.8) containing protease inhibitors. Fractions of 0.5 ml were collected at a flow rate of 3 ml/h and were assessed for radioactivity.

Glycosaminoglycan identification. The types of glycosaminoglycans and their relative proportions in the proteoglycans from the medium and cell layer compartments of LPS-treated and untreated cultures were determined by treatment

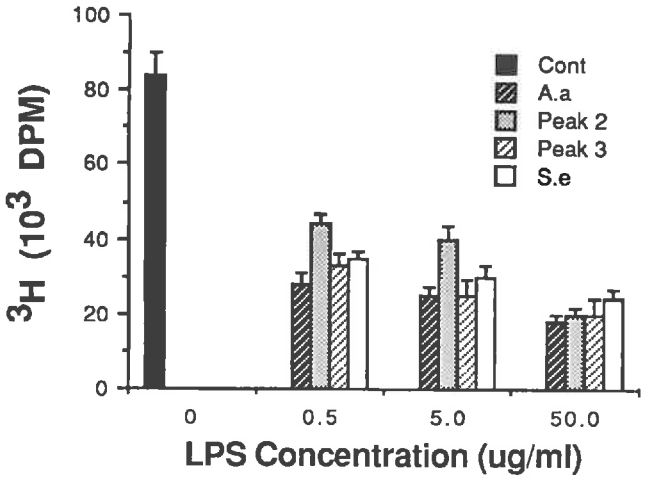

FIG. 1. [^{3}H]thymidine uptake by human gingival fibroblasts in the presence of various concentrations of LPS. The cultures were exposed to LPS for 48 h prior to being pulsed with [^{3}H]thymidine for 3 h. Data represent the means and standard errors (bars) from a representative experiment. Cont, Control; A.a, *A. actinomycetemcomitans*; S.e, *S. enteritidis*.

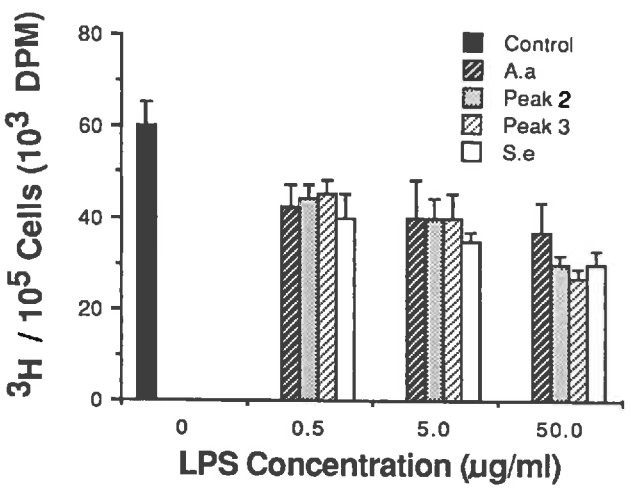

FIG. 2. Incorporation of [^{3}H]proline into protein by human gingival fibroblasts in the presence of increasing concentrations of LPS. Quadruplicate cultures were exposed to LPS for 48 h prior to being labeled with [^{3}H]proline for 24 h. Data represent the means and standard errors (bars) from a representative experiment. A.a and S.e are defined in the legend to Fig. 1.

with chondroitinase ACII, chondroitinase ABC, or nitrous acid (18, 31). The reaction products were then eluted from Sephadex G-50 columns (0.7 by 30 cm) with 0.2% sodium dodecyl sulfate in 0.15 M sodium acetate–1 mM magnesium chloride–1 mM calcium chloride, pH 5.8. Fractions of 0.5 ml were collected at a flow rate of 3 ml/h and were assayed for radioactivity. The relative proportion of each glycosaminoglycan species was determined by calculation of the amount of radiolabeled material resistant to (void volume) or degraded by (included volume) each treatment.

Statistical analysis. All data were subjected to statistical analysis by the method of analysis of variance.

RESULTS

The proliferation rate of human gingival fibroblasts in the presence of increasing concentrations of LPS isolated from *S. enteritidis*, *B. gingivalis*, and *A. actinomycetemcomitans* was assessed by [^{3}H]thymidine uptake (Fig. 1). At all LPS concentrations studied, cell proliferation was significantly reduced ($P < 0.025$). This response appeared to be dose related since the greatest inhibitory effect was noted at the highest concentrations of LPS studied. Within the various LPS preparations studied, the *A. actinomycetemcomitans* preparation consistently inhibited cell proliferation to the greatest extent, while the LPS isolated in the *B. gingivalis* peak 2 fraction had the weakest effect.

To determine whether LPS had any capacity to alter the metabolic activity of human gingival fibroblasts, we monitored total protein synthesis in the presence of increasing concentrations of LPS (Fig. 2). Assuming that the specific activity of the proline pools was not changed by exposure of the cells to LPS, a marked decrease in protein synthesis which was statistically significant ($P < 0.25$) was noted for all the LPS preparations studied. The greatest decrease was noted at the highest LPS concentrations used, and the LPS isolated from *A. actinomycetemcomitans* had the greatest inhibitory effect.

The effect of the various LPS preparations on proteoglycan synthesis was initially monitored by measuring the incorporation of [^{35}S]sulfate into macromolecules released into the medium (Fig. 3). This has previously been shown to be an accurate measure of proteoglycan synthesis (6) since [^{35}S]sulfate is incorporated predominantly into proteoglycans (13). As noted above for protein synthesis, LPS significantly inhibited [^{35}S]sulfate incorporation into macromolecules ($P < 0.025$). This effect was greatest at concentrations of 50 μg/ml at which it accounted for approximately a 50% reduction. At 50 μg/ml, the LPS isolated from *A. actinomycetemcomitans* had the greatest inhibitory effect on proteoglycan synthesis. To determine how quickly LPS inhibited proteoglycan synthesis, we monitored the rate of incorporation of [^{35}S]sulfate into proteoglycans in the presence or absence of *S. enteritidis* LPS over 72 h (Fig. 4). A

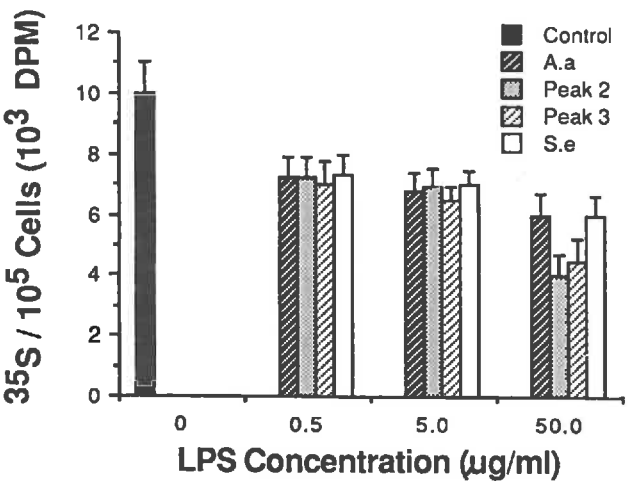

FIG. 3. Proteoglycan synthesis by human gingival fibroblasts exposed to various concentrations of LPS. Quadruplicate cultures were exposed to LPS for 48 h prior to being labeled with [^{35}S]sulfate for 48 h. Incorporation of [^{35}S]sulfate into macromolecules is an accurate measure of proteoglycan synthesis. Data represent the means and standard errors (bars) from a representative experiment. A.a and S.e are defined in the legend to Fig. 1.

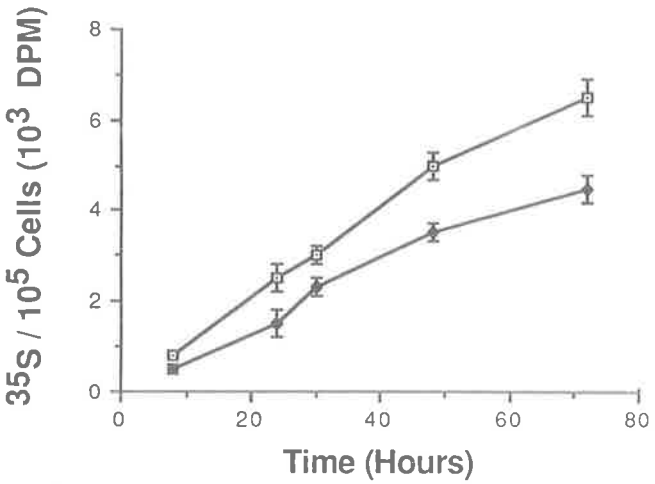

FIG. 4. Time course of effect of LPS on proteoglycan synthesis. Quadruplicate cultures of human gingival fibroblasts were exposed to 50 μg of LPS per ml, and the incorporation of [^{35}S]sulfate into proteoglycans was monitored over various periods. Data represent the means and standard errors (bars) from a representative experiment. Symbols: ⊡, control; ◆, LPS.

steady increase was evident in the release of ^{35}S-labeled proteoglycans into the medium over 72 h for both LPS-treated and untreated cultures. At all time points studied, LPS-treated cells released less ^{35}S-proteoglycans into the medium than the untreated cells did. This difference became statistically significant ($P < 0.05$) after 24 h in culture. To further assess the release and catabolism of prelabeled proteoglycan synthesis in the presence or absence of LPS, we performed pulse-chase experiments (Fig. 5). The release of ^{35}S-labeled proteoglycans from the cell layer into the medium was rapid during the first 8 h of the chase and then leveled off at approximately 30% of the total activity in the medium by 24 to 30 h. The increase in ^{35}S-proteoglycans appearing in the medium was reflected to some extent by a decrease in ^{35}S-proteoglycans within the cell layers. No significant differences were noted between the kinetics of release of ^{35}S-labeled proteoglycans into the medium by LPS-treated or untreated cells.

Since the various LPS preparations studied inhibited gingival fibroblast proliferation (Fig. 1) and because LPS can influence PGE_2 synthesis by fibroblasts in culture, there is a possibility that the effect of LPS on proteoglycan synthesis is a secondary response effected through altered proliferation rate or PGE_2 synthesis. Therefore, the effect of LPS on proteoglycan synthesis was monitored in the presence of agents which can specifically block cell proliferation and PGE_2 synthesis. At 10^{-2} M, hydroxyurea will inhibit gingival fibroblast proliferation by 90% (Bartold, unpublished observation). Despite the exposure of the cells to this agent, proteoglycan synthesis was still depressed in the presence of 50 μg of LPS per ml and was not statistically different from that of cultures treated with LPS in the absence of 10^{-2} M hydroxyurea (Fig. 6). Similarly, if the cells were exposed to a range of concentrations of indomethacin which are known to inhibit PGE_2 synthesis by fibroblasts (12), proteoglycan synthesis remained depressed in the presence of LPS with respect to that of cells cultured in the absence of LPS (Fig. 7). Therefore, the decrease in proteoglycan synthesis by cells cultured in the presence of LPS appears to be independent of either altered cell proliferation or altered PGE_2 synthesis.

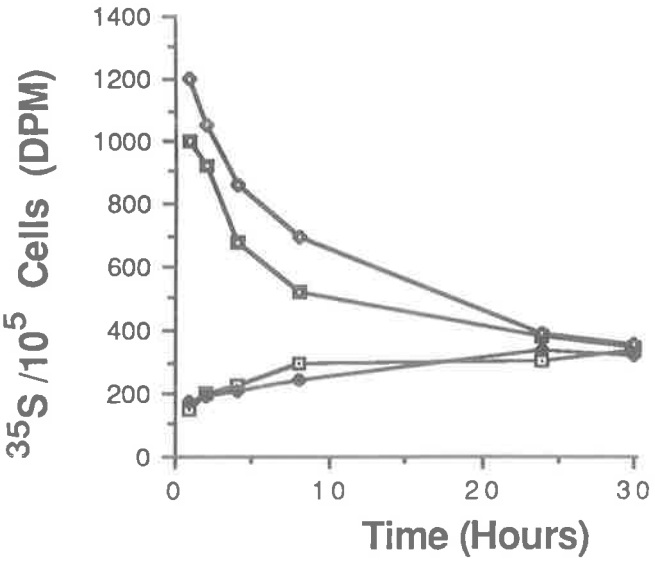

FIG. 5. Effect of LPS on the release of newly synthesized proteoglycans. Human gingival fibroblasts were cultured in the presence or absence of LPS. After the cells were labeled with [^{35}S]sulfate for 24 h, the medium was removed and the cells were incubated in isotope-free medium for various periods. The release of ^{35}S-labeled proteoglycans from the cell layer into the medium was monitored. Data represent the mean values from a representative experiment. Symbols: ⊡, control medium; ◆, LPS medium; ▣, LPS-treated cells; ◈, control cells.

To further define the effect of LPS on the proteoglycans synthesized by human gingival fibroblasts in culture, we determined the molecular size of newly synthesized proteoglycans by gel filtration on Sepharose CL-4B (Fig. 8 and 9). Under the dissociative conditions used (4 M guanidine hydrochloride), various groups of proteoglycans within the medium and cell layer fractions can be identified (4, 6). No remarkable differences between the elution profiles of LPS-treated and untreated cultures could be detected. In addi-

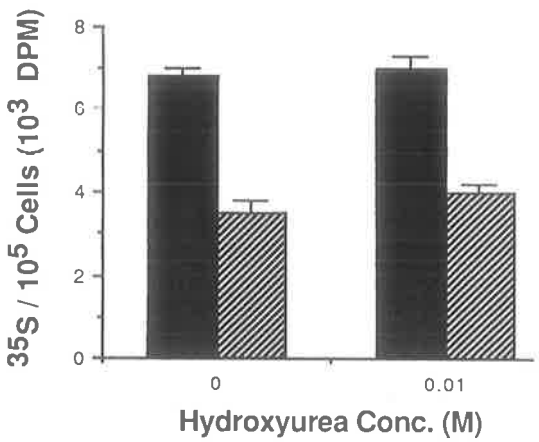

FIG. 6. Effect of inhibition of cell proliferation on synthesis of proteoglycans in the presence of LPS. Quadruplicate cultures of human gingival fibroblasts were cultured in the presence of 10^{-2} M hydroxyurea (a concentration which will inhibit gingival fibroblast proliferation up to 90%), and proteoglycan synthesis in the presence of absence of hydroxyurea and LPS was monitored. Data represent the means and standard errors (bars) from a representative experiment. ■, Control; ▨, LPS.

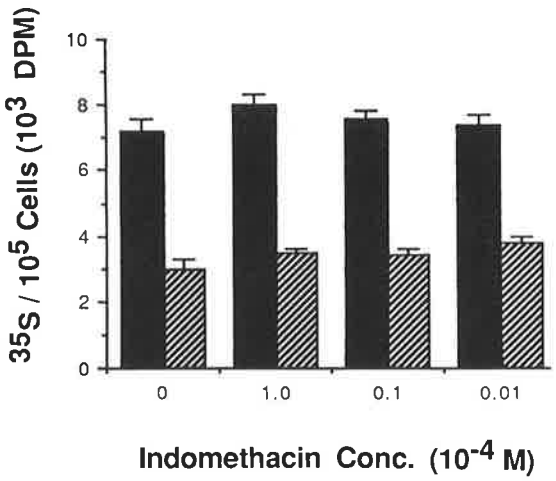

FIG. 7. Effect of inhibition of PGE_2 synthesis on proteoglycan synthesis by fibroblasts in the presence of LPS. Quadruplicate cultures of human gingival fibroblasts were exposed to indomethacin over a concentration range known to inhibit PGE_2 synthesis, and proteoglycan synthesis was monitored in the presence or absence of 50 μg of LPS per ml. Data represent the means and standard errors (bars) from a representative experiment. ■, Control; ▨, LPS.

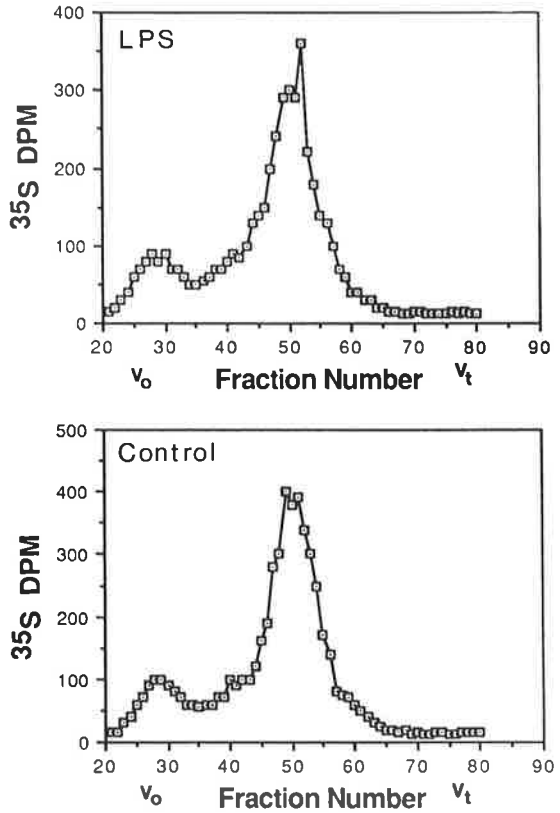

FIG. 8. Sepharose CL-4B gel filtration chromatography of proteoglycans isolated from the medium of human gingival fibroblasts cultured in the presence or absence of LPS. ^{35}S-proteoglycans were isolated from the medium and eluted from Sepharose CL-4B with 4 M guanidine hydrochloride–0.05 M sodium acetate (pH 5.8) containing protease inhibitors. The void volume (V_0) and total volume (V_t) were determined with [^{3}H]DNA and $Na_2{}^{35}SO_4$, respectively.

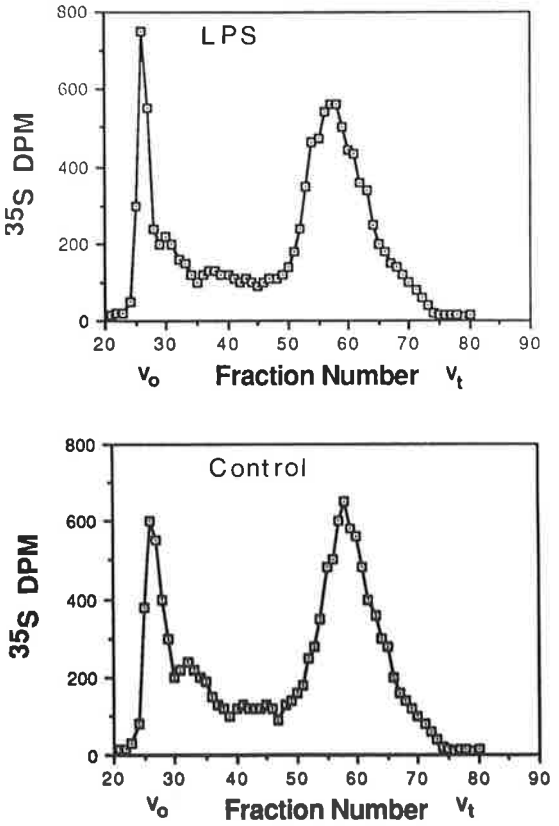

FIG. 9. Sepharose CL-4B gel filtration chromatography of proteoglycans isolated from the cell layer of cultures of human gingival fibroblasts cultured in the presence or absence of LPS. ^{35}S-proteoglycans were eluted from Sepharose CL-4B under the same conditions as described in the legend to Fig. 8.

tion, the relative proportions of proteoglycans within the individual populations did not differ appreciably.

The glycosaminoglycan composition of the newly synthesized proteoglycans was assessed after selective enzymatic and chemical elimination (Table 1). Dermatan sulfate was identified as the predominant glycosaminoglycan species within the medium, and heparan sulfate was the predominant species associated with the cell layers. No significant differences between LPS-treated and untreated cultures were detected.

TABLE 1. Sulfated glycosaminoglycan composition of medium and cell layer proteoglycans[a]

Proteoglycans from:	Percentage of:		
	Chondroitin sulfate	Dermatan sulfate	Heparan sulfate
Medium			
Control	21.8 (± 4)	54.3 (± 4.9)	21.4 (± 2.7)
LPS	29.4 (± 7)	48.5 (± 2.7)	26.6 (± 5.5)
Cell layer			
Control	14.0 (± 5.1)	28.3 (± 4.0)	58.5 (± 2.1)
LPS	15.2 (± 4.5)	22.6 (± 7.0)	60.5 (± 5.6)

[a] Medium and cell layer ^{35}S-proteoglycans were isolated, and the sulfated glycosaminoglycan composition was determined by digestion with chondroitinase ACII, chondroitinase ABC, or nitrous acid. The data represent the mean and standard deviation of the mean of three separate determinations and are expressed as percentages of the total sulfated glycosaminoglycans.

DISCUSSION

LPSs are integral components of the cell wall of gram-negative bacteria. Since several gram-negative species have been implicated in the pathogenesis of the more severe forms of the periodontal diseases, several studies have focused on the biological activity of LPS (1–3, 11). Among the many and varied effects of LPS is an ability to alter fibroblast metabolic activity (9, 19, 20, 37). Gingival fibroblasts are primarily responsible for maintaining the structural components of the periodontium in a state which will permit adequate physiological function. Nonetheless, their role in the pathogenesis of periodontal disease is poorly understood. At present, it appears that phenotypically different subsets of fibroblasts may predominate under inflammatory conditions compared with those found in healthy states (5, 8, 27). Whether or not interactions with inflammatory cells and their products or direct contact with bacterial products are responsible for this phenomenon is not known. In vitro cell systems provide a means of investigating the effects of particular compounds on cells in the absence of the influence of inflammatory cells. Therefore, in the present study we investigated the effect of LPS from a variety of sources on gingival fibroblast proliferation and proteoglycan synthesis.

LPS from all sources studied demonstrated an ability to inhibit gingival fibroblast proliferation and protein synthesis as well as proteoglycan synthesis. With respect to proliferation, these findings are consistent with other studies which have demonstrated an inhibitory effect of LPS or bacterial products on fibroblast proliferation (9, 14, 21, 39). Although the precise mechanism of inhibition of cell proliferation is, as yet, not clear, it seems likely that this results from inhibition of DNA synthesis since endotoxin can be phagocytosed by fibroblasts (14) and is specifically attracted to chromatin, nucleoli, and nuclear membrane (23).

Despite the importance of gingival fibroblasts as principal producers of extracellular matrix components, very little data are available regarding the effect of bacterial components or metabolic products on gingival connective tissue metabolism. Our findings that LPS decreased protein synthesis are in agreement with those of other studies on the effect of whole bacterial extracts or plaque extracts on fibroblast metabolic activity (14, 38, 39). Similar results have also been reported for the effect of LPS on protein metabolism by bone in vitro (24). Nonetheless, Aleo (1) reported that purified LPS from *Escherichia coli* had a stimulatory effect on protein synthesis by 3T6 fibroblasts in vitro. The reason for this discrepancy is not clear, although in our studies *E. coli* LPS had a very weak inhibitory effect on gingival fibroblasts (data not shown). The effects of LPS and bacterial extracts on collagen synthesis have been studied and demonstrate a stimulatory effect of LPS (1) but no discernable effect for whole bacterial extracts (14). Such differences may be accounted for by differences in types of preparations as well as the bacterial source.

To date, no reports, to our knowledge, are available regarding the effect of LPS on proteoglycan synthesis by human gingival fibroblasts. Although proteoglycan synthesis was decreased by LPS, the sizes of the proteoglycans synthesized by cells cultured in the presence of LPS were indistinguishable from those synthesized by control cultures. In this regard, our findings reflect those reported for the effect of LPS on proteoglycan synthesis by articular cartilage (25).

Throughout our studies, we noted that the various LPS preparations differed in the level of inhibition of fibroblast activity. Similar findings have been reported with respect to proliferation in which *B. gingivalis* had a greater inhibitory effect than *Bacteroides intermedius* (21). It is tempting to speculate that such observations indicate that different bacteria have great variance in their potential to affect host tissues. However, it must be emphasized that different LPS preparations and fractions vary in purity and activity (42), and thus additional studies are required before this concept can be confirmed.

The association between LPS and impaired fibroblast function leads us to speculate that this may account, in part, for the tissue disruption noted in gingivitis and periodontitis. Since both cell proliferation and synthetic activity are notably affected by LPS, it seems likely that upon bacterial infection of the gingival sulcus and subsequent ingress of LPS into the tissues (29), there is ample opportunity for the tissues to be affected by compromised fibroblast activity. Indeed, the present findings are interesting in the light of a recent report by Pitaru et al. (28) in which bacterial endotoxin was found to inhibit migration, attachment, and orientation of human gingival fibroblasts in vitro. Proteoglycans and other extracellular matrix components such as fibronectin are considered essential for fibroblast attachment, spreading, and migration (22). Therefore, it is possible that LPS exerts the effect noted by Pitaru et al. (28) by inhibiting synthesis of proteoglycans and other important attachment proteins.

Taken together, these results indicate that LPS from a variety of sources is capable of influencing not only cell proliferation but also extracellular matrix synthesis. These data were obtained from an in vitro system, and thus, extrapolation to the in vivo situation must be made with the appropriate limitations in mind. Nonetheless, the potential significance of these findings lies in two main observations. First, the potency of the various LPS extracts varies, and this could account for the variance in potential virulence of various microbes found to be associated with various forms of periodontal disease. Second, the ability of LPS to alter connective tissue metabolism indicates its potential not only to alter tissue integrity but also to affect the healing capacity of the infected tissues.

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LIPOPOLYSACCHARIDE STIMULATION OF HYALURONATE SYNTHESIS BY HUMAN GINGIVAL FIBROBLASTS *IN VITRO*

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(Accepted 31 May 1991)

Summary—Exposure of gingival fibroblasts to LPS caused a dose-dependent increase in hyaluronate synthesis. Stimulation of hyaluronate synthesis by LPS was significantly greater 24 h after exposure and by 48 h an approx. 50% increase was evident. In parallel, there was an increase in the activity of the hyaluronate synthetase enzyme. Inhibition of PGE_2 synthesis by indomethacin abolished the stimulatory effect of LPS on hyaluronate synthesis. Thus, this stimulatory effect of LPS on hyaluronic acid synthesis may be a secondary response to the induction of PGE_2. The molecular size of newly synthesized hyaluronate was not affected by LPS. The metabolic changes observed may be a primary response of the cells to bacterial toxins and may aid extracellular matrix repair.

Key words: lipopolysaccharide, hyaluronate, fibroblasts.

INTRODUCTION

Lipopolysaccharide, a major component of the outer membrane of Gram-negative bacteria, has received considerable attention in relation to the pathogenesis of the periodontal diseases (Daly, Seymour and Kieser, 1980). In particular, it has been shown to modulate cell growth (Vaheri *et al.*, 1973; Larjava *et al.*, 1987), stimulate bone resorption (Hausman, Raisz and Miller, 1970; Millar *et al.*, 1986) and induce lymphokine release by macrophages (Hanazawa *et al.*, 1985). Thus, it is considered to be a potent biological agent capable of important effects in infected tissues either by direct influences on cell metabolism, or, indirectly, by invoking an inflammatory response.

The connective tissues of the periodontium have both fibrous and non-fibrous elements which, together, impart structural and physiological properties that allow adequate function (Narayanan and Page, 1983; Bartold, 1987). However, upon induction of inflammation, numerous changes occur within the extracellular matrices of the periodontium (Page and Schroeder, 1976). In addition to ultrastructural changes, the metabolism of the resident fibroblasts is also disturbed. The combination of tissue destruction and altered cellular activity leads to compromised function.

In recent years, the metabolism and degradation of periodontal collagens has been well studied (Narayanan and Page, 1983). However, other principal components of the extracellular matrix such as proteoglycans and hyaluronate have received less attention (Bartold, 1987). Therefore, as hyaluronate has been implicated in critical functions such as the regulation of cell–cell interactions, tissue hydration, and control of macrophages and lymphocytes (Comper and Laurent, 1978), my purpose now was to investigate the effect of LPS on hyaluronate synthesis by human gingival fibroblasts *in vitro*.

Abbreviations: CPC, cetylpyridinium chloride; DVM, Dulbecco–Vogt medium; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PG, prostaglandin.

MATERIALS AND METHODS

Materials

Guanidine HCl, indomethacin, glutamine, adenosine 5′ triphosphate (ATP), and UDP-N-acetylglucosamine were all purchased from the Sigma Chemical Co., St Louis, MO, U.S.A. CPC was from Ajax Chemicals Australia, Aulam, N.S.W.; filter paper (3MM) from Whatman, Maidstone, U.K.; *Streptomyces* hyaluronidase from Seikagaku Kogyo Ltd, Tokyo, Japan; *Streptomyces griseus* pronase from Calbiochem, San Diego, CA; Sephadex G-50 and Sepharose CL-2B from Pharmacia Fine Chemicals, North Ryde, Sydney, Australia; DVM, fetal bovine serum, penicillin, streptomycin and non-essential amino acids were all from Flow Laboratories, Irvine, Ayrshire, U.K.; tissue-culture ware was from Nunc, Roskilde, Denmark; D-[6-^{3}H]-glucosamine HCl (33 Ci/mmol) and UDP-D-[U-^{14}C]-glucuronic acid (225 mCi/mmol) were from Amersham (Australia), Sydney, Australia; Ready Solv EP scintillation fluid was from Beckman (Australia), Adelaide, Australia.

LPS preparation

LPS from *Actinobacillus actinomycetemcomitans* was a gift from Dr E. Hausman's laboratory, State University of New York at Buffalo and had been extracted by the phenol–water method (Westphal and Jann, 1965). Although no subsequent purification was carried out, and it was considered a crude LPS preparation, it has significant biological activity

attributable to LPS (Bartold and Millar, 1988). LPS from *Salmonella enteritidis* was purchased from Sigma Chemical Co., St Louis, MO, U.S.A. It had been extracted with phenol and purified by gel filtration chromatography. Both its protein and RNA contents were less than 1%.

Fibroblast cultures

Fibroblasts were obtained by explant culture from biopsies of healthy human gingiva from volunteers, as described by Narayanan, Page and Kuzan (1978); these have been used extensively in previous studies (Bartold and Page, 1986a; Bartold and Millar, 1988). The cells were maintained in DVM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml streptomycin, 2 mM glutamine and non-essential amino acids at 37°C in an atmosphere of 5% CO_2 and 95% air. Cells between the 4th and 8th transfer in culture were used. All experiments, unless otherwise stated, were carried out in complete DVM containing 10% fetal bovine serum.

Radioisotope incorporation studies

Initial studies were designed to investigate the effect of various preparations of LPS on hyaluronate synthesis by the fibroblasts. Fibroblasts were seeded in triplicate at a concentration of 5×10^4 cells per well, into 24-well plates. Upon reaching confluence (1×10^5 cells/well), the cells were incubated in medium containing LPS from either *S. enteritidis* or *A. actinomycetemcomitans* ranging from 50 to 0.5 μg/ml for 24 h and pulsed with [^{3}H]-glucosamine (20 μCi/ml) for the entire period. The medium was then removed and the cell layers washed once with PBS, which was pooled with the medium to make up a fraction for subsequent analyses of hyaluronate content. The cells were released from the culture plates by trypsinization. A sample was taken for counting cell numbers in a haemocytometer and the remainder was frozen until required.

To determine the effect of LPS on the rate of hyaluronate synthesis, confluent cells were cultured as described above. Upon reaching confluence the medium was removed and the cells incubated in the presence or absence of 50 μg/ml *S. enteritidis* LPS for 4, 8, 24, 30 48 and 72 h. The incorporation of [^{3}H]-glucosamine into hyaluronate was determined by selective digestion with hyaluronidase for each time point after the introduction of the radiolabel and 50 μg/ml LPS.

As PGE_2 can influence hyaluronate synthesis and because LPS has been reported to stimulate prostaglandin synthesis in other systems (Yaron *et al.*, 1978; Wahl *et al.*, 1977), the effect of *S. enteritidis* LPS on hyaluronate synthesis in the absence of endogenous PGE_2 was investigated. Gingival fibroblasts were grown to confluence in 24-well plates and incubated in the presence or absence of 50 μg/ml LPS in DVM containing 20 μCi/ml [^{3}H]-glucosamine for 24 h. To inhibit PGE_2 synthesis, indomethacin ranging in concentration from 10^{-6} to 10^{-4} M was also added to some cultures. This concentration range inhibited PGE_2 synthesis by fibroblasts in culture (Dayer *et al.*, 1979) and was without toxic effects on human gingival fibroblasts (Bartold, 1988). Hyaluronate synthesis was monitored by hyaluronidase digestion of molecules labelled with [^{3}H]-glucosamine.

To obtain material for molecular-size analysis, human gingival fibroblasts were grown to confluence in 25-cm^2 culture flasks. Upon attaining confluence (5×10^5 cells per flask) the culture medium was removed and the cells cultured in the presence or absence of 50 μg/ml *S. enteritidis* LPS for 24 h in DVM containing 20 μCi/ml [^{3}H]-glucosamine. After incubation the medium was removed and the cell layers washed with 3 ml PBS which was then pooled with the medium to make up this fraction. The remaining cells in culture vessels were then extracted with 5 ml of 4 M guanidine HCl/0.05 M sodium acetate, pH 5.8, overnight at 4°C. After 24 h at 4°C this cell fraction was centrifuged to remove any debris and the supernatant used for subsequent analyses as the cell-layer fraction. Both the medium and cell-layer extracts were desalted by gel filtration chromatography on Sephadex G-50 to remove unincorporated [^{3}H]-glucosamine (Bartold and Page, 1986a). The labelled macromolecular material which eluted in the void volume of Sephadex G-50 (1×30 cm) was used for subsequent analyses of hyaluronate molecular size.

Determination of ^{3}H-labelled hyaluronate

Hyaluronate synthesis by human gingival fibroblasts *in vitro* was determined as described previously (Castor *et al.*, 1981; Huey, Stair and Stern, 1990). In brief, samples (200 μl) from the culture medium were digested with pronase (10 mg/ml) overnight at 60°C. They were then boiled and split into two portions, each of 50 μl. One portion (designated B) was incubated at 37°C for 12 h with 60 μl *Streptomyces* hyaluronidase in 0.1 M sodium acetate buffer at pH 6.0. The other (designated A) was incubated in the sodium acetate buffer alone under identical conditions. After the incubation, samples (100 μl) from each part were spotted onto filter paper divided into 2-cm squares, allowed to dry at 60°C, and then placed through four washes, each of 30-min duration, in 0.05 M sodium chloride containing 0.1% CPC at room temperature (22°C). The filter paper was then removed and allowed to dry. Each square was then cut into small strips and placed in 14 ml of scintillation fluid before determining the radioactivity in a liquid scintillation counter. The amount of hyaluronate in each sample was calculated as the amount of radioactivity digested by the *Streptomyces* hyaluronidase (i.e. dis/min in portion A – dis/min in portion B.

Pulse-chase experiments

To determine whether or not the effect of LPS on hyaluronate synthesis was due to an increase in rate of synthesis or decrease in rate of breakdown, pulse-chase experiments were made. Fibroblasts were seeded, in triplicate at an initial density of 5×10^4 cells per well, and allowed to grow to confluency (1×10^5 cells/well). The confluent monolayers were then incubated in the presence of 20 μg/ml [^{3}H]-glucosamine and 50 μg/ml LPS for 24 h, washed four times with PBS and then chased in 500 μl of

isotope-free medium containing 50 μg/ml LPS for various times. At each time, the medium was removed and the cell layers scraped from the plates. Hyaluronic determinations were carried out on both the medium and cell-layer fractions.

Hyaluronate synthetase activity

Gingival fibroblasts were grown to confluence in 24-well plates and cultured in the presence or absence of 50 μg/ml *S. enteritidis* LPS for 8, 24 and 48 h. The cells were removed from the culture surface by trypsinization and the cell pellet obtained by gentle centrifugation was washed twice with PBS before freezing at −70°C. The cells were lysed by freezing and thawing the pellet three times. Hyaluronate synthetase activity was determined by incubating the lysed cells in the presence of 0.1 μCi UDP-D-[U-^{14}C]-glucuronic acid and 5 × 10-3 μM UDP-*N*-acetyl-glucosamine as substrates (Appel, Horwitz and Dorfman, 1979). The reaction mixture was incubated for 90 min at 37°C and the amount of hyaluronate thus synthesized was determined by selective digestion with hyaluronidase as described above.

Molecular-sieve chromatography

The molecular size of hyaluronate synthesized in the presence or absence of *S. enteritidis* LPS was assessed after 48 h in culture using Sepharose CL-2B, as described by Bartold and Page (1986a). Approximately the same amounts (40,000 dis/min) of radiolabelled macromolecular material isolated from the medium and cell-layer fractions by Sephadex G-50 chromatography were eluted from Sepharose CL-2B (0.7 × 100 cm) with 4 M guanidine HCl in 0.05 M sodium acetate, pH 5.8. With a flow rate of 3 ml/h, fractions of 0.5 ml were collected and 50-μl samples were taken for radioactivity determination in a liquid scintillation counter. The material which eluted in the void volume was pooled separately from that which eluted in the included volume. These two pools were indicative of the two major molecular-size fractions of [^{3}H]-glucosamine-labelled material eluting from Sepharose CL-2B. After dialysis against 0.1 M sodium acetate, the hyaluronate content in these fractions was determined by selective digestion with *Streptomyces* hyaluronidase (Bartold and Page, 1986a).

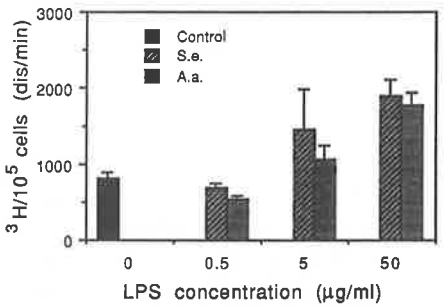

Fig. 1. Effect of LPS from an oral and non-oral source on the release of hyaluronate into the medium by human gingival fibroblasts. Triplicate cultures of human gingival fibroblasts were maintained in the presence or absence of various concentrations of *S. enteritidis* and *A. actinomycetemcomitans* LPS. The incorporation of [^{3}H]-glucosamine into hyaluronate was monitored after 24 h. Data are presented as the mean and SD of the mean from triplicate cultures. Abbreviations: S.e., *S. enteritidis* LPS; A.a., *A. actinomycetemcomitans* LPS.

Statistical analysis

All data were subjected to analysis of variance.

RESULTS

The initial experiments to determine the biological effect of LPS on hyaluronic acid synthesis (Fig. 1), showed that, in all cases, exposure of the fibroblasts to higher (5 and 50 μg/ml) concentrations of LPS increased that synthesis. Concentrations of LPS up to 50 μg/ml had no visible effect on the cells whereas at concentrations of 500 μg/ml, detachment and death occurred (data not shown). Cell counts after the experiments showed no significant change between LPS-treated and -untreated cells (data not shown). Regardless of the source of the LPS, the greatest stimulation of hyaluronate production occurred at the highest concentration which did not cause cell death (50 μg/ml). There was very little statistical variation between the effects of the two different types of LPS used. Due to the limited amount of *A. actinomycetemcomitans* LPS available for analysis, the remainder of the experiments was done with *S. enteritidis* LPS.

Time-course studies to determine how quickly the stimulation of hyaluronate synthesis became effective in the presence of 50 μg/ml LPS showed a steady increase in the release of ^{3}H-labelled hyaluronate into the medium over 72 h for both treated and untreated cultures (Fig. 2). At all times, LPS-treated cells released more ^{3}H-labelled hyaluronate, but this difference was not statistically significant until 48 h ($p < 0.05$). The amount of radiolabelled hyaluronate associated with the cell layers of treated and untreated cultures also increased over time. Although the LPS-treated cells appeared to have greater amounts of ^{3}H-labelled hyaluronate associated with their cell layers, there was little statistical difference between them even after 72 h.

The effect of LPS on newly synthesized, cell-associated, [^{3}H]-glucosamine-labelled hyaluronate, assessed after a pulse chase for various times (Fig. 3), was to

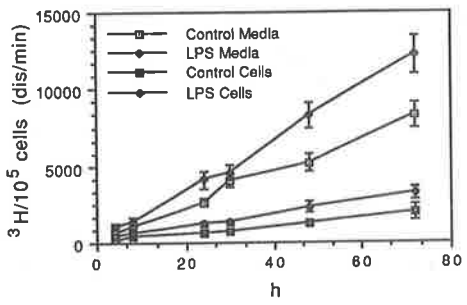

Fig. 2. Time course of the effect of LPS on hyaluronate synthesis. Triplicate cultures of human gingival fibroblasts were maintained in the presence or absence of 50 μg/ml *S. enteritidis* LPS and the incorporation of [^{3}H]-glucosamine into hyaluronate was monitored over 48 h for both the medium and cell layer. Data are presented as the mean and SD of the mean.

produce no remarkable differences in the kinetics of release of labelled hyaluronate between treated or untreated cells. The labelled hyaluronate associated with the cell layer initially decreased rapidly and then more slowly, reaching 50% of the zero-time value by around 15 h. This decrease was accompanied by a concomitant increase of labelled hyaluronate in the medium.

As hyaluronate synthesis is regulated by the enzyme hyaluronate synthetase, the effect of LPS on this enzyme was monitored at various times after introduction of LPS to the cultures (Fig. 4). After isolation of the enzyme from the cells, incubation with the appropriate radioactive substrates showed increased hyaluronate synthetase activity at 48 h in the control cultures. There was greater enzyme activity at all times in those cultures exposed to LPS; by 48 h, these had an approx. 100% increase in activity, while the controls had a 60% increase.

To determine whether the cell response was a direct effect of LPS, or mediated via a secondary signal such as increased PGE_2 production, endogenous PGE_2 synthesis was blocked by exposing the cells to indomethacin (Fig. 5). In the absence of indomethacin, hyaluronate synthesis was significantly greater in the cultures exposed to LPS than in controls. Although indomethacin did not appear to influence hyaluronate synthesis greatly in the controls, it reduced hyaluronate synthesis in LPS-treated cultures to control levels.

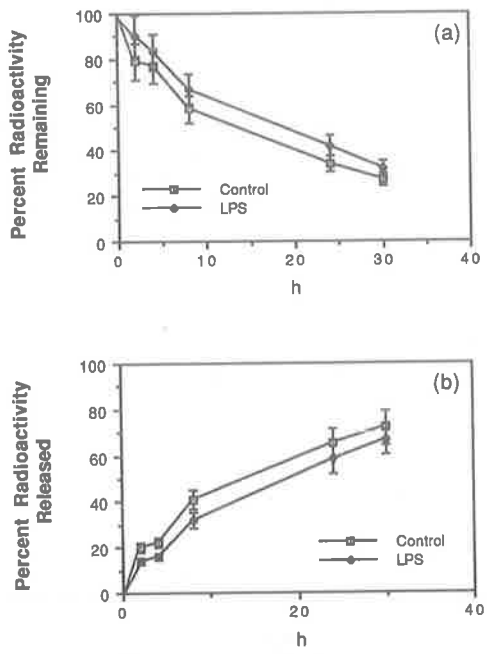

Fig. 3. Pulse chase of newly synthesized hyaluronate. Triplicate cultures of human gingival fibroblasts were maintained in the presence or absence of 50 μg/ml *S. enteritidis* LPS and the incorporation of [^{3}H]-glucosamine into hyaluronate was allowed to proceed for 24 h. The medium was then removed and the cells washed extensively before incubation in isotope-free medium for various periods of time. The presence of [^{3}H]-hyaluronate in both the cell layers (a) and medium (b) was determined by selective digestion with *Streptomyces* hyaluronidase. Data are presented as the mean and SD of the mean.

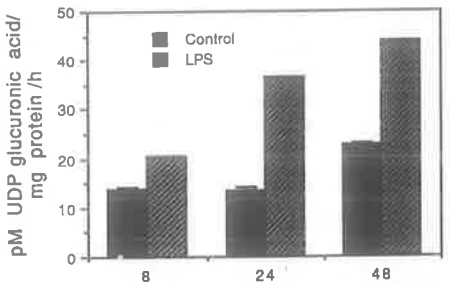

Fig. 4. Effect of LPS on hyaluronate synthetase activity at various time points. Enzyme activity was assessed by the incorporation of UDP-D-[U-^{14}C]-glucuronic acid into hyaluronate at selected time points in either the presence or absence of 50 μg/ml *S. enteritidis* LPS. Data represent the mean and SD of the mean of triplicate cultures.

In the assays of effect of LPS on the molecular size of newly synthesized hyaluronate (Fig. 6), macromolecular material from both the cell layers and the medium eluted from Sepharose CL-2B in two major populations; one which was excluded from the gel and the other which eluted in the included volume. When these two broad molecular populations were digested with *Streptomyces* hyaluronidase and the hyaluronate content determined (Table 1), for the medium, most of the material in the void volume was hyaluronate, whereas a smaller proportion of the included peak was hyaluronate. Although a modest increase in larger molecular-weight hyaluronate was evident in the LPS-treated cultures, the differences were not significant, owing to the large variance. Similar findings were made for material isolated from the cell layers (Table 1).

DISCUSSION

Crude extracts of human dental plaque can alter fibroblast proliferation, metabolism and hyaluronate synthesis (Larjava *et al.*, 1983; Larjava, 1984; Larjava and Jalkanen, 1984). These observations are expanded by my initial findings that LPS, from two different sources, can influence hyaluronate synthesis

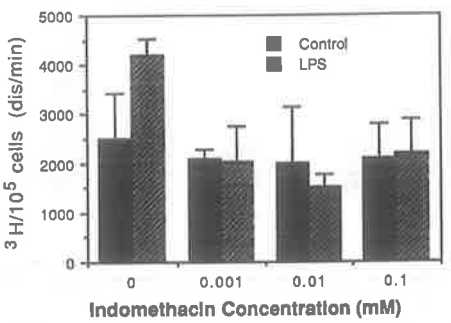

Fig. 5. Effect of inhibition of PGE_2 synthesis on hyaluronate synthesis. Triplicate cultures of human gingival fibroblasts were maintained in the presence or absence of 50 μg/ml *S. enteritidis* LPS and exposed to a range of concentrations of indomethacin known to inhibit prostaglandin synthesis by fibroblasts. Data are presented as the mean and SD of the mean.

by human gingival fibroblasts *in vitro*. Regardless of the source, LPS stimulated the amount of hyaluronate associated with cells that had been exposed to LPS. However, various LPS molecules are known to differ in their biological activity, depending upon their chemical composition (Kotani and Takada, 1990). Therefore, whether these initial observations reflect a general effect of all LPS molecules or are specific for certain types of LPS remains to be established. Because of the limited amount of LPS from *A. actinomycetemcomitans* available, I was able to study only the effects of *S. enteritidis* LPS on hyaluronate synthesis.

The increase in macromolecular hyaluronate associated with cells exposed to LPS appears to be in direct contrast to previous findings that LPS inhibited synthesis of proteoglycans and proteins by human gingival fibroblasts (Bartold and Millar, 1988). Nonetheless, my present findings are in agreement with several other studies in which there was stimulation of hyaluronate synthesis by synovial and lung fibroblasts exposed to various concentrations of LPS from *E. coli* (Buckingham, Castor and Hoag, 1972; Buckingham and Castor, 1975). Furthermore, LPS has a differential effect on proteoglycan and hyaluronate synthesis by lung fibroblasts (Castor *et al.*, 1983).

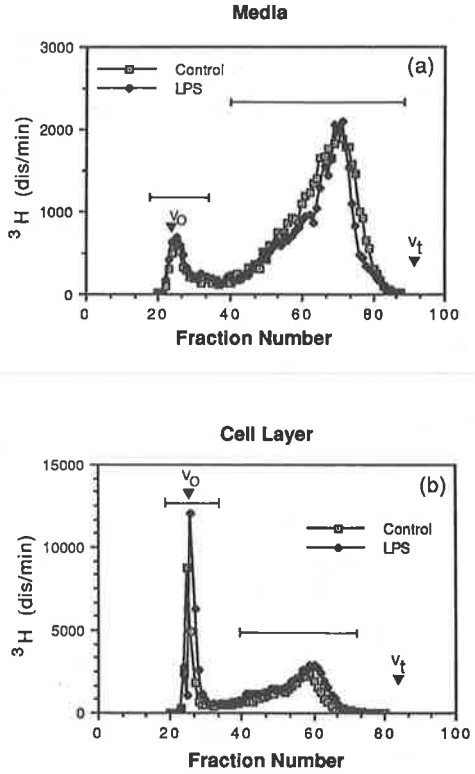

Fig. 6. Sepharose CL-2B gel filtration chromatograms of ^{3}H-labelled macromolecules isolated from (a) medium and (b) cell layer of human gingival fibroblast cultures maintained for 24 h in either the presence or absence of 50 μg/ml *S. enteritidis* LPS. The void volume (v_o) and total volume (v_t) were determined by the elution of [^{3}H]-DNA and [^{35}S]-sulphate respectively and are marked with arrow heads. The bars represent the fractions which were pooled for subsequent hyaluronate determinations in different molecular size pools.

Table 1. Effect of LPS on molecular-size distribution of hyaluronate

	Control*		LPS*	
	Peak 1	Peak 2	Peak 1	Peak 2
Media	69 ± 17	24 ± 4	82 ± 11	18 ± 7
Cell layer	45 ± 9	7 ± 1	49 ± 5	5 ± 1

*Data are the percentage of ^{3}H-labelled material digested by *Streptomyces* hyaluronidase.

The increase in the amount of labelled hyaluronate associated with cells exposed to LPS could reflect either an increase in the rate of synthesis or a decrease in rate of breakdown. From my results, it seems likely that the effect is one of increased synthesis. First, cells exposed to LPS incorporated [^{3}H]-glucosamine into hyaluronate faster than did control cells (time-course studies). Secondly, the release of newly labelled hyaluronate from cells exposed to LPS proceeded at a rate similar to that in the control cultures (pulse-chase experiments). As the breakdown of glycosaminoglycans may be reflected in the rate of loss of newly labelled glycosaminoglycans from cells (Fratantoni, Hall and Neufeld, 1968; Hopwood and Dorfman, 1977), these findings indicate that the alteration in levels of hyaluronate associated with cells exposed to LPS represents an increase in the rate of synthesis as distinct from a decreased rate of breakdown.

LPS at high concentrations is toxic to gingival fibroblasts (Bartold and Millar, 1988), so the response of increased hyaluronate synthesis by cells exposed to LPS may be the harbinger of toxicity. However, although LPS may exert a permissive effect on cells, enabling them to respond more efficiently to serum stimulants (Smith, 1976), the precise mechanism by which stimulation of hyaluronate synthesis occurs is not yet clear. Indeed, hyaluronate synthesis is not regulated in the same way as that of other known glycosaminoglycans (Kleine, 1981), and attempts to influence hyaluronate synthesis by chemicals that affect the synthesis of other glycosaminoglycans have been unsuccessful (Hart and Lennarz, 1978; Hopwood and Dorfman, 1977; Mapleson and Buchwald, 1981). One explanation for such findings may be that the site of hyaluronate synthesis seems to be at the plasma membrane rather than the Golgi (Prehm, 1984). Another explanation of the stimulation of hyaluronate synthesis may lie in the effect of LPS on the proliferation rate of the cells. LPS inhibits gingival fibroblast proliferation (Bartold and Millar, 1988); also, upon inhibition of gingival fibroblast proliferation with hydroxyurea, hyaluronate synthesis is increased (Bartold, 1988). This may reflect a response of the cells to being in a subconfluent state, as hyaluronate production is related not only to proliferation but also cell density (Hopwood and Dorfman, 1977; Toole, 1981). It is possible that a similar response to cell densities may apply when the cells are exposed to LPS, but all my experiments were done with confluent monolayer cultures to reduce such an effect.

The level of hyaluronate synthetase activity was raised in cultures exposed to LPS. This increase was consistent with the observed stimulation of hyaluronate synthesis by LPS and supports the notion that the amount of hyaluronate production varied with time and exposure to LPS. Although an increase in hyaluronate synthetase activity with time has been reported before (Mian, 1986; Brecht *et al.*, 1986), my data are interesting in that they show a cumulative effect of an endogenous agent on the activity of this enzyme.

A further complicating factor in studies concerning the control of extracellular matrix production is the role of secondary mediators. One commonly encountered mediator is PGE_2: PGE_2 synthesis by synovial fibroblasts is increased by LPS (Yaron *et al.*, 1980); PGE_2 can stimulate the synthesis of hyaluronate by fibroblasts (Yaron *et al.*, 1978). Therefore, the LPS-induced enhancement of hyaluronate synthesis could have been mediated through a PGE_2-dependent pathway. I found that inhibition of PGE_2 synthesis by indomethacin abolished the stimulatory effect of LPS on hyaluronate synthesis. Thus the stimulation of hyaluronate by LPS may be secondary to induced endogenous PGE_2 synthesis.

My work confirms the findings of a previous study which showed selective size distribution of hyaluronate between the different cell-culture compartments of fibroblasts (Nakamura *et al.*, 1990). My finding that the molecular size of the newly synthesized hyaluronate was not affected by LPS is important. In chronic periodontal disease, significant alterations occur in both the content and size of hyaluronate (Bartold and Page, 1986b). Whether these changes are the result of exogenous factors (e.g. enzymes and superoxides) or because of altered cell metabolism has been unclear. Although my study does not completely resolve this question, the findings imply that LPS does not contribute directly to the alteration in the molecular size of hyaluronate found in chronic gingival inflammation.

Hyaluronate is considered to play a significant part in the regulation and maintenance of normal tissue functions (Comper and Laurent, 1978). For example, it plays a primary role during wound repair and can significantly influence macrophage, lymphocyte and fibroblast functions (Balazs and Darzynkiewicz, 1973; Forrestor and Balazs, 1980; Goldberg and Toole, 1987). That LPS stimulates hyaluronate synthesis by human gingival fibroblasts *in vitro* suggests that this could be a fundamental response by the cells to produce an extracellular matrix that is conducive to adequate host defence and repair.

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Paper 20.

Bartold, P.M., Gully, N.J., Zilm, P.S. and Rogers, A.H. Identification of components in Fusobacterium nucleatum chemostat-culture supernatants that are potent inhibitors of human gingival fibroblast proliferation. *Journal of Periodontal Research* **26:** 314-322, 1991.

Candidate's Contribution to this paper: 70%

P.M. Bartold's role in this study was:
Provision of research funds
Design of the experiments
Execution of the experiments
Writing of the manuscript

N.J. Gully's role in this study was:
Provision of culture supernatants
Writing of the manuscript

P.S. Zilm's role in this study was:
Provision of culture supernatants
Writing of the manuscript

A.H. Rogers' role in this study was:
Provision of research funds
Provision of culture supernatants
Writing of the manuscript

Identification of components in *Fusobacterium nucleatum* chemostat-culture supernatants that are potent inhibitors of human gingival fibroblast proliferation

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Bartold PM, Gully NJ, Zilm PS, Rogers AH: Identification of components in Fusobacterium nucleatum *chemostat-culture supernatants that are potent inhibitors of human gingival fibroblast proliferation. J Periodont Res 1991; 26: 314–322.*

This present investigation concerned the effect of chemostat-culture cell-free supernatants of *Fusobacterium nucleatum* on the growth and synthetic activity of human gingival fibroblasts *in vitro*. Human gingival fibroblasts were cultured in fetal calf serum supplemented Dulbecco-Vogt medium containing various dilutions of conditioned or unconditioned bacterial culture medium. Cell proliferation was monitored by assessing cell growth over 5 days or incorporation of [^{3}H]-thymidine into DNA. Protein and proteoglycan synthesis were monitored by the incorporation of [^{3}H]-proline and [^{35}S]-sulfate, respectively, into macromolecules. While the conditioned culture medium caused a complete inhibition of cell growth and incorporation of [^{3}H]-thymidine DNA, there was no discernible effect on protein or proteoglycan synthesis. This indicated that the cells remained viable yet unable to divide. Such a view was supported by the observation that the inhibitory effect was reversible upon removal of the conditioned medium. This activity had a molecular size less than 30000, was heat-stable and nonvolatile. Chemical analysis of the conditioned bacterial culture supernatants indicated that high proportions of butyrate, ammonium, and acetate were present. When these components were added to unconditioned medium and tested, most of the inhibitory activity could be attributed to ammonium and butyrate. Since many bacteria which constitute the subgingival microflora release ammonium and butyrate, a very high concentration of these metabolites may well accumulate. Clearly, the potential for inhibition of fibroblast proliferation has ramifications related to diminished tissue repair following bacterially-induced periodontal destruction.

Key words: *Fusobacterium nucleatum* – periodontal disease – gingival fibroblasts – proliferation

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Introduction

With the determination of a close association between dental plaque and the various periodontal diseases, attention has focussed upon the pathogenic features associated with dental plaque microbiota. As a result of recognizing the important ecological relationships between aerobic and anaerobic bacteria in developing periodontal lesions, various groups of bacteria have become implicated in the disease process of periodontitis (1, 2). However it was not until suitable anaerobic culture techniques were developed that studies on the fastidious anaerobic microflora associated with the periodontal disease became possible and thus allowed more precise identification of many so-called "periodontal pathogens" (2). With such identification, there has followed an analysis of the myriad pathogenic and toxic factors elicited by (and associated with) these bacteria which could be implicated in the disease process. Amongst these factors, LPS, various enzymes and leukotoxin have received prominent attention (3–5). Their specific roles seem to be related to disruption and dissolution of the gingival extracellular matrix as well as causing impaired fibroblast, polymorphonuclear leukocyte and lymphocyte function. Although these components should not be considered in iso-

lation, the identification and elucidation of their biological activity is important in understanding the potential pathways for the establishment of periodontal destruction.

By far the most common way of studying bacteria is in a closed, conventional batch culture system. While this method appears to be adequate for many purposes, it has several drawbacks for metabolic and growth studies since the growth characteristics of the bacteria in these systems do not closely resemble those seen *in vivo*. However, by using a chemostat (a continuous culture device) one is able to control stringently both the growth rate of microorganisms and their environment and thus study bacteria in an environments that more closely resembles those found *in vivo* (6).

The purpose of the present investigation was to study the effect of cell-free chemostat culture supernatants of one potential periodontal pathogen (*Fusobacterium nucleatum*) on the growth and synthetic activity of human gingival fibroblasts *in vitro*. Identification of the modulatory factors in these culture supernatants was determined by a series of a deductive experiments.

Material and Methods

Materials

Dulbecco-Vogt medium (DVM), fetal calf serum (FCS), non-essential amino acids, penicillin and streptomycin were purchased from Flow Laboratories Australasia Pty., Ltd., North Ryde, New South Wales; all tissue culture plastic ware was obtained from Nunc, Roskilde, Denmark; sodium pyruvate and L-glutamine were from Sigma Chemical Co., St. Louis, MO; [6-^{3}H]-thymidine (22 Ci/mMol), L-[2,3,4,5-^{3}H]-proline (100 Ci/mMol) and $Na_2[^{35}S]O_4$ (1042 mCi/mMol) were from Amersham (Australia Pty., Ltd., North Ryde, New South Wales; Ready Solv EP liquid scintillation fluid was from Beckman Australia, Adelaide; Sephadex PD-10 columns from Pharmacia Fine Chemicals, North Ryde, New South Wales; chemically inert 0.22 μm Durapore filters were obtained from the Millipore Corporation, Molsheim, France; Antricon 30 Ultrafiltration Concentrators were from Amicon Division, W. R. Grace, Danvers MA.

Cell-free Culture Supernatants of *F. nucleatum*

F. nucleatum (ATCC #10953) was obtained from Dr. H. N. Shah, The London Hospital Medical College, U.K. It was grown in the previously described chemically defined medium. The levels of glucuronate, histidine, serine and lysine were increased to 20, 10, and 10 mM, respectively; glucose was added to 20 mM; Tween 80 (0.2 mg/ml) was added to aid cell dispersion as was thioglycollic acid (0.5 mg/ml) to maintain a low redox potential. Growth in the chemostat (model C-30, Bio-Flo;, New Brunswick Scientific, Edison, NJ) at 37°C, controlled pH of 7.4, 6.8 or 5.8 and imposed dilution of $D = 0.1\ h^{-1}$ ($t_d = 7$ h) was achieved by methods described previously (7). Anaerobic conditions were maintained by continuous gassing of both the culture vessel and medium reservoir with a N_2/CO_2 (90:10) mixture.

Steady-state conditions were established after about 7 volume changes, following which culture samples were centrifuged ($6000 \times g$ for 10 min at 4°C) and the supernatants were filtered through 0.22 μm Durapore filters. These cell-free culture supernatants (CFCS) were then stored at −20°C.

Fibroblast cultures

Human gingival fibroblasts were obtained by explant culture as described previously (8) and tested negatively for mycoplasma infection. The cells were maintained in Dulbecco Vogt Medium (DVM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 Units penicillin, 100 μg/ml streptomycin and non-essential amino acids in a humidified atmosphere of air/CO_2 (9:1) at 37°C. Cells between the 4th and 8th transfer in culture were used.

Fibroblast DNA synthesis and proliferation assays

To measure the effect of the CFCS's on DNA synthesis, human gingival fibroblasts were seeded, in triplicate, into 24-well plates (which hold approximately 10^5 cells at confluence) at an initial density of 20000 cells per well and allowed to attach and spread overnight in DVM containing 10% FCS. The medium was then replaced with 500 μl/well of DVM alone and incubated for a further 48 h. This medium was then removed and replaced with a range of concentrations of CFCS's in medium containing 10% FCS. After 20 h incubation [^{3}H]-thymidine was added to the medium to give a final concentration of 1 μCi/ml. The cells were incubated for a further 4 h after which the medium was removed and the cells were washed three times with 500 μl PBS, and DNA then precipitated with 600 μl TCA at 4°C for 2 h. The cell layers were then lysed with an equal volume of 0.1 M sodium hydroxide for 60 min at 50°C. The radioactivity in the extracted material was determined in a Beckman LS-2800 Liquid Scintillation Counter. This assay has been shown to represent accurately *in vitro* division of fibroblasts (9).

Cell growth was also assessed over a 5-d period

using a colorimetric assay (10). Cells were plated at an initial density of 10000 cells per well into 4-well plates and incubated in the presence or absence of a 1:10 dilution of the CFCS. At daily intervals the medium was removed and the cells fixed in 2.5% (v/v) gluteraldehyde. At the end of 5 d all fixed cells were washed with distilled water and then exposed to 400 μl 1% aqueous crystal violet. After staining for 5 min the plates were washed exhaustively with water and the cells solubilized with 1 ml of 33% (v/v) glacial acetic acid and the absorbance read at 580 nm after 5 min.

Metabolic studies

Protein and proteoglycan synthesis by gingival fibroblasts was assessed as described previously (11, 12). Briefly, triplicate cultures of confluent cells in 24-well plates were incubated in the presence of varying concentrations of CFCS and either 10 μCi/ml [^{3}H]-proline (for total protein synthesis) or 20 μCi/ml $Na_2[^{35}S]O_4$ (for proteoglycan synthesis). After 24 h the medium was removed and the cells washed once with 250 μl PBS. The cell layers were then stored at -4°C prior to total DNA determination by the method of Labarca and Paigen (13). The wash and medium were pooled and 500 μl aliquots were chromatographed on Sephadex PD-10 columns in the presence of 4 M guanidine HCl/0.05 M sodium acetate pH 5.8. Radioactivity in 0.4 ml effluent fractions was determined by liquid scintillation counting. The amount recovered in the void volume provided a measure of incorporation of radiolabel into newly synthesized macromolecules. Recovery from these columns was approximately 85%.

Determination of characteristics of inhibitor

(i) Molecular Size Determination. Preliminary experiments were aimed at determining whether the activity might be related to a protein/peptide or metabolic/degradation product. This was simply determined by using ultrafiltration with a molecular weight cut-off of 30000. The CFCS was filtered and the filtrate and retentate were diluted 1:10 in DVM containing 10% FCS and tested for inhibition of [^{3}H]-thymidine incorporation into cellular DNA as described above.

(ii) Heat Stability. The heat stability of the factor(s) which induced proliferative inhibition was determined by boiling an aliquot of the CFCS for 10 min and then diluting 1:10 prior to assessment of [^{3}H]-thymidine incorporation.

(iii) Volatility. To determine whether the active component(s) was volatile, an aliquot of the CFCS was subjected to lyophilization. The dried residue was then reconstituted to the original volume with deionized water and diluted 1:10 prior to determination of its effect on [^{3}H]-thymidine incorporation.

(iv) Reversibility. To determine whether the inhibitory effect induced upon the gingival fibroblasts by the CFCS was reversible, the cells were first cultured for 24 h in the presence of a 1:10 dilution of the CFCS. This medium was then removed and replaced with DVM containing 10% FCS alone and incubation continued for 20 h at which stage [^{3}H]-thymidine was added and incubation continued for a further 4 or 24 h.

Effect of selective addition of known bacterial metabolic products

Following determination that the active component(s) were most likely of small molecular size, heat-stable and non-volatile, it appeared likely that they were bacterial metabolic end-products. Acidic end-products in the CFCS were assayed by an HPLC method (14). Lactic acid and ammonia were determined enzymatically (Boehringer Mannheim, West Germany). Following these assessments, the most likely candidates were determined on the basis of their high concentration and were added to DVM at the determined concentration. The reconstituted media were then diluted 1:10 and [^{3}H]-thymidine incorporation into cellular DNA was determined as described above.

Statistical analyses

All data were subjected to statistical analysis using the method of analysis of variance.

Results

The effect of various dilutions of CFCS of *F. nucleatum* on [^{3}H]-thymidine incorporation into DNA by human gingival fibroblasts can be seen in Fig. 1. Control cultures of fibroblasts grown in the presence or absence of 10% FCS show the expected stimulatory effect on growth-arrested cells. However, when *F. nucleatum*-conditioned medium was added to growth-arrested fibroblasts in DVM containing 10% FCS, significant inhibition of incorporation of [^{3}H]-thymidine into DNA by the cells was observed at both 1:10 and 1:100 dilutions, representing inhibitions of approximately 98% and 37% respectively. Visual assessment of the fibroblasts under phase contrast light microscopy indicated no morphological changes upon exposure to the CFCS's for up to 5 d. At no stage was cell death or detachment from the culture plates observed (results not shown). Although not a principal part

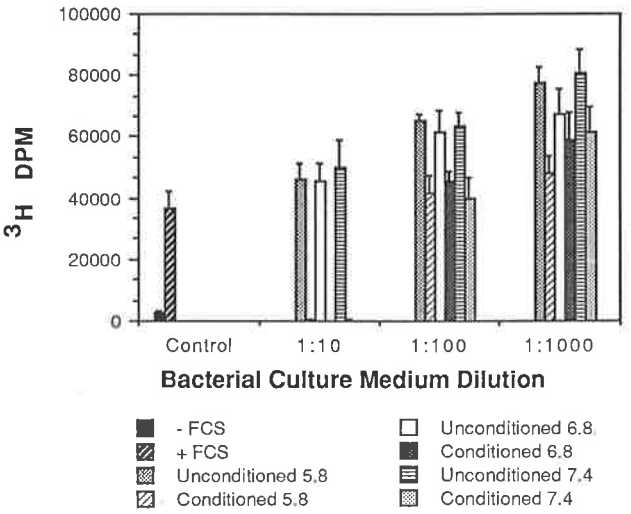


Fig. 1. Effect of *Fusobacterium nucleatum* cell-free culture supernatants on DNA synthesis by human gingival fibroblasts. [^{3}H]-thymidine incorporation by the cells was monitored 20 h after exposure to varying dilutions of the culture media in DVM containing 10% FCS. Control cultures were incubated in the presence or absence of 10% FCS only to establish the stimulatory effect of FCS. Data represent the mean and standard deviation of the mean from triplicate cultures.

of this study, the pH conditions of the bacterial culture medium were varied to determine what role, if any, pH played in bacterial metabolism. While slight variations were noted over the pH ranges studied, the differences were not statistically significant. Therefore, most of the later studies were performed using conditioned medium from bacteria grown at pH 7.4. Furthermore, the addition of unconditioned bacterial culture medium to DVM supplemented with 10% FCS appeared to stimulate the incorporation of [^{3}H]-thymidine into DNA by the gingival fibroblasts. Although the mechanisms involved in this stimulation are not clear, the data obtained with the CFCS's over the same dilutions further highlight the production of inhibitory component(s) by *F. nucleatum* with respect to thymidine uptake by the cells.

Although the inhibition of [^{3}H]-thymidine incorporation into DNA was very dramatic, in the absence of other confirmatory proliferative studies, care must be taken in correlating this observation with cellular proliferation. Therefore, the effect of

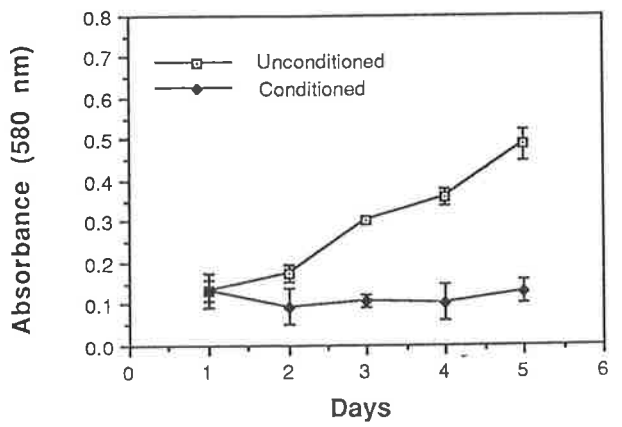


Fig. 2. The effect of *Fusobacterium nucleatum* cell-free culture supernatants (CFCS) on cell growth. Cells were cultured in the presence of 1:10 dilution of either conditioned or unconditioned CFCS over a 5-d period. Cell growth was monitored by the uptake of crystal violet by cells cultured over 5 d. Data are expressed as the mean and standard deviation of the mean from triplicate cultures.

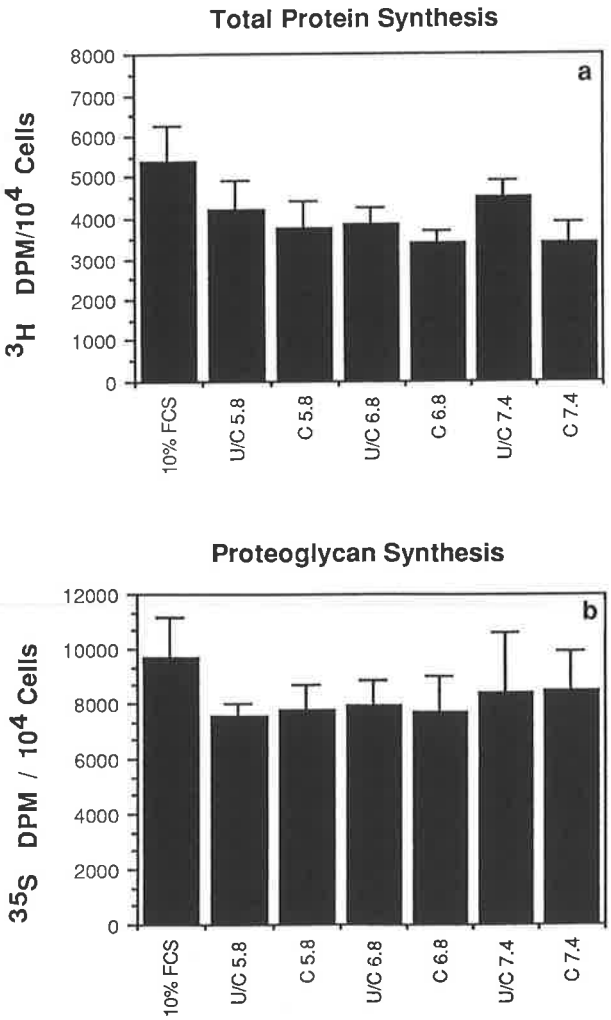
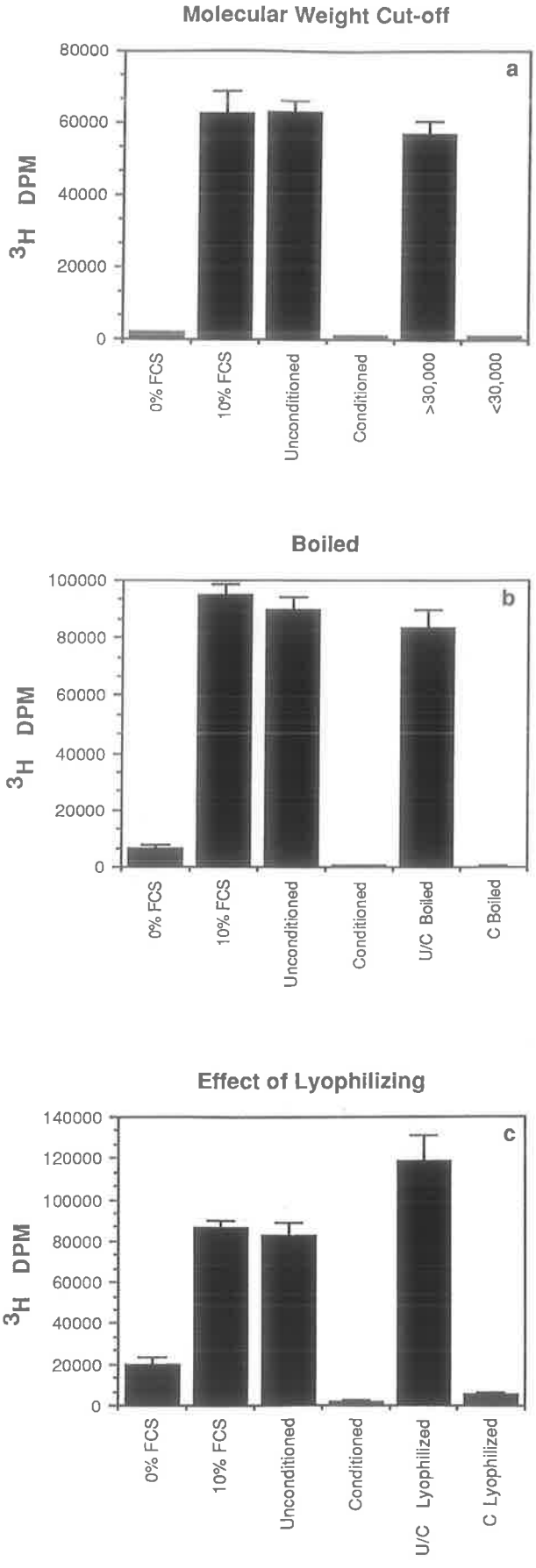


Fig. 3. The effect of *Fusobacterium nucleatum* cell-free culture supernatants on (a) total protein synthesis and (b) proteoglycan synthesis. Conditioned and unconditioned bacterial culture media over a range of pH values were tested for their effect on total protein (^{3}H-proline incorporation into macromolecules) and proteoglycan (^{35}S-sulfate incorporation into macromolecules). Control cultures were incubated in the presence of DVM containing 10% FCS only. Data represent the mean and standard deviation of the mean of triplicate cultures. Abbreviations used: U/C: unconditioned medium; C: conditioned medium.



a 1:10 dilution of the CFCS on cell growth over a 5-d period was monitored (Fig. 2). From these studies it was apparent that the CFCS's inhibited fibroblast growth *in vitro* and this effect was significant by d 2 ($p < 0.05$).

Since many inhibitors of fibroblast proliferation also interfere with synthetic activity of the cells, the effect of the CFCS on total protein and proteoglycan synthesis was determined (Fig. 3 a & b). A slight decrease in both protein and proteoglycan synthesis was observed between control cultures grown in DVM supplemented with 10% FCS alone and those exposed to bacterial conditioned and unconditioned media. Variation of the bacterial culture medium pH had no significant effect on the release of inhibitory products towards gingival fibroblasts. Since few differences were detected between fibroblasts exposed to conditioned and unconditioned bacterial media, it is unlikely that the observed difference between control and treated cultures were due to products released by the bacteria. Rather, the effect most likely lies in the culture medium used for the culture of the bacteria.

In order to determine the nature of the inhibitory factor(s) in the CFCS's, a series of experiments was performed to assess the molecular size, heat stability and volatility of the active component(s) (Fig. 4). Following ultrafiltration through a 30000 molecular weight cut-off membrane, all of the inhibitory activity was recovered in the filtrate indicating it to be less that 30000 (Fig. 4a). After boiling the samples for 10 min, the inhibitory activity of the CFCS was retained while that of the unconditioned medium was unaffected (Fig. 4b). Similarly, following lyophilization to remove any volatile components, the inhibitory activity was retained in the CFCS and that of the unconditioned medium was unaffected (Fig. 4c). On the basis of these findings it was concluded that the inhibitory activity was of a molecular weight less than 30000, was not labile to heating and was not volatile.

Since there was no discernible effect of the CFCS on total protein or proteoglycan synthesis and no toxic effects in the form of cell death or detachment were observed, it seemed that although proliferat-

Fig. 4. Characterization of the inhibitory factor in *Fusobacterium nucleatum* cell-free culture supernatants. (a) Molecular size was determined by filtering the media through a 30000 molecular weight cut-off ultrafiltration membrane; (b) Heat stability was determined by boiling the media for 10 min; (c) volatility was determined by lyophilization of the media. Data represent the mean and standard deviation of the mean of triplicate cultures. Abbreviations used: U/C: unconditioned medium; C: conditioned medium.

ive activity was depressed the cells remained vital. In order to determine whether the cells could recover from their exposure to the CFCS, [^{3}H]-thymidine incorporation by gingival fibroblasts was monitored by FCS stimulation after removal of the CFCS's (Fig. 5). Under these conditions, proliferative activity was noticeably elevated after 24 h and restored very close to the control values 48 h after removal of the CFCS from the culture medium.

To characterize further the agent(s) responsible for the inhibition of fibroblast profileration, the CFCS's were analyzed qualitatively and quantitatively for bacterial metabolic products (Table 1). High proportions of butyrate, ammonium and acetate were present. By determining the concentrations of these metabolic products in the conditioned bacterial medium we were able to re-introduce purified commercial preparations of these products into unconditioned bacterial medium and test them either individually or in combination for their effects on gingival fibroblast proliferation (Fig. 6). In doing so it was determined that the bulk of the inhibitory activity could be attributed to ammonium and butyrate.

Discussion

This study has demonstrated the ability of *F. nucleatum* to secrete metabolites that are extremely potent inhibitors of human gingival fibroblast proliferation. Such a finding is not surprising since plaque extracts (15–18), sonicates, homogenates or chemical extracts of periodontal pathogens (19–22) and some bacterial culture products (23–25) have all been shown to possess factors which are toxic or inhibitory to fibroblasts. However, careful analysis of these previous studies indicates some possible limitations with respect to their relevance to *in vivo* situations. First, the majority of studies have relied upon mechanical homogenization, sonication or chemical extraction of dental plaque or whole bacteria to obtain material for testing in bio-assays. While these approaches have provided useful information regarding the presence of potentially toxic components associated with bacteria, they largely ignore the extreme extraction procedures used which bear minimal relevance to *in vivo* disruption of oral bacteria. Secondly, of those studies which have addressed the ability of bacteria to synthesize and secrete toxic components, most have used batch culture systems in which the growth and environment is largely uncontrolled and bacterial growth rates are higher than those encountered *in vivo*. This study has, therefore, attempted to overcome some of these problems by assessing the bio-activity of bacterial products

Table 1. Concentration of *Fusobacterium nucleatum* metabolic products in culture supernatants

End Products	pH 5.8	pH 6.8	pH 7.4
Acetate	6.46	23.10	12.79
Butyrate	11.18	26.64	11.24
Ammonia	31.21	41.38	64.78
Lactate	0.35	0.49	0.76
Formate			
Proprionate	between 0.1 & 0.5 mM		
Succinate			

All values are expressed as mmol/l.

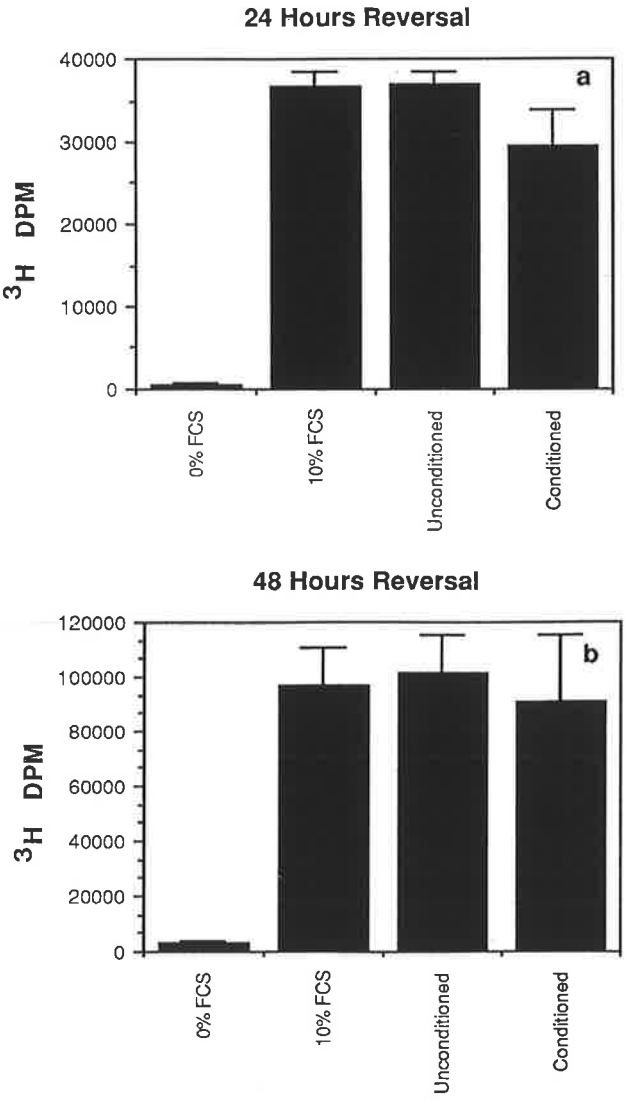


Fig. 5. Recovery of fibroblasts exposed to *Fusobacterium nucleatum* cell-free culture supernatants. Fibroblasts were cultured for 24 h in the presence of conditioned and unconditioned bacterial culture media. The media were replaced with DVM containing 10% FCS only and DNA stimulation was monitored 20 h later by monitoring [^{3}H]-thymidine incorporation for either 4 or 24 h, thus making the total time of medium reversal 24 or 48 h. Data represent the mean and standard deviation of the mean from triplicate cultures.

released into culture medium during growth under the closely controlled conditions provided by the chemostat. In this way, the effects on bacterial metabolism of environmental factors such as pH and nutrient limitation can be studied (26). While care must always be taken in extrapolating data obtained from *in vitro* studies to the *in vivo* situation, this system does permit the study of specific oral bacteria in a manner more relevant to the oral environment than has previously been possible.

Over the years many studies have attempted to assess the influence of bacterial components and products on human gingival fibroblasts. However, apart from LPS, few modulatory bacterial components have been specifically identified or characterized to the point of precise determination of their molecular nature and composition. For example, although the ability of *Actinobacillus actinomycetemcomitans* to secrete a leukotoxin is well recognized (5, 27), this molecule has no effect on human gingival fibroblasts (28). Similarly, the production and release of many enzymes capable of degrading the gingival extracellular matrix has been well documented (5, 29), but their effect on gingival fibroblast function is poorly-defined. In general, factors which have been found to alter fibroblast activity have only been identified under loose descriptions such as "heat-labile substances" or "small non-protein molecules" (21, 23, 25).

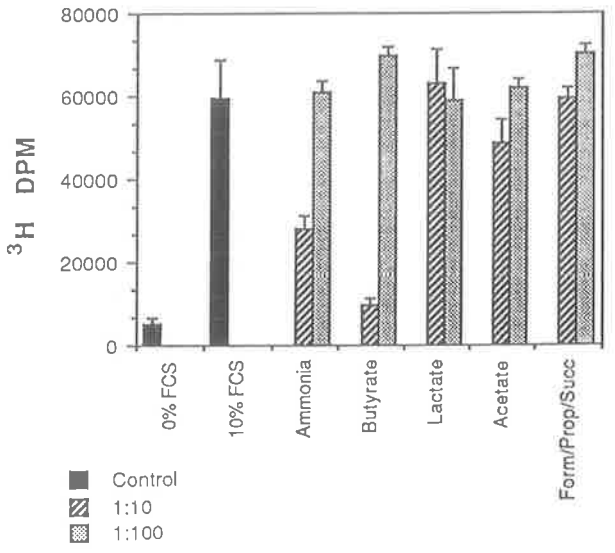


Fig. 6. Determination of active inhibitory factor(s) in *Fusobacterium nucleatum* cell-free culture supernatants. Selective addition of bacterial metabolic products determined to be present in highest concentrations (see Table 1) were added to fibroblast cultures and the effect of such additions on DNA synthesis monitored. Original concentrations used prior to dilution: Ammonium (65 mM); Butyrate (10 mM); Lactate (1 mM); Acetate (10 mM); Formate/Proprionate/Succinate (0.5 mM). Data represent the mean and standard deviation of the mean from triplicate cultures.

From the present study we have identified butyrate, and to a lesser extent ammonium, as the major factors in the CFCS's of a strain of *F. nucleatum* responsible for the inhibition of human gingival fibroblast proliferation. The finding that butyrate inhibits gingival fibroblast proliferation is in partial agreement with that of Singer and Buckner (30). However, these workers also reported that proprionate was a significant component of dental plaque extracts which inhibited mouse fibroblast proliferation. In our studies we found little effect for proprionate. Rather, we determined that ammonium was the only other major inhibitory factor identified. This may reflect the fact that we studied the CFCS's of only one bacterial species whereas Singer and Buckner analyzed whole-plaque culture supernatants. Our finding that ammonium is an important contributor to fibroblast inhibition is in agreement with a previous study concerning the effect of bacterial culture supernatants on tissue culture cells (31).

With regard to butyrate, it is noteworthy that the effects recorded in our studies occurred at concentrations less than those reported for butyrate in dental plaque *in vivo* (32, 33). Thus, one could expect the potential for butyrate to be effective *in vivo* should be high. Indeed, such a situation seems especially relevant when one considers that *Fusobacterium nucleatum* is not the only bacterium present that is capable of producing butyrate. Therefore, it is conceivable that very high levels of butyrate could be attained in heavily populated periodontal pockets.

The reversible nature of the effect of CFCS's on the inhibition of fibroblast proliferation further implicates butyrate as a prime factor since another study has also shown reversible inhibition of fibroblast proliferation by butyrate (34). The mechanism by which butyrate affects cell proliferation is still unclear. For example, although butyrate causes hyperacteylation of histones, several other short-chain fatty acids have a similar effect; yet only butyrate inhibits proliferative activity (35). It has been postulated that butyrate is able to hold cycling cells in the S-phase or prevent cells from entering S-phase and thus effectively synchronizes all cells prior to chromosome condensation (34).

An important consideration regarding the pathogenicity of periodontal bacteria is their ability to influence the gingival connective tissue extracellular matrix either directly via enzymatic destruction or indirectly via alteration of fibroblast synthetic activity. Therefore, the inability of the components in the *Fusobacterium nucleatum* culture supernatants to affect extracellular matrix synthesis *in vitro* may be important in relation to its

pathogenicity. Such an observation indicates that the effect is not toxic to the point of causing cell death. Rather, although the cells are unable to divide they can still function in an apparently "normal" manner with respect to synthesis of matrix proteins. Similar findings that both butyrate and ammonium have little effect on total protein and proteoglycan synthesis by human gingival fibroblasts and chick chondrocytes *in vitro* have been reported (25, 36).

In conclusion, the present study highlights the toxic nature of small molecular weight metabolic products of one potential periodontal pathogen. Given the propensity for accumulation of this metabolite in infected periodontal pockets, and the likely easy access to the underlying gingival connective tissues (37), a significant role for these components in the pathogenesis of periodontal disease is proposed. Furthermore, although extracellular matrix synthesis appears to be unaffected by these components, the fact that cell proliferation is so dramatically inhibited indicates that the potential for rapid wound healing would be severely compromised if such products are present.

Acknowledgments

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Paper 21.

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Regulation of human gingival fibroblast growth and synthetic activity by cyclosporine-A *in vitro*

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Bartold PM: Regulation of human gingival fibroblast growth and synthetic activity by cyclosporine-A in vitro. *J Periodont Res 1989; 24: 314–321.*

Gingival overgrowth is an adverse side-effect seen in a proportion of patients taking cyclosporine-A which indicates that cyclosporine-A may modulate the activities of cells other than T lymphocytes. Therefore, the effect of cyclosporine on human gingival fibroblasts has been studied *in vitro*. Cyclosporine-A was found to stimulate DNA synthesis and the proliferative activity of these cells with maximal stimulation noted at a concentration of 10^{-9} g/ml. Although this stimulation was most noticeable in the presence of 10% fetal calf serum, proliferation still occurred in serum-free medium. In the presence of lipopolysaccharide, at a concentration which normally inhibits ingival fibroblast proliferation, cyclosporine retained its capacity to stimulate proliferative activity. Fibroblasts isolated from overgrown gingival tissue responded to a greater extent than those isolated from a healthy site from the same individual. This stimulatory effect was not restricted to gingival fibroblasts, since human foreskin fibroblasts responded in a similar fashion. Cyclosporine-A did not significantly alter protein or proteoglycan production by these cells. These responses are considered to reflect the *in vivo* response of gingival overgrowth in patients taking cyclosporine-A. The reversal of lipopolysaccharide inhibition of gingival fibroblast proliferation by cyclosporine-A may explain, in part, why gingival overgrowth is most prominent in areas of heavy dental plaque accumulation.

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Introduction

Cyclosporine-A (CSA), is an immunosuppressant commonly used for patients receiving organ transplants. The principal mode of action of this drug has been determined to be one of blocking the production of, and responsiveness to, interleukin-2 by T lymphocytes (1, 2). In addition, CSA indirectly influences monocyte function by suppressing the release of several T-cell lymphokines (3–5) which in turn influences interleukin-1 production by monocytes. Thus, it is not surprising that the administration of this drug is often associated with several side-effects. These include nephrotoxicity (6, 7), hepatotoxicity (8, 9), neurotoxicity (10) lymphoproliferative neoplasms (11, 12) and gingival overgrowth (13–20).

Many studies on the histological features of the gingival response have reported massive plasma cell infiltration but have made little comment on the changes in fibroblast numbers (15, 17–20). Recently, fibroblasts isolated from overgrown gingiva from patients receiving CSA were found to possess different metabolic and proliferative activities when compared with fibroblasts from healthy gingival sites from the same donor (13). These findings raise the possibility that, in these lesions, CSA affects not only T cells but also the fibroblastic cells responsible for the production of the extracellular matrix. Whether this occurs via the direct action of CSA on fibroblasts, or reflects a secondary response due to immuno-modulation, is not clear. Therefore, this study has been designed to examine the effect of CSA on gingival fibroblast proliferation and synthetic activity *in vitro*.

Material and Methods

Materials

Dulbecco-Vogt medium (DVM), RPMI-1640 medium, fetal calf serum (FCS), non-essential amino acids, penicillin and streptomycin were all purchased from Flow Laboratories Australasia Pty Ltd., North Ryde, New South Wales; all tissue culture plastic ware was obtained from Nunc, Roskilde, Denmark; sodium pyruvate, L-glutamine, guanidine HCl and lipopolysaccharide (*Salmonella enteritidis*, TCA extract) were from Sigma Chemi-

cal Co., St. Louis, MO: phytohemagglutinin was from Wellcome Reagents Ltd., Beckton, U.K., [6-^{3}H]-thymidine (22 Ci/mMol), L-[2,3,4,5-^{3}H]-proline (100 Ci/mMol) and $Na_2[^{35}S]O_4$ (1042 mCi/mMol) were from Amersham (Australia Pty Ltd., Surrey Hills, New South Wales); Ready Solv EP liquid scintillation fluid was from Beckman Australia, Adelaide; Sephadex PD-10 columns from Pharmacia Fine Chemicals, North Ryde, New South Wales. CSA was a generous gift from Sandoz Australia Pty Ltd.

Cell cultures

Gingival fibroblasts were isolated from explant cultures of healthy gingiva as well as from CSA-enlarged gingiva as described previously (13, 21). Human foreskin fibroblast cultures were a generous gift from S. Hay (Department of Pathology, University of Adelaide). The cells were maintained in Dulbecco-Vogt medium supplemented with 10% fetal calf serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2 mM glutamine, 10 mM sodium pyruvate and non-essential amino acids at 37°C in a moist atmosphere of 5% CO_2 and 95% air. Cells between the 4th and 10th transfer in culture were used for this study.

Mouse thymocytes were derived from the thymus glands of 9- to 11-week-old C3H/HeJ mice as described previously (22) and were a gift from D. R. Haynes (Department of Pathology, University of Adelaide). These cells were maintained in RPMI-1640 medium with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 5×10^{-5} M 2-mercaptoethanol in a moist atmosphere of 5% CO_2 and 95% air at 37°C.

Preparation of cyclosporine-A for *in vitro* studies

Due to its highly hydrophobic nature, CSA is insoluble in culture media. Therefore, prior to its introduction into the culture system, a 1 mg/ml solution was made by dissolving 1 mg CSA in 100 μl ethanol, then 20 μl Tween 80 was added while vigorously shaking. Finally, 880 μl of the culture medium to be used was added. The sample was then diluted 1:1000 to reach the starting concentration of 10^{-6} g/ml. Appropriate solvent controls were always run and no detectable effect on the cells was noted (results not shown).

Fibroblast DNA synthesis and proliferation assays

To measure the effect of CSA on DNA synthesis, human gingival fibroblasts and foreskin fibroblasts were seeded, in triplicate, into 24-well plates (which hold approximately 10^5 cells at confluence) at an initial density of 20 000 cells per well and allowed to attach and spread overnight in DVM containing 10% FCS. The medium was then replaced with 500 μl/well of DVM alone and incubated for a further 48 h. This medium was then removed and replaced with a range of concentrations of CSA in medium containing 10% FCS. After 48 h incubation the medium was replaced with 500 μl of medium containing cyclosporine and 1 μCi/ml [3H]-thymidine. The cells were incubated for a further 15 h after which the medium was removed and the cells were washed three times with 500 μl PBS. The cells were then lysed with 0.1 M sodium hydroxide for 60 min at 50°C, an equal volume of 10% TCA was added and the radioactivity in the precipitated material was determined in a Beckman LS-2800 Liquid Scintillation Counter. This assay has been shown to accurately represent *in vitro* division of fibroblasts (23).

In some experiments the above protocol was used, except that the cells were incubated in mdium containing cyclosporine, 50 μg/ml lipopolysaccharide and either 0% or 10% FCS.

The effect of CSA on the time course of DNA synthesis by human gingival fibroblasts was assessed as described by Ko *et al.* (24). Briefly, the cells were plated at an initial density of 15 000 cells per well in complete medium and allowed to attach and spread in 24-well plates overnight. The medium was then removed and replaced with DVM alone and the cells incubated for a further 48 h after which the medium was removed and replaced with complete medium with or without 10^{-9} g/ml CSA. The cells were cultured for 12, 18, 24, 30, 42 and 48 h prior to a 3-h pulse with 1 μCi/ml [3-H]-thymidine. The radioactivity in the newly synthesized DNA was determined as described above.

Cell growth was also assessed over a 5-d period using a colorimetric assay (25). Cells were plated at an initial density of 10 000 cells per well into 4-well plates and incubated in the presence or absence of 10^{-9} g/ml CSA. At daily intervals the medium was removed and the cells were fixed in 2.5% (v/v) glutaraldehyde. At the end of 5 d all fixed cells were washed with distilled water and then exposed to 400 μl 1% aqueous crystal violet. After staining for 5 min, the plates were washed exhaustively with water and the cells solubilized with 1 ml 33% (v/v) glacial acetic acid and the absorbance read at 580 nm after 5 min.

T-cell proliferation assays were performed using [^{3}H]-thymidine incorporation into DNA as described above, except that medium RPMI-1640 was used and the cells were incubated either in the presence or absence of 3 μg/ml phytohemagglutinin (26).

Metabolic studies

Protein and proteoglycan synthesis by gingival fibroblasts was assessed as described previously (27, 28). Briefly, triplicate cultures of confluent cells in 24-well plates were incubated in the presence of varying concentrations of CSA and either 10 μCi/ml [^{3}H]-proline (for total protein synthesis) or 20 μCi/ml $Na_2[^{35}S]O_4$ (for proteoglycan synthesis). After 24 h the medium was removed and the cells washed once with 250 μl PBS. The cell layers were then stored at -4°C prior to total DNA determination by the method of Labarca and Paigen (29). The wash and medium were pooled and 500 μl aliquots were chromatographed on Sephadex PD-10 columns in the presence of 4 M guanidine HCl/0.05 M sodium acetate pH 5.8. Radioactivity in 0.4 ml effluent fractions was determined by liquid scintillation counting. The amount recovered in the void volume provided a measure of incorporation of radiolabel into newly synthesized macromolecules. Recovery from these columns was approximately 85%.

Statistical analyses

All data were subjected to statistical analysis using the method of analysis of variance.

Results

In order to determine the biological activity of CSA, initial experiments were directed towards the inhibitory effect of this drug on T-cell proliferation (Fig. 1). With increasing concentrations of CSA, T-cell proliferation was inhibited. At a concentration of 10^{-6} g/ml, the proliferative activity of these cells was reduced to approximately that of unstimulated cells. The concentration which caused 50% inhibition (IC_{50}) was determined to be 5 ng/ml.

Since CSA-associated gingival overgrowth could be due to a proportional increase in both tissue mass and cell numbers, the effect of CSA on gingival and foreskin fibroblast proliferation was initially assessed by monitoring DNA synthesis (Fig. 2). In the absence of FCS, fibroblast DNA synthesis was minimal and in the presence of 10% FCS significant activation was noted ($p<0.01$). At a concentration of 10^{-6} g/ml, cyclosporine appeared to have an inhibitory effect on DNA synthesis ($p<0.01$). However, at lower concentrations stimulation of DNA synthesis was evident. Maximal stimulation of foreskin fibroblasts was noted at 10^{-10} g/ml and at 10^{-9} g/ml for the gingival fibroblasts. At concentrations less than 10^{-10} g/ml,

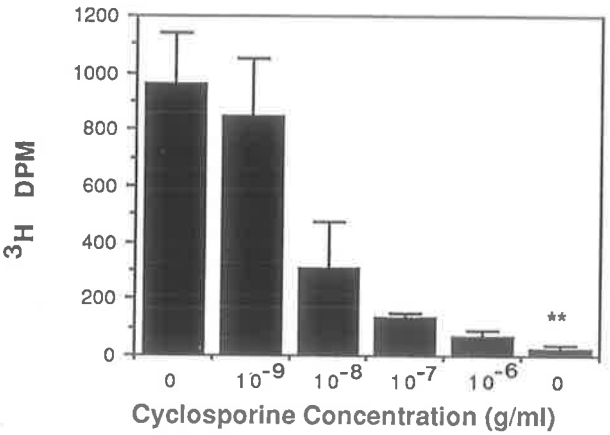

Fig. 1. Effect of CSA on the proliferation of thymus-derived mouse thymocytes. [^{3}H]-thymidine incorporation by the cells was monitored 72 h after exposure to various concentrations of CSA in the presence of PHA. Data are represented as the mean and standard deviation of the mean of triplicate cultures. ** Represents results in medium containing no PHA.

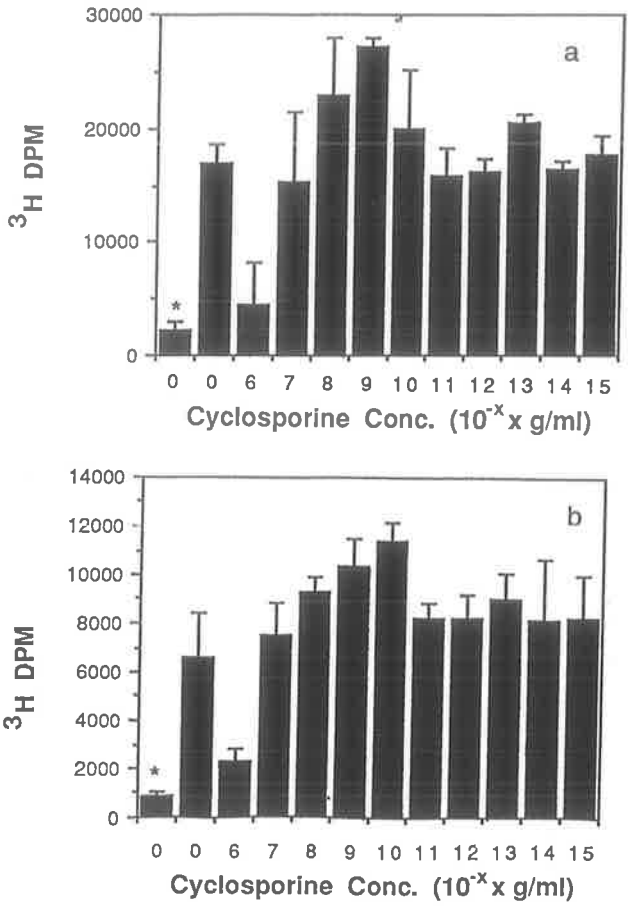

Fig. 2. Effect of CSA on DNA synthesis by (a) human gingival fibroblasts, and (b) human foreskin fibroblasts. ^{3}H-thymidine incorporation by the cells was monitored after 48 h exposure to CSA in the presence of 10% fetal calf serum. Data are represented as means and standard deviations of the mean of triplicate cultures. * represents results for medium containing 0% FCS.

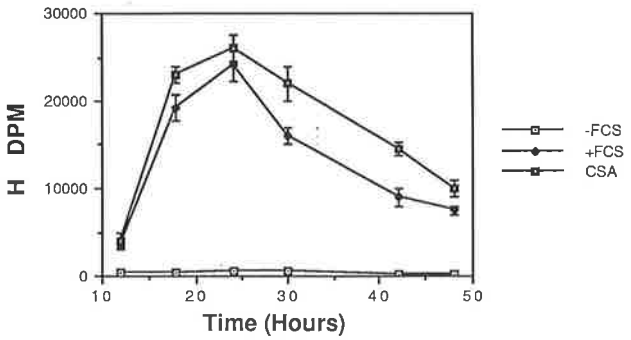

Fig. 3. The effect of CSA on DNA synthesis by quiescent cells activated by serum exposure. [^{3}H]-thymidine incorporation into DNA was monitored after a 3-h pulse at the indicated time intervals. Data represent the mean and standard deviation of the mean from triplicate cultures.

DNA synthesis by both types of fibroblast returned to the level of that seen for 10% FCS alone.

To determine whether [^{3}H]-thymidine incorporation into DNA was a valid means of assessing the effect of CSA on gingival fibroblast proliferation, DNA synthesis was monitored at 12-, 18-, 24-, 30-, 42-, and 48-h intervals (Fig. 3). Regardless of the presence of CSA, DNA synthesis was cyclical and peaked at around 24 h. During the early time points no significant difference in the amounts of ^{3}H-DNA synthesized by the cells was detected. However, from 30 h onwards the amount of ^{3}H-thymidine incorporated into DNA was slightly elevated in cells exposed to CSA compared to those cultured in the absence of CSA.

The effect of CSA on gingival fibroblast proliferation was also monitored using a direct measure of cell numbers (Fig. 4). Although maximum proliferation occurred around d 2 and 3, the cells cultured in the presence of CSA showed a significant increase in growth over the control cultures between d 1 and 2.

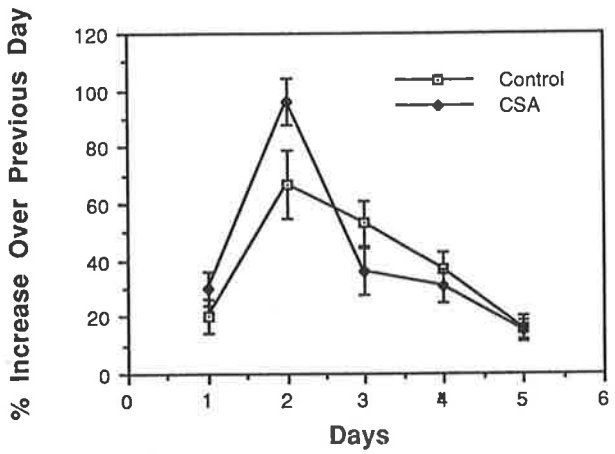

Fig. 4. The effect of CSA on cell growth. Cell growth was monitored by the uptake of crystal violet and is expressed as the percentage increase in cell density over the previous day.

Because the above experiments were done in the presence of 10% FCS, which may augment the response to CSA, the effect of cyclosporine on fibroblast proliferation in the absence of FCS was assessed (Fig. 5b). CSA caused significant stimulation of DNA synthesis at all concentrations tested ($p < 0.05$). However, only at the concentration of 10^{-9} g/ml was stimulation greater than that seen for cells grown in 10% FCS.

Since it has been suggested that bacterial plaque plays an important role in CSA-induced gingival overgrowth (17, 20), and lipopolysaccharide (LPS) has been implicated in the development of gingivitis (30, 31), DNA synthesis was assessed in the presence of 10% FCS, and 50 μg/ml of LPS (a concentration which normally inhibits gingival fibroblast proliferation). Although stimulation of DNA synthesis above that noted for cells treated with 10% FCS alone was not seen, CSA did stimulate DNA synthesis by gingival fibroblasts ($p < 0.01$) grown in the presence of LPS (Fig. 5c).

The response to CSA of fibroblasts from CSA-induced gingival overgrowth and cells from healthy tissue was also assessed (Fig. 6). CSA stimulated both groups of cells to synthesize DNA, although the cells from the overgrown tissue appeared to respond to a greater extent than the cells from normal tissue.

In addition to an assessment of cellular proliferative activity, the effect of CSA on the biosynthesis of total proteins and proteoglycans was also studied. In contrast to its effect on proliferation, CSA, at concentrations as high at 10^{-6} g/ml caused only marginal apparent stimulation of protein synthesis (Fig. 7). Although this stimulation was not statistically significant ($p > 0.05$), it was observed over a very wide range of concentrations (10^{-6}–10^{-9} g/ml). Furthermore, an insignificant change in proteoglycan synthesis by cultures exposed to CSA corroborated these findings (Fig. 8). Experiments with cells derived from overgrown and normal sites within the same individual revealed similar findings (results not shown).

Discussion

Gingival overgrowth is an adverse side-effect associated with the administration of drugs such as phenytoin, nifedipine and cyclosporine-A (14–20, 32, 33). Of these, phenytoin is the only drug which has been extensively studied and it has provided most of the information relating to the etiology and management of drug-induced gingival overgrowth. Although the gross histological appearance of the lesions is similar, the modes of action of these

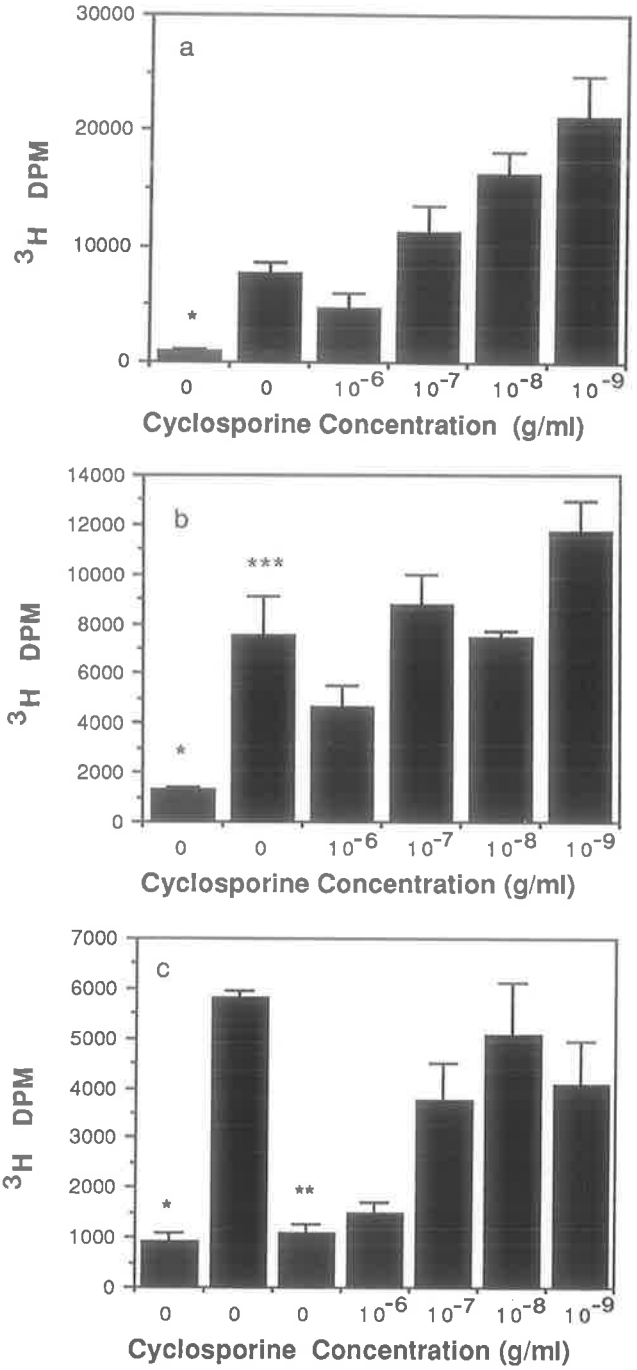

Fig. 5. Effect of CSA on human gingival DNA synthesis in (a) 10% fetal calf serum, (b) 0% fetal calf serum, and (c) 50 μg/ml lipopolysaccharide in 10% fetal calf serum. Proliferation rates were monitored as described for Fig. 2. Data represent the means and standard deviations of the mean of triplicate cultures. * Represents results for medium containing 0% FCS. ** Represents cells grown in the presence of 10% FCS and 50 μg/ml LPS but no CSA. ***Represents results for medium containing 10% FCS alone.

drugs are very different. Therefore, besides routine histological assessment of such conditions, understanding the mechanisms behind these reactions requires additional experimental investigations at

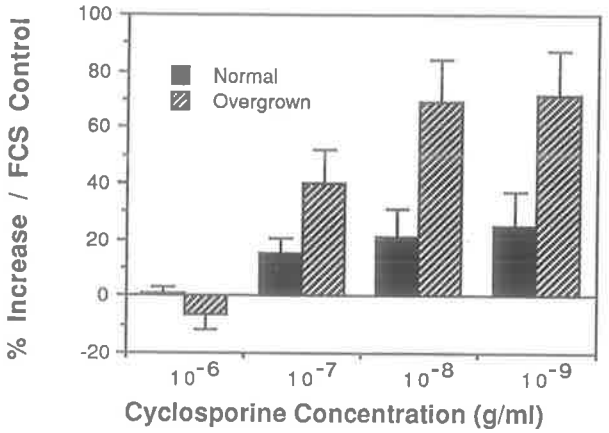

Fig. 6. Effect of CSA on DNA synthesis by fibroblasts derived from normal and overgrown gingivae from a patient taking CSA. Proliferation was monitored as described for Fig. 2. Data represent the means and standard deviation of the mean of triplicate cultures.

the cellular level. In the present study, the effect of CSA on gingival fibroblast proliferation and synthetic activity has been studied.

Hyperplasia is defined as an increase in the size of an organ or tissue due to an increase in the number of its specialized constituent cells. However, in cyclosporine-induced gingival overgrowth, as well as a probable increase in the absolute numbers of fibroblasts, there is also a significant increase in the number of inflammatory cells which contribute to the increase in tissue mass (15, 17). Therefore there is some uncertainty as to whether the lesion should be classified as a true hyperplastic response (17, 20). Nevertheless, these observations imply that stimulation of fibroblasts could be a major contributory factor in the pathogenesis of CSA-induced gingival overgrowth. In this respect,

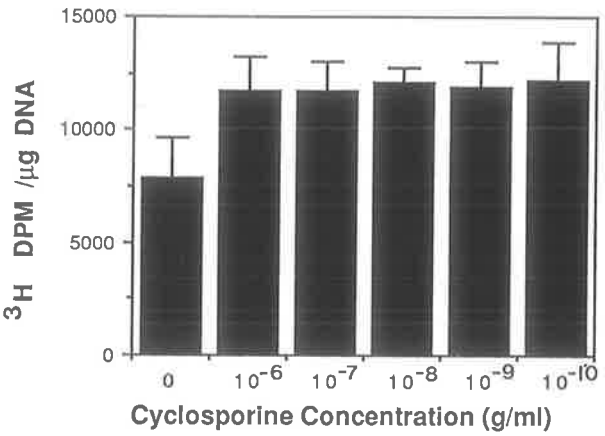

Fig. 7. Effect of CSA on total protein synthesis by human gingival fibroblasts. [^{3}H]-proline incorporation into proteins by the cells was monitored over a 24-h period of exposure to various concentrations of CSA. Data represent the means and standard deviations of the mean of triplicate cultures.

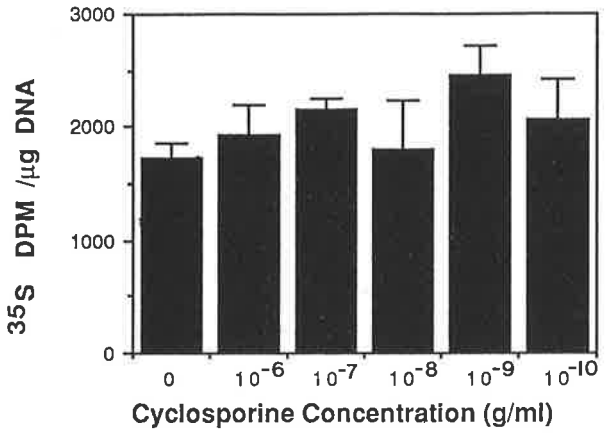

Fig. 8. Effect of CSA on proteoglycan synthesis. [^{35}S]-sulfate incorporation into proteoglycans was monitored during 24 h exposure of the cells to various concentrations of CSA. Data represent the means and standard deviations of the mean of triplicate cultures.

it is significant that this study has demonstrated gingival fibroblast proliferation in response to exposure to CSA. Furthermore, this stimulation occurred within the concentration ranges found in plasma and tissues of patients taking CSA (34) and thus highlights the likelihood of biological activity of CSA towards gingival fibroblasts *in vivo*.

Human foreskin fibroblasts responded to CSA in a similar fashion to the gingival fibroblasts. Thus, stimulation of fibroblast proliferation may be a general feature of the biological activity of CSA. Ostensibly, this finding appears to be opposite to that of Yocum *et al.* (35) who reported no effect of CSA on the proliferative activity of rat synovial fibroblasts or guinea pig dermal fibroblasts. However, closer examination of the experimental protocol reveals that Yocum *et al.* only studied cells which had been previously exposed to the mitogen, fibroblast-activating factor. Thus the two studies are not comparable and the new data may more accurately reflect tissue behaviour *in vivo*. Since an association between dental plaque and the development of CSA-induced gingival overgrowth has been reported (17, 20), and because LPS from bacteria in dental plaque has been implicated in the development of gingivitis (30, 31), the effect of LPS on CSA stimulation of fibroblasts was studied. Under normal conditions, LPS at the concentrations used in the present study inhibits gingival fibroblast proliferation (30). However, in the presence of CSA, inhibition of DNA synthesis by LPS was significantly diminished and, at some concentrations of CSA, stimulation of DNA synthesis was noted. This indicates that CSA may override the inhibitory effect of LPS on fibroblast proliferation. Thus, the potential exists for overgrowth of tissue adjacent to heavy plaque deposits in patients taking CSA.

Recently, the responses of human gngival fibroblasts to CSA have been found to be heterogeneous whereby 35% of the cells do not bind CSA while 41% avidly bind the drug and are more responsive in terms of synthetic and proliferative behavior (37, 38). These findings may explain why, in the present study, cultures exposed to CSA exhibited a slightly higher level of ^{3}H-thymidine incorporation into DNA at the latter time points of the DNA synthesis time course experiments. For example, in the presence of CSA, not only may similar cells be activated by serum (possibly the 35% which do not bind CSA) in which DNA synthesis peaks at 24 h and then decreases, but an additional group of cells (possibly the 45% which do bind CSA) could be activated to either begin DNA synthesis at a later time point or are slower to complete the phase of DNA synthesis prior to cell division. Despite this, it is perplexing that, although there is greater DNA synthetic activity in some cells exposed to CSA between 24–48 h, there is no significant increase in proliferative activity (in terms of increase in cell numbers) between control and treated cultures 24 h later (i.e. d 3). Whether this reflects further heterogeneity of the cells with respect to their ability to complete the cell cycle in the presence of CSA, or reflects an overlap between cells cycling at different rates, remains to be established.

In all types of tissue overgrowth there exists a relationship between cell numbers, cell proliferation rates and extracellular matrix production. In the case of CSA-induced gingival overgrowth it appears that an increase in fibroblast cell number is accompanied by a proportional increase in tissue matrix (19). This implies that, in these lesions, the synthetic activity of the fibroblastic cells remains unchanged. If this balance was disrupted (i.e. increased or decreased matrix production) the histological picture would be quite different. To determine whether stability in matrix production was reflected *in vitro*, the synthetic activity of the gingival fibroblasts was assessed. Total protein synthesis was found to be marginally increased by CSA. However, this increase could not be accounted for by an increase in proteoglycan synthesis. Compared to the ultrastructural findings of Yamasaki *et al.* (36), in which they reported an increase in rough endoplasmic reticulum in fibroblasts from CSA-induced gingival overgrowth the above findings of little change in protein synthesis by cells exposed to CSA are difficult to explain. Although it is possible that CSA changes the delicate balance between synthesis and degradation of extracellular matrix, and this should be investigated further, it is unlikely to be the case since no remarkable

changes in the ratio of protein DPM/μg DNA were seen. Therefore, while extrapolation from *in vitro* to *in vivo* conditions must be made with appropriate caution, the present findings could explain the apparently unchanged proportion of cells in these lesions despite the increase in tissue mass.

Although phenytoin-induced and CSA-induced gingival overgrowth exhibit similar histological appearances (16, 18, 19), this study indicates that, at the cellular level, the two lesions differ in several respects. Firstly, CSA appears to act directly on fibroblasts by influencing their proliferative behavior, whereas phenytoin does not appear to act directly on fibroblasts (39–41). In addition, CSA does not overtly affect protein or extracellular proteoglycan synthesis while phenytoin stimulates up to a two-fold increase in protein synthesis (33). Moreover, while the active component of phenytoin appears to be its metabolic products, the present study indicates a direct action of cyclosporine on fibroblasts *in vitro*. Such an effect is probably *in vivo* since cyclosporine is highly bound to plasma proteins, red cells and lipoproteins (42) and therefore would be readily available in a highly vascularized tissue such as gingiva. This availability would be increased in inflammatory conditions where there is significant release of plasma proteins into the local tissues as a result of increased vascularity.

In conclusion, the present findings corroborate the histological appearance of CSA-induced gingival overgrowth. By assessing cell proliferation under a variety of *in vitro* conditions, and concurrently monitoring protein synthesis, CSA has been shown to be capable of inducing the fibroblasts to proliferate without drastically altering their synthetic activity. This, in part, accounts for the proportional increase in tissue mass and total cellularity. Nonetheless, the question as to why only a percenage of patients taking CSA react with gingival overgrowth remains unresolved and indicates that additional factors are involved.

Acknowledgments

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Paper 22.

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Substance P: An immunohistochemical and biochemical study in human gingival tissues. A role for neurogenic inflammation?
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Candidate's Contribution to this paper: 50%

P.M. Bartold's role in this study was:
Provision of research funds
Design of the experiments
Supervision of the experimental work
Writing of the manuscript

A. Kylstra's role in this study was:
Student vacation scholar
Execution of the experiments

R Lawson's role in this study was:
Student vacation scholar
Execution of the experiments

Substance P: An Immunohistochemical and Biochemical Study in Human Gingival Tissues. A Role for Neurogenic Inflammation?*

P.M. Bartold, A. Kylstra, and R. Lawson

SUBSTANCE P HAS BEEN STUDIED in relation to its distribution within gingival tissues as well as its effect on cultured human gingival fibroblasts. The tissue distribution was varied depending on the degree of inflammation present. In healthy tissues substance P was found in the connective tissues interspersed between the collagenous elements and was particularly prominent in the areas immediately subjacent to the epithelial rete pegs. In inflamed tissues, substance P was markedly increased particularly around the blood vessels as well as in close association with much of the inflammatory cell infiltrate. The effect of substance P on human gingival fibroblast proliferation was monitored by [^{3}H]-thymidine incorporation and indicated substance P to be mitogenic for these cells at low concentrations (1×10^{-9} M) and tended towards an inhibitory effect at higher concentrations (1×10^{-4} M). Substance P did not have any effect on the release of either total proteins or proteoglycans into the culture medium. However, exposure of the cells to substance P did cause a greater accumulation of both total protein and proteoglycan with the cell layer material. These findings suggest a potential role for substance P on gingival tissues and in particular their resident fibroblastic cells and can therefore be used as a basis for more detailed studies into the relationship between neuropeptide release associated with neurogenic inflammation and periodontal pathology. *J Periodontol 1994; 65: 1113–1121.*

Key Words: Fibroblasts; gingiva; inflammation; substance P; connective tissue; blood vessels.

The term ''periodontal disease'' is an all encompassing term used to describe inflammatory disorders of the periodontium, ranging from the relatively benign form of gingivitis, in which the inflammation is confined to the marginal tissues, to more aggressive forms such as rapidly progressive periodontitis, in which the disease process leads to loss of connective tissue attachment to the root surfaces, loss of alveolar support, and impaired function of the dentition.[1] Experimental studies have demonstrated the primary cause of gingival inflammation is bacterial plaque and the pathological features associated with various stages of periodontitis have been well documented.[2,3]

Despite intense microbiological, immunological, and biochemical studies into the pathogenesis of the various periodontal diseases several enigmas remain. For example, although dental plaque is considered essential for the development of gingivitis and periodontitis, its presence (often in relatively large amounts) does not always result in progression of gingivitis to periodontitis. This implies that differences may lie either within the nature of the plaque or the nature of the ''host response.'' With respect to the host response, the periodontium is influenced significantly by poorly defined local and systemic factors including anatomy, hormonal, metabolic, immunological, nutritional, and other ''environmental'' factors.

Since there is general agreement in the literature that mental, physical, and biological stress have an important impact on the body's ability to resist disease,[4,5] stress has been added to the evergrowing list of factors which may modify various manifestations of periodontal disease.[6-8] Experimentally, stress has been demonstrated to negatively influence the status of the periodontium and may be significantly correlated with the severity of periodontal disease in humans,[9] associated with changes in bone in stressed laboratory animals,[10] and changes in the cellular and fibrous components of the periodontium.[11,12] As a result of such observations it has been suggested that the link between

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inflammatory diseases (perhaps such as periodontal disease) and stress may lie in the release of neurotransmitters from sensory nerve fibers upon stimulation by external stimuli.[13]

Recently, substance P has been implicated as one such neurotransmitter which may be associated with inflammation. Substance P is a neuropeptide released from the nerve endings of substance P immunoreactive nerves.[13-18] It is an undecapeptide that is stored in the secretory granules of sensory neurones, whose C- and N-terminals appear to have separate functions both of which may affect the inflammatory process. Substance P immunoreactive nerves are afferent, small diameter, unmyelinated polymodal, C-type fibers with dual functions. First, for central transmission of nociceptive information, substance P is released from the spinal tract upon orthodromic stimulation by noxious stimuli. The second function is release of substance P and other neuropeptides from collateral nerve terminals and peripheral tissue following antidromic noxious stimulation, resulting in "neurogenic inflammation."

Peripheral release of substance P has been implicated as a neurogenic promoter in various inflammatory processes (e.g., asthma, rhinitis, conjunctivitis, inflammation of the skin, and mucosa).[16,19-21] Although it is not known if substance P causes inflammation by acting directly upon blood vessels or by activating other mediators,[16,20] this neuropeptide is a potent vasodilator and increases vascular permeability. Thus substance P may contribute to the increased blood flow and plasma extravasation—the "hallmarks of inflammation"—in tissues innervated by substance P immunoreactive nerves. Substance P also has proinflammatory effects on neutrophils, macrophages, mast cells, lymphocytes, and endothelial cells.[12-24] The neurogenic component of substance P-induced inflammation has been established by observations of denervated tissue and pretreatment of tissues with substance P antagonists, both of which showed no response to noxious stimuli.[21,22,25]

In light of the above, we hypothesize that neurogenic inflammation may be a complicating or modifying factor in periodontal diseases. The aim of this study was to determine the presence of substance P in normal and inflamed human gingival tissues and to establish whether substance P can influence gingival fibroblast function in vitro.

The multifactorial nature of periodontal diseases has been recognized for some time. Although it is likely that no single factor will act in isolation, it is important to initially dissect potential players and determine their role in the disease process. For these reasons, this study was designed to determine whether the neuropeptide substance P could be detected in human gingiva and whether its effect on gingival fibroblasts could indicate a potential role for neurogenic inflammation in the periodontal diseases.

MATERIALS AND METHODS

Materials

Dulbecco's modification of eagle's medium (DMEM), fetal calf serum (FCS), nonessential amino acids, penicillin, and streptomycin were purchased from Flow Laboratories Australasia Pty. Ltd., North Ryde, New South Wales; all tissue culture plastic ware was obtained from Nunc, Roskilde, Denmark; sodium pyruvate and L-glutamine were from Sigma Chemical Co., St. Louis, MO; [6-^{3}H]-thymidine (22 Ci/mMol), L-[2,3,4,5-^{3}H]-proline (100 Ci/mMol) and $Na_2[^{35}S]O_4$ (1042 mCi/mMol) were from Amersham (Australia Pty. Ltd., Surrey Hills, New South Wales); Ready Solv EP liquid scintillation fluid was from Beckman Australia; Sephadex PD-10 columns from Pharmacia Fine Chemicals, North Ryde, New South Wales; substance P and anti-substance P antibodies were from Auspep, Parkville, Victoria.

Source of Tissues

Healthy human gingival tissue was obtained from volunteers with their informed consent by a minimally invasive procedure removing the interdental papilla between the upper right premolar teeth causing little postoperative discomfort with complete regeneration of tissue occurring within 4 weeks. Inflamed gingival specimens were obtained during routine periodontal surgery for the management of patients with advanced periodontal destruction requiring surgical intervention. For this study, the precise periodontal disease status was not classified as the purpose was to establish the presence or absence of substance P in normal and inflamed tissues with no attempt made to correlate disease activity and type of periodontitis.

Following surgical removal, the gingiva were placed immediately into 2% paraformaldehyde, 2% glutaraldehyde and 0.3% cetylpyridinium chloride, and 30 mM NaCl in 0.1 M phosphate buffer, pH 7.4 and fixed for 2 hours at 4°C. After fixation, the tissues were embedded in paraffin and 5 μm sections were cut. Although the surgical specimens were initially judged on clinical criteria as being derived from normal or inflamed sites, routine staining with hematoxylin and eosin was carried out to confirm the inflammatory status of the tissues.

Primary Antibodies

Polyclonal rabbit anti-substance P serum was used to detect the presence of substance P in human gingival tissues. This antibody was originally raised against the peptide sequence substance P 1-11, has no cross reactivity with other known neuropeptides, and has been well characterized and used for substance P localization within a variety of tissues.[25]

Immunohistochemical Staining

Sections were deparaffinized, rehydrated through graded alcohol solutions, and then exposed to methanol containing 2.5% hydrogen peroxide for 20 minutes to block any endogenous peroxidase activity in the sections. After the sections were washed with phosphate-buffered saline (PBS), the primary antibody was then applied at a 1:1,000 dilution to the sections and allowed to incubate 30 minutes at room temperature. After removal of the unbound antibody by

washing in PBS, the sections were incubated with a 1:50 dilution biotinylated swine anti-rabbit immunoglobulins for 30 minutes at room temperature. The bound antibody was then detected using a 1:50 dilution of streptavidin-biotinylated horseradish peroxidase complex for 30 minutes at room temperature prior to color development with 3,3'-diaminobenzidine tetrahydrochloride (120 mg in 200 ml tris-buffered saline) containing 0.08% nickel chloride to enhance the staining intensity.[26] Following mounting in xylene, the sections were evaluated under a light microscope. Control studies were carried out by omitting the primary antibody, by use of an irrelevant secondary antibody (substituted the swine anti-rabbit immunoglobulin with biotinylated rabbit anti-mouse antibody), or by absorbing the primary antibody with substance P overnight at 4°C with peripheral blood lymphocytes and substance P.

Fibroblast Cultures

Human gingival fibroblasts were obtained by explant culture as described previously.[29,30] The cells were maintained in Dulbecco's modification of eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units penicillin, 100 µg/ml streptomycin, and non-essential amino acids in a humidified atmosphere of air/CO_2 (9:1) at 37°C. Cells between the fourth and eighth transfer in culture were used.

Effect of Substance P on Gingival Fibroblast DNA Synthesis

To measure the effect of the substance P on DNA synthesis, human gingival fibroblasts were seeded, in triplicate, into 24-well plates (which hold approximately 10^5 cells at confluence) at an initial density of 20,000 cells per well and allowed to attach and spread overnight in DMEM containing 10% FCS. The medium was then replaced with 500 µl/well of DMEM alone and incubated for a further 48 hours. This medium was then removed and replaced with one of the following: DMEM + 0% FCS; DMEM + 0.2% FCS; DMEM + 10% FCS; DMEM + substance P (10^{-4} – 10^{-9} M) in either 0% or 0.2% FCS. After 20 hours incubation in the test media, [^{3}H]-thymidine was added to each well to give a final concentration of 1 µCi/ml. The cells were incubated for a further 4 hours after which the medium was removed and the cells washed three times with 500 µl PBS. The cells were then washed twice with PBS and DNA, then precipitated with 600 µl TCA at 4°C for 2 hours. The cell layers were then lysed with an equal volume of 0.1 M sodium hydroxide for 60 minutes at 50°C. The radioactivity in the extracted material was determined in a liquid scintillation counter. This assay has been shown to represent accurately in vitro division of fibroblasts.[29]

Metabolic Studies

Protein and proteoglycan synthesis by gingival fibroblasts was assessed as described previously.[27,28] Triplicate cultures of confluent human gingival fibroblasts in 24-well plates were incubated in the presence of varying concentrations of substance P in medium containing 0.2% FCS and either 10 µCi/ml [^{3}H]-proline (for total protein synthesis) or 20 µCi/ml $Na_2[^{35}S]O_4$ (for proteoglycan synthesis). After 24 hours the medium was removed and the cells washed once with 250 µl PBS. The wash and medium were pooled and 500 µl aliquots were chromatographed on Sephadex PD-10 columns in the presence of 4 M guanidine HCl/0.05 M sodium acetate pH 5.8. Radioactivity in 0.4 ml effluent fractions was determined by liquid scintillation counting. The amount recovered in the void volume provided a measure of incorporation of radiolabel into newly synthesized macromolecules. Recovery from these columns was approximately 85%. The cell layers were subjected to extraction with 500 µl 4 M guanidine HCl in 0.05 M sodium acetate, pH 5.8 containing the following as protease inhibitors: 6-aminohexanoic acid, benzamidine HCl, N-ethymaleimide, phenyl-methylsulfonyl fluoride. The extraction was allowed to proceed overnight at 4°C prior to application to Sephadex PD-10 columns as described above. Data were normalized to the total numbers of cells in each well.

Statistical Analyses

All data were subjected to statistical analysis using the method of analysis of variance.

RESULTS

The results of immunolocalization of substance P in normal and inflamed human gingivae are shown in Figure 1. Control sections exposed to antibody preabsorbed with substance P showed no detectable staining. In healthy gingiva the antibody localized in the connective tissues specifically within the rete peg region as well as around blood vessels. This distribution could be seen at higher power to be either fibrous varicosities within the fiber network of the connective tissue often running parallel with the orientation of the rete pegs or specifically associated with the outline of vascular walls. In inflamed tissues the distribution was similar to the normal tissues with fibrous networks and extravascular distributions as well as localizing specifically to inflammatory foci. The distribution in inflamed tissues was notable in that it appeared to be closely associated with inflammatory cell infiltrates.

The effect of substance P on DNA synthesis by human gingival fibroblasts was monitored by uptake of [^{3}H]-thymidine. In all circumstances the control cultures responded normally with no DNA synthesis detected in cultures exposed to medium containing 0% FCS. In the presence of 0.2% FCS, there was a small amount of DNA synthesis while in the presence of 10% the rate of DNA synthesis was very high. When the cells were exposed to substance P in media containing 0% FCS there was very little detectable DNA synthesis (Fig. 2). However, if the substance P was introduced to the cultures in the presence of 0.2% FCS, there appeared to be a significant increase in DNA synthesis compared to cells exposed to media containing

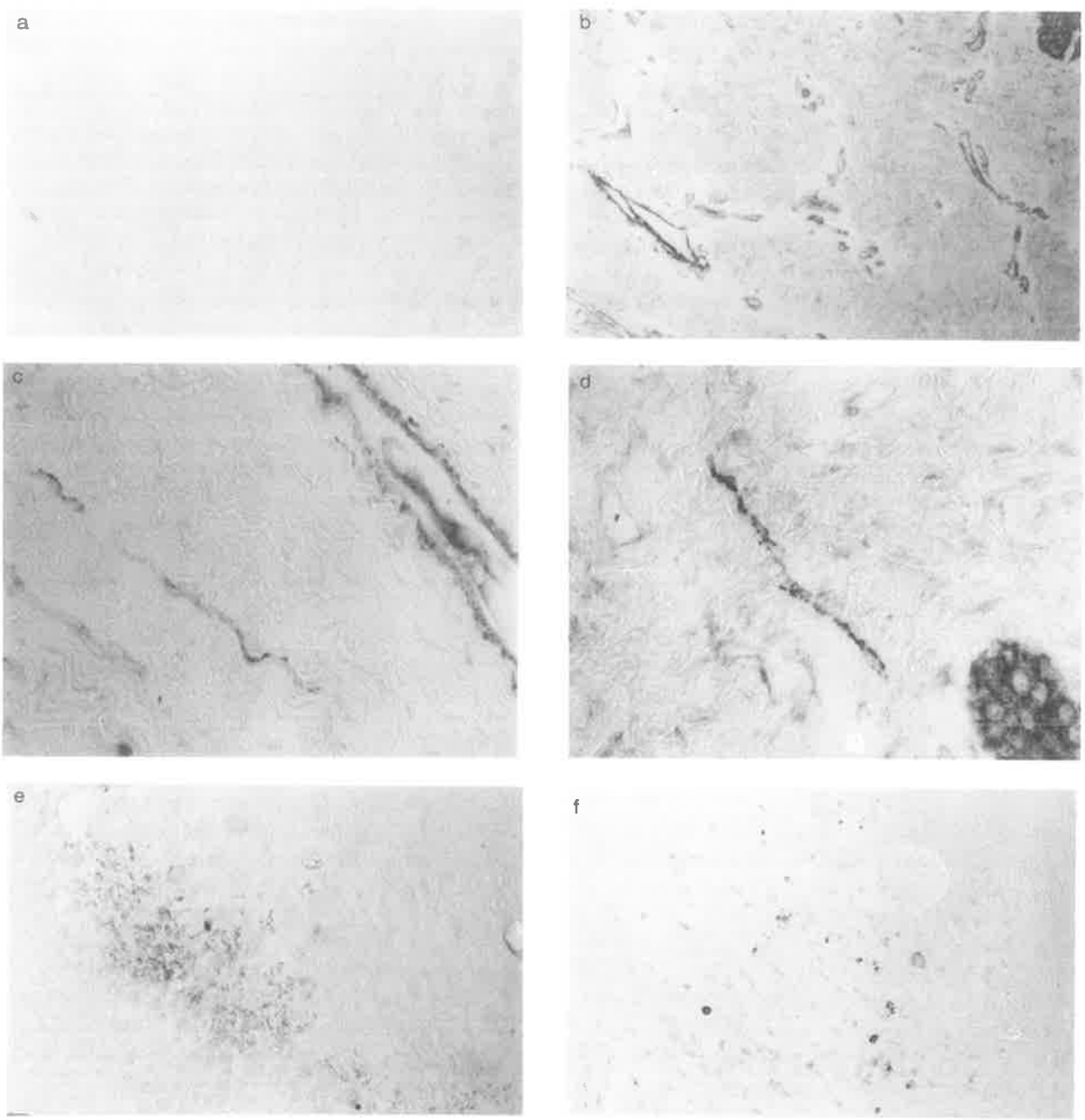

Figure 1. Immunohistochemical distribution of substance in human gingiva. ***a****: control immunoperoxidase staining in which the sections were stained with antibody preabsorbed with substance P;* ***b****: normal human gingival tissue showing strong localization of substance P to the perivascular tissues;* ***c****: normal human gingiva showing localization of substance P within the fibrous connective tissue as well as surrounding a large vessel;* ***d****: normal human gingiva showing a substance P reactive fiber running across collagen bundles;* ***e*** *and* ***f****: inflamed human gingiva showing localization of substance P to foci of inflammatory cells (E = epithelium; CT = connective tissue; original magnification = ×40).*

0.2% FCS alone (Fig. 3). In the presence of substance P and 0.2% FCS, maximum stimulation was noted at 10^{-9} M and was found to approach those levels noted for cells exposed to 10% FCS.

To monitor the effect of substance P on extracellular matrix formation and general synthetic activity of the cells, total proteoglycan as well as total protein synthesis were monitored (Figs. 4, 5, 6, and 7). In all cases cells exposed to 10% FCS produced significantly greater amounts of protein and proteoglycan. The amount of total protein and proteoglycan released into the culture by the cells exposed to substance P did not differ significantly from the controls. However, for both protein and proteoglycan synthesis, the cells exposed to the substance P appeared to retain more material within the cell layer with respect to their comparable control cultures (0.2% FCS).

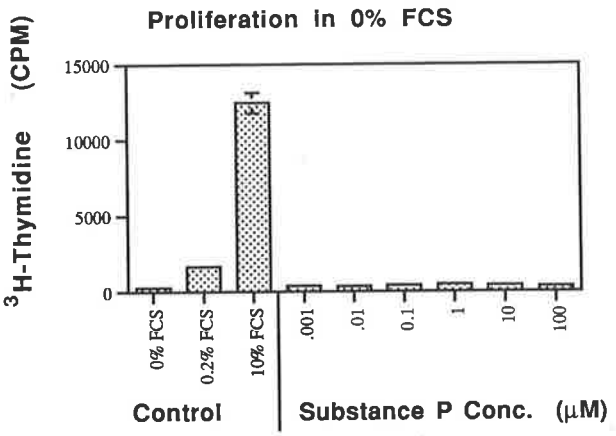

Figure 2. Proliferative activity of human gingival fibroblasts cultured in the presence of 0% FCS and varying concentrations of substance P. The proliferative activity of cells was assessed after culture in the presence of [^{3}H]-thymidine. Data represent the mean and standard deviation of the mean of triplicate cultures.

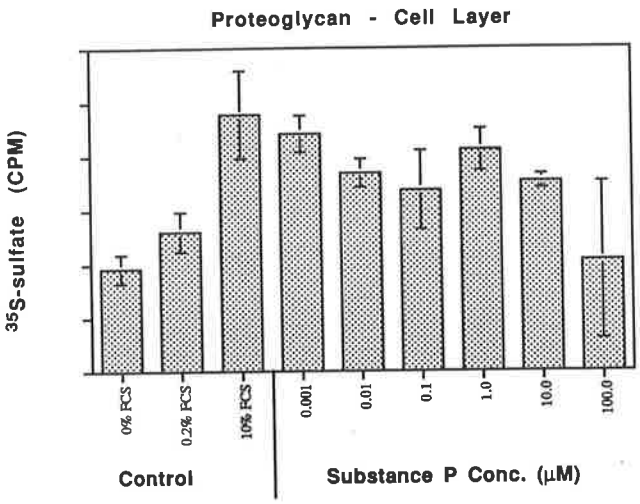

Figure 4. Total amounts of proteoglycans synthesized and retained within the cell layer by human gingival fibroblasts exposed to varying concentrations of substance P in DMEM containing 0.2% FCS. Proteoglycan synthesis was monitored by [^{35}S]-sulfate uptake by the cells. Data represent the mean and standard deviation of the mean of triplicate cultures.

DISCUSSION

There is accumulating evidence that interactions between the nervous system, immune cells, and cells such as fibroblasts are critical to the development and persistence of a variety of inflammatory disorders. While a considerable body of experimental and clinical evidence supports the potential role for neural modulation of infectious and inflammatory diseases of the skin and gastrointestinal tract,[30-32] little is known regarding the importance of neural modulation of host responses in the periodontium. Although networks of neurofilament-immunoreactive fibers have been noted in normal and inflamed gingiva,[33] their relationship to the inflammatory process is unclear.[33,34] Nonetheless, results of several functional and morphological studies appear to implicate neuropeptides as important neurogenic components of inflammatory changes induced by various chemical and mechanical stimuli in oral mucosa.[16] Therefore, neural modulation of inflammatory events in the gingiva, a site in which continuous tissue reactivity is generated in response to the accumulation of dental plaque, is of particular interest in light of the recognized potential for stress to serve as a modifying factor in inflammatory lesions which affect the periodontium.[6-8]

Several neuropeptides have been identified as being involved in the transmission of sensory information from peripheral tissues to the central nervous system. Of these, substance P has been well characterized. Interestingly, some neurons conveying afferent information to the central nerv-

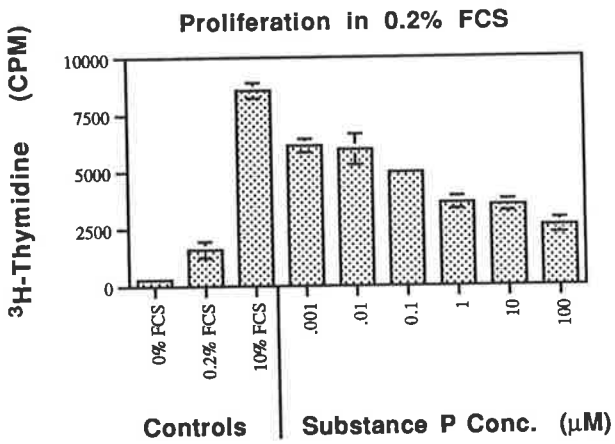

Figure 3. Proliferative activity of human gingival fibroblasts cultured in the presence of 0.2% FCS and varying concentrations of substance P. The proliferative activity of cells was assessed after culture in the presence of [^{3}H]-thymidine. Data represent the mean and standard deviation of the mean of triplicate cultures.

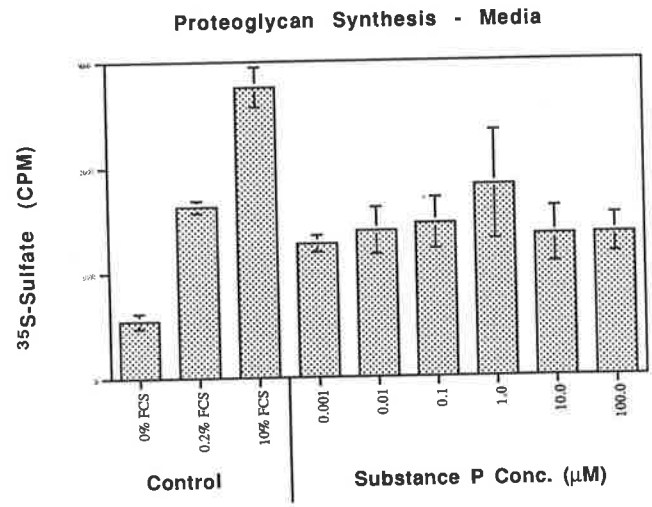

Figure 5. Total amounts of proteoglycans synthesized and released into the culture medium by human gingival fibroblasts exposed to varying concentrations of substance P in DMEM containing 0.2% FCS. Proteoglycan synthesis was monitored by [^{35}S]-sulfate uptake by the cells. Data represent the mean and standard deviation of the mean of triplicate cultures.

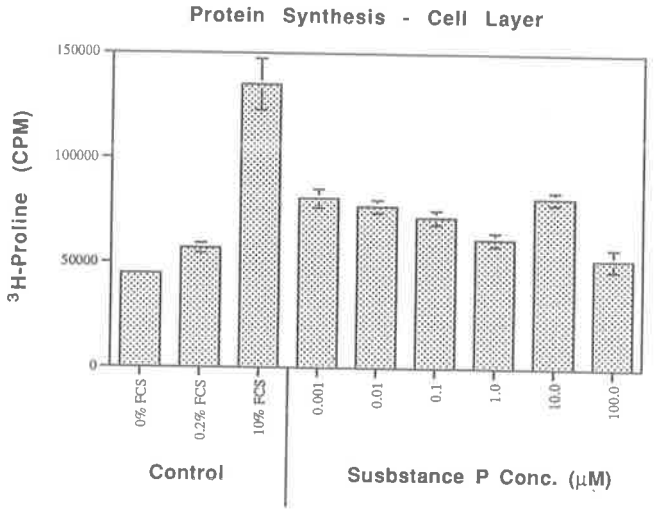

Figure 6. Total amounts of protein synthesized and retained within the cell layer by human gingival fibroblasts exposed to varying concentrations of substance P in DMEM containing 0.2% FCS. Protein synthesis was monitored by the uptake of [³H]-proline. Data represent the mean and standard deviation of the mean of triplicate cultures.

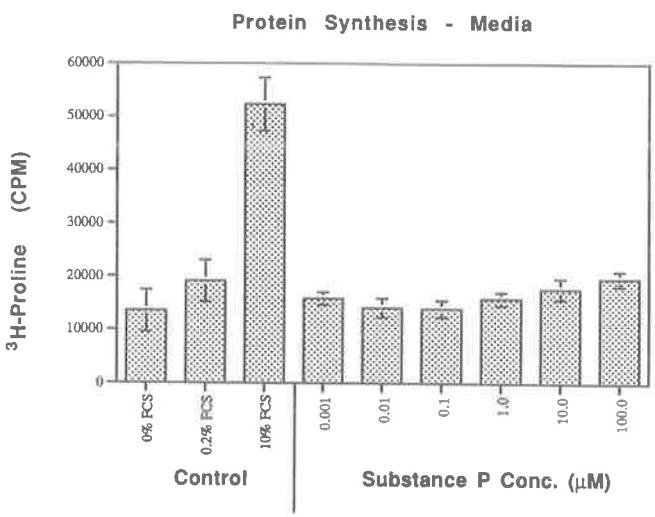

Figure 7. Total amounts of protein synthesized and released into the culture medium by human gingival fibroblasts exposed to varying concentrations of substance P in DMEM containing 0.2% FCS. Protein synthesis was monitored by the uptake of [³H]-proline. Data represent the mean and standard deviation of the mean of triplicate cultures.

ous system from peripheral tissues are also involved in the efferent regulation of the peripheral tissues they innervate. Indeed, substance P-containing neurons have been implicated in the efferent regulation of inflammation and sensitization of joint sensory endings in arthritis.[35] Thus several mechanisms exist by which substance P may play a role in inflammation: substance P is synthesized by dorsal root ganglia neurons and is released at the peripheral terminals rather than the central nervous system;[36] substance P is a potent vasodilator;[37] terminals of nerves releasing substance P are found in close approximation to blood vessels;[38] and substance P has proinflammatory effects on neutrophils, macrophages, mast cells, lymphocytes, and endothelial cells.[12-24]

Additional evidence for the role of neuropeptides in neurogenic inflammation arises from the use of capsaicin or denervation of sensory nerves. For example, systemic pretreatment of rats with capsaicin leads to the selective irreversible degeneration of C-type afferent sensory neurons. As a result, neurogenic inflammation cannot be induced in these animals.[39,40] Capsaicin-sensitive sensory neurons have been noted in oral mucosa and proposed to be important in the development of neurogenic inflammation in these tissues.[41,42] The role of neuropeptides in oral inflammation has also been investigated by studying inflammatory responses in denervated tissues. Surgical laceration of the inferior or superior alveolar nerves in laboratory animals has been shown to significantly affect hemodynamic reactions in the pulp,[43,44] periodontal ligament,[45] gingiva,[46] and oral mucosa.[16,47]

In order to elucidate the mechanisms by which nerves may influence inflammatory reactions in the gingiva, we have determined the presence of substance P in normal and inflamed tissues. From the results of our study and others, gingiva from a variety of species appear to have a significant distribution of neural networks.[48-53] In particular, substance P appears to localize as a fine fiber network in the connective tissues in close proximity to the epithelium and blood vessels.[33] Although few studies have addressed the issue of the distribution of substance P in inflamed gingiva, studies from other tissues would indicate an expected increase in the amount of substance P in such tissues.[54,55] In the present study, the distribution of substance P was found to be largely similar to that noted in healthy and inflamed tissues and this would seem to be in agreement with previous reports.[33] However, of note was our observation of localized areas of substance P immunoreactivity not in a fibrous distribution but associated with collections of inflammatory cells. Such a finding may be of significance in light of previous reports indicating a role for substance P in the activation of lymphocytes.[56] In particular, substance P has been implicated in the activation of B lymphocytes, modulating primary antibody responses as well as stimulating the production of cytokines by monocytes.[23,57,58] In light of the role of B lymphocytes in periodontal disease progression and the role of various cytokines in exacerbation of the inflammatory response, the association between substance P and the immune response in periodontitis should be investigated further.

As a result of the localization of substance P in normal and inflamed gingival tissues, and its previously reported effects on some mesenchymal cells, the second part of this study focused on the effects of substance P on gingival fibroblasts in vitro. Substance P stimulated human gingival fibroblast proliferation up to four times the values found for their basal proliferative level. This finding is in accord with Nilsson, et al.[59] who reported that substance P stimulated DNA synthesis in cultured human skin fibroblasts.

The concentration at which maximum proliferation of mesenchymal cells occurs is variable. For example skin fibroblasts have been reported to respond maximally at 1×10^{-7} M substance P and human synovial cells at 10^{-8} M.[60] In the present study, maximum proliferation of human gingival fibroblasts was noted 1×10^{-9} M which is the same concentration at which substance P causes maximum proliferation of B-lymphocytes.[57] The biphasic nature of substance P being slightly inhibitory at high concentrations and stimulatory at low concentrations may be in line with an hypothesis postulated by Mantyh[17] who suggested that initially substance P has the action of promoting and directing the inflammatory and immune response to produce a hyperinflammatory state in which destruction of the surrounding tissues is the main outcome (i.e., a predominantly catabolic mode). At this early stage it is feasible that substance P should be present locally at high concentrations because antidromic stimulation of substance P immunoreactive fibers will cause great quantities of substance P (which was stored in varicosities at the nerve terminal) to be released into the surrounding tissue. However, after the infection and damaged tissues are cleared, substance P may continue to be released and operate as a mitogen to promote cell proliferation and therefore have a role in tissue remodelling. It is plausible at this stage that the concentration of substance P in the tissues will be much lower due to depletion of substance P supplies from the varicosities of the terminal nerve endings. In the present study it was also noted that substance P did not stimulate proliferation of gingival fibroblasts in media containing 0% FCS. This, therefore suggests that substance P requires co-stimulation from other factors present in the tissue or, in this case, in the serum. In this context, it is interesting to note that substance P can enhance the proliferation of fibroblasts when added to suboptimal concentrations of platelet derived growth factor.[59] It has also been postulated that substance P can act in both a stimulatory and inhibitory fashion towards the same cell, depending on what other chemical signals were present.[17] Therefore the action of substance P could be switched from a catabolic mode (e.g., pro-inflammatory) to an anabolic mode (e.g., tissue regeneration) depending on the presence of other factors. The notion that small sensory neurons may play roles not directly related to nociception, but rather regulatory and reparative processes has been suggested previously. For example, denervation of the inferior alveolar nerve has indicated that while sensory denervation modified fibroblast responses in gingival wound healing, sympathetic innervation played an important role in regulating not only fibroblast activity but also cells in the epithelium and alveolar crest.[61] Thus, the nervous system may play a greater role in co-ordinating growth and differentiation in normal, inflamed and regenerating periodontal tissues than previously considered.

With respect to proteoglycan synthesis, substance P appeared to have a slight stimulatory effect on the cultured human gingival fibroblasts. These findings are in contrast with those of others who have found that substance P has no direct effect on proteoglycan synthesis in either the cell extract or the cell supernatant when applied to articular bovine chondrocytes in culture.[62] Whether this reflects differences between various cell types or merely reflects the difference in principle proteoglycans made by chondrocytes (principally aggrecan) and fibroblasts (principally decorin and biglycan) remains to be established. A small rise in the rate of total protein synthesis was also noted, with up to a 40% increase in synthesis rate being reflected in the values obtained. Our finding of stimulated protein synthesis by substance P, although not as striking, is in accord with the findings of Lotz et al.[60] in which the addition of substance P to synoviocytes stimulated a 5-fold increase in protein synthesis. It is also interesting to note that a corresponding increase in collagenase synthesis was observed in the Lotz et al. study. As such it would be useful to investigate whether the rise in total protein synthesis seen in this study also included a significant rise in collagenase production as this would also lend support to the hypothesis that substance P at high concentrations has a predominantly catabolic effect on surrounding tissues, as the maximum stimulation of protein synthesis was seen when cultured with substance P at a concentration of 1×10^{-4} M, the highest concentration tested.

In conclusion this study has demonstrated the presence of substance P in normal and inflamed human gingiva as well as the ability of substance P to influence human gingival fibroblast proliferative and synthetic activity. The immunohistochemical localization of substance P in gingival tissues implicates it as a potential mediator of inflammation, however, whether our in vitro findings correlate with those found in vivo is not clear. Furthermore, issues such as whether substance P acts directly or indirectly on the cells, the effect of secondary signals such as PGE_2[46] and the role of substance P on immune cell function all remain to be established.

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Paper 23

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Platelet-derived growth factor stimulates hyaluronate but not proteoglycan synthesis by human gingival fibroblasts *in vitro*.
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Platelet-derived Growth Factor Stimulates Hyaluronate but not Proteoglycan Synthesis by Human Gingival Fibroblasts *in vitro*

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The effect of PDGF-BB on human gingival fibroblasts was monitored in an *in vitro* system. PDGF was found to be mitogenic for these cells, although it required the presence of low concentrations of fetal calf serum to be active. Proteoglycan and hyaluronate synthesis was analyzed by labeling newly synthesized macromolecules with [^{35}S]-sulfate or [^{3}H]-glucosamine, respectively. Identification of specific glycosaminoglycans was achieved by selective enzymatic or chemical degradations. It was found that cells cultured in the presence of PDGF showed no discernible differences in proteoglycan synthesis relative to the control cultures. There were no alterations in amounts of proteoglycans synthesized, types of sulfated glycosaminoglycans synthesized, or relative hydrodynamic sizes of the proteoglycans. In contrast to the proteoglycans, hyaluronate synthesis was significantly increased in the presence of PDGF. The increase in [^{3}H]-glucosamine incorporation into newly synthesized hyaluronate correlated with an increase in the activity of the enzyme hyaluronate synthetase but could not be accounted for entirely by changes in the specific activity of sugar nucleotide precursors, which did alter slightly under differing culture conditions. It is concluded from these results that PDGF stimulates gingival fibroblasts to proliferate and is associated with a differential effect on proteoglycan and hyaluronate synthesis. These observations may correlate with the observed early events associated with wound healing and repair.

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Introduction.

The regenerative events of wound healing require the recruitment of connective tissue cells to the site, proliferation of the cells, and synthesis of the specialized components of the connective tissue which they are attempting to repair (Ross, 1968). These processes may be co-ordinated by mediators originating from cells involved in coagulation or inflammation such as platelets, macrophages, polymorphonuclear leukocytes, lymphocytes, and fibroblasts (Wahl *et al.*, 1989). By virtue of the many different cells involved, there is a complex interplay between cells of a variety of lineages mediated *via* the local release of cytokines.

The nature of the extracellular matrix synthesized by fibroblasts during wound healing of soft connective tissues has been defined somewhat, although the precise controlling mechanisms and molecular interactions remain to be elucidated. In broad terms, the early events of extracellular matrix deposition in wound healing can be divided into the synthesis and deposition of the ground substance, followed by the production of various fibrillar components (Bentley, 1966). From our own studies, the earliest events with respect to matrix synthesis and repair in experimental inflammatory lesions are the initial production of hyaluronate, which is shortly followed by the appearance of proteoglycans prior to collagen deposition (unpublished observations).

The mechanisms associated with the healing of periodontal tissues destroyed by inflammation have been the subject of intense investigation. In recent times, manipulation of the healing response by "biological response modifiers" has been pursued as a possible means of improving periodontal wound healing (Terranova *et al.*, 1989). In particular, the use of various growth factors shows promise (Terranova and Wikesjö, 1987; Lynch *et al.*, 1987, 1989). Of these, epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and transforming growth factor (TGF) have been proposed to be of potential significance in relation to their regulatory effects on immune function, epithelium, bone, and soft connective tissues.

Despite these proposals, the basic cellular and biochemical phenomena involved in improving wound repair are still poorly understood. Indeed, *in vivo* there is an extremely complex interplay among various growth factors, cytokines, cell surface molecules, and components of the extracellular matrix (Wahl *et al.*, 1989). However, in order for such processes to be understood, it is still necessary for individual events to be targeted and studied in isolation before more complex interactions can be addressed.

In light of recent reports concerning improved periodontal wound healing in the presence of various growth factors, the aim of this investigation was to determine the effect of one such growth factor, platelet-derived growth factor (PDGF), on gingival fibroblast function *in vitro*. In particular, the ability of PDGF to influence the synthesis of proteoglycans and hyaluronate was assessed.

Materials and methods.

Materials.—Human recombinant platelet-derived growth factor-BB was purchased from Genzyme Corporation (Boston, MA). Dulbecco's modification of Eagle's Medium (DMEM), fetal calf serum (FCS), non-essential amino acids, penicillin, and streptomycin were all purchased from Flow Laboratories (Australasia Pty Ltd., North Ryde, New South Wales). Tissue culture plasticware was obtained from Nunc (Roskilde, Denmark). L-glutamine, urea, guanidine HCl, UDP-N-acetylglucosamine, and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). D-[6-^{3}H]-glucosamine HCl (33 Ci/mmol), UDP-

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D-[U-^{14}C]-glucuronic acid (225 mCi/mmol), and Na[^{35}S]O_4 (1042 mCi/mmol) were obtained from Amersham Australia (Sydney, NSW). Ready solv™ scintillation fluid was obtained from Beckman Australia (Adelaide, S.A.). *Streptomyces* hyaluronidase, chondroitinase ACII (*Arthrobacter aurescens*), and chondroitinase ABC (*Proteus vulgaris*) were purchased from Seikagaku Kogyo Pty. Ltd. (Tokyo, Japan); Zeta probe was from BioRad Laboratories Australasia (Sydney, NSW); and Sephadex G-50, Sepharose CL-4B, and DEAE Sephacel were purchased from Pharmacia (Australia) (North Ryde, N.S.W.).

Human gingival fibroblasts.—Human gingival fibroblasts were obtained essentially by explant culture of healthy gingival tissue derived from healthy donors, as described previously (Bartold and Page, 1987). Cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% FCS, penicillin, streptomycin, glutamine, and non-essential amino acids at 37°C in a moist atmosphere of 5% CO_2 and 95% air. Cells between the 4th and 10th transfers in culture were used.

Fibroblast DNA synthesis.—For confirmation of the mitogenicity of PDGF for gingival fibroblasts previously reported (Bartold *et al.*, 1992), cells were seeded, in triplicate, into 24-well plates (which hold approximately 10^5 cells at confluence) at an initial density of 20,000 cells *per* well and allowed to attach and spread overnight in DMEM containing 10% FCS. The medium was then replaced with 500 µL/well of DMEM alone and incubated for a further 72 h in DMEM containing 0% FCS to achieve cell quiescence at G_o of their cell cycle. This medium was then removed and replaced with a range of concentrations of PDGF in medium containing 10% FCS. After 20 hours of incubation, [^{3}H]-thymidine was added to the medium to give a final concentration of 1 µCi/mL. The cells were incubated for a further 4 h, after which the medium was removed and the cells washed three times with 500 µL PBS. The cells were then washed twice with PBS and DNA, then precipitated with 600 µL TCA at 4°C for 2 h. The cell layers were then lysed with an equal volume of 0.1 mol/L sodium hydroxide for 60 min at 50°C. The radioactivity in the extracted material was determined in a Beckman LS-2800 Liquid Scintillation Counter. This assay has been shown to represent accurately *in vitro* division of fibroblasts (Wahl *et al.*, 1978).

Proteoglycan synthesis.—Proteoglycan synthesis by gingival fibroblasts was assessed by means of a solid-phase assay (Rapraeger and Yeaman, 1989). Briefly, dose-response experiments were carried out on triplicate cultures of confluent cells in 24-well plates which were incubated in the presence of DMEM containing 0.2% FCS, 10% FCS or 0.2% FCS, and 5 ng/mL PDGF, together with 20 µCi/mL Na_2[^{35}S]O_4 for 24 h. The cumulative synthesis of proteoglycans over a 48-hour period was assessed by incubation of cells in the presence or absence of PDGF and sampling at various time intervals (2, 4, 8, 24, and 48 h). At the conclusion of each experiment (dose response or time course), the medium was removed and the cell layers washed with 500 µL PBS. The medium and wash were pooled to make up the medium fraction (total volume of 1 mL). The cell layers were then extracted overnight at 4°C in 1 mL of 8 mol/L urea containing 10 mM Tris HCl, 1 mmol/L sodium sulfate, and 0.1% Triton X-100. Aliquots (200 µL) from both the medium and cell-layer fractions were applied onto Zeta probe membranes assembled in a Bio-Dot apparatus and filtered through under vacuum. The membranes were removed from the apparatus and washed twice each in Tris-buffered saline, de-ionized water, and 95% ethanol. After drying, the membrane was cut into small pieces and added to 5 mL of scintillation fluid; the amount of radioactivity present was determined by liquid scintillation counting.

Sulfated glycosaminoglycan identification.—Gingival fibroblasts were seeded into 25-cm² flasks and allowed to reach confluence. Each flask was then washed three times with PBS prior to incubation of the cultures in various test media and 20 µCi/mL Na_2[^{35}S]O_4. The culture medium was then removed from the flasks, and macromolecular labeled material was recovered following chromatography on Sephadex G-50 (2.5 x 50 cm). Aliquots of the excluded medium and cell-layer extract peaks obtained after Sephadex G-50 chromatography were dialyzed against 7 mol/L urea in 0.05 mol/L Tris HCl and 0.1 mol/L sodium chloride, pH 7.0, and eluted from a DEAE-Sephacel column (0.9 x 7 cm) equilibrated with the same buffer. Prior to analytical ion-exchange chromatography, Triton X-100 was added to give a final concentration of

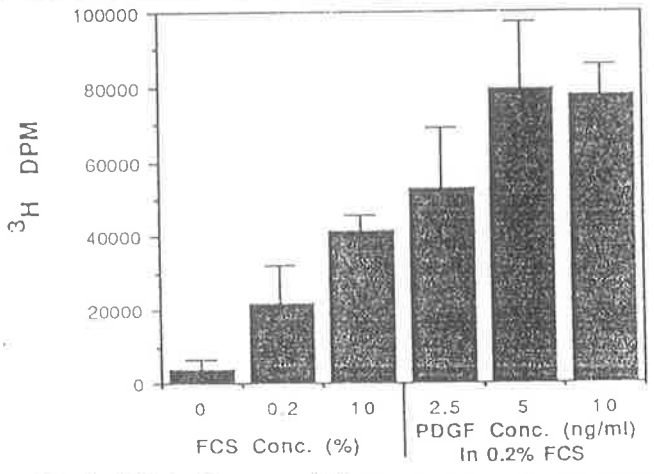

Fig. 1—Effects of serum and PDGF on [^{3}H]-thymidine incorporation into DNA by human gingival fibroblasts. Cells were cultured in the presence of various concentrations of fetal calf serum or PDGF, and the incorporation of [^{3}H]-thymidine into precipitable DNA was monitored 48 h later. Data are expressed as the mean ± standard deviation of the mean from triplicate cultures of a representative experiment.

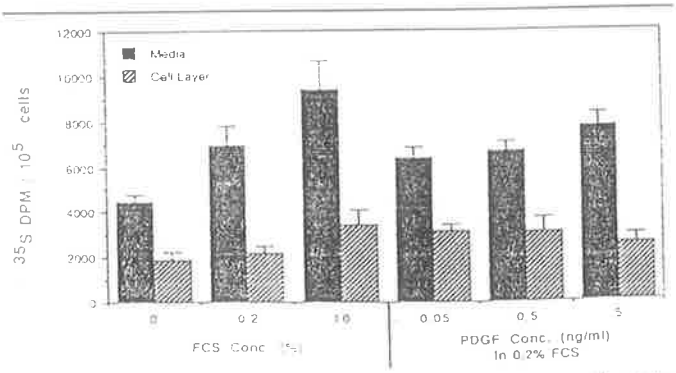

Fig. 2—Effects of serum and PDGF on proteoglycan synthesis by human gingival fibroblasts. Cells were cultured in DMEM containing 0.2% FCS, 10% FCS, or 0.2% FCS and 5 ng/mL PDGF, and the incorporation of [^{35}S]-sulfate into macromolecules was monitored 48 h later. Data are expressed as the mean ± standard deviation of the mean from triplicate cultures of a representative experiment.

0.5%. After elution of the unbound material, a continuous gradient from 0.1 to 0.9 mol/L NaCl gradient was applied in a total volume of 50 mL. Fractions of 0.5 mL were collected at a flow rate of 5 mL/hr, and the whole fractions were assessed for radioactivity.

The types of glycosaminoglycans and their relative proportions in the proteoglycans from the medium and cell-layer compartments of PDGF-treated and untreated cultures were determined by treatment with either chondroitinase ACII, chondroitinase ABC, or nitrous acid (Bartold and Page, 1985; Saito *et al.*, 1968; Shively and Conrad, 1976). The reaction products were then eluted from Sephadex G-50 columns (0.7 cm x 30 cm) with 0.2% sodium dodecyl sulfate in 0.15 mol/L sodium acetate, 1 mmol/L magnesium chloride, and 1 mmol/L calcium chloride, pH 5.8. Fractions of 0.5 mL were collected at a flow rate of 3 mL/h and assayed for radioactivity. The relative proportion of each glycosaminoglycan species was determined by calculation of the amount of radiolabeled material resistant to (void volume) or degraded by (included volume) each treatment.

Analytical column chromatography.—The molecular size distribution of [^{35}S]-sulfate-labeled proteoglycans was analyzed by gel filtration from Sepharose CL-4B. Aliquots containing approximately similar amounts of radioactivity (10,000 dpm) of the [^{35}S]-sulfate-labeled material which was excluded from Sephadex G-50 were concentrated in dialysis tubing against Aquacide to 200 µL. The concentrate was then eluted from columns of Sepharose CL-4B (0.7 cm x 100 cm) with 4 mol/L guanidine HCl/0.05 mol/L sodium acetate containing protease inhibitors, pH 5.8. Fractions of 0.5 mL were collected at a flow rate of 3 mL/h and assessed for radioactivity.

Hyaluronate synthesis.—Hyaluronate synthesis was assessed by the seeding of fibroblasts, in triplicate at a concentration of 5 x 10^4 cells *per* well, into 24-well plates coated with various concentrations of PDGF. Upon reaching confluence (1 x 10^5 cells/well), the cells were incubated in medium containing [^{3}H]-glucosamine (20 µCi/mL) for 24 h. The medium was then removed and the cell layers washed once with phosphate-buffered saline (PBS), which was pooled with the medium to make up the medium fraction for subsequent analyses of hyaluronate content. To determine the effect of PDGF on the rate of hyaluronate synthesis, we cultured confluent cells as described above. Upon reaching confluence, the medium was removed and the cells incubated for 4, 8, 24, 30, 48, and 72 h. The incorporation of [^{3}H]-glucosamine into hyaluronate was determined by selective digestion with hyaluronidase for each time point after the introduction of the radiolabel.

Determination of [^{3}H]-labeled hyaluronate was carried out as described previously (Castor *et al.*, 1983; Huey *et al.*, 1990). Briefly, aliquots (200 µL) from the culture medium were digested with pronase (10 mg/mL) overnight at 60°C. The samples were then boiled and split into two aliquots of 50 µL each. One group (designated aliquot B) was incubated at 37°C for 12 h with 60 µL *Streptomyces* hyaluronidase in 0.1 mol/L sodium acetate buffer at pH 6.0. The other (designated aliquot A) was incubated in the sodium acetate buffer alone under identical conditions. After the incubation period, aliquots (100 µL) from each sample were spotted onto filter paper divided into 2-cm squares, allowed to dry at 60°C, and then placed through 4 washes, each of 30-minute duration, in 0.05 mol/L sodium chloride containing 0.1% cetylpyridinium chloride at room temperature (22°C). The filter paper was then removed and allowed to dry. Each square was then cut into small strips and placed into 14 mL of scintillation fluid prior to determination of radioactivity in a liquid scintillation counter. The amount of hyaluronate present in each sample was calculated as the amount of radioactivity digested by the *Streptomyces* hyaluronidase (*i.e.*, d.p.m. in aliquot A - d.p.m. in aliquot B).

Hyaluronate synthetase determinations.—Levels of hyaluronate synthetase activity were determined on lysates of whole cells. Confluent monolayers of human gingival fibroblasts in 24-well plates were cultured in either 0.2% FCS or 0.2% FCS and PDGF for 8, 24, and 48 h. The cells were released by trypsinization, and the cell pellet was washed in PBS prior to being frozen at -70°C. The cells were lysed by repeated freezing and thawing of the cell pellet (three times), and hyaluronate synthetase activity was determined as described previously (Appel *et al.*, 1979). The lysed cells were incubated in the presence of 0.1 µCi UDP-D-[U-^{14}C]-glucuronic acid and 5 x 10^{-3} µmol/L UDP-N-acetylglucosamine for 90 min, and the amount of hyaluronate thus synthesized was determined by selective digestion with hyaluronidase as described above.

Specific activity determinations.—Since differences in the specific activity of sugar nucleotide precursors may vary between differing culture conditions (Morales *et al.*, 1984), the specific activity of radiolabeled products was assessed by use of a dual labeling protocol (Bartold and Page, 1986). Fibroblasts were incubated for 48 h in the presence of [^{35}S]-sulfate (50 µCi/mL) and [^{3}H]-glucosamine (20 µCi/mL). Labeled macromolecules were separated from unincorporated radiolabel by Sephadex G-50 chromatography, and the proteoglycans were partially purified by ion-exchange chromatography on DEAE-Sephacel. The proteoglycans were then subjected to digestion with chondroitinase ABC as described previously. The digestion products were then eluted from a Sephadex G-50 column. The liberated disaccharides eluted near the total volume of the column, and thus the ratio of ^{3}H/^{35}S in this peak could be readily determined.

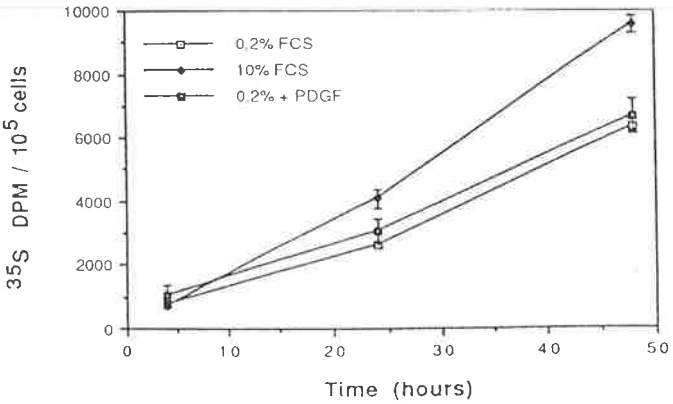

Fig. 3—[^{35}S]-sulfate incorporation into proteoglycans with time. Human gingival fibroblasts were cultured in DMEM containing 0.2% FCS, 10% FCS, or 0.2 % FCS and 5 ng/mL PDGF, and proteoglycans released into the medium were monitored by assessment of the incorporation of [^{35}S]-sulfate into macromolecules over a 48-hour period. Data are expressed as the mean ± standard deviation of the mean of triplicate cultures from a representative experiment.

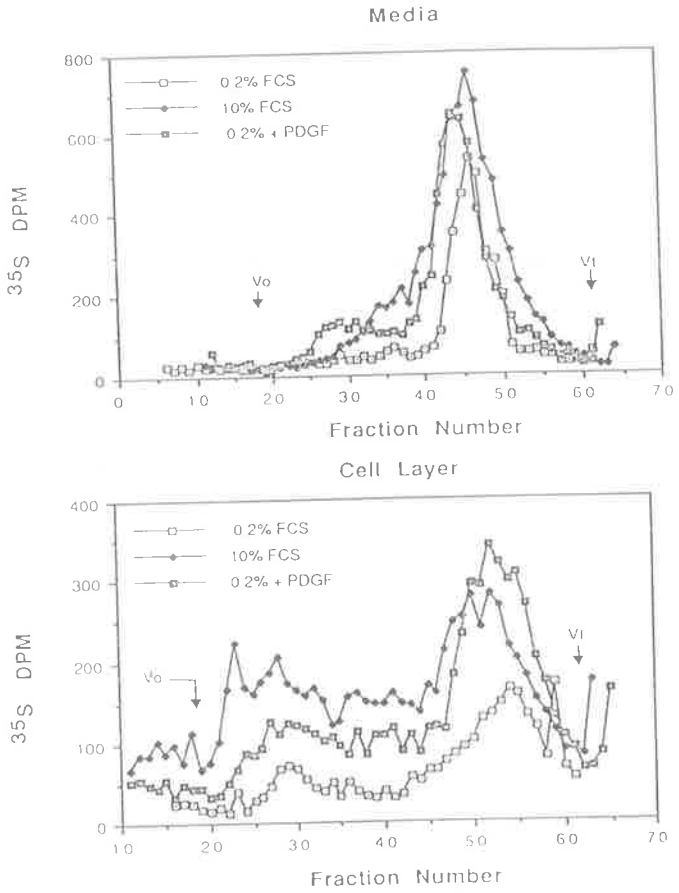

Fig. 4—Gel chromatography on Sepharose CL-4B of proteoglycans isolated from (a) the medium and (b) cell pellets of human gingival fibroblasts cultured in DMEM in the presence of various concentrations of fetal calf serum or PDGF. The v_o and v_t were determined with [^{3}H]-DNA and [^{35}S]-sulfate, respectively.

Results.

Mitogenicity of PDGF.—The effects of various concentrations of serum and PDGF on [^{3}H]-thymidine incorporation into DNA are shown in Fig. 1. In the control cultures, which were not exposed to PDGF, there was a dose-dependent response to increasing concentrations of serum, with 0% FCS inducing very little proliferative activity and 10% FCS resulting in a five-fold increase. Cultures exposed to 0.2% FCS showed approximately a two-fold increase in proliferative activity. The proliferative responses of the cells to various concentrations of PDGF were monitored in 0.2% FCS, because no response was noted in the absence of serum, and at higher concentrations of serum (*e.g.*, 10% FCS) the effects were masked by the proliferative response to the serum components (results not shown). At all concentrations of PDGF studied, a marked increase in proliferative activity was noted over the controls. At the highest concentration studied, PDGF appeared to stimulate the proliferative response 10-fold over the control conditions of 0.2% FCS.

Effect of PDGF on proteoglycan synthesis.—Proteoglycan synthesis by human gingival fibroblasts was monitored by the incorporation of [^{35}S]-sulfate into macromolecules in the presence of various concentrations of FCS and PDGF (Fig. 2). Increasing concentrations of FCS appeared to stimulate the amounts of proteoglycans secreted into the culture medium, with cells cultured in the presence of 10% FCS showing an almost two-fold increase in amounts compared with cells cultured in the absence of FCS. Cells cultured in low serum (0.2%) demonstrated a 60% increase over the control cells cultured in 0% FCS. The presence of PDGF in 0.2% serum appeared to have no stimulatory effect on the amounts of proteoglycans synthesized by human gingival fibroblasts as measured by cell-associated material or material released into the culture medium. At all concentrations of PDGF studied, the levels of proteoglycan synthesis remained at levels close to those noted in the control cultures exposed to 0.2% FCS alone.

Because the initial experiments were carried out after 48 hours' exposure to PDGF and [^{35}S]-sulfate, it was possible that alterations in synthesis at earlier time points may have been overlooked. Therefore, time-course observations at specified time intervals over 48 h were also carried out (Fig. 3). At all time points studied, cells cultured in the presence of 10% FCS synthesized greater proportions of labeled proteoglycans. The amounts of proteoglycans synthesized by cells cultured in the presence of either 0.2% FCS or 0.2% FCS and PDGF were similar.

Effect of PDGF on glycosaminoglycan species.—Although no discernible differences in total proteoglycans synthesized were detected, the glycosaminoglycan content was studied to determine whether PDGF had any selectivity for these components of the proteoglycans.

The results of analysis of sulfated glycosaminoglycans by selective enzyme and chemical elimination are shown in the Table. Regardless of the culture conditions, no significant differences in the distribution of sulfated glycosaminoglycans could be detected. In all cases, dermatan sulfate was the predominant species in the culture media, and heparan sulfate predominated in the cell layers.

Effect of PDGF on proteoglycan hydrodynamic size.—Chromatography with Sepharose CL-4B was used in order to establish whether PDGF had any effect on the sizes of proteoglycans synthesized by human gingival fibroblasts (Fig. 4). For the material secreted into the medium, two principal peaks were noted to elute at around Kav values of 0.56 and 0.29 for the cells cultured in either 10% FCS or 0.2% FCS and PDGF. However, cells cultured in the presence of low serum (0.2%) appeared to synthesize only the species eluting at Kav 0.56. For the cell-layer-associated material, no significant differences were noted in the relative distributions of the various proteoglycan elution profiles on Sepharose CL-4B.

Effect of PDGF on hyaluronate synthesis.—Hyaluronate synthesis by human gingival fibroblasts was assessed in various concentrations of FCS and PDGF (Fig. 5). The greatest amount of hyaluronate synthesis was noted in the presence of 10% FCS, although significant increases in synthesis were also noted for cultures exposed to PDGF compared with the control cultures of 0% and 0.2% FCS. The response to PDGF appeared to be dose-dependent and represented a significant increase over the control cultures exposed to 0.2% FCS alone. Determinations of hyaluronate synthesized at various time points indicated that the stimulatory effect of PDGF became evident by 24 h after exposure to the PDGF, although some trend toward stimulation in the presence of 10% FCS could be seen as early as 4 h (Fig. 6).

The activities of the enzyme hyaluronate synthetase were also assessed in the presence or absence of PDGF (Fig. 7). A steady increase in enzyme activity over time in culture could be observed for both conditions. However, the levels of activity were greater at all time points studied for the cells exposed to PDGF.

Specific activity determinations.—Because various culture conditions can alter the specific activities of sugar nucleotide precursor pools, increases in amounts of labeled sugar incorporation may not necessarily reflect true increases in synthesis of hyaluronate. Therefore, the specific activity of the glycosaminoglycans was assessed by dual labeling and analysis of the $^{35}S/^{3}H$ ratios in digested disaccharides. The results from these analyses indicated that the $^{35}S/^{3}H$ ratio increased in proportion to the levels of FCS and PDGF present in the culture medium. The values for 0.2% FCS, 10% FCS, and 0.2% FCS with PDGF were 0.28 (± 0.03), 0.38 (± 0.04), and 0.40 (± 0.05), respectively. Thus, the ratio for chondroitinase ABC disaccharides derived from cultures exposed to PDGF in 0.2% FCS or 10% FCS was approximately 1.4 times greater than that for cultures exposed to 0.2% FCS alone.

Discussion.

The effects of growth factors on mesenchymal cells are considered to be fundamental to maintenance and repair of tissues (Kingsworth and Slavin, 1991; Pierce *et al.*, 1991; Deuel *et al.*, 1991). However, the precise mechanisms involved in such regulation are still equivocal. This study has focused on aspects of the role of growth factors in modulating some functions of human gingival fibroblasts. Specifically, the ability of PDGF to regulate cell proliferation and the synthesis of proteoglycans and hyaluronate by human gingival fibroblasts were studied with the view to shedding some light on the possible mechanisms by which these agents might augment tissue repair and regeneration.

PDGF occurs predominantly as a dimer of related but distinct polypeptide chains (termed A and B) in several forms, including homodimers of A and B chains (PDGF AA and PDGF BB) as well as heterodimers (PDGF AB) (Heldin, 1992). Many of the biological effects of PDGF result from its variety of processed forms and different localizations after synthesis (Heldin and Westermark, 1990; Heldin, 1992). Various forms of PDGF can localize to cell surfaces to act in an autocrine or juxtacrine manner. Alternatively, PDGF may accumulate in the extracellular matrix to become available at a later stage to induce cell proliferation and migration. Regulation of the cellular responses to PDGF is *via* receptors on the the surfaces of target cells. Several receptors have been identified, including the α-receptor, which binds both A- and B-containing forms of PDGF, while the β-receptor binds only PDGF-B (Hart *et al.*, 1988). Variability in biological response to the various forms of PDGF may be related to signal transduction through the α- and β-receptors. For example, PDGF AA is not as potent as PDGF BB with respect to mitogenesis (Bywater *et al.*, 1988; Raines *et al.*, 1989), and cell migration appears to be mediated through the β-receptor (Eriksson *et al.*, 1992).

The mitogenic effect of PDGF on many different types of mesenchymal cells has been recognized for several years (Westermark and Wasteson, 1975; Ross and Vogel, 1978; Floege *et al.*, 1991). Nonetheless, when working in particular model systems (in this study, periodontal connective tissues), it is essential that one confirm that agents known to operate on cells of a similar nature (*e.g.*, fibroblasts) but in tissues of vastly different functions are, indeed, effective on the particular cells being studied (*e.g.*, gingival fibroblasts). In this context, the present study has demonstrated that PDGF is, indeed, mitogenic for human gingival fibroblasts. Such a finding confirms earlier reports concerning the effects of PDGF on gingival fibroblast proliferation (Nakae *et al.*, 1991; Bartold *et al.*, 1992). The range of concentrations over which PDGF has been found to be effective on gingival fibroblasts was similar to that reported for many other cells of mesenchymal origin but different locale (Raines and Ross, 1985). Although PDGF elicits its mitogenic response *via* attachment to specific cell-surface receptors (Bowen-Pope and Ross, 1982), it is considered only a competence factor in that progression factors are required for completion of cell division (Stiles *et al.*, 1979). As further confirmation that

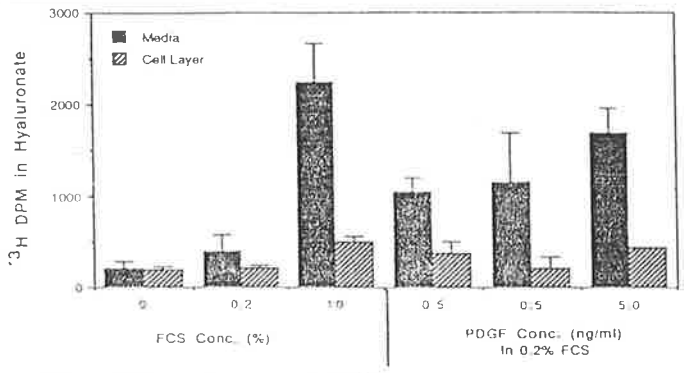

Fig. 5—Effects of serum and PDGF on hyaluronate synthesis by human gingival fibroblasts. Cells were cultured in DMEM containing 0.2% FCS, 10% FCS, or 0.2% FCS and 5 ng/mL PDGF, and the incorporation of [^{3}H]-glucosamine into macromolecules identifiable as hyaluronate on the basis of susceptibility to *Streptomyces* hyaluronidase digestion was monitored 48 h later. Data are expressed as the mean and standard deviation of the mean from triplicate cultures of a representative experiment.

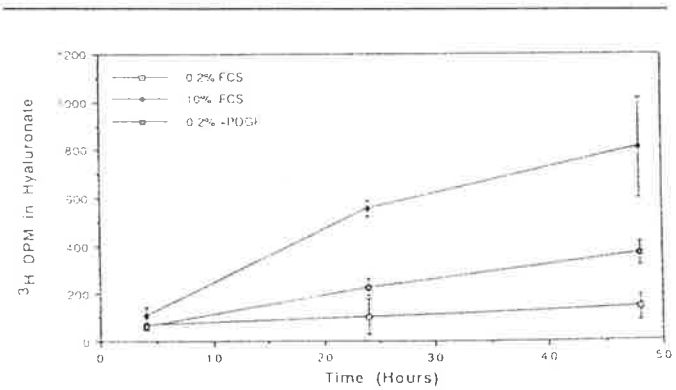

Fig. 6—[^{3}H]-glucosamine incorporation into hyaluronate with time. Human gingival fibroblaasts were cultured in DMEM containing 0.2% FCS, 10% FCS, or 0.2% FCS and 5 ng/mL PDGF, and hyaluronate released into the medium was monitered by assessment of the incorporation of [^{3}H]-glucosamine into hyaluronate over a 48-hour period. Data are expressed as the mean and standard deviation of the mean of triplicate cultures from a representative experiment.

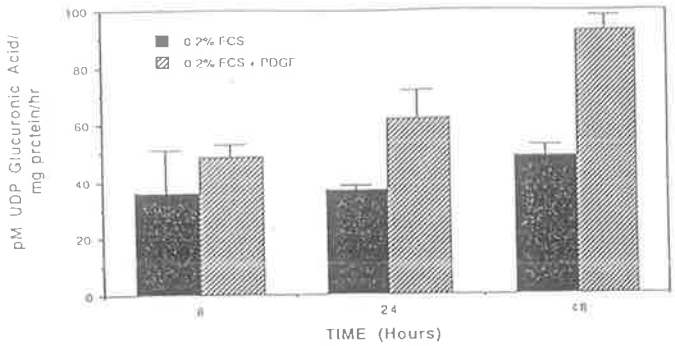

Fig. 7—Effect of PDGF on hyaluronate synthetase activity at various time points. Enzyme activity was assessed by the incorporation of UDP-D-[^{14}C]-glucuronic acid into hyaluronate at selected time intervals in either the presence or absence of 5 ng/mL PDGF in DMEM containing 0.2% FCS. Data represent the mean and standard deviation of the mean of triplicate cultures from a representative experiment.

PDGF acts in a recognized manner on human gingival fibroblasts, it is of note that, in the absence of serum, PDGF was unable to stimulate thymidine uptake by gingival fibroblasts (data not shown), and that mitogenic activity was noted only in the presence of low (0.2%) concentration of serum. Thus, all experiments in this study were performed in the presence of 0.2% FCS. The precise components in FCS which act as progression factors have yet to be identified but likely will result from a complex interplay among various growth factors, cytokines, and other glycoproteins (Pledger *et al.*, 1978).

Despite assertions that PDGF has an ubiquitous distribution throughout tissues, is synthesized by a large number of cells, and affects extracellular matrix synthesis (Ross, 1989), there have been surprisingly few studies addressing the direct effect of PDGF on proteoglycan and hyaluronate synthesis. Of the studies which have addressed this problem, most have focused on the hard connective tissues such as cartilage and bone, with relatively few studies concerned with the soft connective tissues. Furthermore, there appears to be only one report considering the effect of PDGF on both proteoglycan and hyaluronate synthesis (Imai *et al.*, 1992), in which PDGF was found to influence both proteoglycan and hyaluronate synthesis by retro-ocular fibroblasts derived from patients suffering from Graves' ophthalmopathy. The direct relevance of retro-ocular fibroblasts to gingival fibroblasts is unclear, but it would seem reasonable that these cells may have quite different functions and responses to various growth factors. Such an apparent lack of detailed analysis of PDGF with respect to extracellular matrix biology is curious, especially in light of the well-documented effects of other growth factors on proteoglycan synthesis, such as TGF-β (Wahl, 1991), and the important role of proteoglycans in acting as receptors for growth factors as well as modulators of growth factor activities (Ruoslahti and Yamaguchi, 1991).

The findings of this study that PDGF had little effect on proteoglycan synthesis may be surprising in light of previous reports of other growth factors having significant effect on proteoglycan synthesis. Nonetheless, they are consistent with other studies in which PDGF has been found to have little or no effect, *in vitro*, on proteoglycan synthesis by chondrocytes (Hamerman *et al.*, 1986), liver cells (Border *et al.*, 1987), smooth muscle cells (Chen *et al.*, 1987), cartilage (Manin *et al.*, 1991; Harrison *et al.*, 1991), and skin fibroblasts (Savage *et al.*, 1987; Westergren-Thorsson *et al.*, 1991).

Despite the negligible effect on proteoglycan synthesis, PDGF was found markedly to increase the amount of [^{3}H]-glucosamine labeled hyaluronate. However, interpretation of such data can be difficult, since the specific activity of glucosamine in the nucleotide sugar precursor pool can vary, depending upon the culture conditions (Hascall *et al.*, 1990). Consequently, differences in the uptake of

TABLE

GLYCOSAMINOGLYCAN QUANTITATION[1]

	Chondroitin Sulfate	Dermatan Sulfate	Heparan Sulfate
MEDIA			
0.2% FCS[2]	29 (± 3)	55 (± 7)	16 (± 3)
10% FCS[3]	32 (± 2)	57 (± 3)	11 (± 3)
PDGF[4]	34 (± 5)	46 (± 4)	20 (± 5)
CELL LAYER			
0.2% FCS[2]	21 (± 4)	25 (± 4)	54 (± 9)
10% FCS[3]	11 (± 7)	29 (± 5)	60 (± 5)
PDGF[4]	17 (± 5)	26 (± 3)	57 (± 6)

[1]Glycosaminoglycans were quantitated after selective degradation with chondroitinase ACII, chondroitinase ABC, or nitrous acid for determination of the relative contributions of chondroitin sulfate, dermatan sulfate, and heparan sulfate, respectively. Data are expressed as percentages (mean ± standard deviation of the mean of triplicate experiments) of the total radiolabeled material digested by each degradative procedure.

[2]Represents data for cells cultured in DMEM containing 0.2% FCS.

[3]Represents data for cells cultured in DMEM containing 10% FCS.

[4]Represents data for cells cultured in DMEM containing 0.2% FCS and PDGF.

labeled glucosamine in differing culture conditions (*e.g.*, presence or absence of growth factors) may not reflect the identical differences noted in amounts of hyaluronate synthesized. However, by demonstrating that the specific activities of labeled glycosaminoglycans cannot account for the increase as well as elevated levels of hyaluronate synthetase activity, this study provides good evidence for a stimulatory role of PDGF on hyaluronate synthesis. Indeed, it may be that, for the reasons outlined above, of the few studies which have addressed the effect of PDGF on hyaluronate synthesis, the conclusions have been varied. Nonetheless, the present study is in agreement with several other well-controlled studies concerning the *in vitro* effects of PDGF on hyaluronate synthesis in which a stimulatory effect has been noted (Heldin *et al.*, 1989; Heldin *et al.*, 1991).

The finding that PDGF has no discernible effect on proteoglycan synthesis but did stimulate hyaluronate synthesis is interesting from a number of aspects: First, it indicates that the synthesis of these two components is under separate control. Second, it has significant ramifications for the likely events occurring during wound healing. With respect to the control of synthesis of hyaluronate and proteoglycans, it has been reported that the metabolism of these two components is correlated in cartilage organ cultures (Morales and Hascall, 1988). Furthermore, a growth factor from cartilage has been found to enhance hyaluronate synthesis and diminish sulfated glycosaminoglycan synthesis in chondrocytes in a manner similar to that used for the results reported in the present study (Hamerman *et al.*, 1986). The precise mechanisms involved in such control are not entirely clear, since proteoglycans are synthesized through normal channels associated with glycoprotein synthesis (Kimura *et al.*, 1984), whereas hyaluronate appears to be synthesized by the enzyme hyaluronate synthetase located on the plasma membrane (Prehm, 1983). Another complicating factor in studies concerning growth factors is the close association between cell proliferation and the synthesis of proteoglycans and hyaluronate. Although confluent cultures were used in order to minimize the effects of cell proliferation, PDGF has been found to elicit a mitogenic response in confluent cultures (Clemmons and VanWyck, 1981; Williams *et al.*, 1983). Therefore, whether the various responses noted in the present study reflect a general trend of proliferating fibroblasts to synthesize increased proportions of hyaluronate and decreased levels of proteoglycans (Lembach, 1976; Hopwood and Dorfman, 1977; Preston *et al.*, 1985) needs to be investigated further.

In conclusion, this study shows that PDGF has several biological effects on human gingival fibroblasts which may be of significance in the complex events associated with repair and regeneration of the periodontal tissues damaged by inflammation. Further investigations into these effects are warranted to determine the effectiveness of PDGF and other growth factors as potential therapeutic agents. Although extrapolations from *in vitro* studies to *in vivo* studies must be made with caution, it is tempting to speculate that observations such as increased hyaluronate synthesis and decreased proteoglycan synthesis closely reflect the events occurring during early wound healing and repair.

Acknowledgment.

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Design of the experiments
Supervision of the experimental work
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Growth factor modulation of fibroblasts in simulated wound healing

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Growth factors are potent bioactive molecules responsible for the co-ordination of many cell functions and interactions. Of these agents PDGF and IGF have shown particular promise as agents which may be used to stimulate periodontal regeneration. In order to further understand the mechanisms by which growth factors may work, a simple model of *in vitro* wound healing has been utilized to assess the effects of PDGF on human periodontal ligament fibroblasts and its potential to stimulate wound healing. Human periodontal ligament fibroblasts were plated into 24-well plates and upon reaching confluence were wounded by creating uniform discoid lesions stripped of cells. The influence of various concentrations of PDGF on cell proliferation, cell migration and extracellular matrix synthesis was monitored. The results of this study indicated that in the presence of 10 ng/ml PDGF and 0.2% fetal calf serum, both cell proliferation and cell migration were significantly stimulated. In the wounded cultures, PDGF appeared to cause a moderate stimulation of proteoglycan synthesis compared to unwounded cultures. In conclusion, the model system tested appears to be useful for studying fundamental cellular and biochemical events associated with wound healing. The effects of PDGF in this system confirm that it is capable of modulating fibroblasts in a manner compatible with the events associated with wound repair.

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Key words: growth factor; wound healing; cell proliferation; cell migration

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Despite intense microbiological, immunological and biochemical investigations in recent years to determine the precise aetiological and pathological events associated with the periodontal diseases, treatment regimes have not always advanced at a commensurate rate. Once tissue destruction has occurred, the ultimate goal of periodontal therapy should be regeneration of the affected tissues to restore their original architecture and function (1). To date, the principal therapy for teeth affected by periodontal destruction has been removal of root surface deposits and control of the bacterial infection. This may be achieved via conservative (closed-pocket) subgingival debridement, open surgical debridement, the prescription of specific antimicrobial agents, or any combination of all three procedures. Thus, the elementary procedures of thorough root surface debridement and removal of soft tissue infection still remain the hub of current therapies. Nonetheless, considerable efforts to develop treatments which will produce satisfactory results have included advanced surgical procedures (2), the use of autologous and synthetic bone grafting materials(3), root surface treatment and etching (4–6) and, more recently, the use of barrier membranes (7). Although the use of barrier membranes demonstrated good potential for regeneration of the root surface cementum, alveolar bone and periodontal ligament, the clinical results using this method are still, to some extent, unpredictable and variable (8). Probably the single most important explanation for this lies in the fact that while the histological events associated with these processes have been well documented, fewer studies have addressed the cell biological and biochemical events associated with periodontal regeneration (9–11).

Despite many of the shortfalls of current therapies, each development has been useful and has permitted

a better understanding of the complex events associated with periodontal healing and regeneration.

The regenerative events of wound healing require recruitment (migration) of connective tissue cells to the site, proliferation of these cells, and synthesis of the specialized components of the connective tissues which they are attempting to repair (12). These processes may be co-ordinated by mediators originating from cells involved in coagulation or inflammation such as platelets, macrophages, polymorphonuclear leukocytes, lymphocytes and fibroblasts (13). By virtue of the many different cells involved, there is a very complex interplay between cells of a variety of lineages mediated via the local release of cytokines. Thus, dissecting these processes is essential not only to better understand the events of tissue regeneration but also to provide a rationale for augmenting or directing wound healing.

In recent years, the application of tissue growth factors to aid periodontal regeneration has received prominent attention (14). Among the myriad growth factors currently characterized and available, epidermal growth factors (EGF), fibroblast growth factor (FGF), insulin-like growth factors (IGF), platelet derived growth factors (PDGF) and transforming growth factors (TGF) have been proposed to be of potential use in relation to their regulatory effects on immune function, epithelium, bone and soft connective tissues.

Two of these growth factors, PDGF and IGF-1, have recently been shown to enhance periodontal regeneration in beagle dogs and monkeys with artificially induced periodontal disease (15, 16). In a variety of cell culture systems, PDGF and IGF have been reported to stimulate cell proliferation, proteoglycan synthesis and collagen synthesis (17–19). In particular, PDGF has been shown to influence periodontal cell proliferation, migration and synthetic activity (20–23). Furthermore, PDGF and IGF-1 have been found to act synergistically to accelerate the healing of skin wounds (24) as well as to stimulate metabolism of bone cells and fibroblasts (25, 26). Therefore, in conjunction with the improved periodontal healing noted in animal studies, these findings suggest that various growth factors may be able to facilitate the early events of periodontal healing and stimulate periodontal regeneration via the formation of new cementum, periodontal ligament and bone.

The events associated with periodontal wound healing will include (i) initial surgical incision, tissue debridement and flap replacement, (ii) appearance of numerous growth factors and cytokines from the vasculature and extravasated leukocytes, (iii) migration and proliferation of fibrobloasts induced by the cytokines and growth factors, and (iv) new matrix synthesis. Thus, creation of a wound is a fundamental step in the sequences leading to regeneration. While numerous models have been developed to study chemotaxis of periodontal cells to a variety of compounds (22, 27–29), none have considered the trilogy of events following wound healing; namely proliferation, migration and matrix synthesis.

In light of the recent reports concerning improved periodontal wound healing in the presence of various growth factors, the aim of this investigation was to determine the effect of one such growth factor, platelet derived growth factor (PDGF) on periodontal ligament fibroblasts in a simple *in vitro* model of wound healing.

Materials and methods

Materials

Dulbecco's modification of Eagle's medium (DMEM), fetal calf serum (FCS), non-essential amino acids, penicillin and streptomycin were purchased from Flow Laboratories Australasia Pty Ltd, North Ryde, New South Wales, all tissue culture plastic ware was obtained from Nunc, Roskilde, Denmark; L-glutamine was from Sigma Chemical Co., St Louis, MO; [6-^{3}H]-thymidine (22 Ci/mMol), L-[5-^{3}H]-proline (25 Ci/mMol) and $Na_2[^{35}S]O_4$ (1042 mCi/mMol) were from Amersham (Australia Pty Ltd, Surrey Hills, New South Wales; Ready Safe EP liquid scintillation fluid was from Beckman Australia; Sephadex PD-10 columns from Pharmacia Fine Chemicals, North Ryde, New South Wales; recombinant human PDGF-BB from Genzyme Corporation, USA. Kodak D19 developer and Rapid Fix were purchased from Kodak Australia, Sydney, NSW; Ilford K2 emulsion was from Ilford Australia, Sydney, NSW.

Fibroblast cultures

The fibroblasts used for this experiment were obtained from the periodontal ligament of premolar teeth extracted from a young healthy patient for orthodontic reasons. Fibroblasts were isolated according to the procedures described by Ragnarsson et al. (1985). Tissue explants were cultured using Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) supplemented with penicillin (100U/ml), streptomycin (100U/ml) and fungizone (2.5 μg/ml). Cultures were incubated at 37°C in a humidified atmosphere of 5% C°2 and 95% air. The media was replaced every 2 or 3 days until confluency was reached. At confluency, the cells were split 1:3 and this was denoted as the first passage. The periodontal ligament fibroblasts used for this study were between

their 5th and 10th passage in culture and for all experiments cells of the same passage number were used.

Fibroblast DNA synthesis

To confirm the mitogenicity of PDGF-BB on periodontal ligament fibroblasts, DNA synthesis was measured by the uptake of [^{3}H]-thymidine (27). Human periodontal ligament fibroblasts were seeded, in triplicate, into 24-well plates (which hold approximately 10^5 cells at confluence) at an initial density of 20,000 cells per well and allowed to attach and spread overnight in DMEM containing 10% FCS. The medium was then replaced with 500 µl/well of DMEM alone and incubated for a further 48 h. This medium was then removed and replaced with the one of the following: DMEM+0% FCS; DMEM+0.2% FCS; DMEM+10% FCS; DMEM+PDGF-BB (2.5, 5.0 and 10 ng/ml) in 0.2% FCS. After 20 h incubation in the test media, [^{3}H]-thymidine was added to each well to give a final concentration of 1 µCi/ml. The cells were incubated for a further 4 h after which the medium was removed and the cells washed three times with 500 µl PBS. DNA was then precipitated with 600 µl TCA at 4°C for 1 h. The cell layers were then lysed with an equal volume of 0.1 m sodium hydroxide for 30 min at 60°C. The radioactivity in the extracted material was determined in a Beckman LS-2800 Liquid Scintillation Counter.

In vitro wounding

Fibroblasts were plated into 24-well plates at an initial density of 50,000 cells per well in DMEM containing 10% FCS and cultured for 72 h until confluent. A sterile 7 mm rubber bung was placed in the centre of each well and, under slight pressure, was gently rotated. This served to produce a discoid shaped lesion void of cells of approximately 8 mm in diameter. The increase in diameter to 8 mm compared to the 7 mm diameter of the rubber bung was due to some concentric slipping of the bung during wounding. The slipping could be minimized by placing a nylon washer 0.5 mm narrower than the diameter of the cell culture wells. After wounding the medium was removed and the remaining cells washed 3 times with PBS prior to the addition of fresh media containing various concentrations of PDGF-BB.

Cell proliferation

For experiments analysing cell proliferation after *in vitro* wounding 8 wells of cells were used for each time point and treatment – a total of 144 wells. After wounding, the cells were cultured for varying times up to 16 days and the rate at which the discoid wound filled with cells was monitored. In order not to "saturate" the system with PDGF the cells were cultured in 1 ml of test or control media which was replenished only at d 10 (50:50 v/v) with fresh test or control media. At each time point the medium was removed and the cells fixed in gluteraldehyde and later stained with crystal violet. Following solubilization of the stained cells in acetic acid, the absorbance of each solute was read at 620 nm. In this assay dye concentration correlates positively with cell number (31).

Cell migration

Prior to solubilizing the cells with acetic acid for absorbance measurements as described above, the visible wound edge was traced by image analysis using a Zeiss microprojector onto the digitizing table of a Hipad Plus 9000 series digitizer and the readings converted into a measurement of wound area. This allowed quantification of the rate at which the wounded areas were filled with migrating cells.

Proteoglycan synthesis

Triplicate cultures of confluent human periodontal ligament fibroblasts in 24-well plates were either wounded as described and then incubated in various concentrations of PDGF-BB for 6, 24 and 48 h. At 6 h prior to termination of the incubation (i.e 0, 18 and 43 h) 20 µCi/ml $Na_2[^{35}S]O_4$ was added to the culture medium. The medium was removed from each well and the cells washed once with PBS. The wash and media were pooled. Labelled macromolecules (proteoglycans) were quantitated by Sepharose G-25 (PD-10) column chromatography in the presence of 4 m guanidine HCU0.05 m sodium acetate pH 5.8. Radioactivity in 0.4 ml effluent fractions was detemmined by liquid scintillation counting. The amount recovered in the void volume provided a measure of incorporation of radiolabel into newly synthesized macromolecules. Recovery from these columns was approximately 85%.

Autoradiography

Autoradiography was carried out to establish variability in [^{3}H]-thymidine-labelling indices at various times after wounding. Triplicate cultures for each treatment and time point were labeled for 4 h (at 1 day, 2 d, 4 d and 6 d post-wounding) with 1 µCi/ml

[^{3}H]-thymidine. The cultures were fixed in 2% gluteraldehyde and 1% paraformaldehyde in PBS, pH 7.2 and then washed 5 times with deionized water to remove most of the unincorporated radiolabel and left to dry thoroughly. The wells were then cut out from the plates, attached onto glass slides and then coated with Ilford K2 emulsion which had been diluted with deionized water (1:1 v/v). The slides were then dried and exposed for 2 wk at 4°C. The autoradiographs were developed for 5 min in D-19 developer and fixed with rapid Fix and counterstained with Giemsa stain. The labelling indices were determined by counting the number of labelled cells in various fields within the culture wells. A cell was assessed to be actively proliferating if a minimum of 3 silver grains were noted within the nuclear region. At least 200 cells in each of 3 fields were counted at sites designated as (i) the wound edge, (ii) the mid-distance between the wound edge and the periphery of the culture and (iii) the periphery of the culture well.

Statistical analyses

All experiments were repeated 3 times and representative examples from these replicate experiments are shown in the results section. All data were subjected to statistical analysis using the method of analysis of variance. To determine the interaction between time and treatment on the dependent variable under question, 2-way ANOVA was carried out. The data relating to effects of PDGF-BB on either cell proliferation, cell migration or wound closure were analysed by a 1-way ANOVA. The data relating to proteoglycan synthesis in wounded and unwounded cultures as a function of time and treatment were analysed by a 2-way ANOVA. All statistical analyses were performed using StatView® v4.02 (Abacus ConceDts, Inc., Berkley, CA).

Results

Mitogenicity of PDGF-BB

The effect of various concentrations of serum and PDGF-BB on [^{3}H]-thymidine incorporation into DNA is shown in Fig. 1. The maximal proliferative response of the cells to various concentrations of PDGF-BB was noted in 0.2% FCS, with reduced responsiveness noted in the absence of serum or at high (10%) concentrations of serum (results not shown). At all concentrations of PDGF-BB studied, a significant ($p<0.01$) increase in proliferative activity was noted over the 0.2% FCS controls.

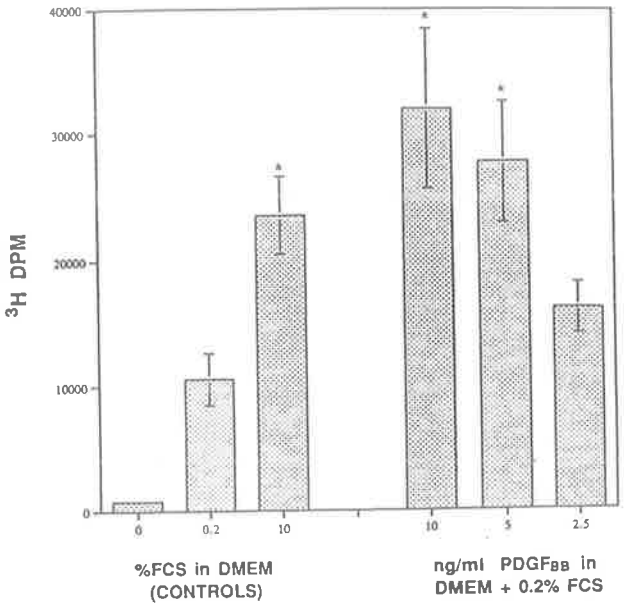

Fig. 1. Triplicate cultures were exposed to varying concentrations of PDGF-BB and the proliferative responses of fibroblasts under a variety of culture conditions as monitored by [^{3}H]-thymidine incorporation into precipitable DNA. Each experiment was repeated 3 times and representative data from one of these is shown. Data are represented as means and standard deviations of the mean. *Represents data which are significantly increased ($p < 0.05$) over the 0.2% FCS controls.

Cell proliferation, migration and wound closure

The effect of various experimental conditions on the wounded cultures at the macroscopic level is shown in Fig. 2. For illustrative purposes this figure shows a representative appearance at d 6. In the presence of 0% FCS the wounded cultures showed very little wound closure. Indeed, the reason for including 0% FCS as a control was to observe the rate of migration in the absence of proliferation since these cells do not divide in the absence of serum (see Fig. 1). On the other hand wounded cultures exposed to 10 ng/ml PDGF-BB in 0.2% FCS or 10% FCS showed progressive wound closure. This is, presumably, a combination of both cell migration and proliferation. The microscopic appearance of the cultures at various stages over the experimental period is shown in Fig. 3–5. At 24 h after wounding very little migratory behaviour was evident between any of the experimental conditions (Fig. 3). At all time points very little cell migration was noted in the cells cultured in the absence of serum and the cultures exposed to 0.2% FCS demonstrated some migration with little evidence of cell proliferation and wound closure. By d 4 the cultures exposed to 0% FCS began to detach and die. While the cells exposed to 0.2% FCS remained viable, they did not migrate substantially after d 4. Over the whole of

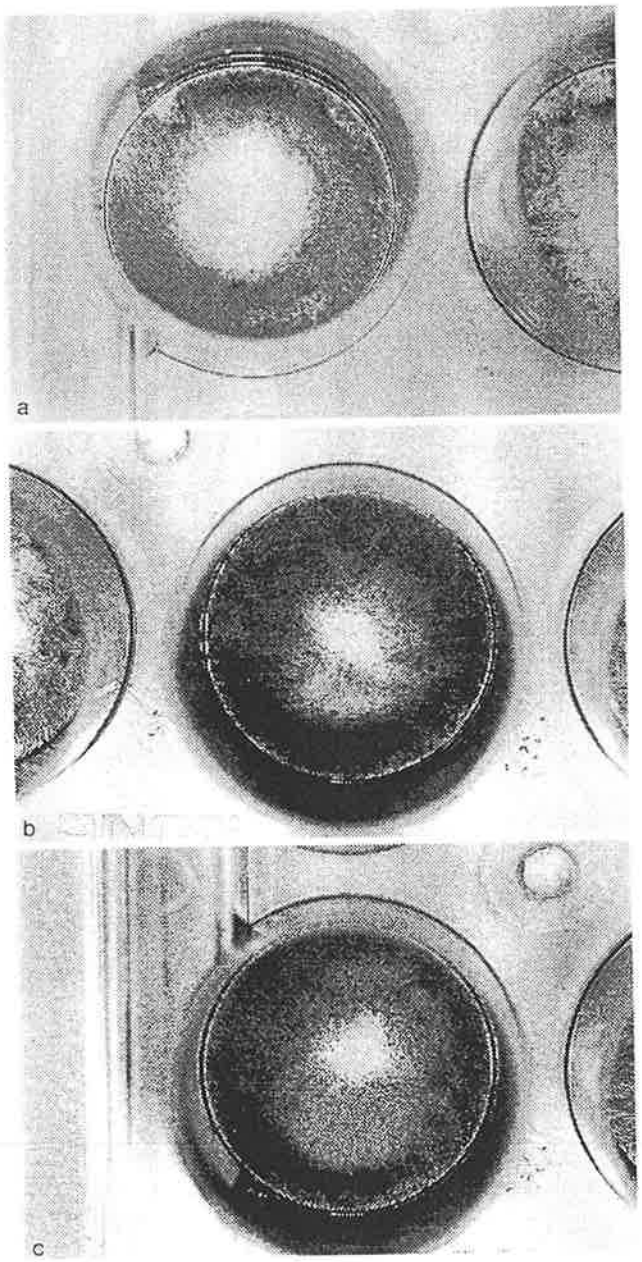

Fig. 2. Macroscopic appearance of wounded cell cultures stained with crystal violet at d 6 post-wounding. (a) 0% FCS; (b) 10 ng/ml PDGF-BB: and (c) 10% FCS.

the experimental period the cells cultured in either 10% FCS or 0.2% FCS and 10 ng/ml PDGF-BB showed the greatest and fastest cell migration. By d 12 the cells exposed to 10% FCS had reached the centre of the wound defect and had almost completely filled the entire culture plate (Fig. 5 c, d). The cells exposed to PDGF-BB lagged behind the cells exposed to 10% FCS somewhat and reached the centre of the plate by d 16.

To determine the early proliferative responses to the various treatments the numbers of cells in each well were monitored over the first 3 d (Fig. 6). After this stage the cell numbers become sufficiently high to saturate the limits of detection of the dye binding assay. The correlation between dye and cell number was routinely checked – a typical standard curve is described by the formula $y = 2.184x + 19.257$ ($r = 0.983$) where x = cell number and y = absorbance value at 260 nm and r = the correlation coefficient. By d 2 detectable increases in cell numbers were evident in the cultures exposed to 10% FCS, 10 ng/ml PDGFBB and 5.0 ng/ml PDGF-BB. As expected the cell numbers did not change appreciably between d 2 and 3.

More detailed analysis of the wound closure process was obtained by image analysis of the wound area (Fig. 7). From these studies it can be seen that the cells exposed to 10% FCS migrated fastest and filled the wound area quicker than other treatments. The cultures exposed to 5.0 and 10 ng/

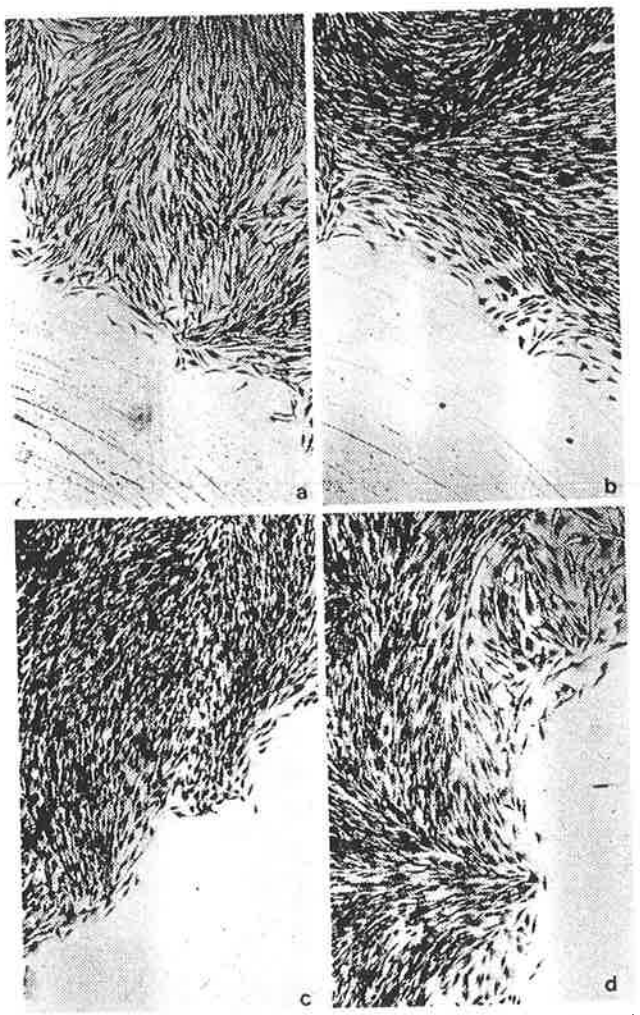

Fig. 3. Microscopic appearance of the wounded cultures stained with crystal violet 24 h after wounding. (a) 0% FCS: (b) 0.2% FCS; (c) 10 ng/ml PDGF-BB. and (d) 10% FCS. Original magnification ×10.

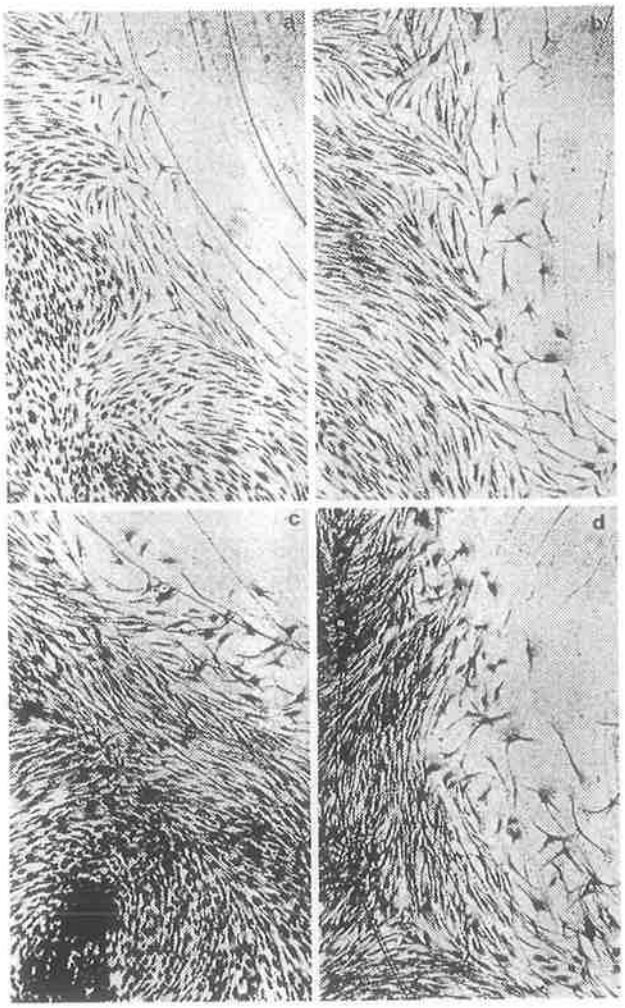

Fig. 4. Microscopic appearance of wounded cultures stained with crystal violet 4 d after wounding. (a) 0% FCS; (b) 0.2% FCS; (c) 10 ng/ml PDGF-BB. and (d) 10% FCS. Original magnification ×10.

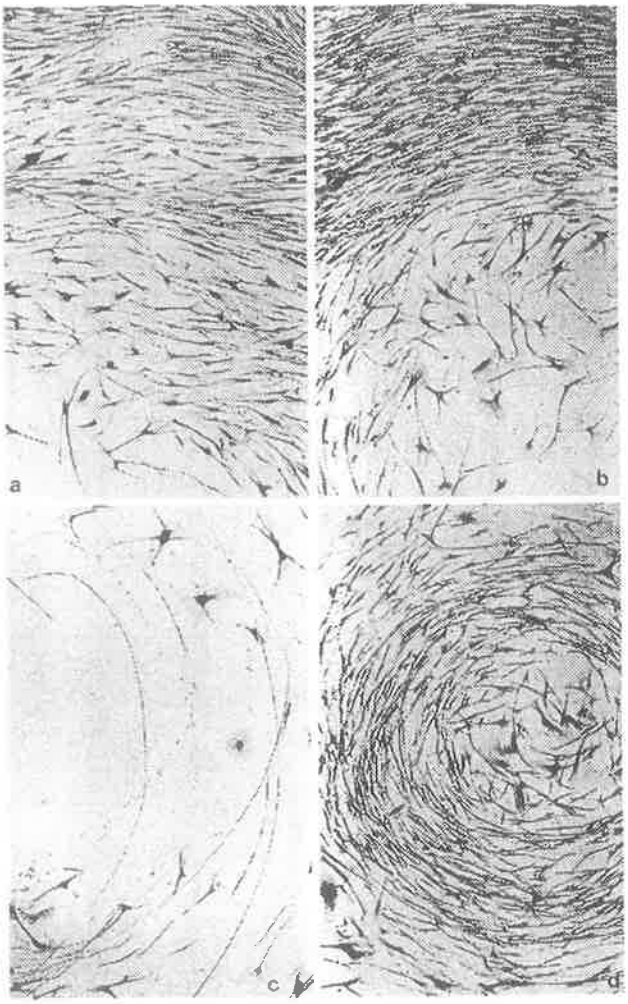

Fig. 5. Microscopic appearance of wounded cultures stained with crystal violet at 8 d (a,b) and 12 d (c,d). (a,c) 10 ng/ml PDGF-BB, and (b,d) 10% FCS. Original magnification ×10.

ml PDGF-BB also filled the wound area over the experimental period and did so in a faster period than the controls of 0.2% FCS. To avoid confusing Fig. 7 with error bars the means, standard deviations and the p-values for all treatments over the time periods studied are shown in Tables 1 and 2.

Proteoglycan synthesis

Proteoglycan synthesis was monitored as a measure of extracellular matrix synthesis during the reparative phase of cell migration, cell proliferation and wound closure (Fig. 8). In the unwounded cultures, PDGF-BB had a significant effect on proteoglycan synthesis only at the earliest time point studied (6 h). However, in the wounded cultures, PDGF-BB did cause a significant ($p<0.05$) increase in proteoglycan synthesis over the 0.2% FCS controls at all time points. This increase in proteoglycan synthesis was in the same order as that observed for cells cultured in the presence of 10% FCS. Since similar values for labelled proteoglycans were obtained at various time points for both wounded and unwounded cultures the effect of growth factors on proteoglycan synthesis would appear to be greater than the wounding effect.

Autoradiography

In order to determine whether all cells in the model system were responding to the application of PDGF-BB, the uptake of [^{3}H]-thymidine was monitored by autoradiography (Table 3). In all cases the cells in the confluent portion of the cultures had low labelling indices. Therefore the data recorded relate to those cells labelled at the periphery of the wound edge. After 24 h the cells cultured in the presence of 10% FCS or 10 ng/ml PDGF-BB showed a signifi-

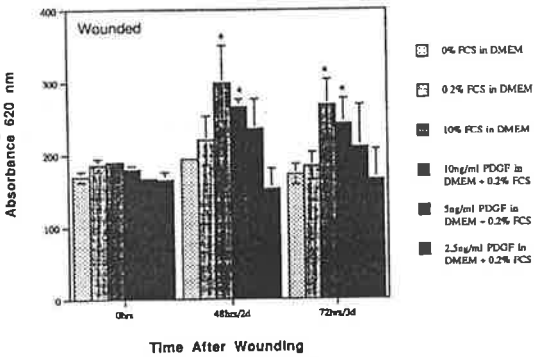

Fig. 6. Cell proliferation of wounded cultures as assessed by staining with crystal violet. Confluent monolayers of cells were wounded and then cultured under a variety of conditions. At specified time points the cultures were fixed, stained with Crystal Violet and the amount of dye bound determined by spectrophotometric analysis. Each experiment was repeated 3 times and representative data from one of these is shown. Data are represented as means and standard deviations of the mean. Represents data which are significantly increased ($p < 0.05$) over the 0.2% FCS controls within each time point grouping.

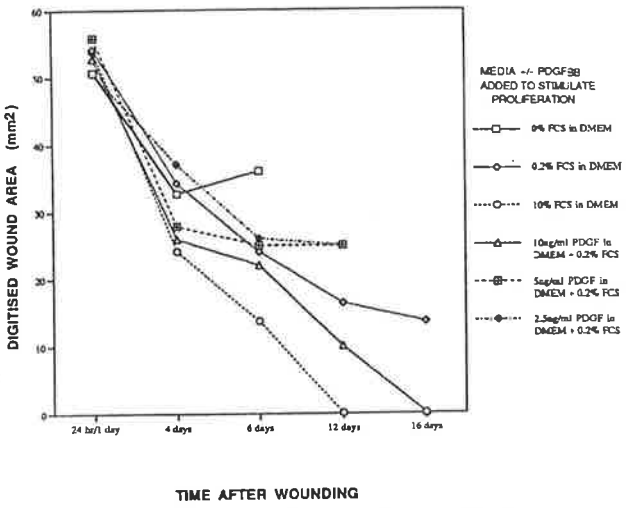

Fig. 7. Wound surface area closure of wounded cultures as assessed by image analysis. Each experiment was repeated 3 times and representative data from one of these is shown. The mean data, standard deviations of the mean and p-values for this analysis are shown in Tables 1 and 2.

cant increase in the number of labelled nuclei compared to the 0% and 0.2% controls. Cells cultured in the absence of FCS showed negligible labelling while those cultured in the presence of 0.2% FCS had a moderate number (5% of total number of cells) labelled. At this early stage, only those cells immediately adjacent to the artificially induced wound edge showed evidence of thymidine uptake. Similar results were noted at the 6 d time point. Cells cultured in the absence of FCS had no cells labelled with thymidine. In the presence of 0.2% FCS, and where there had been a slight amount of

Table 1. Wound surface area closure of wounded cultures as assessed by image analysis

Treatment	d 1	d 4	d 6	d 12	d 16
0% FCS	49.85 (±5.34)	31.8 (±2.62)	40.25 (±7.44)	n.d.	n.d.
0.2% FCS	51.79 (±2.59)	35.87 (±5.54)	24.38 (±1.69)	16.07 (±2.74)	14.75 (±2.49)
10% FCS	54.04 (±4 51)	23.75 (±3.20)	13.25 (±2.43)	0.00	0.00
10 ng PDGF	52.81 (±4.81)	27.37 (±2.67)	21.31 (±6.08)	11.63 (±2.72)	0.00
5 ng PDGF	51.25 (±5.48)	37.5 (±2.45)	25.60 (±4.41)	25.0 (±1.5)	n.d.
2.5 ng PDGF	55.00 (±4.13)	26.0 (±5.63)	23.35 (±4.29)	25.8 (±1.96)	n.d.

Values are expressed as means and standard deviation of the mean. n.d.= not determined.

Table 2. Assessment of p-values for wound surface area closure of wounded cultures as determined by image analysis

Comparison	d 1	d 4	d 6	d 12	d 16
0% FCS, 0.2% FCS	0.374	0.081	<0.0001	n.d.	n.d.
0% FCS,10% FCS	0.112	<0.0001	<0.0001	n.d.	n.d.
0% FCS,10 ng PDGF	0.269	0.004	<0.0001	n.d.	n.d.
0% FCS, 5 ng PDGF	0.077	0.0004	0.0003	n.d.	n.d.
0% FCS, 2.5 ng PDGF	0.566	0.021	<0.0001	n.d.	n.d.
0.2% FCS,10% FCS	0.241	0.0001	<0.0001	<0.0001	<0.0001
0.2% FCS,10 ng PDGF	0.604	0.001	0.191	0.005	<0.0001
0.2% FCS, 5 ng PDGF	0.156	0.0004	0.457	<0.0001	n.d.
0.2% FCS, 2.5 ng PDGF	0.759	0.570	0.539	<0.0001	n.d.
10% FCS,10 ng PDGF	0.607	0.027	0.003	<0.0001	No Diff
10% FCS, 5 ng PDGF	0.707	0.136	<0.0001	n.d.	n.d.
10% FCS, 2.5 ng PDGF	0.218	<0.0001	<0.0001	<0.0001	n.d.
10 ng PDGF, 2.5 ng PDGF	0.497	0.302	0.128	<0.0001	n.d.
10 ng, PDGF, 5 ng PDGF	0.410	0.0004	0.453	<0.0001	n.d.
5 ng PDGF, 2.5 ng PDGF	0.144	0.0001	0.318	0.227	n.d.

n.d. = not determined; No Diff = no difference detected (wound fully closed).

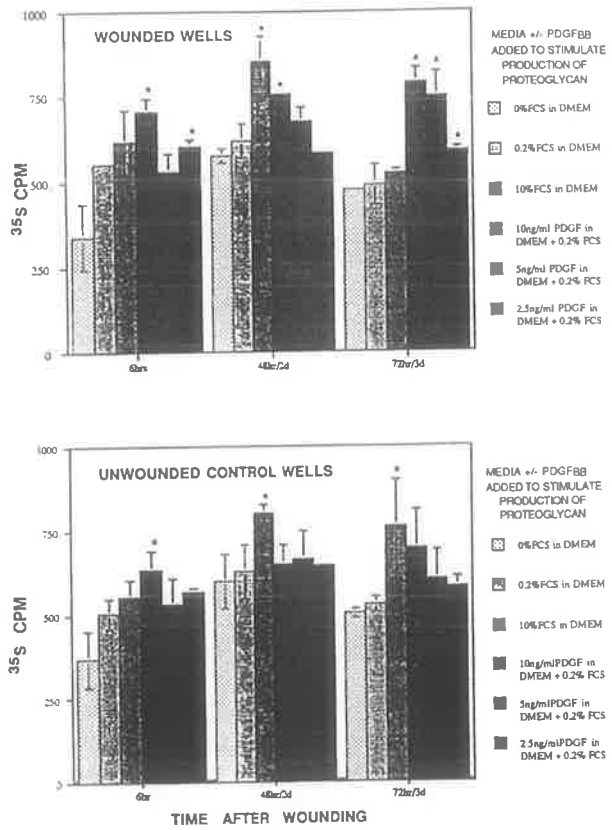

Fig. 8. Synthesis of sulphated proteoglycans in wounded cultures. Cells were wounded at d 0 and then labelled 6 h prior to the termination of the experiment at the noted time point (6, 24 and 48 h). The amount of labelled proteoglycans released into the culture medium was determined by PD-10 gel exclusion chromatography. Each experiment was repeated 3 times and representative data from one of these is shown. Data are represented as means and standard deviations of the mean. Represents data which are significantly increased ($p < 0.05$) over the 0.2% FCS controls within each time point grouping.

cell migration, up to 20% of the migrating cells showed some labelling. In the presence of 10 ng/ml PDGF-BB approximately 80% of the migrating cells had labelled nuclei while nearly 100% of the cells exposed to 10% FCS were labelled at this time point.

Relative interactions of time, type of growth factor and dependent variable

Since 2 factors (time and type of growth factor) and 1 dependent variable (either cell proliferation, wound closure or proteoglycan synthesis) were under consideration within specified experiments, 2-way ANOVA was carried out on all datasets to determine the interaction terms. From Table 4 it can be seen that time and treatment have significant influences on the variables of wound area closure and proteoglycan synthesis but not cell proliferation. Thus, for wound closure and proteoglycan synthesis treatment differences change across time whereas cell proliferation changes are the same at each time point. On the basis of these findings, all descriptive statistical analyses were carried out by 1-way ANOVA within each time point to determine differences between treatment groups and controls.

Discussion

Periodontal wound repair is an extraordinarily complex process requiring the deposition of at least 4 distinct connective tissues including gingiva, periodontal ligament, bone and cementum (32). The cellular and biochemical processes by which repair and regeneration of these tissues takes place are still largely poorly understood. Nonetheless, it is clear that in order for regeneration to proceed, specific cells must be recruited to the damaged site, cell proliferation must occur and new extracellular matrix consistent with the lost tissues must be deposited. The repair of damaged tissues begins as soon as tissue damage occurs with the release of a multitude of growth factors, cytokines and other (possibly as yet unidentified) bioactive agents by the injured cells and inflammatory cells.

In recent years the use of various growth factors for enhancing periodontal regeneration has shown considerable clinical promise (33). However, despite numerous histological studies detailing the likely clinical outcomes of the treatments, only a few studies have begun to address the cellular and

Table 3. [^{3}H]-Thymidine labelling indices of cells in wounded cultures

	24 h			10 d		
	edge	middle	outer	edge	middle	outer
0% FCS	1.0 (±0.5)	-	-	-	-	-
0.2% FCS	10.5 (±5.0)	-	-	16 (±3.5)	-	-
10% FCS	64.5 (±20.5)	2.5 (±0.2)	-	76 (±17.5)	10 (2.5)	
PDGF	35.5 (±6.5)	5.0 (±0.2)	-	60 (±14)	7.5 (±1.5)	-

Labelling indices were determined by counting the number of labelled cells in various fields within the culture wells. A cell was assessed to be actively proliferating if a minimum of 3 silver grains were noted within the nuclear region. At least 200 cells in each of 3 fields were counted at sites designated as (i) the wound edge, (ii) the mid-distance between the wound edge and the periphery of the culture and (iii) the periphery of the culture well. Data are expressed as mean percentages (± standard deviations of the mean).

Table 4. Two-way ANOVA of all data to assess the interactions between time and growth factor

	DF	F-value	p-value
Cell proliferation			
Time	2	9.9	0.0004
Treatment	5	8.0	<0.0001
Time & treatment	10	1.4	2.032
Wound closure			
Time	4	579.9	<0.0001
Treatment	5	146.0	<0.0001
Time & treatment	20	28	<0.0001
Proteoglycan synthesis – wounded			
Time	2	26.3	<0.0001
Treatment	5	29.7	<0.0001
Time & treatment	10	8.1	<0.0001
Proteoglycan synthesis – unwounded			
Time	2	21.0	<0.0001
Treatment	5	13.4	<0.0001
Time & treatment	10	2.4	0.022

molecular events associated with the use of such agents. PDGF has been shown to stimulate periodontal cell proliferation at concentrations ranging from 0.1 ng/ml to 50 ng/ml with 10 ng/ml being the optimal dose (20–23, 34, 35). The results of this study confirm this response and, although higher doses were not studied, indicate that significant proliferative responses can be achieved with doses as low as 5 and 10 ng/ml PDGF-BB. In addition to stimulating proliferation, PDGF-BB has been found to influence hyaluronate synthesis (21) and collagen synthesis (19, 22). In the present study we have extended these studies to consider the influence of PDGF-BB on cell proliferation and proteoglycan synthesis under conditions which simulate wound healing and require cell migration, cell proliferation and new matrix synthesis.

This study was initiated to provide some understanding of the fundamental events associated with fibroblast activation at wounded sites by the application of an exogenous growth factor. In this context, the system was purposely kept very simple by utilizing an *in vitro* model of wound healing which specifically excludes the influence of inflammatory and other cells associated with the complex repair process of the periodontal tissues and by studying only one growth factor, namely PDGF-BB. We acknowledge the artificial nature of the model and recognize the limitations of extrapolating *in vitro* conditions to the *in vivo* situation.

Although several models of mechanical injury of cultured cells have been described (36–39), the model system chosen for the present study was a modification of that developed by Klein-Soyer et al. (40) and allowed for a uniform discoid shaped lesion to be made. This lesion had the advantage in that it was of such a size that it could be followed over a relatively extended period of time in culture to follow the cellular proliferative and migratory behaviour. In other systems such as the multi-scratch system the cells divide and migrate so rapidly that the lesions are "repaired" in a very short period and thus makes quantitative and qualitative assessment difficult (41).

Despite the relative abundance of *in vitro* model systems used to study wound healing, few studies have investigated the factors regulating the migration of fibroblasts into a denuded area of a confluent monolayer (42). In our study it was noted that both PDGF-BB and FCS stimulated the migration of human periodontal ligament fibroblasts while serum depletion (0% or 0.2% FCS) decreased the migratory capacity of the cells. The results reflect, in part, some of the observations of Kondo et al. (42) in that the migration of adult fibroblasts requires a factor(s) present in FCS. While we demonstrated that the cells were responsive to PDGF-BB alone, Kondo et al. (42) found that in their system PDGF was only active in the presence of collagen. A possible explanation for these discrepancies may lie in the fact that our studies were done in the presence of low concentrations of FCS whereas Kondo et al. (42) carried out their studies in the absence of serum. The requirement of low concentrations of serum for bioactivity of PDGF is well established with PDGF being recognized as a initiation or competence factor requiring certain progression factors (such as IGF-1) for it to effect its mitogenic capacity.

Cell proliferation was noted to be an important part of the reparative process in our *in vitro* system.

As judged by autoradiographic analysis, the majority of the proliferative activity which resulted in increased cell numbers in the denuded areas came from the wound edges. Cell proliferation in conjunction with cell migration led to complete fill of the denuded areas in the cultures exposed to PDGF-BB or 10% FCS. Of particular interest here was the observation that in the cultures exposed to PDGF-BB, not all cells were labelled. This indicates that a small proportion of cells were not responding proliferatively to this mitogen yet were responding by migration. Whether this indicates that our cultures consist of a heterogeneous population of cells with different capacities to respond to various growth factors remains to be established. There is, however, good evidence to suggest that not all cells in a mixed explant culture (such as used in this study) will respond to growth factors (43). The significance of this to periodontal regeneration is interesting in that it indicates that cell migration may be a universal phenomenon but the necessary response to specific growth factors may be more discrete, sensitive and restricted to a smaller subset of cells.

The phenomenon and significance of abundant cell division occurring during regeneration is an important consideration. For example, dividing cells will alter their synthetic capacity quite significantly during cell division. Indeed, after wounding of the cultures, an increase in the incorporation of ^{35}S-sulphate into proteoglycans was noted in the present study. This response is most probably associated with migratory activity of the cells since cells undergoing active replication do not seem to actively synthesize proteoglycans (44, 45). Additional evidence which would support the observed increase in matrix synthesis being associated with cell migration comes from the observation that the rate of incorporation of ^{35}S-sulphate into proteoglycans was elevated between 8–16 h after wounding, during which time the cells had begun to migrate from the edge to fill the denuded area. Furthermore, cell proliferation, as monitored by [^{3}H]-thymidine uptake and autoradiography, was maximal by 48 h after wounding and continued at near maximal levels for at least 72 h after wounding, during which time the ^{35}S-sulphate incorporation levels declined.

The significance of proteoglycan production in the early phases of wound healing should not be overlooked. Both proteoglycan and hyaluronate synthesis precede collagen synthesis by several days in experimental wound healing models *in vivo* (46). The initial newly formed extracellular matrix is an important regulator of cell migration (47). Cell surface proteoglycans can bind a number of growth or cell motility factors, while others may be associated with "de-adhesion" of motile cells (48, 49). Additional support for a role of proteoglycans in early cell movement has come from analysis of focal adhesion sites in which significant quantities of both the proteoglycans and hyaluronate have been noted (50). While hyaluronate was not monitored in the present study, this large uronic acid containing macromolecule will most probably be very important in the migratory activity of fibroblasts and requires further investigation. Indeed, hyaluronate accumulation coincides with the onset of embryonic cell migration and coincides with the migration of macrophages and fibroblasts into sites of tissue injury (51, 52).

In conclusion this study has utilized a simple model system for studying cell migration, proliferation and matrix under *in vitro* conditions simulating wound healing. By using such a system, particular events and agents associated with the complex events of growth factor-mediated periodontal regeneration may be analysed. In particular, the events of initial extracellular matrix production at a wounded site with proliferating and migrating cells can now be monitored under well controlled culture conditions. Here we report that the migratory and proliferative response of periodontal ligament fibroblasts in response to PDGF–BB is accompanied by modulation of proteoglycan synthesis which is consistent with the early biochemical events of cell mediated wound repair and tissue regeneration.

Acknowledgements

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IX. SUMMARY REVIEWS

Paper 25. Bartold, P.M.
Connective tissues of the periodontium. Research and clinical implications.
Australian Dental Journal **36:** 255-268, 1991.

Paper 26. Bartold, P.M.
Modulation of gingival fibroblast function by lipopolysaccharides.
In: *Periodontal Disease: Pathogens and Host Immune* Responses. Editors: S. Hamada, S.C. Holt and J.R. McGhee, Quintessence Publishing Co, 1991

Paper 27. Bartold, P.M. and Narayanan A.S.
Biochemistry of the Periodontium
In: *Fundamentals of Periodontics*. Editors: T. Wilson, and K. Kornman, Quintessence Publishing Co.
Chapter 6, 1996

These selected review articles serve to place the preceding works in perspective. Paper 25 was prepared to highlight the clinical ramifications of basic research concerning the composition of connective tissues of the periodontium. By drawing the current scientific findings together, the importance of connective tissue reactions in relation to inflammatory periodontal disease, medication-related alterations to the periodontal tissues, periodontal diagnosis and periodontal regeneration was discussed. In paper 26 the role that lipopolysaccharide plays in modulating fibroblast function is reviewed. This paper illustrates how the elucidation of biological activities of individual factors implicated in the pathogenesis of periodontitis is fundamental to understanding the potential pathways for development and establishment of periodontal destruction. Finally, paper 27 is an invited contribution to a new textbook on Periodontics. This review is a summation of our knowledge of the matrix composition of the periodontium which is current right to the time of submission of the collection of published works.

Paper 25.

Bartold, P.M.
Connective tissues of the periodontium. Research and clinical implications.
Australian Dental Journal **36**: 255-268, 1991.

Connective tissues of the periodontium. Research and clinical implications

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Key words: Collagen, connective tissues, periodontal diseases, proteoglycans.

Abstract

The periodontium is a complex anatomical site composed of a variety of hard and soft connective tissues. The site is subjected to enormous daily chemical and mechanical abuse, yet, for the most part remains relatively intact. Nonetheless, when breakdown does occur, the ability of the periodontium to regenerate is sometimes limited. This review considers the various components of the periodontium and how they might be involved in not only the pathology of periodontal diseases but also the various reparative and regenerative processes required during wound healing. In addition, the importance of external factors such as a variety of commonly prescribed medications on these events is discussed.

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Introduction

The periodontium is a unique anatomical site which includes four discrete connective tissues, namely: gingiva, periodontal ligament, cementum and alveolar bone. By virtue of the constant masticatory forces and bacterial presence in the mouth, these tissues are under continual mechanical and chemical abuse yet, for the most part, they manage to maintain their functional and structural integrity. However, if the delicate balance between host defence and bacterial virulence is upset then disease and associated tissue destruction ensues. Upon removal of the offending agents, tissue repair follows and health may be restored to the periodontium.

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Although the important functional role played by connective tissues in wound healing and repair has been recognized for decades, it is only recently that research into their structure, assembly and metabolism has been actively pursued. A principal factor leading to this has been the recognition that many diseases common in our society are associated with defects in either the connective tissue matrices, their resident cells, or both.

The periodontal diseases are classic examples of chronic inflammatory lesions in which connective tissue changes are evident. Since the responsiveness of these diseases to treatment is paramount to the successful outcome of our various therapies, an understanding of the basic biology of these systems (as well as establishing the mechanisms involved in their failure) will permit us to be better positioned to influence these adverse processes during clinical management.

The variety of responses elicited by the periodontal tissues under different conditions is striking and highlights the variability in connective tissue responses (Fig. 1). For example, what factors are in play which permit the relative health observed in an 85-year-old compared with the disfiguring gingival overgrowth seen in a younger individual. Furthermore, the effects of medication, uncontrolled fibrosis, genetic factors and immune deficiency are all accompanied by different changes

Table 1. Components of the extracellular matrix

Fibrous:	Collagen
	Elastin
Ground substance:	Proteoglycans
	Glycoproteins
	Lipids
	Minerals
	Water

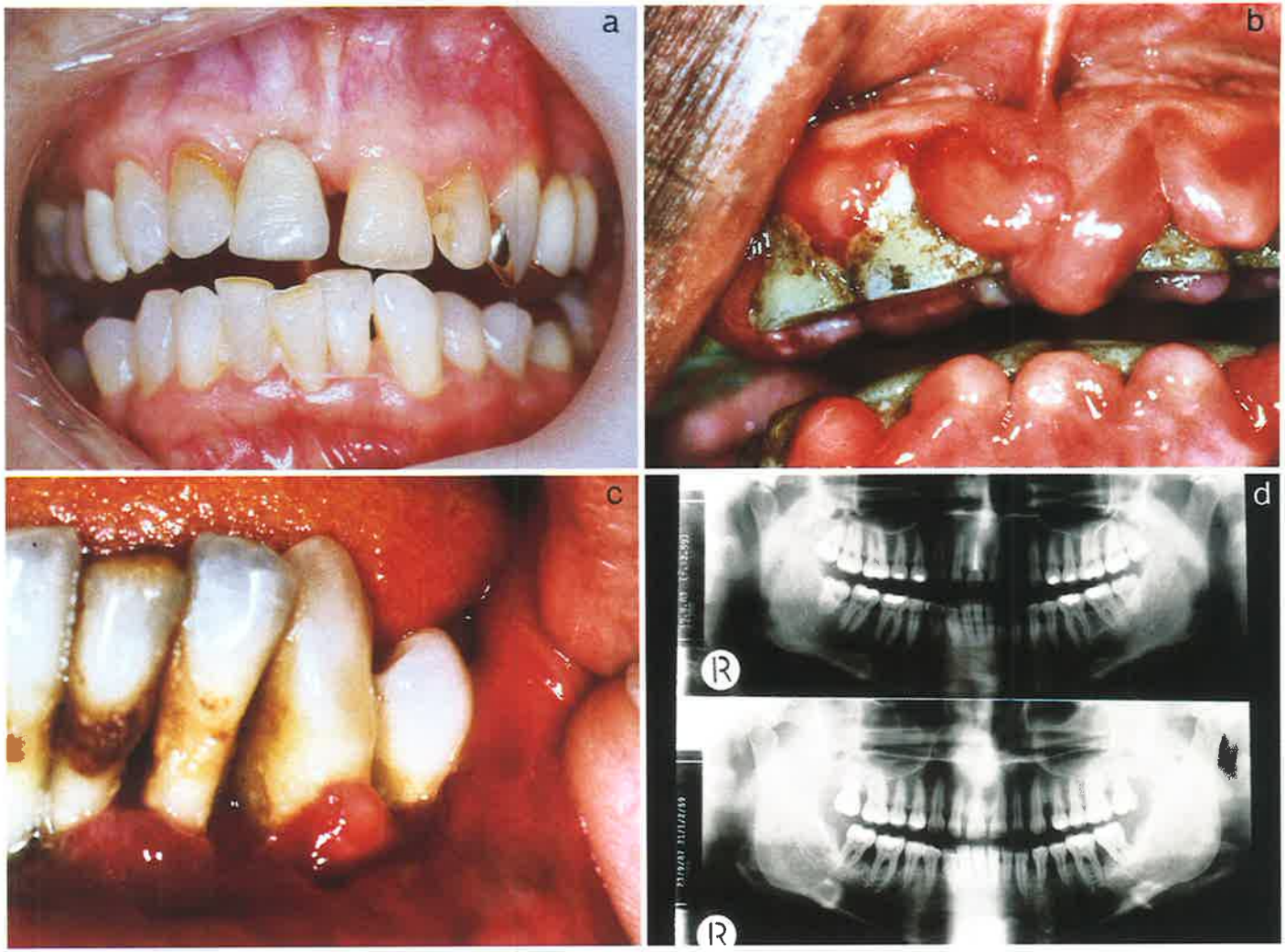

Fig. 1.—Various manifestations of connective tissue changes in the periodontium. (a) Appearance of gingival tissues of 80-year-old female. (b) Plaque-induced gingival overgrowth in 35-year-old male. (c) Changes in the periodontal support tissues associated with Acquired Immune Deficiency Syndrome. (d) Radiographic changes in alveolar bone associated over a four-year period of rapidly progressing periodontitis.

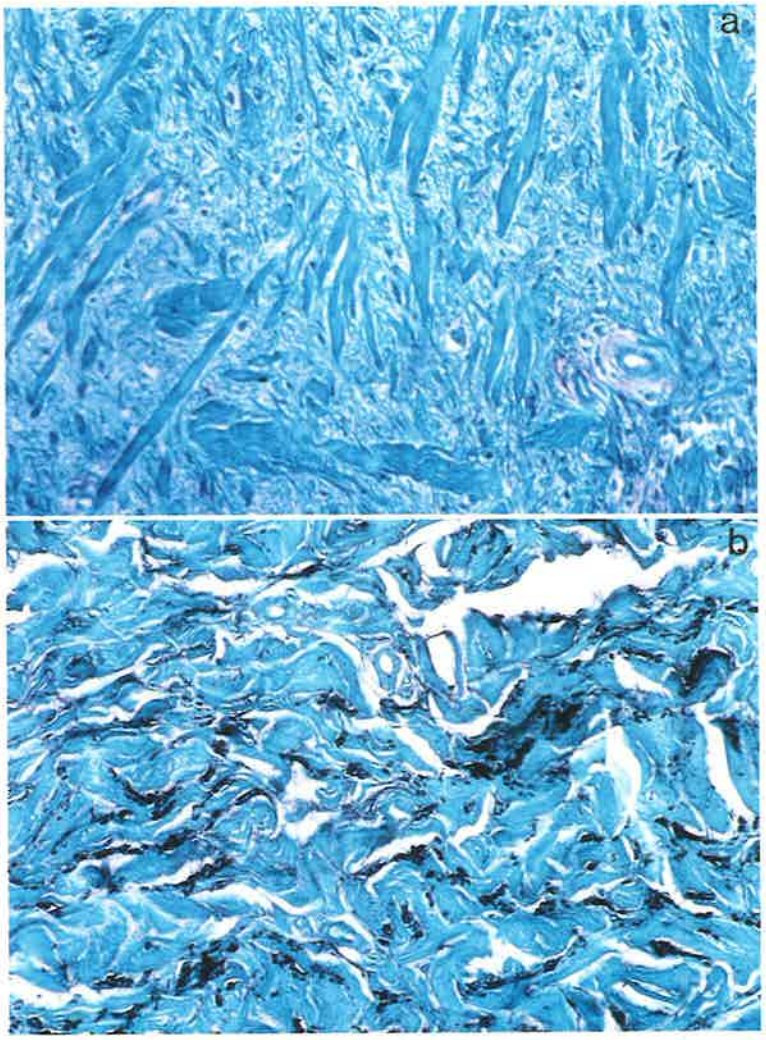

Fig. 2. – Deposition of elastin in oral tissues. (a) Attached gingiva, and (b) alveolar mucosa. Note heavy deposits in the alveolar mucosa (black amorphous material between collagen fibres) and virtual absence of material reactive for elastin in attached gingival connective tissue. With Miller's Stain. Orig. ×100.

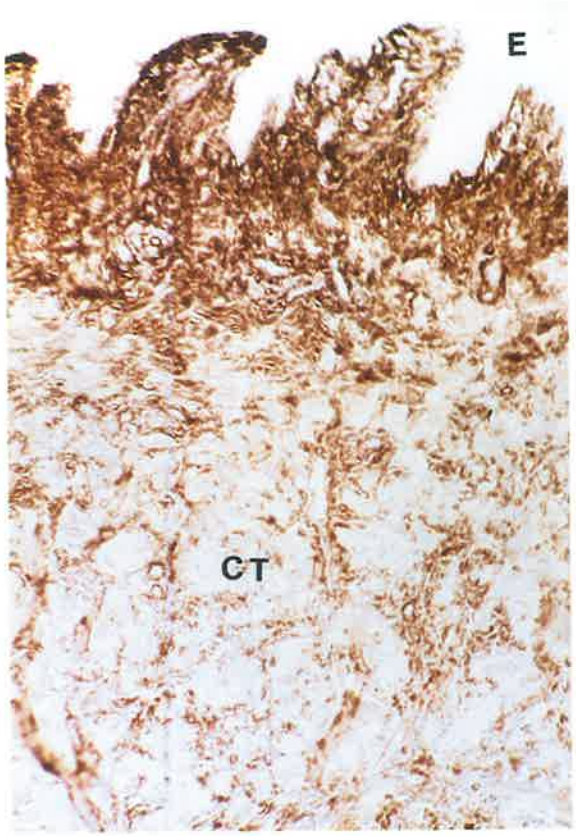

Fig. 3. – Distribution of dermatan sulphate proteoglycan in gingival connective tissue. Note intense staining adjacent to the subepithelial tissues. Sections were reacted with monoclonal antibody to chondroitinase-ABC digested dermatan sulphate (9-A-2) and visualized utilizing immunoperoxidase staining. E, Epithelium; CT, connective tissue. Orig. ×75.

Table 2. Distribution of major matrix components of the periodontium

Site	Collagens	Proteoglycans
Gingiva	I*, III*, IV, V	HA, HS, DS*, CS
Periodontal ligament	I, III*, V	HA, HS, DS*, CS
Alveolar bone	I*	HA, HS, DS, CS*
Cementum	I, III	HA, HS, DS, CS*

Abbreviations: HA (hyaluronate); HS (heparan sulphate); DS (dermatan sulphate); CS (chondroitin sulphate).
*Indicates the predominant species present at each site.

in the appearance of the periodontal connective tissues.

Components of the periodontal connective tissues

Most connective tissues can be divided into fibrous and non-fibrous elements (Table 1). Of these components, the collagens and proteoglycans have been reasonably well characterized.[1,2]

The collagens are a major constituent of a variety of skeletal and soft connective tissues. It has been estimated that 30 per cent of the total protein in the human body is collagen.[3] These fibrous proteins are responsible principally for the maintenance of the framework and tone of the tissues. To date, eleven different types of collagen have been identified on the basis of their molecular composition.[4] Various tissues have differences in type, size, distribution and alignment of the collagen fibres and this is related to the different physical and functional properties of the tissues. The periodontium is no exception in which differences in type and quantity vary depending upon the location (Table 2).

The elastic properties of tissues requiring an ability to stretch, twist or bend are due to the presence of the protein elastin.[5] Although elastin appears to be a minor component of the periodontal tissues, with small amounts being noted in the periodontal ligament and gingivae,[6,7] the more mobile and loose connective tissue of the adjacent alveolar mucosa has significant elastin deposits (Fig. 2).

The ground substance of connective tissues provides the gel in which the structural fibrous components and cells are embedded. While there are several principal components of the ground substance (Table 1), to date, only the proteoglycans, hyaluronate and some glycoproteins (for example, fibronectin and laminin) have been well characterized. The proteoglycans are a polydisperse family of macromolecules which vary in both their protein and carbohydrate composition.[8] The carbohydrate components are termed glycosaminoglycans and

Table 3. Properties of extracellular matrix macromolecules

Physiochemical properties	Function
Osmotic properties	Tissue hydration
Flow resistance	Flow barrier
Polyelectrolyte properties	Distribution and activity of ions Charge interactions
Exclusion properties	Distribution of plasma proteins Precipitation of macromolecules Stabilization of macromolecular conformation Driving force in transport
Sieve effects	Diffusion barriers
Dissipation of forces	Tissue protection

may be used as a primary source of identification. Thus, on the basis of their glycosaminoglycan content a proteoglycan may be initially identified as a chondroitin sulphate-, dermatan sulphate-, heparan sulphate- or keratan sulphate proteoglycan. As shown in Table 3, these large polyanionic macromolecules are responsible for maintaining a wide variety of physiological properties of tissues.[9] As noted with the collagens, some site specificity appears to be present in the periodontium (Fig. 3) and this is most likely associated with the different functional roles of the various sites within the periodontium (Table 1).

Molecular interactions of the connective tissues

Although it is important to recognize the individual components of the extracellular matrix, it is essential to remember that they do not exist in isolation. Rather, they are capable of interacting with each other to contribute to the formation and organization of a comprehensive network forming the tissues in question.

To date, molecular interactions between proteoglycans and collagen,[10] proteoglycans and hyaluronate,[11] proteoglycans and other glycoproteins (for example, fibronectin and laminin[12,13]) as well as between different proteoglycan species[14] have been reported. It is believed that such interactions result in a stabilizing effect allowing the matrix to assemble as a single functional unit.

In addition to macromolecular interactions, components of the extracellular matrix can interact on the surface of cells. Cell surface associated proteoglycans and glycoproteins may act as receptors for intercellular communication and location. In addition, they may act as adhesive receptors or mediators. For example, heparan sulfate proteo-

glycan plays a direct role in forming adhesive bonds between plasma fibronectin while hyaluronate and chondroitin sulfate proteoglycan facilitate cell detachment and locomotion.[15]

Another important function of extracellular components with respect to the periodontium is in relation to calcification. Although this process is still poorly understood, a role for proteoglycans similar to those isolated from cementum and alveolar bone has been proposed for the calcification of growth plate cartilage.[16]

Connective tissue changes with inflammation

One fascinating feature of the periodontal diseases is that they occur in a relatively small and well localized region. As a result, studies of the local reactions have been easily performed following excisional biopsy of the affected areas and histological assessment. The general features of normal and inflamed periodontal tissues have thus been examined and four stages of periodontal destruction have been defined on the basis of the histological appearance.[17] The initial, early and established lesions are representative of the development of gingivitis and are reversible. The advanced lesion relates to destruction of the deeper periodontal tissues and indicates the development of periodontitis.

There is little doubt that the components of the extracellular matrix of the periodontium are significantly affected during inflammation. During the development of gingivitis the changes are confined to the gingiva and include altered histochemical staining patterns of affected tissue,[18] depletion of collagen,[19] changes in the arrangement of collagen fibres[20] as well as infiltration of inflammatory cells such as polymorphonuclear leukocytes, macrophages, lymphocytes and plasma cells.[17]

With the introduction of biochemical assays, more precise data concerning these changes were obtained. Up to 70 per cent of the collagen content of gingiva is lost during the establishment of gingival inflammation and this is closely associated with the infiltration of polymorphonuclear leukocytes.[21] In addition, the ratios of collagens change with more Type V becoming evident as well as the appearance of Type I trimer collagen which is not present in healthy gingiva.[22] Under inflammatory conditions, changes occur to the proteoglycans as well. Although few quantitative changes are seen, subtle variations in the types of proteoglycan present in inflamed gingiva may be noted with a decrease in dermatan sulphate proteoglycan and an increase in chondroitin sulphate proteoglycan occurring.[23] In addition, there appears to be significant degradation of the protein core of proteoglycans leading to alterations in both molecular size and structure of the proteoglycans.[23,24] Hyaluronate is significantly depolymerized also in inflamed gingiva.[23]

With development of the advanced lesion indicative of periodontitis, damage to the periodontal ligament, cementum and alveolar bone becomes evident clinically and radiographically. Although few studies have addressed the biochemical changes involved in alveolar bone resorption, it is likely that processes similar to those involved in gingival tissue degradation may be involved.[25] However, there are some specific problems associated with understanding the mechanisms of bone resorption. For example, whether alveolar bone must be demineralized before removal of the organic components can occur has been a long standing debate.[26,27] Furthermore, although the osteoclast has historically been regarded as the principal instigator of bone resorption, recent evidence indicates that the osteoblast (the major bone-forming cell) is intimately associated with the regulation of bone resorption.[28,29]

Although the majority of changes associated with inflammation of the periodontal connective tissues can be accounted for by enzymatic degradation, the precise source of these enzymes is not clear since both endogenous and bacterial enzymes possess the ability to cause significant matrix destruction.[30] However, from recent investigations, it seems likely that endogenous collagenases (derived from polymorphonuclear leukocytes, macrophages and fibroblasts) are responsible for collagen destruction[31,32] while both bacterial and endogenous enzymes are responsible for proteoglycan destruction.[33,34] Furthermore, oxygen-derived free radicals which are highly reactive metabolic products released by activated polymorphonuclear leukocytes may also contribute to the tissue disruption.[35]

In addition to changes in the matrix itself, significant changes are brought about by inflammatory mediated effects on the resident fibroblasts. Studies analysing fibroblasts isolated from normal and inflamed gingival tissues indicate good correlation between collagen and proteoglycan types synthesized by gingival fibroblasts *in vitro* and the types present in either normal or inflamed tissues.[36-39] Of particular importance here is that gingivitis very often is a stable lesion and, in fact, may never progress to develop into periodontitis. This indicates that, in gingivitis, a balance exists between destruction and replacement of the matrix. If, however this balance is disrupted then periodontitis develops and advanced tissue destruction of both the hard and soft tissues becomes evident which, for the most part, is not reversible.

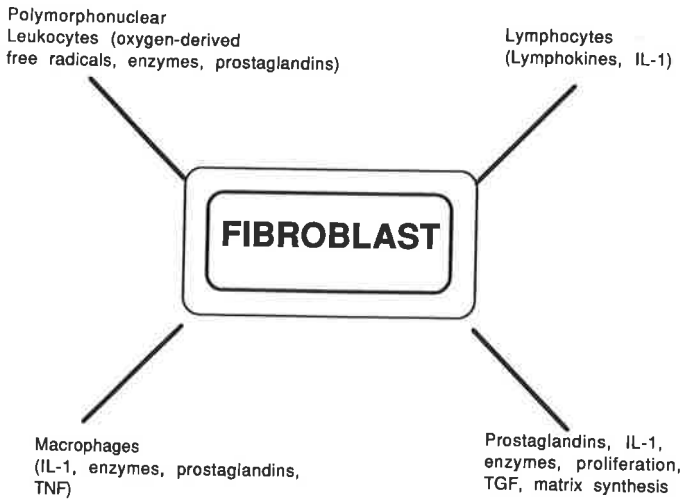

Fig. 4.—Factors capable of modulating fibroblast function.

In addition to enzymatic degradation of the extracellular matrix, interactions between the various cells in inflamed tissues are of central importance to understanding both the destructive and reparative phases. These include modulation of fibroblast function by soluble mediators released by the inflammatory cells, effects of cytotoxicity and modulation of inflammatory cell function by fibroblasts.[40] Although relatively simplistic in its presentation, Fig. 4 illustrates many of these features. These are very complex interactions involving numerous different cells, systems and processes which are only now beginning to be fully appreciated.

Although these seem to be basic biological phenomena the fact that these reactions occur within a specialized environment, namely the extracellular matrix of the periodontal connective tissues, is often overlooked. Indeed, all materials passing to and from cells, as well as the movement of cells themselves, must occur within the extracellular matrix. Therefore, any changes in the composition and architecture of the matrix would be expected to have a profound influence on both the resident cells and the tissue as a whole.

Role of the extracellular matrix in wound repair and regeneration

A major biologic response critical to survival of the dentition is repair of damaged tissue. In the case of gingivitis, this is achieved easily. However, once the destructive phase reaches the deeper periodontal structures, then repair in the form of regeneration of destroyed components is less likely to occur predictably.[41] This is a feature not restricted to the periodontium since mammals in general evidence little regeneration of organs or appendages in response to tissue destruction. Commonly, the repair process in humans, where severe tissue damage has occurred, leads to additional problems of scarring.[42] In this respect the periodontium is different compared with its anatomically similar counterpart skin, since scarring of the gingival tissues is a very rare occurrence following surgical excision (Fig. 5).

Obtaining anatomical repair and regeneration of the deeper periodontal tissues (periodontal ligament, bone and cementum) is now a fundamental aim of both periodontal therapy and research. At present, significant advances are being made leading towards treatments which will permit repair and regeneration of pathologically damaged periodontal structures.

Connective tissue newly synthesized at the repair site is composed of cells, vasculature and matrix. The synthetic phase of healing is preceded by an inflammatory response which occurs as a result of injury (mechanical or infective) and is necessary for the protection of the host. In this phase, mesenchymal cells (fibroblasts) produce new connective tissue components and organize them into new matrix (Fig. 6).

In the case of the periodontium, a principal negative factor in repair and regeneration is the rapid re-epithelialization of the damaged interface between the root surface and the gingival connective tissue.[43] Following recognition of this response, a new treatement philosophy evolved and the term 'guided tissue regeneration' was introduced for a

procedure in which epithelium is physically kept distant to the healing connective tissues by insertion of a membrane barrier between the root surface and the healing periodontal tissues.[44] As shown in Fig. 7 all of the necessary ingredients for tissue repair appear to be present in this procedure – a distant epithelium, inflammatory cells and matrix producing fibroblasts.

Currently, the histological events of guided tissue regeneration are well documented and it is now timely that the biochemical events of periodontal regeneration should be dissected. A significant disadvantage of current guided tissue regeneration procedures is the need for a staggered two-step surgical procedure. With adequate understanding of the biochemistry and cell biology of the events involved it is likely that biodegradable membranes will be developed in the future. This should lead to a more streamlined procedure and better clinical results based upon sound biological principles. In addition to this, the role of 'growth factors' released at the site of wound healing and tissue regeneration such as Transforming Growth Factor β (TGFβ), Platelet Derived Growth Factor (PDGF), Platelet Factor 4 (PF4) and Fibroblast Growth Factor (FGF) are likely to take prominence as agents of importance. These are not only mitogenic but also chemo-attractants capable of recruiting leukocytes (mainly monocytes) and fibroblasts to the healing site.[45] Considerable interest in the properties of these agents had led some to predict that future periodontal therapy may include their use to aid periodontal repair and regeneration.[46]

Connective tissue elements as diagnostic markers

At present, there are no reliable diagnostic tests that accurately measure periodontal destruction with active periodontitis.[47] Possible parameters which could provide this information would include ongoing loss of attachment or loss of alveolar bone. The gingival crevicular fluid contains many components of breakdown products from the periodontium arising from periodontal disease. As a result many studies have focused on analysing components of this inflammatory exudate in the hope of finding an indicator of active periodontal breakdown. Specifically, plasma proteins, bacterial and leukocytic enzymes, and inflammatory mediators have been amongst the many components studied in detail.[48] Unfortunately, one serious drawback of each of these 'potential markers' is that they reflect the processes occurring in the gingiva. Thus, no distinction can be made between gingivitis and periodontitis. However, it is now apparent that there is some site specificity of various connective tissue extracellular matrix components within the periodontium. Therefore, the development of an assay which could detect components unique to either bone or periodontal ligament only would be of significant use.[49] In the long term, such studies may help us determine whether we are dealing with active or remissive periodontitis.

Effects of medications on the periodontal connective tissues

Regardless of the development of the above tests, one of the greatest aids in assessing periodontal status will still be an accurate compilation of the medical history of our patients. Indeed, although the developments of new treatment regimes and diagnostic tests based upon biological principles are exciting developments, another area which will become increasingly relevant in the field of periodontics is that of medication-related periodontal responses. Although only three of the most common reactions will be presented here (Fig. 8), the dentist must be cognizant of the fact that any agent which has the ability to alter fundamental host responses can potentially cause side effects at sites unrelated to the organ being targetted. Due to their high metabolic activity and susceptibility to acute and chronic inflammation the connective tissues of the periodontium are likely targets for the manifestation of many drug-related effects.

Hormonal effects

A close correlation between normal physiological changes in the sex hormone levels and periodontal changes has been well documented. For example, gingivitis is a frequent finding during puberty and pregnancy.[50] Indeed, the potential for the sex steroids to exert potent effects on the gingival tissues is high since gingival fibroblasts possess receptors for testosterone[51] and oestrogen.[52] In addition, the sex hormones are actively metabolized in gingival tissues with chronically inflamed gingiva accumulating greater amounts of progesterone and androgen metabolites compared with normal gingiva.[53,54] These agents have been shown to affect the cellularity, vascularity, as well as the collagen and proteoglycan content of connective tissues significantly.[55-57] In addition to changing the make up of the extracellular matrix, the sex hormones can be effectively utilized as growth factors by several of the putative periodontal pathogens.[58,59] The steroid hormones of the adrenal cortex also may be correlated with changes in the periodontium. Their adverse effects on connective tissues are decreased

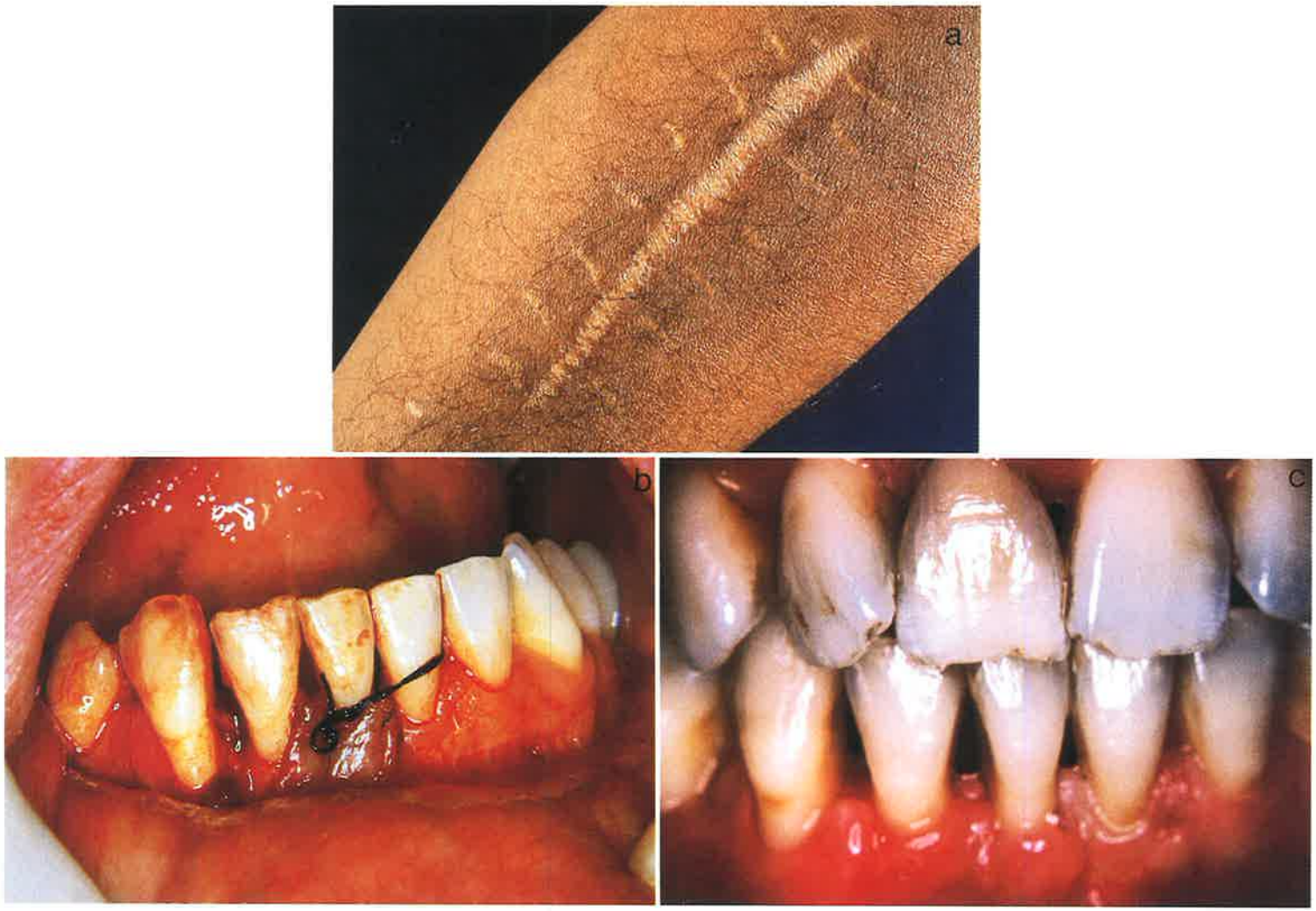

Fig. 5.–Different healing responses between different tissues. (a) Scar in skin of the arm following surgery. (b) Immediate postoperative view of lateral sliding pedicle graft from 42 to 41 leaving denuded bone adjacent to 42. (c) 3-weeks postoperative view of the site showing healing and lack of scar formation in gingival tissues.

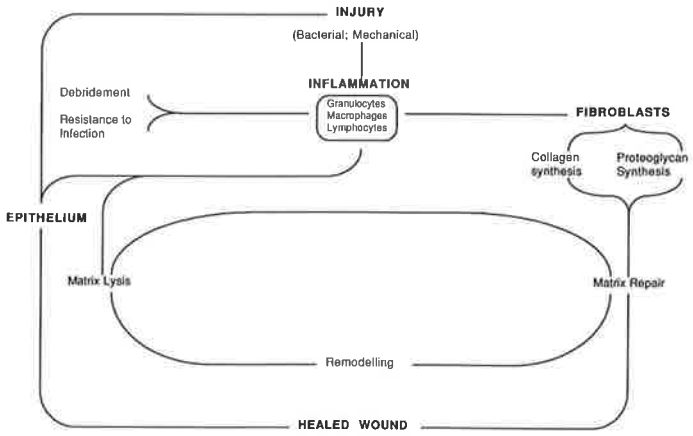

Fig. 6.—Factors involved in the connective tissues during wound healing.

fibroblast proliferation, as well as altered collagen and proteoglycan synthesis.[60] It is, therefore, not surprising to find that both the sex and adrenal hormones significantly affect wound healing.[57] The effects of various hormones are not restricted to the soft connective tissues of the periodontium. For example, osteoporosis of the jaws, retarded formation of the periodontal bone and reduction in cementum formation have been recognized as being associated with altered levels of sex and adrenal hormones for a long time.[61] Although other hormones such as insulin, thyroid hormones and pituatory hormones also produce changes in connective tissues, prescription of these as medications is usually restricted to specific medical problems and they are not as widely prescribed as the steroid hormones. However, the prescription of steroids and their analogues is very prevalent in western society for a wide range of treatments (for example, oral contraceptives, osteoporosis, menopausal therapy, as well as anti-inflammatory agents), and therefore the possibility of side effects from these medications manifesting in the periodontium is high. These may range from mild gingivitis and stomatitis to 'pregnancy' epulis and granuloma formation as well as gingival overgrowth.

Gingival overgrowth

This is frequently reported as a side effect of many medications commonly prescribed. Of these, phenytoin has been the most widely recognized and studied.[62] The effects of this drug on connective tissue metabolism are well documented. It has been suggested that phenytoin can selectively allow a subpopulation of gingival fibroblasts to proliferate which are capable of high protein and collagen metabolism.[63] This may lead to uncontrolled deposition of extracellular matrix and resultant tissue overgrowth. Until recently, phenytoin was the only well recognized agent associated with causing gingival overgrowth. However, in the last ten years there has been an 'explosion' of agents reported in the literature to have similar effects in relation to gingival overgrowth. A current listing of such drugs is shown in Table 4; however, it is very likely that with the development of new drugs further additions will be necessary. Despite the experimental observations of altered fibroblast function associated with both phenytoin and cyclosporin gingival overgrowth,[53,64] the fact that not all patients taking these medications develop gingival overgrowth, and that the lesions are usually confined to particular areas of the mouth, indicates that additional, unrecognized factors are involved. From several reports it seems that a crucial factor in the development of gingival overgrowth is the presence of dental plaque, since most lesions are reversible upon restoration of adequate plaque control.[64-66] This being the case, it is interesting to note a similarity between plaque-induced gingival overgrowth and drug-induced gingival overgrowth. One of the presumed pathogenic components of plaque, bacterial lipopolysaccharide, is capable of altering cell membrane permeability to ions. Thus, whether the drugs which induce gingival overgrowth act to exacerbate localized plaque-induced gingival overgrowth needs to be assessed.

Non-steroidal anti-inflammatory drugs (NSAIDS)

These are very commonly prescribed agents which have potent anti-inflammatory and anti-analgesic properties. They are commonly prescribed for patients with long-standing chronic inflammatory

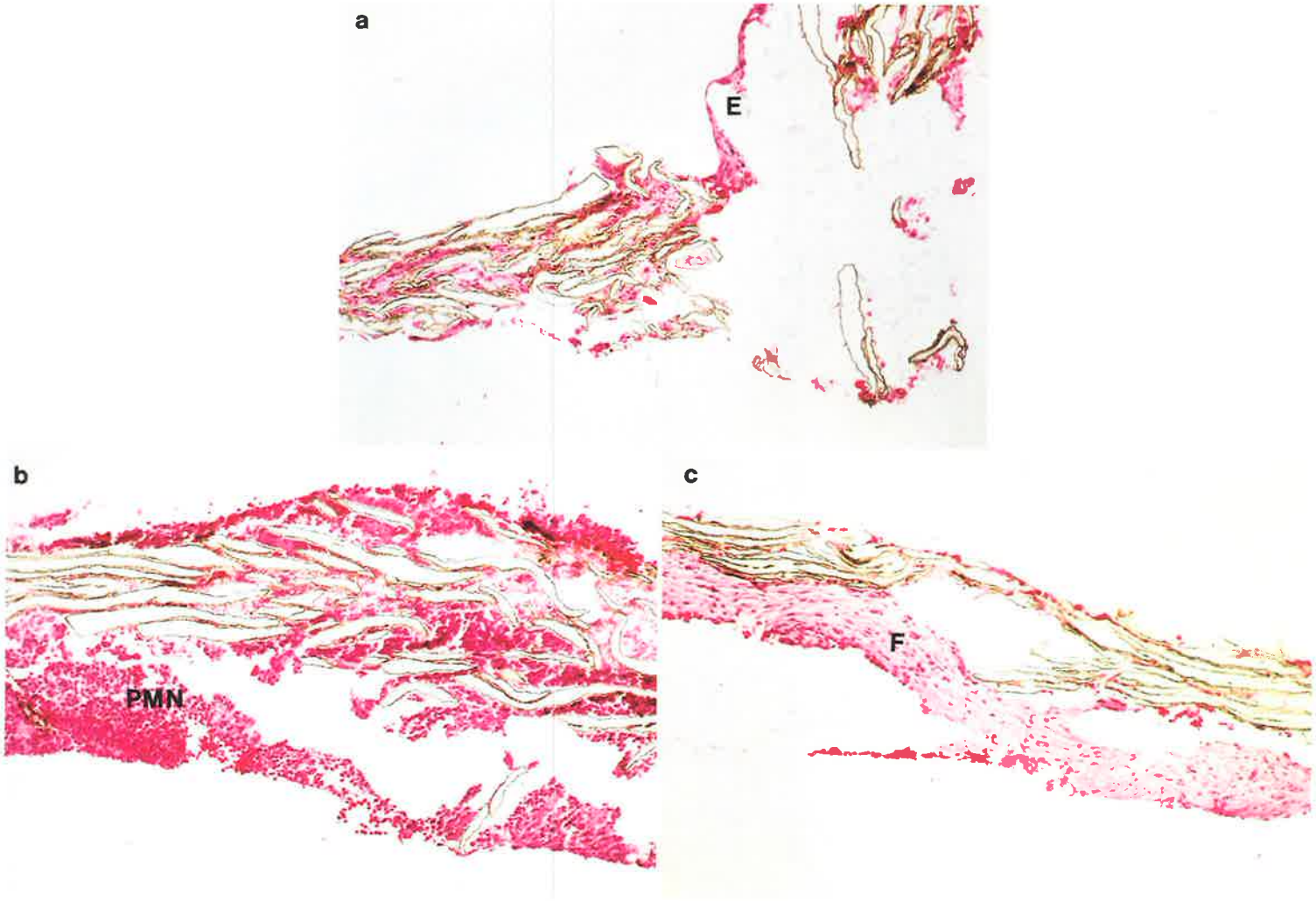

Fig. 7.—Connective tissue elements associated with membrane used for 'guided tissue regeneration'. The membrane was removed 6 weeks after placement. Three separate portions were sectioned: (a) coronal portion containing the open microstructure collar; (b) middle portion which was adjacent to a furcation defect; and (c) apical portion which had been overlying alveolar bone. Note that all of the necessary ingredients for successful periodontal regeneration are present. Epithelium (E) is distant to the activity inflamed and repairing tissue containing polymorphonuclear leukocytes (PMN) and fibrous connective tissue (F).

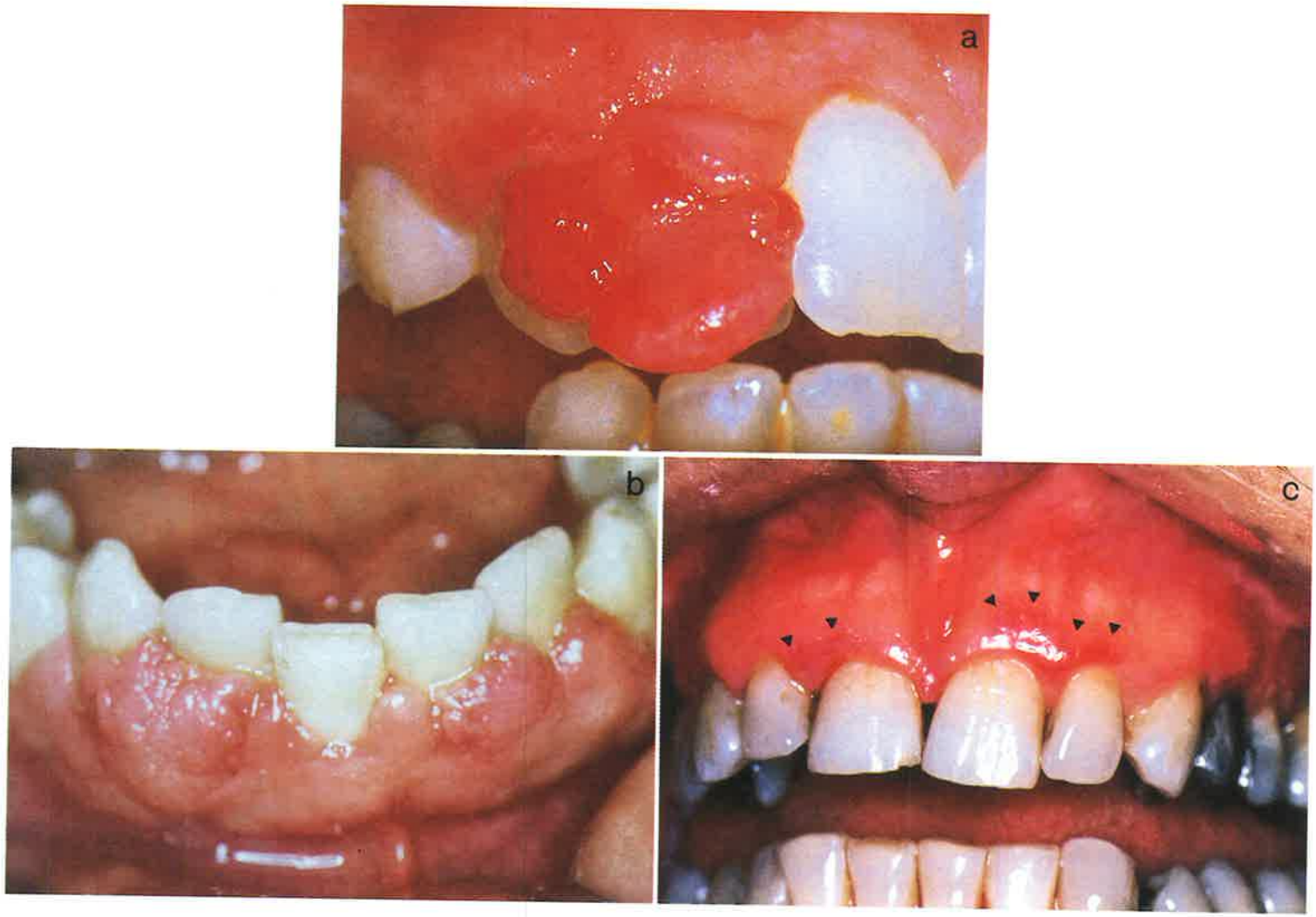

Fig. 8. – Three common forms of medication-induced gingival changes. (a) Epulis which appeared shortly after change in contraceptive pill prescription. (b) Cyclosporine-A-induced gingival overgrowth. (c) Gingival reaction (marked with arrowheads) associated with non-steroidal anti-inflammatory drug 'cocktail'.

Table 4. Medications capable of inducing gingival overgrowth

Generic name	Proprietary name	Treatment	Mode of action
Phenytoin	Dilantin	Anti-convulsant	Affects Na^+ ion efflux
Sodium valproate	Epilim	Anti-convulsant	Diminishes transmittors
Nitrendipine	Bayotensin	Anti-hypertensive	Ca^{++} ion influx inhibitor
Nifedipine	Adalat Anpine Procardia	Anti-hypertensive Anti-arrhythmic Anti-angina	Ca^{++} ion influx inhibitor
Verapamil	Verapamil	Anti-arrhythmic Anti-hypertensive Anti-angina	Ca^{++} ion influx inhibitor
Diltiazem	Cardizem	Angina Atherosclerosis	Ca^{++} ion influx inhibitor
Cyclosporin	Sandimmum	Organ transplants Arthritis	Immunosuppressant (Depresses T cell function)
Oxodipine	–	Vasodilator	Ca^{++} ion influx inhibitor

disorders (for example, rheumatoid arthritis, osteoarthritis) as well as for postoperative pain control. More recently the use of NSAIDs in the treatment of chronic inflammatory periodontal diseases has been advocated.[67] However, as will be evident below, their use in these circumstances should be with caution due to their ability to adversely affect connective tissues. The propensity of NSAIDs to cause ulceration of the gastrointestinal tract (stomach, duodenum, and oesophagus) is well known.[68] Therefore, it is not surprising that the oral cavity (which is also part of the gastrointestinal tract) also manifests complications – these may be as glossitis, stomatitis or tongue ulcerations. The NSAIDs dramatically alter not only inflammatory cell function but also influence mesenchymal cell function by disturbing the metabolism of collagens, proteoglycans and other structural proteins.[69] Thus, the potential for NSAIDs to cause compromised tissue status exists and this may lead to poor resistance to chemical and mechanical abuse common of the gingival tissues. In a recent report,[70] the incidence of oral reactions to NSAIDs was found to be between 1-2.5 per cent of all adverse reactions reported (Table 5). Given the ever-increasing wide prescription of these drugs, as well as an expected number of similar new products entering the market, it can be predicted the number of patients presenting with NSAID-related gingival problems will become increasingly prevalent.

Table 5. Oral reactions to non-steroidal anti-inflammatory drugs*

Drug	% adverse reactions manifesting in mouth
Phenylbutazone	1.7
Ibuprofen	1.0
Indomethacin	0.7
Naproxen	1.8
Sulindac	1.5
Piroxicam	2.5
Diflusinol	2.4
Diclofenac	1.8

*Adapted from reference 70.

Conclusion

In this review an attempt to highlight the overriding importance of the periodontal connective tissues in the manifestation, pathology and responses of the periodontal diseases. From a large body of fundamental and applied research it is now apparent that all reactions (pathological, reparative and regenerative) occur within a complex environment which is exquisitely sensitive to disruption as a result of matrix destruction or interference of fibroblast metabolic activity. The fundamental responses of treatment rely on a sound understanding of these responses and allow us to recognize what constitutes an acceptable clinical result.

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20 MODULATION OF GINGIVAL FIBROBLAST FUNCTION BY LIPOPOLYSACCHARIDES

P. M. Bartold

INTRODUCTION

A close interplay between host defense mechanisms and bacterial plaque during the initiation of gingivitis and periodontitis is now well established (Slots and Genco, 1984). Due to the apparent requirement of the presence of dental plaque for development of the various periodontal diseases, attention has focused upon the potential pathogenic features of the microflora which comprise dental plaque. With the development of suitable microbiological techniques, studies on the anaerobic micro-organisms residing within periodontal pockets were carried out and several groups of organisms were identified as being closely associated with different forms of periodontal disease. Amongst these, many Gram-negative anaerobic bacteria have been classified as potential periodontal pathogens. As a result of these identifications, there has followed an analysis of the many toxic and pathogenic factors synthesized by (and associated with) these bacteria which could be implicated in the pathogenesis of the periodontal diseases. Amongst these factors, lipopolysaccharide (LPS), peptidoglycan, outer membrane polypeptides, various enzymes and a leukotoxin have received prominent attention (Daly, Seymour and Kieser, 1980; Hunter, 1984; DeRienzo, Nakamura and Inouye, 1978; Tipler and Embery, 1985; Taichman, Dean and Sanderson, 1980). Although their specific effects appear to be related to impairment of cellular function and disruption to the extracellular matrix of the gingivae, these components should not be considered in isolation but rather part of a group of potential pathogenic mechanisms. Nonetheless, the identification and elucidation of their individual biological activities is fundamental to understanding the potential pathways for development of periodontal destruction.

Since LPS appears to be a common factor to all gram negative bacteria and it has well documented biological effects, it has received prominent attention. This chapter aims to

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review the role that LPS modulation of fibroblast function might play in the infectious periodontal diseases.

LIPOPOLYSACCHARIDE STRUCTURE

General Structure

LPS consists primarily of covalently bound lipids and polysaccharides and a small amount of non-covalently bound protein. The lipid moiety, known as lipid A, contains fatty acids which range in length from C_{10} to C_{22} and are responsible for the expression of the molecule's endotoxicity. β-hydroxymyristic acid is usually present in the highest concentration and is considered unique to LPS. The polysaccharide portion can be divided into two portions, the O- and R-polysaccharides. The O-polysaccharide, which determines the antigenic specificity of bacteria, is composed of repeating sequences of neutral sugars which may be either linear or branched and variable in their length. The composition and structural arrangements of these sugars is very diverse and is considered unique for a given LPS as well as the parental bacterial strain (Lüderitz et al., 1982). The R-polysaccharide links the lipid moiety to the O-polysaccharide portion and is subdivided into the O-chain proximal outer and the lipid A proximal inner core. The O-chain is composed of variable proportions of 5 basal sugars, phosphate and O-phosphorylethanolamine (Elin and Wolf, 1973). Of the core sugars in the R-chain, ketodeoxyoctonate (KDO) is a very common component (yet not always present) which is specific to LPS and links the R-chain to Lipid A.

Oral Bacterial Lipopolysaccharide Structures

Given the potential for significant structural and compositional diversity between bacterial species, it has been proposed that many of the differences in bacterial pathogenicity could be attributed to differences in LPS composition (Kotani and Takada, 1990). Thus many investigations have studied the LPS composition of oral bacteria. Indeed, one of the earliest studies concerning LPS from oral bacteria demonstrated variability in both LPS composition and biological activity (Mergenhagen, Hampp and Scherp, 1961). More recently, investigators have analyzed, in detail, the chemical composition of LPS molecules from periodontal pathogens and found them to be very heterogeneous (Mashimo, et al., 1985).

When compared to *Enterobacteriaceae*, LPS from *Porphyromonas (Bacteroides) gingivalis* has been reported to be unique in that it lacks heptose and β-hydroxytetradecanoic acid (Mansheim, Onderdonk and Kasper, 1978; Nair et al., 1983) and has variable amounts of KDO present (Mansheim et al., 1978; Koga et al., 1984; Bramanti et al., 1989; Johne, Olsen and Bryn,1988). Such differences have been related to the low level of endotoxicity associated with various *Bacteroides* species (Sveen, 1977; Nair et al., 1983). In addition intra-species differences are evident since electrophoretic differences have been noted for LPS isolated from the virulent and avirulent strains of *P. gingivalis* (Bramanti et al., 1989). The complexity of differences in LPS structure within the same species has been

highlighted further by the isolation of two chemically distinct LPS molecules from the same strain of *Bacteroides gingivalis* which possess different biological activities (Millar et al., 1986).

For *Actinobacillus actinomycetemcomitans*, the sugar and fatty acid compositions of its LPS are immunochemically and biochemically of the *Enterobacteriaceae* type, containing β-hydroxymyristic acid, heptose and KDO (Killey and Holt, 1980; Dahlén and Mattsby-Baltzer, 1983; Hofstad, 1984; Nishihara, et al., 1986). Nevertheless, some intraspecies heterogeneity with respect to molecular size and structure has been noted (Hoover, 1988).

Similar findings of species specific features of LPS, together with some intra-species heterogeneity, have also been reported for *Eikenella corrodens* and *Wolinella recta* (Kato, Okuda and Takazoe, 1987; Gillespie et al., 1988).

BIOLOGICAL PROPERTIES OF LPS

When injected into experimental animals, LPS elicits a range of non-specific pathological reactions manifesting at both the systemic and local level. Some of the systemic effects include fever, changes in leukocyte counts, stimulation of the hypothalamic-pituatory system, hypotension, and if administered in high doses, shock or even death. While these represent significant effects on the animal as a whole, their significance to periodontal disease is equivocal. Rather, it would seem more likely that the ability of LPS to induce specific local reactions is of more significance to the pathological changes associated with periodontal disease.

The effect of LPS on neutrophils is of central concern to the immediate host response to periodontal infections. Thus many investigators have studied the interaction between LPS and neutrophils (Wilson, 1985). A principal mode of action is believed to be via binding of LPS to neutrophil membrane (Springer and Adye, 1975) which results in altered neutrophil adhesion, aggregation and locomotion (Dahinden, Galanos and Fehr, 1983). In addition, LPS may induce lysosomal degradation as well as altered production of reactive oxygen species and bactericidal properties of the neutrophil (Modrzakowski and Spitznagel, 1979; Proctor, 1979; Cybulsky, Chan and Movat, 1988).

LPS influences the immune system locally by virtue of its strong antigenicity as well as being a strong B-cell mitogen causing polyclonal B-cell activation (Anderson, Coutinho and Melchers, 1977; Morrison and Ryan, 1979). Since B-cells and plasma cells are the major inflammatory cell types present in periodontal lesions a role for them in destruction of periodontal support has been proposed (Tew, Engel and Mangan, 1989). Thus the presence of bacterial LPS in gingival tissues could lead to polyclonal B-cell activation which in turn may result in release of lymphokines such as interleukin-1, autoantibodies, immune complexes and complement activation (Allison, Schorlemmer and Bitter-Suermann, 1976; Hanazawa et al., 1985; Bramanti et al., 1989).

In addition to the above, other local effects of LPS are mediated by activation of monocytes/macrophages resulting in the release of many inflammatory mediators such as prostaglandins, interleukin-1 and tumor necrosis factor (Ohmori et al., 1988; Koga et al.,

1985; Bramanti et al., 1989). The relevance of these inflamammatory mediators to local tissue destruction has been extensively studied since they have potent effects on both osteoclasts and fibroblasts mediating the production of a wide variety of enzymes capable of inducing connective tissue degradation (Miekle, Heath and Reynolds, 1986).

LPS ASSOCIATED WITH THE DENTAL TISSUES

The likely presence of large quantities of LPS associated with the periodontal tissues is of significance with respect to the pathogenesis of periodontal disease. Indeed, if LPS is capable of gaining access to the gingival tissues, then a strong correlation between disease progression and presence of LPS could exist. The presence of LPS at various periodontal sites will thus be considered..

LPS in dental plaque

A potential role for LPS in the pathogenesis of periodontal disease would be supported by its presence in dental plaque. Following the initial observation of Mergenhagen et al. (1961) regarding the presence of LPS in oral bacteria, toxic factors found in plaque extracts were attributed to LPS (Hattfield and Baumhammers, 1971). Such an association was subsequently confirmed by Selvig, Hofstad and Kristofferson (1971) who demonstrated the electronmicroscopic localization of bacterial LPS in dental plaque matrix. According to this study, the LPS had a morphology similar to LPS from other gram-negative bacteria appearing as either short rods or circular particles comprised of an outer electron dense zone surrounding an electron lucent inner portion. Later, using the Limulus Amebocyte Lysate test the presence of LPS in dental plaque was confirmed (Fine et al., 1978a).

Today, LPS has been isolated from a wide variety of oral gram-negative anaerobic bacteria and appears to be an ubiquitous component of these bacteria. As discussed above, there are fine structural and quantitative differences between different bacteria isolated from periodontal pockets which may account for differences in virulence.

The presence of LPS has been noted in gingival crevicular fluid (Simon et al., 1969). This most likely has origins within the subgingival microflora being released from autolysed bacteria or even secreted by living microbes. Following this initial observation, it was established that the concentration of LPS in gingival crevicular fluid correlated positively with the severity of gingival inflammation both clinically and histologically (Simon et al., 1970, 1971). A further positive correlation between the development of dental plaque from a gram-positive to gram-negative flora over 2-3 days and an increase in crevicular fluid content of LPS has been reported (Tzamouranis et al., 1979).

Few quantitative studies concerning the LPS content of dental plaque have been carried out. Sonicated supragingival plaque has been reported in one study to contain between 0.2-8.2 μg/mg plaque (Shapiro et al., 1972), while in another (Johnson et al., 1976), approximately 0.01% of the dry weight of plaque (i.e. 10 μg/mg) was reported to be accounted for by LPS. From other studies it appears that the highest concentrations of LPS are found in subgingival loosely adherent plaque (Fine et al., 1978 a & b) which may

facilitate diffusion of LPS into the gingival tissues. When the LPS content of saliva, crevicular fluid, gingival tissues and plaque was studied and correlated with gingival inflammation, that of plaque contained the highest concentration of LPS.

LPS in gingival tissues

The finding of detectable levels of LPS (0.001-0.1 μg/mg) in gingival tissues, which increased with the severity of gingival inflammation, provided early evidence that LPS could, indeed, penetrate the gingival tissues (Shapiro et al., 1972). Despite this, the question of LPS access to gingival tissues has been one of some controversy. The principal concern with this concept has been whether LPS can penetrate an intact sulcular epithelial barrier and thus be responsible for the initiation of gingival inflammation. Using the localized Schwartzman reaction, it has been demonstrated that LPS at low concentrations does not diffuse through intact sulcular epithelium but LPS is capable of passing through damaged sulcular epithelium (Rizzo, 1968, 1970). Furthermore, using immunofluorescence techniques, the O-antigen of LPS has been demonstrated in gingival connective tissues (Courant and Bader, 1966).

Subsequent to these studies, LPS was found to penetrate through intact sulcular epithelium of dog gingiva showing a higher penetration in the more coronal regions of the sulcular epithelium (Schwartz, Stinson and Parker, 1972). Additional evidence of LPS penetration through healthy sulcular epithelium was presented by Ranney and Montgomery (1973) in which vascular leakage was noted within gingivae following topical application of LPS within the gingival sulcus.

Regardless of the above findings, the arguments regarding the integrity of the sulcular epithelium may be mute since there exists within the gingival sulcular environment ample enzymatic and cellular events which, with time, have the potential to significantly disrupt tissues and alter tissue permeability and thus provide adequate avenues of access to the gingival connective tissues for LPS.

LPS associated with cementum

Although an alteration occurring within cementum exposed to the periodontal pocket environment has always been considered to be important to the disease process of periodontitis, it was not until relatively recently that the toxic nature of elements associated with such cementum has been addressed. The first suggestion that cementum toxicity to the periodontal tissues could be related to the presence of LPS was forwarded by Hatfield and Baumhammers (1971). Following this, numerous studies tested for the presence of LPS within cementum from periodontally healthy and diseased root surfaces (Aleo et al., 1974; Aleo, De Renzis and Farber, 1975) and resulted in general acceptance of the presence of LPS in cementum exposed to periodontal pockets (Ruben and Shapiro, 1978). A specific role for LPS removal by root planing was thus advocated for restoration of periodontal health (Jones and O'Leary, 1978; McCoy et al., 1987).

Despite these studies, some doubts existed over the presence and toxicity of LPS in

cementum. For example, not only was LPS recovered from diseased cementum in very low quantities (Ito et al., 1985) but tests based on the Limulus amebocyte lysate assay had limitations which did not allow specific identification. In fact, in the early studies, LPS was only assumed to be present in the extracts based upon the supposed preferential specificity for LPS by the Limulus amebocyte lysate assay (Fine et al., 1980). Furthermore, some studies had suggested that gingival fibroblasts attached and grew on root surfaces irrespective of root surface detoxification (Adelson et al., 1980).

Nevertheless, in recent times, convincing evidence has accrued to support the concept of a close relationship between root surface cementum and LPS. Histochemical and immunohistochemical techniques have clearly demonstrated the presence of LPS on the surface of cementum (Nakib et al., 1982; Daly et al., 1982). Although these studies demonstrated little penetration of cementum by the LPS, they did, however, raise the question of whether LPS was firmly bound or merely associated loosely with the cementum surface (Daly et al., 1982). In view of the identification of most of the detectable LPS being present in loosely adherent plaque on the root surface (Fine et al., 1980), and the relative ease with which LPS can be removed from the cementum surface (Moore, Wilson and Kieser, 1986; Blomlöf et al., 1989), it would appear that LPS is not firmly bound to the root surface. The LPS content of cementum has been studied and variable levels have been reported. For example, Jones and O'Leary (1978) reported an extraction recovery of 148 ng LPS/periondontally involved tooth compared to 0.25 ng LPS/uninvolved tooth. In this study, root planing was found to reduce the LPS content of root surfaces close to those of uninvolved teeth. In more recent studies, the level of LPS associated with periodontally involved roots was reported to range between 19-394 ng/tooth (Maidwell-Smith, Wilson and Kieser, 1987) to as high as $4.3x10^3$ -10.4 x 10^3 ng/tooth (Moore et al. 1986; Wilson, Moore and Kieser, 1986). Although this appears to be a significant increase over the earlier report, several methodological differences most likely account for the differences. For example, in the latter studies no prior washing of the teeth was done and the whole root surface was assessed which together would account for a significant increase in recovery compared to the washed and selected root area studied by Jones and O'Leary (1978). Washing of the teeth prior to extraction procedures would have a marked effect since as much as 39% may be removed by gentle rinsing of the teeth, while a further 60% may be removed by light rotary brushing of the root surface (Moore et al., 1986). The remaining 1% most likely remains embedded within the cementum since bacterial product penetration of up to 10 μm into cementum has been previously reported (Daly et al., 1982). The association of LPS with the surface of the root surface has been further confirmed by both light and electronmicroscopic immunohistochemistry (Hughes and Smales, 1986; Hughes, Auger and Smales, 1988).

EFFECT OF LPS ON GINGIVAL FIBROBLASTS

The effect of plaque or bacterial extracts on mammalian cells in vitro has been the subject of numerous studies over the past 20 years (Levine and De Luca, 1978; Duguid, Al-Makadsi and Cowley, 1980; Shenker, Kushner and Tsai, 1982; Stevens and Hammond, 1988).

Unfortunately, many of these have been merely observational studies concerned with obvious cytotoxic effects on cells without addressing the chemical nature of the toxic component(s). Nonetheless, some biologically active components associated with oral bacteria have been identified of which various enzymes (Tipler and Embery, 1985), a leukotoxin (Taichman et al., 1980) and LPS (Daly et al., 1980) appear to be the most prominent. Although the leukotoxin and various enzymes are important modifiers of biological events their potential effects on fibroblast activity appear to be minimal (Taichman et al., 1980; Cogen and Taubman, 1982). On the other hand, LPS demonstrates numerous potent effects towards fibroblasts which require further discussion.

LPS and gingival fibroblast proliferation

Although not positively identified, LPS associated with periodontally involved root surfaces was first implicated as an agent which could alter mitotic rates of mammalian cells in 1971 (Hatfield and Baumhammers, 1971). Following this initial observation, several studies reported the extraction of LPS from human teeth and dental plaque and its inhibitory effect on fibroblast proliferation (Aleo et al., 1974, 1975; Singer and Buckner, 1980; Olson, Adams and Layman, 1985). By measuring the incorporation of ^{3}H-thymidine into the DNA of human gingival fibroblasts, *E coli* LPS was reported to suppress proliferation up to 49% while extracts from periodontally diseased root surfaces (which tested positively for LPS) suppressed proliferation up to 58% (Olson et al., 1985). Although substances other than LPS were extracted from the root surfaces, strong circumstantial evidence was accruing to indicate a strong biological effect of LPS against fibroblasts. With the identification of the so-called periodontal pathogens, and the development of successful culturing methods, a crude LPS preparation from *P. gingivalis* was subsequently prepared and found to cause inhibition of human gingival fibroblast proliferation similar to a native extract of the whole bacteria (Larjava et al., 1987). In addition, LPS purified from *P. gingivalis* and *Prevotella (Bacteroides) intermedia* were also found to inhibit gingival fibroblast proliferation up to 50% (Layman and Diedrich, 1987). Subsequently similar findings have been reported for other LPS preparations from *A. actinomycetemcomitans* and *P. gingivalis* (Bartold and Millar, 1988; Layman and Landreneau, 1989).

LPS and cell morphology

Apart from inhibiting cellular proliferation, LPS may also induce morphological changes which could impair normal cellular functions. One of the earliest studies to address morphological and functional status of gingival fibroblasts following exposure to oral bacteria (*Actinomyces viscosus*) noted that although the cells appeared to have engulfed the bacterial substances, little cytopathic changes were noted (Engel, Schroeder and Page 1978). In addition, some binding of the bacteria to the cell surfaces was noted. In this study the contribution of LPS, or other bacterial components, to the observed changes was not determined.

However, given the potential of fibroblasts to engulf LPS particles, and the documented

effect of LPS on lysosomal enzyme leakage (McGivney and Bradley, 1978), mitochondrial respiration (White et al., 1971; Kilpatrick-Smith and Ericinska, 1983), and other cellular organelles (Lucas, Chen and Aleo, 1979), a great potential exists for LPS-induced cellular changes. Indeed, the ingestion of LPS by fibroblasts has been studied and localization of LPS to the cell nuclei was noted with the nuclear chromatin and nucleoli being the principal sites of location (Lucas, Subramoniam and Aleo, 1985). Despite LPS affecting other cellular organelles (De Renzis and Chen, 1983), it does not appear to localize to the mitochondria, lysosomes, Golgi, endoplasmic reticulum or ribosomes. The entry to the nuclear compartment is very rapid (approximately 2 minutes). The specific localization to nuclear chromatin indicates that the primary effect of LPS may be related to an alteration of the transcription and translation of DNA and RNA.

LPS and cell attachment

An important event in periodontal repair is the attachment of fibroblasts to the root surface. From the above discussion it is clear that not only is there an abundance of LPS associated with roots of periodontally involved teeth, but also a strong indication that LPS could affect the mechanisms of fibroblast attachment. Early studies addressing this issue demonstrated that introduction of LPS to fibroblasts cultured on glass surfaces resulted in detachment (Neiders and Weiss, 1973). Although the precise mechanisms involved were not elucidated, it was suggested that the evoked cell detachment was not related to toxicity of LPS towards the cells.

The presence of inhibitors of attachment for fibroblasts cultured on human root surfaces was first demonstrated by Aleo et al. (1975). Although LPS was not identified in these studies, it was suggested as a possible candidate for inhibition of fibroblast attachment. Further observations that fibroblasts could attach to "clean" root surfaces, while little or no attachment occurred on root surfaces previously exposed to the oral environment and not cleaned, have corroborated the likely presence of attachment inhibitors such as LPS on periodontally involved teeth. However, in contrast, some studies have been unable to demonstrate any difference in fibroblast attachment or cytotoxicity on instrumented or non-instrumented root surfaces (Wirthlin and Hancock, 1980; Cogen, Garrison and Weatherford, 1983; Gilman and Maxey, 1986). Since all of these studies were done on heat sterilized surfaces to prevent bacterial contamination the interpretation of the results is difficult and their ultimate clinical applicability is doubtful. The complexity of such interactions has been highlighted by two recent investigations. Although initial attachment of fibroblasts to diseased and non-diseased root surfaces is similar (Fardal et al., 1986), longer term culture assays indicate that in the presence of LPS not all initial attachment events lead to long term adhesion of cells to root surfaces nor permit adequate growth and proliferation (Pitaru et al., 1987).

LPS AND THE EXTRACELLULAR MATRIX

Regardless of the above findings, whether the attachment of fibroblasts to diseased or non-

diseased root surfaces is similar may have no bearing on the ability of the cells to initiate connective tissue regeneration. Indeed, it would seem probable that events occurring after attachment of the cells to the root surface will be responsible for dictating regenerative responses. Furthermore, altered synthetic activity of the fibroblasts residing within the periodontally affected tissues will be of importance with respect to both the pathogenesis of periodontal disease as well as the controlling mechanisms involved in periodontal wound repair.

Collagen

Surprisingly, the effect of LPS on collagen synthesis by human gingival fibroblasts has received little attention and thus much of the information related to this topic has been obtained from other systems or inferential data. While culture filtrates of various bacteria have been studied and found to have some effect on general protein synthesis, these studies have not provided specific details of the agent(s) involved or types of proteins affected (Touw et al., 1982; Van Kampen et al., 1984; Larjava and Jalkanen, 1984).

Studies which have directly assessed the effect of LPS on collagen synthesis by fibroblasts have provided conflicting results. While one report describes stimulation of total protein as well as collagen synthesis by fibroblasts exposed to LPS (Aleo, 1980), others have reported an inhibitory effect (Singer and Dutton, 1979). Similar inhibitory effects have been reported for protein synthesis by chondrocytes (Morales, Wahl and Hascall, 1984) and bone (Millar et al., 1986). In addition, in bone, LPS may selectively inhibit collagen up to 40% yet a 25% increase in collagen synthesis in wounds exposed to LPS has been reported (Kanta et al., 1981). More recently, although LPS has been found to inhibit total protein synthesis by human gingival fibroblasts approximately 40-50% (Bartold and Millar, 1988), little effect has been noted on collagen synthesis by similar cells exposed to LPS (Kamin et al., 1986). These apparently discordant findings may highlight differences between types of cells studied as well as source of LPS and purity of LPS preparations used.

In addition to modulation of collagen synthesis, a potential role for LPS in modulating collagen fibrillogenesis has also been proposed (Sauk, Johnson and Rozkowski, 1982). In view of the relative importance of collagen to the structural integrity of the periodontal tissues as well as its role in periodontal regeneration the influence of LPS on collagen synthesis has been a poorly studied area.

Proteoglycans and hyaluronic acid

Despite collagen being regarded as the major structural component of the extracellular matrix of the periodontal tissues, the proteoglycans and hyaluronic acid are the major macromolecular components of the ground substance and are responsible for maintaining a suitable environment for cellular metabolism and molecular interactions. Thus several studies have investigated the effects of LPS on proteoglycan and hyaluronic acid synthesis.

Early studies using bacterial culture supernatants and plaque extracts indicated that the *in vitro* synthesis of both glycosaminoglycans and proteoglycans could be altered by

unidentified agents (Van Kmapen et al., 1984; Larjava et al., 1983; Larjava, 1984). In particular, hyaluronic acid synthesis by gingival fibroblasts has been demonstrated to be stimulated by components in dental plaque while proteoglycan synthesis by chondrocytes appears to be decreased in the presence of *P. gingivalis* culture supernatants. Although LPS was not positively identified in these studies as being the responsible agent, its participation cannot be discounted. In this regard, the observation that LPS stimulates hyaluronic acid synthesis by synovial cells has been of particular significance (Buckingham, Castor and Hoag, 1972).

Modulatory effects of LPS on hyaluronic acid and proteoglycan synthesis on human gingival fibroblasts has been demonstrated recently . LPS from several oral and non-oral sources appear to inhibit proteoglycan synthesis yet stimulate hyaluronic acid synthesis by fibroblasts in vitro (Bartold and Millar, 1988; Bartold, 1990, unpublished observations). Although these appear to be opposite findings, it is important to note that these macromolecules have different biosynthetic pathways and probably serve different cellular functions. Thus they may represent different responses by the cells to LPS exposure.

CONCLUDING REMARKS

While it may be recognized now that LPS can act directly upon gingival fibroblasts as discussed above, we must also be cognizant of the fact that such a bioactive molecule will also affect many other cellular systems. Indeed, LPS is a multipotential initiator of inflammation. In addition to modulation of fibroblasts, these macromolecules also influence platelets, neutrophils, mast cells, macrophages, lymphocytes and endothelial cells. Furthermore, at least 2 major humoral systems (complement and coagulation) are activated by LPS. As a result of the multiple cell systems which are activated by LPS, endogenous mediators are overproduced. Of these, prostaglandins, interleukin-1 and tumor necrosis factor have received prominent attention because of their ability to modulate fibroblast proliferative behavior, extracellular matrix synthesis as well as release of matrix degrading enzymes such as collagenase and stromelysin (Miekle et al., 1986).

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Paper 27.

Bartold, P.M. and Narayanan A.S.
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P.M. Bartold's role in this manuscript was:
Conceptualization
50% of text

A.S. Narayanan's role in this manuscript was:
Conceptualization
50% of text

Note: This chapter is divided into two parts, one authored by Bartold and Narayanan (pages 61-87) on the Connective Tissue, and the other authored by Schwartz, Dean and Boyan (pages 87-99) on The Physiology of Bone.

6

The Biochemistry and Physiology of the Periodontium

Connective Tissue

Biochemistry of Matrix Constituents

The periodontium comprises the supporting tissues around the teeth, consisting of gingiva, periodontal ligament, cementum, and alveolar bone.[1] Although it is not strictly a connective tissue, due to its unique relationship and functional demands the epithelium (junctional, sulcular, and oral epithelia in normal periodontium and pocket epithelium in diseased tissue) must be considered as a periodontal component; however it will not be discussed in this section.

Several features distinguish the periodontium from other organs. It is constantly subjected to mechanical and bacterial stress, yet it is remarkably efficient in maintaining its structure and functional integrity; this is due to the fast turnover and efficient remodeling of its component structures. The periodontium functions as a single unit, even though each of its components has a distinct composition and connective-tissue architecture. Recent research has revealed that matrix constituents of one periodontal structure can influence the cellular activities of adjacent structures.[1] Finally, the periodontium's unique composition dictates that its maintenance, repair, and regeneration require a variety of complex processes coordinating the synthesis of soft and hard tissues.

The connective tissues of the periodontium consist of fibrous and nonfibrous molecular constituents. Fibrous components are collagens and elastin, and nonfibrous constituents are fibronectin, laminin, tenascin and other proteins, proteoglycans, hyaluronate, and lipid. In addition, calcified structures also contain osteopontin, bone sialoprotein-II (BSP-II), and minerals. The objective of this chapter is to summarize currently available information on periodontal connective-tissue matrix in health and disease. To provide a perspective of matrix structure and function, first the biochemistry of matrix macromolecules is reviewed. Emphasis is on collagens, as these are the major constituents of all periodontal structures. This is followed by a description of connective-tissue architecture in the normal periodontium (pages 76 to 81) and how it is affected by inflammation and fibrotic diseases. Factors that cause pathologic connective-tissue alterations are outlined and, finally, emerging biologic principles of wound healing relevant to periodontal therapy are discussed.

Collagens. Collagens are the most abundant proteins in the animal kingdom. The word collagen is derived from the French *collagene* (from the Greek *kolla*-glue + *gen*-birth) to designate connective-tissue constituents that produce glue. The collagen molecule is rigid and resists stretching; therefore, it is utilized in tissues such as tendon, skin, and periodontal ligament where mechanical force must be transmitted without loss. The organization of collagen depends upon the specific functional requirements in various tissues. For example, it forms branching and anastomosing fibers in skin, thick fibers oriented parallel to the long axis in tendon, and laminated sheets in corneal tissue.[2]

Structure and types. Several unique structural properties distinguish the collagen molecule from other proteins. 1) Three polypeptide chains called α *chains* form the collagen molecule. The α chains are left-handed helices that assemble into a "triple helix" with a right-handed twist, and the molecule may be a homotrimer or a heterotrimer made up of same or different α chains, respectively.[2,3] 2) A repeating gly-X-Y amino acid sequence, in which X and Y are usually amino acids other than glycine, forms the triple helix. In fibril-forming collagens, the helical domain is flanked at both ends by short, nonhelical *telopeptides.*[2] Short gly-X-Y repetition is also present in complement component C1q, lung surfactant protein, and several other proteins; however, they are not considered collagens because they do not form part of the extracellular matrix. 3) Collagen contains two unique amino acids, hydroxyproline (hyp) and hydroxylysine (hyl). In vertebrate collagens, these amino acids are present in the "Y" position.[2] 4) Collagen molecules are covalently linked through lysine-derived intra- and interchain crosslinks. The hierarchical organization of collagen molecules into banded fibrils is schematically illustrated in Fig 6-1.

More than fifteen different collagen types have been described so far.[5] These are divided into three groups. The first group includes those collagens that form banded fibrils in tissues; type I, II, III, V, and XI collagens belong to this group. In these collagens, called *fibril-forming collagens,* the triple-helical domain contains an uninterrupted stretch of 338–343 gly-X-Y triplets in each chain, and the molecule has the dimension 15 Å × 3,000 Å. The second group of collagens associate with the first group, forming connective-tissue elements between banded fibrils and other components. This group is called *fibril-associated collagens* with interrupted triple helices (FACITS), and includes types IX, XII, and XIV. *Nonfibrillar collagens* form the third group. Molecules belonging to this family form sheets or protein membranes enclosing tissues and organisms. Examples are type IV (basement membranes), type VIII (Descemet's membrane), type VI (microfibrils), type VII (anchoring fibrils), and invertebrate collagens (these form the cuticle of worms).

Tissues contain a mixture of collagen types. Type I collagen is the predominant type in all connective tissues except cartilage and accounts for 65% to 95% of total collagens. Type III is the second largest species, accounting for 5% to 30% of adult-tissue collagen. The percentage of type III may be much higher in fetal and granulation tissues.[6] These two collagens are co-distributed with types V, VI, and XII. In cartilage, type II is the major fibril-forming collagen and it is present with FACIT types IX, XI, and XII. Type X is a short collagen with 1,380-Å helix, and its distribution is limited to growing bones in the zone of hypertrophic chondrocytes.[5]

In type IV collagen, which is found only in basement membranes, triple-helical domains are interrupted by short nonhelical sequences. This collagen forms a fine spiderlike network of cords, and this structure is created by head-to-head assembly of N-terminal ends of four type IV molecules and tail-tail assembly of two nonhelical C-terminal ends. The type IV collagen binds to anchoring fibrils, which are structures attached by their extremities to epithelial basement membranes on one end and to anchoring plaques on the other end. The anchoring fibril consists of type VII collagen that contains a 4,240-Å-long triple-helical region.

The features of various vertebrate collagen types and their tissue distribution are summarized in Table 6-1.

Biosynthesis of collagens. The collagen molecule is insoluble under physiological conditions. It contains several modified amino acids; the amino-acid modifications can occur only on free-α chains because the enzymes responsible cannot act on the triple-helical molecule. For these and other reasons, collagen is first synthesized as a precursor in which the α chains have extra amino acid sequences at N- and C-terminal ends. These "pro-α" chains undergo a series of well-coordinated biosynthetic reactions in the nucleus, cytoplasm, and extracellular space (reviewed in Byers[4]). These reactions have been characterized for type I collagen and most of them apply to other collagens as well. The biosynthetic events can be divided roughly into three groups associated with 1) gene expression, 2) translation and posttranslational cytoplasmic events, and 3) extracellular reactions.

Gene expression. The genes for collagens are large, ranging in size from 5 kb for COL10A1 to 100 kb for

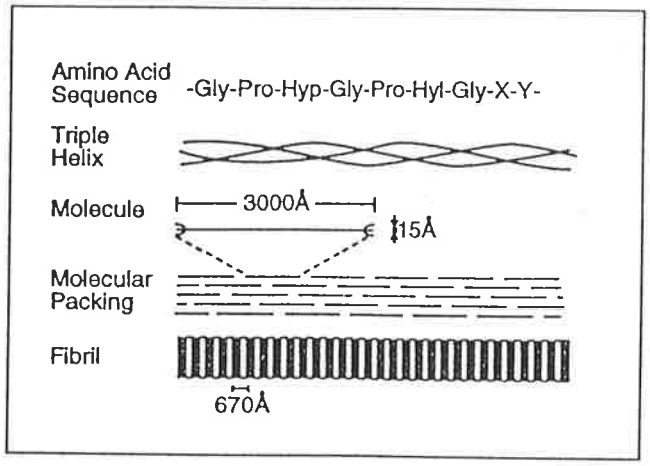


Fig 6-1 Structure of collagen fibrils formed from the basic gly-X-Y triple-helical unit. Three "α" chains with this amino acid sequence assemble into the triple helix. Each triple-helical collagen molecule, represented as a line, is of size 3000 x 15 Å. The molecules aggregate with a quarter stagger, which results in *hole* and *overlap* zones. These zones are alternating regions where there is gap between ends of molecules and no gaps, respectively, and are responsible for the characteristic banding pattern in collagen fibers. The bands of a typical collagen fiber have a periodic spacing of 670 Å. (Figs 6-1 and 6-2 from Byers.[4] Redrawn with permission.)

Table 6-1 *Vertebrate Collagen*

Collagen Type	Chain Composition*	Gene†	Distribution
Fibrillar collagens			
Type I	$[(\alpha1)_2\alpha2]$	COL1A1, COL1A2	Soft tissues, bone
Type II	$[(\alpha1)_3]$	COL2A1	Cartilage, vitreous humor
Type III	$[(\alpha1)_3]$	COL3A1	All soft tissues
Type V	$[(\alpha1)_2\alpha2]$ $[\alpha1,\alpha2,\alpha3]$	COL5A1, COL5A2, COL5A3	Most soft tissues
Type XI	$[(\alpha1)_2\alpha2]$	COL11A1, COL11A2	Cartilage
FACIT collagens			
Type IX	$[\alpha1,\alpha2,\alpha3]$	COL9A1, COL9A2, COL9A3	Cartilage
Type XII	$[(\alpha1)_3]$	COL12A1	Soft tissues
Type XIV	$[(\alpha1)_3]$	COL14A1	All tissues
Nonfibrillar collagens			
Type IV	$[(\alpha1)_2\alpha2]$	COL4A1, COL4A2	Basement membranes
Type VI	$[\alpha1,\alpha2,\alpha3]$	COL6A1, COL6A2, COL6A3	Soft tissues
Type VII	$[(\alpha1)_3]$	COL7A1	Anchoring fibrils
Type VIII	$[(\alpha1)_3]$	COL8A1	Descemet's membrane
Type X	$[(\alpha1)_3]$	COL10A1	Cartilage

* Collagen molecule consists of three polypeptides called "α" chains. Each chain is identified by the arabic number that follows. The collagen molecule may be composed of same (homotrimers) or different (heterotrimers) "α" chains. Roman numerals indicate the collagen type. Thus, $[\alpha1(II)3]$ represents type II collagen composed of three α1(II) chains.

† "COL" represents collagen, and the arabic number that follows indicates its type. "A" and the number after it designates the α chain and its type. For example, COL5A2 is the gene for α2(V) chain.

COL9A1 (see the footnote in Table 6-1 for terminology), and are approximately ten times the size of functional messenger ribonucleic acid (mRNA).[7] More than 27 genes have been described for collagen types I to XVII and their location identified in human chromosomes. Although differences exist among various collagen genes, those for fibril-forming collagens have a similar arrangement of coding sequences (exons). These genes contain approximately 40 exons, which are separated by introns (non-coding sequences) 80 to 2,000 nucleotides long. The exons for the triple-helical domain are of 54-bp size or its integral multiples, and all these exons start with an intact codon for glycine. This organization is conserved independent of the species or type of fibrillar collagen.[8]

However, the organization of other collagen genes varies. For example, exons for the basement membrane (type IV) collagen gene do not conform to the 54-bp

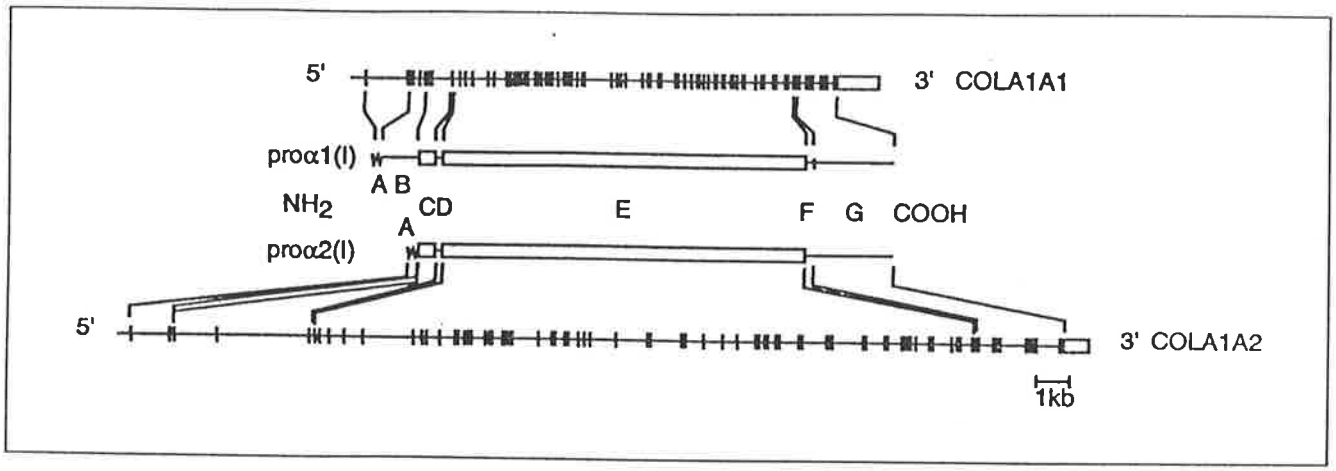


Fig 6-2 Structure of fibrillar collagen genes. The intron-exon organization of the prototype fibrillar collagen genes that encode for pro-α1(I) and pro-α2(I) are shown. The exons are designated by solid boxes or vertical lines. Exons 7 to 48 encode the triple-helical domain, and these start with a glycine codon and end with the codon for the "Y" amino acid. (A) to (G) designate various polypeptide domains in the molecule: (A) signal peptide; (B) N-terminal globular domain; (C) triple-helical domain of N-propeptide; (D) N-terminal telopeptide; (E) triple helix; (F) C-terminal telopeptide; (G) C-terminal propeptide.

rule; they are multiples of 9 bp, and frequently these exons start with split codon for glycine. In type IX collagen, the sizes of exons range in size from 21 to 400 bp depending upon their location. Type VI collagen gene is composed of 34 exons. Exons for these collagens are also multiples of 9 bp. The type X collagen gene is unique because its triple-helical domain consists of a single exon of 2,136 bp.[7]

The gene structure for type I collagen is schematically shown in Fig 6-2.

Translation and posttranslational events. Collagen mRNAs are transcribed as precursors and undergo usual processing reactions of intron removal by splicing, capping, and polyadenylation. These are nuclear events; the product is then translocated to the cytoplasm where collagen mRNA binds to ribosomes and is translated. The translated product is one and one half times as long as α chains and has approximately 1,500 amino acids. The additional sequences are present at both N- and C-terminal ends. Signal sequences are cleaved from these "prepro-α" chains during chain elongation as they are transported through the membrane into the lumen of the rough endoplasmic reticulum (RER). As the pro-α (prepro-α minus signal peptides) chains are being translated, certain prolyl and lysyl residues at "Y" position are oxidized to hyp and hyl, respectively, by enzymes prolyl hydroxylase and lysyl hydroxylase. The hydroxylation occurs predominantly at C-4 position of proline by prolyl-4-hydroxylase; however, it also occurs at C-3. The latter reaction is catalyzed by a separate enzyme, prolyl-3-hydroxylase.

Hydroxylation of both prolyl and lysyl residues requires molecular O_2, Fe^{++}, α-ketoglutarate, and ascorbic acid as cofactors. Only nascent pro-α chains, not triple-helical molecules, are substrates for the hydroxylation reaction, and the minimum sequence requirement for prolyl hydroxylation is -X-pro-gly. Not all prolyl and lysyl residues are hydroxylated, and the extent of hydroxylation depends upon availability of substrate, tissue, and other factors. Fully hydroxylated triple helix has a T_m (melting temperature, temperature at which the triple helix is denatured to individual α chains) of 39°C, while unhydroxylated molecules melt at 25°C;[9] therefore, prolyl hydroxylation is an essential step in collagen biosynthesis because underhydroxylation results in denaturation and subsequent degradation of the collagen molecule. The role of 3-hyp is unknown, however. Prolyl-4-hydroxylase is a tetramer consisting of two α and β subunits each, which have molecular size of 64 kDa and 60 kDa, respectively. Lysyl hydroxylase is a homodimer of 85-kDa subunits. The relative rates of hydroxylation vary with collagen types; for example, type IV collagen has a higher degree of 3-prolyl hydroxylation and up to 90% lysyl hydroxylation relative to types I and III.

The pro-α chains are also glycosylated during translation at hyl and asparagine residues. Glycosylation of peptidyl hyl occurs on oxygen at C-5 and is carried out by enzymes hydroxylysylgalactosyl transferase and gal-

actosylhydroxylysylglucosyl transferase. These enzymes transfer uridine-5′-pyrophosphate (UDP)-galactose and UDP-glucose, respectively. This reaction, like hydroxylation, occurs on non–triple-helical structures. Glycosylation occurs in the RER-lumen and continues in the Golgi.

As soon as synthesis of pro-α chains is completed, globular domains at their C-terminal end fold and are stabilized by intrachain disulfide bonds of cysteines. Then, two pro-α1 and one pro-α2 chains in type I, and three pro-α1 chains in type II and III collagens, associate at the C-terminal ends aided by interchain disulfide bonds. Triple-helix formation is thus initiated and proceeds to the N-terminal end.

The assembled *procollagen* molecule is then translocated to the Golgi where additional glycosylation, sulfation, and phosphorylation occur. Sulfation occurs in some collagens, notably in type V, at tyrosyl residues of amino terminal propeptide, and phosphorylation occurs at serine. Completed molecules are packaged into vesicles, which fuse with the cell membrane and release their content into extracellular space.

Extracellular events. Three key events occur in the extracellular space that convert procollagen into functional molecules. First, procollagen is converted to collagen. This is done by removal of propeptide sequences at the N- and C-terminal ends. The enzymes responsible are N- and C-procollagen peptidases. The N-propeptidase cleaves pro-gln bond in pro-α1(I) and pro-α1-(III) chains and ala-gln in pro-α2(I). This enzyme requires a triple-helical substrate and removes pro-N-domains *en masse.* At the carboxy end, cleavage occurs at ala-asp bond in both pro-α1(I) and pro-α2(I) chains. While removal of propeptides is necessary for type I, II, and III collagens, other collagens may not be processed similarly and some not at all. The "collagen" molecules formed then aggregate spontaneously into ordered fibrils, dictated by the charged and hydrophobic regions on the molecule. Aggregation occurs in an ordered parallel, overlapping lateral array such that adjacent molecules are staggered by approximately ¼ of the length of the molecule (670 Å) (see Fig 6-1). Although the aggregation is nonenzymatic, the rate of fiber formation and diameter of the fibrils appear to be regulated by type V and III collagens and by other macromolecules (decorin proteoglycan, for example). Removal of propeptide extensions is necessary for ordered fibril formation, and their retention, especially the N-terminus, results in poor fibril organization in an inherited disease, dermatosparaxis, in cattle, sheep, and cats. Absence of this step is believed to be the reason type IV collagen does not form fibrils in basement membranes.

The collagen fibrillar array is then stabilized by crosslinking, which is initiated by the enzyme *lysyl oxidase.* This enzyme requires Cu^{++} and pyridoxal phosphate as cofactors.[10,11] Lysyl oxidase oxidatively deaminates ε-amino groups to aldehydes converting lysyl and hydroxylysyl residues to allysine and hydroxyallysine, respectively. In collagen types I, II, and III, four crosslinking loci exist, one lys at the N-terminal telopeptide, two hyl in the triple helix, and one hyl at C-terminal telopeptide. Aldehyde residues in allysines and hydroxyallysines condense spontaneously with each other or with unmodified lys or hyl residues in adjacent α chains, forming divalent crosslinks norleucine, hydroxynorleucine, and aldol condensation products. More complex crosslinks, merodesmosines and hydroxypyridiniums, are formed as adducts with additional residues[12] (Fig 6-3).

The reactions involved in collagen biosynthesis are summarized in Fig 6-4.

Regulation of collagen biosynthesis. Collagen synthesis is tightly regulated during normal development and homeostasis in a cell- and tissue-specific manner. Regulation is effected by several mediators. These substances affect collagen gene transcription either directly or through other factors capable of interacting with gene sequences. Growth factors and cytokines are key regulators of collagen production during inflammation and wound repair,[6,13] and aberrations in the regulation lead to connective-tissue alterations during pathological conditions.

Regulation of collagen synthesis can occur at the level of gene transcription or posttranslational modification. The most significant point of regulation is that of gene transcription, and changes in its rate are reflected in mRNA levels. The latter is also affected by the stability of mRNA. Posttranslationally, collagen production may be affected by the extent of prolyl hydroxylation because underhydroxylation results in decreased stability of the collagen molecule, which is degraded. The prolyl hydroxylation is impaired in scurvy due to deficiency of vitamin C, which is a cofactor for prolyl hydroxylase. Because the N-propeptide, released by pro-N-peptidase action, inhibits protein translation, it may also inhibit collagen systhesis[13]; however whether this inhibition occurs physiologically or not is not clear. The biosynthetic steps at which regulation of collagen biosynthesis may occur are indicated in Fig 6-4.

The collagen genes, like other protein genes, contain promoters and enhancers of transcription. The first intron of COL1A2 contains short promoter and enhancer sequences, which act in concert, and at least three sequence domains bind to transacting factors (these are

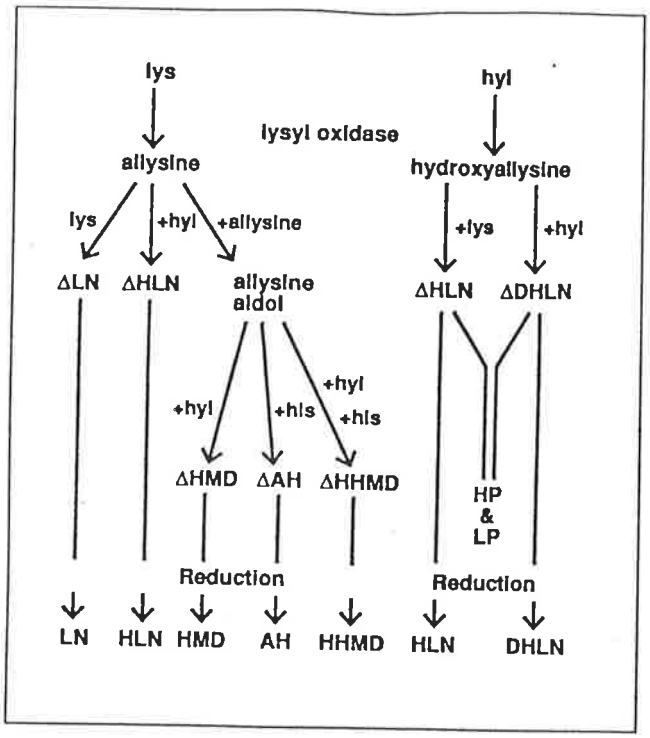


Fig 6-3 A scheme of lysine-derived crosslink formation in collagens. Only the lysyl oxidase step is enzymatic, and all other reactions occur by spontaneous condensation dictated by the type of neighboring amino acid (lys or hyl), access, and age. (LN) lysinonorleucine; (HLN) hydroxylysinonorleucine; (DHLN) dihydroxylysinonorleucine; (HMD) hydroxymerodesmosine; (AH) aldolhistidine; (HHMD) histidinohydroxymerodesmosine; (HP) hydroxylysyl pyridinoline; (LP) lysyl pyridinoline. The prefix "Δ" indicates dehydro forms. For chemical formulas and more details, see Eyre et al.[12] Elastin crosslinks also form in similar reactions, except that this protein does not have hydroxylysine-derived crosslinks.

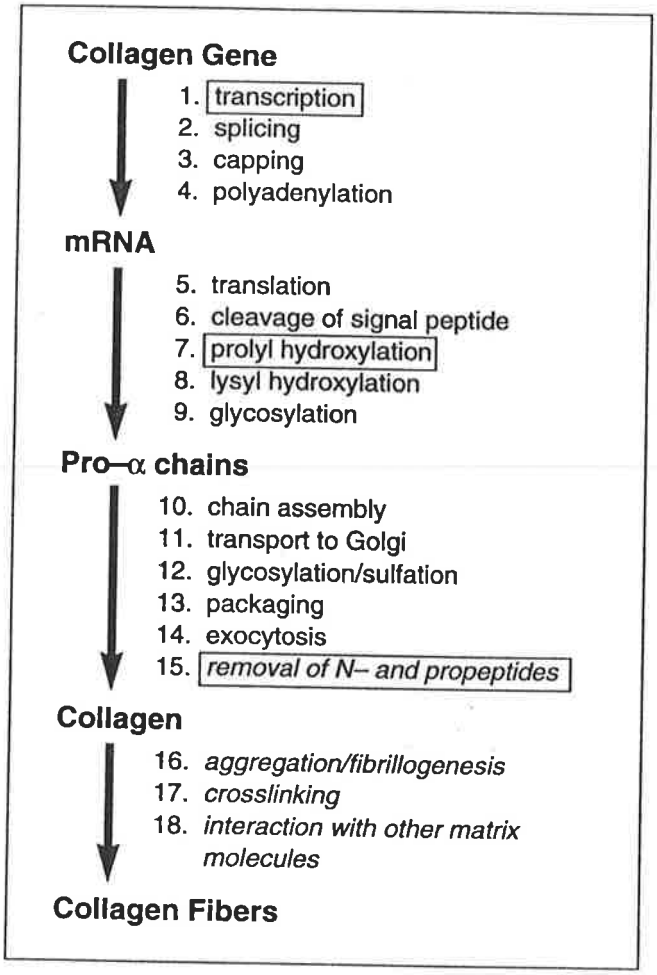


Fig 6-4 Biosynthesis of collagen. Reactions occurring in the nucleus, cytoplasm, and extracellular space are shown in normal characters, bold and italics, respectively, and boxed reactions are those associated with regulation of synthesis. Most common regulation is at the gene transcription. Prolyl hydroxylation determines the stability of the collagen molecule. N-propeptide can inhibit translation, causing a feedback inhibition of collagen biosynthesis. These reactions have been characterized for fibrillar collagens. Although this general scheme is common to all collagens, variations occur, especially in the removal of propeptide.

proteins that interact with specific DNA sequences). In the mouse COL1A2, these sequences are located between -84 and -80 (CCAAT Motif), -250 and -247 (CAGA), and between -315 and -295 (GCCAAG) relative to the transcription initiation site.[14,15] The last sequence represents a binding site for the nuclear factor NF1, which is an intracellular mediator for DNA replication and a transcription factor. Transforming growth factor-β (TGF-β) activates collagen gene transcription through this sequence.[16] In the human COL1A1 gene, the first intron contains a negative regulatory-sequence domain. This is located at +820 to +1093 and contains a binding motif for the transcription factor SP1 (GCCCCGCCCC) and a viral core enhancer sequence (GTGGTTAGC).[13,17]

The manner in which collagen genes are regulated differs for different collagen types. For example, in type IV collagen, genes for COL4A1 and COL4A2 are arranged head to head, separated by a 130-bp segment. At the center of the intervening region is a binding site for SP1. The 130-bp sequence interacts with enhancer and negative regulatory elements located in COL4A1 and COL4A2 genes, respectively, and regulates the expression of both α1(IV) and α2(IV) genes.[18]

Developmental expression of collagens is regulated in temporal, tissue, and cell type-specific manner. For example, interruption of the first intron of the COL1A1 gene in the mouse germ line by integration of the MoMLV viral gene results in transcriptional inactivation of the COL1A1 gene and, as a result, type I collagen is not made. The embryos die between 12 and 14 days. However, tooth rudiments synthesize normal amounts of type I collagen, indicating that in odontoblasts, unlike fibroblasts, this collagen is regulated by a site different from intron 1.[19] Thus, collagen synthesis appears to be regulated differently in skin and teeth. In type IX collagen, tissue-specific expression appears to be achieved by utilizing different transcription-initiation sites, which are located 20 kb apart. In cartilage, COL9A1 transcription starts in exon 1. In cornea, however, it starts downstream at the 3′ end of intron 6; after splicing, in corneal COL9A1, exons up to 7 are skipped. As a result, the corneal α1(IX) chain is shorter than cartilage α1(IX).[20]

A variety of growth factors and cytokines regulates collagen production during development, inflammation, and wound repair[6,13] and, in virtually all cases, this is reflected in mRNA levels. The collagen mRNA levels may also increase or decrease due to a change in either the rate of collagen gene transcription or in mRNA stability. Among the various mediators that affect collagen synthesis, TGF-β is an important mediator as it enhances the synthesis of collagen and other matrix components. This polypeptide is believed to play a major role in wound repair and fibrosis.[18] In contrast, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) suppress collagen gene expression.[11,22,23] During inflammation and wound healing, these substances are secreted by platelets, macrophages, and other inflammatory cells. The action of some of these mediators on collagen synthesis is summarized in Table 6-2.

Table 6-2 *Some Mediators Affecting Collagen Synthesis*

Mediator	Effect
TGF-β*	↑
PDGF	↑
IFN-γ	↓
IL-1α,β†	↑
TNF-α	↓
PGE_2	↓
Glucocorticoids‡	↓
Parathyroid hormone	↓
Vitamin D	↓

* Also decreases MMP synthesis and increases TIMP and proteoglycan production.
† Enhances matrix degradation by stimulating MMP synthesis.
‡ Increases elastin and fibronectin synthesis.

Inflammatory mediators may also regulate matrix composition through their effect of matrix degrading enzymes.

Degradation and remodeling of collagens. Collagen undergoes degradation and remodeling during development, inflammation, and wound repair, and during resorption in bone. Collagen degradation requires special enzymes because the crosslinked molecules in fibers are resistant to most common proteinases. These enzymes, called "collagenases," initiate collagen degradation by cleaving gly-ileu and gly-leu bonds in α1(I) and α2(I), respectively. These peptide bonds are located approximately one quarter of the length from the C-terminus, thus fragments of ¾ and ¼ size are released. The released fragments have a lower T_m than that of the native molecule, and thus become denatured and further degraded by other common tissue proteinases. Alternatively, collagens may be ingested by phagocytosis by macrophages and fibroblasts and hydrolyzed by lysosomal enzymes.

The enzymes responsible for degradation of collagen and other matrix molecules are a family of nine or more metal-dependant enzymes called matrix metallo-

Table 6-3 *Matrix Metalloproteinases*

Enzyme	MMP*	Substrates
Interstitial collagenases		
Fibroblast type	MMP-1	Collagen types I to III, VII, VIII
PMN type	MMP-8	Same as MMP-1
Gelatinases/type IV collagenases		
72 kDa	MMP-2	Gelatin, collagen types IV to VII, X, XI, elastin
92 kDa	MMP-9	Same as MMP-2
Stromelysins		
Stromelysin 1	MMP-3	Proteoglycan core proteins, laminin, collagen types IV, V, IX, X
Stromelysin 2	MMP-10	Same as MMP-3
Stromelysin 3	MMP-11	Fibronectin, gelatins, types II, III, V collagens
Other enzymes		
Putative metalloproteinase (PUMP-1)	MMP-7	Fibronectin, laminin, type IV collagen, gelatin
Telopeptidase	MMP-4	Collagen C-propeptide

* Classification based on Nagase et al.[24] Also see Birkedal–Hansen et al.[26]

proteinases (MMPs). The MMPs have a highly conserved and related gene structure and broad specificity.[24,25] These enzymes are divided into four groups. The first group, interstitial collagenases, includes fibroblast- (MMP-1) and PMN-type (MMP-8) collagenases. Collagen types I, II, III, VII, VIII, and X and gelatin are substrates for these enzymes. The fibroblast enzyme hydrolyzes type III fibers faster than type I, while the PMN enzyme hydrolyzes type I faster. The second group includes 72-kDa (MMP-2) and 92-kDa (MMP-9) gelatinases (also called type IV collagenases). These enzymes have a high affinity for gelatin, and degrade gelatin, collagen types IV, V, VII, X, and XI, and elastin. They cleave gly-X-peptide bond where X = val, leu, glu, asn, or ser. Stromelysins 1 and 2 (MMP-3 and 10, respectively), form the third group, and these enzymes hydrolyze core protein of proteoglycans, type IV, V, IX, and X collagens and elastin. The last group includes other enzymes such as putative metalloproteinase 1 (PUMP-1, MMP-7) (Table 6-3). Fibroblasts, keratinocytes, monocytes/macrophages, and several other cells produce MMP, although MMP-2 has not been detected in PMN. The fibroblast and keratinocyte enzymes are regulated transcriptionally and synthesized in response to external stimuli, therefore there is a time lag between exposure to an agent and release of active enzyme. Many inflammatory mediators and bacterial substances influence the MMP production by these cells.[25] In contrast, the PMN enzyme is released readily on demand as it is stored in storage granules.

MMP activity is controlled in vivo in three ways.[25] First, the MMPs are synthesized and secreted as inactive precursors and conversion to active form requires activation by plasmin, trypsin, or other proteinases. Second, production of MMP is regulated by several growth factors and cytokines. Interleukin-1 (IL-1) and TGF-β are key regulators of MMP production in inflamed tissues. The IL-1 increases and TGF-β decreases MMP synthesis. Finally, activity of MMPs is neutralized by serum and tissue inhibitors. A major serum inhibitor is α2-macroglobulin, which covalently crosslinks with susceptible proteolytic enzymes and inactivates them. The α2-macroglobulin is a potent inhibitor because it binds to MMP-1 with even greater avidity than its substrate, collagen.[26] Tissues contain another group of protein inhibitors to MMP. Two such inhibitors, tissue inhibitor of metalloproteinases 1 and 2 (TIMP-1 and TIMP-2), have been characterized. These inhibitors act by inactivating active-MMPs and by preventing their conversion to active forms from precursors. The TIMP-1 and TIMP-2 are more effective towards interstitial collagenases and gelatinases, respectively.[27] The TIMPs are distributed widely in many tissues and fluids.

Unlike vertebrate collagenases, collagenases from bacteria can degrade native collagen molecules to small peptides.

Diseases associated with collagen alterations. Alterations either in the molecular structure or composition of collagens lead to functional abnormality of

connective tissues. Collagen molecular structure is affected by mutations in both the collagen gene and genes of posttranslational processing enzymes. Such mutations could arise by nucleotide substitution, deletion, or insertion. The severity of diseases caused by collagen molecular defects will depend on several factors. Mutations that affect the structure of pro-α1(I) genes are often lethal, whereas those affecting pro-α2 chains are not; this is because when α2(I) cannot be produced due to a defect in its primary structure, homotrimers of α1(I) can form and these are stable. However, homotrimers of α2(I) chains are not stable and they cannot assemble into functional trimers without α1(I). Therefore, large deletions, insertions, and mutations near C-terminus of pro-α1(I), which affect the assembly of pro-α chains, are lethal. For example, in osteogenesis imperfecta-II (OI-II), α1[I] $glycine_{988}$ is converted to cysteine; the mutated molecules cannot form trimers, therefore it is lethal.[4] The OI-II is often associated with dentinogenesis imperfecta because dentin contains type I collagen as a major component. In contrast to those of collagen genes, mutations of processing enzymes are usually not lethal, even though they could result in functional abnormalities of constituent tissues. Thus, defective lysyl oxidase and lysyl hydroxylation lead to loss of tensile strength of connective tissues and hypermobility of joints, respectively, in cutis laxa and Ehlers-Danlos syndrome.[4]

While diseases due to collagen molecular defects are inherited and rare, acquired diseases are much more common. The acquired diseases include chronic inflammatory diseases and fibroses. Many of these, especially systemic diseases affecting connective tissues, have manifestations in the periodontium (Crohn's disease and progressive systemic sclerosis, for example). Connective-tissue alterations in acquired diseases differ from inherited diseases in several respects. For instance, in acquired diseases gene expression is affected, not gene structure. The underlying cause of these diseases is often unknown and it is multifactorial, with changes occurring in virtually all matrix components, including proteoglycans.[6] Proportions of collagen types may be affected in these diseases; for example, in atherosclerosis, type V collagen is enriched, and in periodontal disease type I and III collagens decrease, and type V increases (see pages 81 and 82).

Connective-tissue alterations in acquired diseases are brought about by the interaction of connective-tissue cells, chiefly fibroblasts, with numerous inflammatory mediators and cytokines present at the site of injury. These substances are released from damaged tissue and inflammatory cells.[6,28] Important among these are platelet-derived growth factor (PDGF) and TGF-β, which enhance cell growth and matrix synthesis respectively, and IFN-γ, TNF-α, and PGE_2, which suppress collagen synthesis.[6,13] These substances may affect the synthesis activities of all resident cells. Alternatively, they may interact with a subpopulation of resident cells and selectively enrich this subpopulation. Presence of such cells is believed to be one reason for the alterations of matrix constituents during wound repair in periodontal diseases and in scleroderma.[6] These mechanisms are discussed in the section of this chapter on periodontal connective tissue, under "Repair and regeneration."

Noncollagenous proteins. The extracellular matrices of the periodontium and other connective tissues contain elastin, fibronectin, laminin, tenascin, thrombospondin, entactin, and other noncollagenous proteins. Quantitatively the noncollagenous proteins are minor constituents relative to collagens, nevertheless they play a significant role in connective-tissue integrity and function. Many of these proteins share several common features. For example, they are large molecules composed of multiple functional domains with distinct binding properties; they influence a variety of cellular activities, and they exist in multiple forms.

Elastin. Elastin is a unique, rubber-like protein that is present, in vertebrates, in virtually every organ of the body. It is a major component of large arteries, vocal cords, elastic cartilage, and lungs, and in nuchal ligaments in cows. Ultrastructurally, elastin fibers are composed of two morphological components—an amorphous elastin component constituting 90% of the mature fiber, and a 10- to 12-nm diameter microfibrillar component. The latter is located around the periphery of the amorphous component.[29]

Elastin is the most insoluble protein known. Glycine comprises approximately 33% of the amino acid in elastin, but is not found regularly (as every third amino acid) as in collagen. Elastin molecules are organized in such a manner that crosslink regions alternate between hydrophobic regions. The pentapeptide val-pro-gly-val-gly and hexapeptide pro-gly-val-gly-val-ala repeat several times in the hydrophobic regions. In the crosslink regions lysine residues occur in sequences lys-ala-ala-ala-lys and lys-ala-ala-lys.[29]

Like collagen, elastin is first synthesized as an uncrosslinked precursor, tropoelastin. The elastin gene is ~40-kb long and contains 34 to 36 exons.[30] While organization of the exons may differ, there is considerable homology between human, porcine, bovine, and chicken genes. The tropoelastin mRNA is 3.5-kb long,

and the overall size of tropoelastin is approximately 750 amino acids.

Elastin crosslinking is mediated by the enzyme lysyl oxidase, the same enzyme that acts on collagens.[10,11] The enzyme prefers the insoluble form of substrates and it oxidatively deaminates lysines to allysines[31]; the latter spontaneously condenses to crosslinks.[12] Elastin does not have hydroxylysine- and histidine-derived crosslinks, but contains other crosslinks found in collagens. In addition, it has two unique crosslinks derived from four lysines each, desmosine and isodesmosine. The desmosines connect two peptide chains each. The synthesis of elastin is regulated primarily at the transcriptional level.

Fibronectin. Fibronectin is a multifunctional adhesive glycoprotein that is co-distributed with type I and III collagens in fibers. It is present in the extracellular matrix and various body fluids. It binds to fibroblasts and many other cell types and mediates their attachment, spreading, and migration. It also binds to collagens, heparin, fibrin, DNA, and bacteria. These properties allow fibronectin to participate in many biological processes during growth, development, and repair. Fibronectin plays a prominent role in phagocytosis, hemostasis, thrombosis, and oncogenic transformation.[32]

Fibronectin is a large 540-kDa dimer of two similar 230- to 270-kDa polypeptide subunits, which are connected by disulfide bonds at the C-terminus. Two forms of fibronectins, plasma (pFN) and cellular (cFN), have been characterized. Both are heterogenous mixtures of molecules, cFN assembled from eight different subunits, and pFN from four. While pFN is a major blood protein synthesized by hepatocytes, cFN is produced by many cell types and incorporated into extracellular matrices.[32]

The fibronectin gene and protein structures are highly conserved among species. The molecule is made up of three internally homologous repeats known as type I, II, and III, which are assembled into globular domains with distinct biological activities. The fibronectin gene ranges between 48 and 70 kb in size in different species and generates an 8-kb mRNA.[33] The gene contains 48 exons of similar size. The protein contains 12 type I repeats of approximately 45 amino acids coded by 12 exons. These repeats make up the fibrin-binding regions present at both N- and C-termini of the fibronectin molecule, and two repeats code for part of the gelatin/collagen-binding domain. These repeats contain four cysteines each. However, no intrachain disulfide-bonds are present in the type III repeats. There are 15–17 type III repeats, each with approximately 90 amino acids and coded by two exons. This domain contains binding sites for cells, heparin, and DNA and is highly conserved.

Fibronectin subunit variation is due to alternative mRNA splicing. Three variants in rat and five in human fibronectin arise due to a novel pattern of alternative splicing from a single exon in the "V" segment. This segment, which contains two splice sites, resides within the type III domain. This region is structurally different from other domains in amino acid distribution, and contains glycosylation sites and an arg-gly-asp (RGD in one-letter amino acid code) recognition sequence for cell attachment. Subunits also arise by alternative splicing at another type III repeat site designated as EIIIa and EIIIb; in this case an extra domain is either excluded or included in the fibronectin molecule. The inclusion occurs only in cFN.[33]

Vitronectin. Vitronectin is a glycoprotein found in both serum and the extracellular matrix.[34,35] It is present on elastin fibers and in the matrix of loose connective tissues of several organs in a fibrillar pattern. However, it is not considered an integral matrix constituent as it is a serum protein produced in the liver. Vitronectin is a 70- to 78-kDa protein and has multiple functional domains. It attaches cell surfaces at focal adhesion sites and sites of intracellular cytoskeletal elements, indicating a mode of transmembrane signalling to the cellular cytoskeleton.[36,37] The protein has a heparin binding site, but this is a cryptic site, hidden within a fold of the protein. Vitronectin facilitates the binding of bacteria to host cells and phagocytosis, and inhibits complement-mediated cell lysis. It also binds plasminogen activator-inhibitor, and may be involved in the regulation of this protease.[34,35]

Thrombospondins. Thrombospondins are a family of large multidomain glycoproteins produced in platelets and by many cell types. The thrombospondin molecule participates in platelet aggregation and it affects the migration, adhesion, and growth of many cells, especially the PMN and macrophages.[38,39] It regulates angiogenesis and is expressed during wound healing. It interacts with several extracellular matrix components and cell surfaces. Four different thrombospondin genes have been distinguished and described.

Tenascin. Tenascin (also called cytotactin and hexabrachion) is a glycoprotein that consists of six disulfide-linked subunits assembled into a "star-shaped" complex.[40] It has EGF- and fibronectin-type-III–like sequences and binds to cells and matrix constituents. During development, tenascin is expressed selectively at mesenchyme condensations at sites of mesenchymal-

Table 6-4 *Integrin Receptors that Bind to Matrix Proteins and Serum Proteins*

Class	Integrin	Binds to
β_1	$\beta_1\alpha_1$	Type I and IV collagens, laminin
	$\beta_1\alpha_2$	Same as above
	$\beta_1\alpha_3$	Type I–IV, VI collagens, laminin
	$\beta_1\alpha_4$	Fibronectin
	$\beta_1\alpha_5$	Fibronectin
	$\beta_1\alpha_6$	Laminin
	$\beta_1\alpha_v$	Vitronectin, fibronectin
β_2	$\beta_2\alpha_2$	ICAM-1, ICAM-2
	$\beta_2\alpha_M$	C3bi, factor X, fibrinogen
	$\beta_2\alpha_X$	P130/95 glycoprotein
β_3	$\beta_3\alpha_v$	Vitronectin, fibrinogen, von Willebrand factor, osteopontin, BSP-II
	$\beta_3\alpha_{IIb}$	Fibrinogen, vitronectin, fibronectin, von Willebrand factor
β_4	$\beta_4\alpha_6$	Basement membrane
β_5	$\beta_5\alpha_v$	Vitronectin

epithelial interaction, and its presence almost always correlates with morphogenetic events.[41] The distribution of tenascin becomes restricted in adult tissues, but it is actively expressed during wound healing and tumorigenesis.[42] This molecule can promote as well as inhibit cell adhesion. It interacts specifically with some proteoglycans and fibronectin and is believed to influence cell-fibronectin interactions.[40,43,44] A strong relationship between tenascin expression and epithelial-mesenchymal interactions has been noted during tooth morphogenesis.[45]

Laminin. Laminin is a large 900-kDa glycoprotein that is present only in basement membranes. It is composed of one heavy (400 kDa) and two light (200 kDa) chains. The laminin molecule has a crosslike structure with three short arms and one long arm, which are formed by N- and C-termini of subunits, respectively. Five laminin subunits have been described; these include two heavy chains, A and merosin M, and three light chains, B1, B2, and S. Unlike fibronectin, which results from alternative splicing to form multiple variants, laminin variants appear to be the result of assembly of different gene products to form a single heterogeneous family of molecules. Laminin mediates many biological functions associated with basement membranes, including cell attachment, migration, and differentiation. It is expressed early in embryogenesis, indicating its importance in development. Like fibronectin, laminin interacts with other matrix components and cell surface molecules. The laminin molecule has different domains with distinct functions, and these domains bind to cells, heparin, collagen, entactin, and growth factors.[46,47]

Entactin. Entactin (nidogen) is a glycoprotein of approximately 150 kDa. It is ubiquitously expressed in basement membranes, where it is present as a noncovalent complex with laminin. Structurally, entactin has been described as having a "dumb-bell" appearance with two globular domains separated by a linear, cysteine-rich domain.[48] In addition to binding with laminin, entactin can interact with cell surfaces via RGD sequences near its carboxy terminus.[49] Entactin, alone and as a complex with laminin, binds to type IV collagen within basement membranes, and contributes to the "sieve-like network" in these membranes.[50]

Cell-surface/matrix interactions and integrins. Within the primary structure of matrix proteins, the arg-gly-asp (RGD) amino acid sequence appears to be responsible for many attachment interactions.[51] Attachment to the RGD recognition sequence is mediated by specific cell-surface receptors called *integrins.* This term was first proposed in 1986 to describe those molecules on cell surfaces intimately involved in linking the extracellular matrix with the cytoskeleton. Integrins are heterodimers of α and β subunits and are classified on the basis of their β-subunit composition[52] (Table 6-4). Each chain is composed of three domains: a large extracellular domain, a membrane-spanning domain, and a short

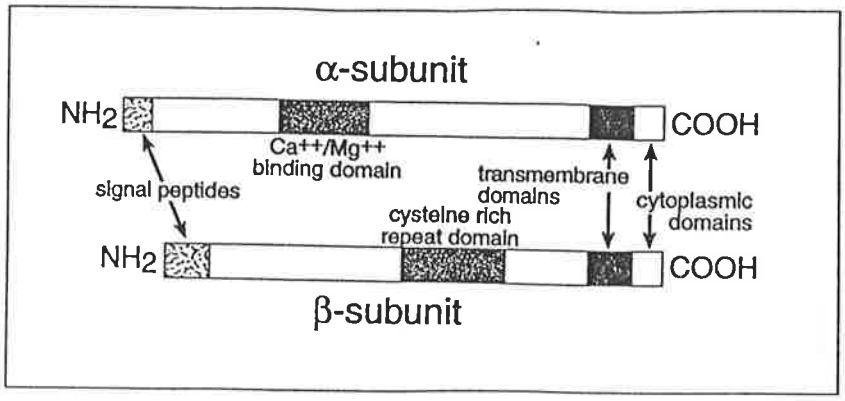


Fig 6-5 Structure of integrin α and β subunits showing the distribution of key sequence domains.

cytoplasmic domain. The amino acid sequence of the extracellular domain of α subunits contains Ca^{++} (or Mg^{++}) binding sites that are homologous to similar sites in calmodulin (Fig 6-5). To date at least 11 α subunits and 6 β subunits have been described. Binding of integrins to matrix proteins is specific and dictated by the α- and β-subunit composition (see Table 6-4), and depends on divalent cations. The binding induces a series of signalling events such as activation of protein kinases, which are believed to mediate cell migration, attachment, growth, and other functions. The integrins provide a valuable link between the extracellular matrix and the cytoskeleton, and are implicated in white blood-cell diapedesis, leukocyte migration, T-cell–macrophage interactions, clot formation, epithelial cell migration, and fibroblast migration. Integrins are expressed actively during wound healing, angiogenesis, and tumorigenesis.[53,54]

While the presence of the RGD sequence is necessary for many cell/matrix interactions, hundreds of molecules that contain RGD sequences play no significant role in cell adhesion. RGD-containing domains have also been identified in bone-matrix proteins, osteopontin, and bone sialoprotein-II (BSP-II), which presumably function in regulating osteoblast adhesion. These proteins are discussed in the section on bone.

Proteoglycans. Proteoglycans are highly anionic complexes in which one or more hexosamine-containing polysaccharides called glycosaminoglycans (GAGs) are covalently attached to a protein core.[55] They are ubiquitous to all connective tissues, and are located within the matrix as integral components, on cell surfaces and within cell organelles.[56] By virtue of their high charge, they have been ascribed a variety of functions, including tissue hydration, retention and regulation of water flow, lubrication of synovial and mesothelial surfaces, regulation of collagen-fiber formation, growth-factor binding, and cell adhesion and growth.[57-60] These macromolecules were first described in the late 1950s, and later called *mucopolysaccharides*[61,62]; the term *proteoglycan* was introduced in 1967.[63]

Glycosaminoglycans. The GAGs are composed of repeating, unbranched disaccharide units of uronic acid (either D-glucuronic acid or L-iduronic acid), and D-galactose or a hexosamine (either D-glucosamine or D-galactosamine) (Table 6-5). The GAG types include hyaluronic acid, chondroitin sulfates, dermatan sulfate, keratan sulfate, heparan sulfate, and heparin.[64] Hyaluronic acid, which is the only nonsulfated GAG, is a large molecule varying in size from 10^5 daltons to 10^6 daltons, and it consists of N-acetylglucosamine and D-glucuronic acid. Chondroitin sulfate consists of D-glucuronic acid and N-aceteylgalactosamine, it is sulfated at C-4 or C-6, and it is found in most tissues, especially cartilage. Dermatan sulfate is made up of D-glucuronic acid, L-iduronic acid, and N-acetylgalactosamine, and it is a component of fibrous tissues including gingiva and periodontal ligament. Heparan sulfate is a component of basement membranes and epithelium. It is composed of D-glucuronic acid, L-iduronic acid, and N-acetylglucosamine, and it is N- and O-sulfated. Heparin has a structure similar to heparan sulfate and is present in mast cells. Keratan sulfate is the only GAG in which D-galactose replaces uronic acid in the repeating disaccharide unit. It has a limited distribution, being present in cornea and skeletal tissues.

Table 6-5 *Disaccharide Composition of Glycosaminoglycans*

Glycosaminoglycan	Disaccharide subunit	Sulfate*
Hyaluronic acid	D-glucuronic acid, D-glucosamine	None
Chondroitin 4– and 6–sulfate	D-glucuronic acid, D-galactosamine	O-
Dermatan sulfate	D-glucuronic acid/L-iduronic acid, D-galactosamine	O-
Heparan sulfate	D-glucuronic acid/L-iduronic acid, D-glucosamine	N-, O-
Heparin	D-glucuronic acid/L-iduronic acid, D-glucosamine	N-, O-
Keratan sulfate	D-galactose, D-glucosamine	O-

* O-, O-sulfated through sugar; N-, N-sulfated through asparagine.

Core proteins. The exceptional diversity of proteoglycans is due to the number and posttranslational modifications of the *core proteins*, which covalently bind to GAG units. These polypeptides range in size from 10 to 300 kDa[65] and are rich in amino acids serine, glycine, proline, and glutamic acid.[66,67] In early studies to characterize these proteins, conventional protein sequencing was difficult to perform due to their complex structure; however, recent advances in molecular biology have made it possible to deduce their amino acid sequences. Various hydrophobic, hydrophilic, and globular domains have been identified within the molecules and these correlate closely with the tissue location and function of proteoglycans.[67-70] However, they do not appear to form a single supergene family.

Glycopeptide linkages. The attachment of GAG chains to protein cores is relatively consistent among most of the proteoglycans. Attachment of a trisaccharide sequence xyl-gal-gal to serine is usually via an O-glycosidic link.[71] Such a linkage sequence has been identified for chondroitin sulfate-4 and -6, dermatan sulfate, and heparan sulfate. The linkage of keratan sulfate to core proteins appears to be unique. Corneal keratan sulfate links to its protein via a N-glycosidic bond between N-acetylglucosamine and asparagine,[72] while skeletal keratan sulfate binds through an O-glycosidic bond between N-acetylglucosamine and either serine or threonine.[73]

In addition to GAG chains, most proteoglycans contain variable proportions of N- or O-linked oligosaccharides.[74] In this case, the O-linked oligosaccharide linkage is between a glycoside bond of terminal N-acetylglucosamine and the hydroxyl group of serine or threonine. The N-linked oligosaccharides attach by N-glucosamine bonds to asparagine residues. The function of these oligosaccharides is not entirely clear.

Proteoglycan synthesis. Like all proteins, the core proteins of proteoglycans are synthesized in the RER. All of them have a hydrophobic signal sequence at the N-terminal, which is removed as the protein is being translated. The addition of sulfated GAGs to the core protein occurs in the Golgi and is initiated by xylosyltransferase; this enzyme adds xylose to hydroxyl groups in acceptor serines. For GAG chain elongation to occur, up to 6 glucosyltransferases and 2 sulfotransferases are required.[64] First xylose is added to the serine residue; this may occur either late in the RER or early in the Golgi. This is followed by sequential addition of galactose, glucuronic acid, and N-acetylglucosamine. Then the uronic acid and hexosamine are added sequentially to the nonreducing end of the growing chain. Sulfate esters are then added as the chain elongates. Additional modifications occur to dermatan sulfate, heparin, and heparan sulfate, either during or immediately after chain elongation.[75,76] The modifications include epimerization of D-glucuronic acid to L-iduronic acid, 2-O-sulfation of L-iduronic acid, and N-deacetylation and N-sulfation of heparin and heparan sulfate.

Proteoglycan types. Historically, the proteoglycans had been designated by their GAG content and size, and called by names such as "small dermatan sulfate proteoglycans" and "large aggregating chondroitin sulfate proteoglycans." However, in recent years the nomenclature is based on core proteins, amino acid sequence, and tissue location. Irrespective of the individual names, it is still convenient to classify proteoglycans

Table 6-6 *Composition and Distribution of Some Proteoglycans*

Proteoglycan	GAG*	Tissues	Interacts with
Extracellular proteoglycans			
Aggrecan	CS, KS	Cartilage	HA
Versican	CS	Soft connective tissues, fibroblasts, gingiva, periodontal ligament, cementum	HA
Perlecan	HS	Basement membranes	bFGF
Decorin	DS/CS	Soft connective tissues, bone, gingiva, periodontal ligament, cementum	Collagens types I, II, TGF-β, FN
Biglycan	DS/CS	Soft connective tissues, bone	Matrix components, not collagen, cell surface
Centoglycan	CS	Striated muscle, fibroblasts, bone	
Cell surface proteoglycans			
Syndecan-1	CS/HS	Epithelium	Various growth factors (bFGF, EGF)
Syndecan-2	HS	Fibroblasts	Various growth factors (bFGF, EGF)
Syndecan-3	HS	Schwann cells, cartilage	Various growth factors (bFGF, EGF)
Syndecan-4	HS	Fibroblasts, endothelial and epithelial cells	Various growth factors (bFGF, EGF)
CD-44	CS/HS	Lymphocyte homing factor	HA)
Betaglycan	HS/CS	Cell surfaces	TGF-β receptor
Proteoglycans in hemopoietic cells			
Serglycine	CS	Mast cell granules	Intracellular enzymes

* CS, chondroitin sulfate; KS, keratan sulfate; HA, hyaluronic acid; DS, dermatan sulfate; HS, heparan sulfate; FN, fibronectin; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor.

into three separate groups based on their location as: 1) extracellular proteoglycans, which are matrix organizers and tissue-space fillers; 2) cell-surface proteoglycans; and 3) intracellular proteoglycans of the hematopoietic cells (Table 6-6). The following is a brief overview of those proteoglycans that are relevant to periodontal tissues. A schematic representation of the structures of proteoglycans is provided in Fig 6-6.

Extracellular proteoglycans. These may be further subdivided into large and small species. Large proteoglycans include aggrecan, versican, and perlecan. Aggrecan is a large cartilage-specific proteoglycan bound to hyaluronic acid,[77,78] and it consists of approximately 100 chondroitin-sulfate chains and approximately 100 keratan-sulfate chains, together with numerous N- and O-linked oligosaccharides. Versican is the fibroblast equivalent of aggrecan, but it does not contain keratan sulfate. The protein core of this proteoglycan has a multidomain structure with EGF-like repeats, a lectin domain, a complement-regulatory domain, and a hyaluronate-binding region.[79] Perlecan is a basement-membrane proteoglycan containing heparan sulfate as the GAG. It has structural homology to neural cell adhesion molecule, "A" chain of laminin and other components, and it can aggregate with type IV collagen and laminin.[80]

Biglycan, decorin, and centoglycan are small extracellular proteoglycans. Biglycan is a small dermatan sulfate–containing proteoglycan.[81] It has two sites for GAG attachment and it is found in developing bone and cartilage. It is localized in close association with keratinocytes and fibroblasts, and it binds to some matrix molecules, but not to collagens. The production of biglycan is modulated by TGF-β1 and growth hormone. Decorin is similar to biglycan in structure except that it has only one attachment site for GAG chain[81]; this proteoglycan shows a close association with collagen fibers and localizes in the gap region of collagen fibrils. Centoglycan (PG-100) is synthesized by osteoblasts and fibroblasts, and it contains N- and O-linked oligosaccharides and a single chondroitin-6-sulfate chain.[82]

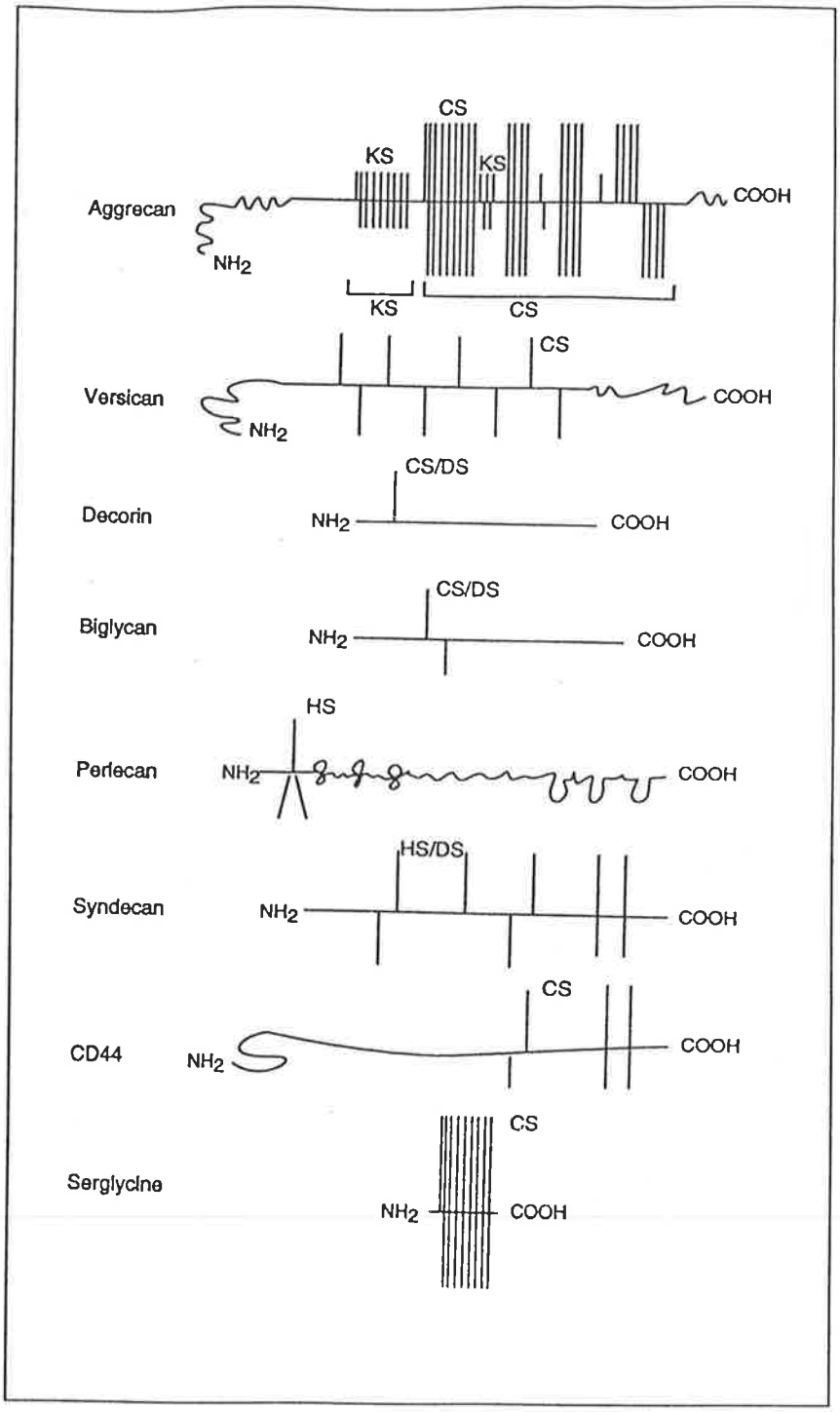


Fig 6-6 Composition of various proteoglycans. Each proteoglycan consists of a core protein (horizontal lines) covalently linked to GAG chains (vertical lines). (KS) keratan sulfate; (CS) chondroitin sulfates; (DS) dermatan sulfate; (HS) heparan sulfate.

Cell-surface proteoglycans. These proteoglycans contain heparan sulfate GAG. Cell-surface proteoglycans are ubiquitous cell-surface components of all mammalian cells,[83] and they are present on cell surfaces as integral components of membrane proteins spanning the lipid bilayer, by partial insertion into the lipid bilayer of a phosphatidyl inositol component of the proteoglycan, or by binding of a GAG side chain to specific plasma membrane receptors.[56,84]

Among cell-surface proteoglycans, the syndecans are the best studied. These molecules have a unique protein core composed of both hydrophobic and hydro-

philic domains and substituted with either heparan sulfate or chondroitin sulfate, or both.[85] To date four syndecans have been identified on the basis of their cDNA-derived amino acid sequence. Syndecan-1 is present in many cells including epithelial cells, lymphocytes, and embryonic dental and lung mesenchyme.[86] Syndecan-2 (fibroglycan) is like syndecan-1, and it can form large dimers or multimers.[87] Syndecan-3 (N-Syndecan) shares several structural features with syndecan-1 and fibroglycan, but differs in extracellular domain in both amino acid sequence and location of the GAG attachment sites.[88] It is expressed in high amounts during chondrogenesis and in neonatal rat brain, heart, and Schwann cells. Syndecan-3 has numerous potential sites for mediating cell/matrix/cytoskeleton interactions. Syndecan-4 (ryudocan/amphiglycan) is an important component of most endothelial cells, epithelial cells, and fibroblasts, and it has three putative GAG attachment sites.[89,90]

The syndecans have several functions. By virtue of their strategic location on the cell surface, they are believed to influence cell/cell, cell adhesion, and cell/matrix interactions.[91] The heparan-sulfate chains interact with growth factors, cytokines, extracellular matrix components, and protease inhibitors, and they even self-aggregate.[76] The syndecans are differentially expressed during development and wound healing.[92,93]

Other cell-surface proteoglycans include thrombomodulin, a transmembrane protein, which may be substituted with a single chondroitin-sulfate chain and has anticoagulation properties[94]; CD44, the lymphocyte homing factor, which is expressed on most cell types and may exist in a proteoglycan form containing either chondroitin sulfate, heparan sulfate, or both GAGs[95]; epican, a specialized form of CD44, containing both chondroitin-sulfate and heparan-sulfate chains and expressed on keratinocytes[96]; glypican, which is intercalated through the cell membrane via a glycosylphosphatidylinositol membrane anchor[97]; and betaglycan, which is a specific cell-surface proteoglycan that binds to TGF-β.[98]

Intracellular proteoglycans of hemopoietic cells. These proteoglycans are often found in secretory granules and are distinct from those residing in the matrix or cell surface.[99,100] Serglycine is the major proteoglycan of this type. It has a unique repeat sequence of serine and glycine residues in its protein core and is located in the secretory granules of mast cells, basophils, neutrophils, platelets, lymphocytes, and natural killer (NK) cells. It is resistant to protease attack and is released in response to specific stimuli. The functions of proteoglycans belonging to this group remain largely unknown, although it has been speculated that they may be involved in enzyme packaging or in the mediation of cellular activity.[99]

Hyaluronic acid. Also called *hyaluaronan* or *hyaluronate,* this acid has two distinguishing features: it is the only nonsulfated GAG, and it does not covalently associate with a protein to form proteoglycan.[101,102] Still, it is considered part of the proteoglycan family by virtue of its ability to interact with the core proteins of several proteoglycans to form large aggregates. Hyaluronic acid also differs from proteoglycans in that it is synthesized in the plasma membrane by the addition of sugars to the reducing end of the molecule, with the reducing end projecting into the pericellular environment.[103] Hyaluronic acid is ubiquitous to all tissues and is synthesized by most cells. Its functions are many and varied, being most importantly associated with tissue hydration, cell-surface matrix interactions, cell migration, tissue development, and aggregation with aggrecan, CD44, and other matrix components.[101,104]

Periodontal Connective Tissues

Like all other connective tissues, the extracellular matrix of periodontal structures is made up of collagens, noncollagenous proteins, and proteoglycans.[1,6] However, the proportion of various matrix constituents, especially collagen types, and their organization differs in each periodontal component. These differences determine the characteristic structure and function of each component and of the periodontium as a whole.

The integrity of the periodontium must be maintained during tooth eruption and mesial drift, and to combat occlusal forces and microbial challenge; this is achieved by the high turnover rate of connective-tissue constituents. Experiments using marmoset and rat models have shown that the periodontal ligament and gingiva have a high collagen turnover rate, much higher than the rate in skin and other connective tissues.[105,106] The primary cells responsible for synthesizing collagen and other matrix components are fibroblasts in soft tissues and osteoblasts in mineralized structures. Biosynthesis of collagens and proteoglycans by gingival and periodontal-ligament fibroblasts have been studied extensively, and these cells have served as a model to examine mechanisms of connective-tissue alterations in periodontal diseases and other acquired disease.[6]

Recent ultrastructural studies by electron microscopy and immunocytochemistry have revealed that connective tissues are organized into distinct architectural patterns in the gingiva, periodontal ligament, and cemen-

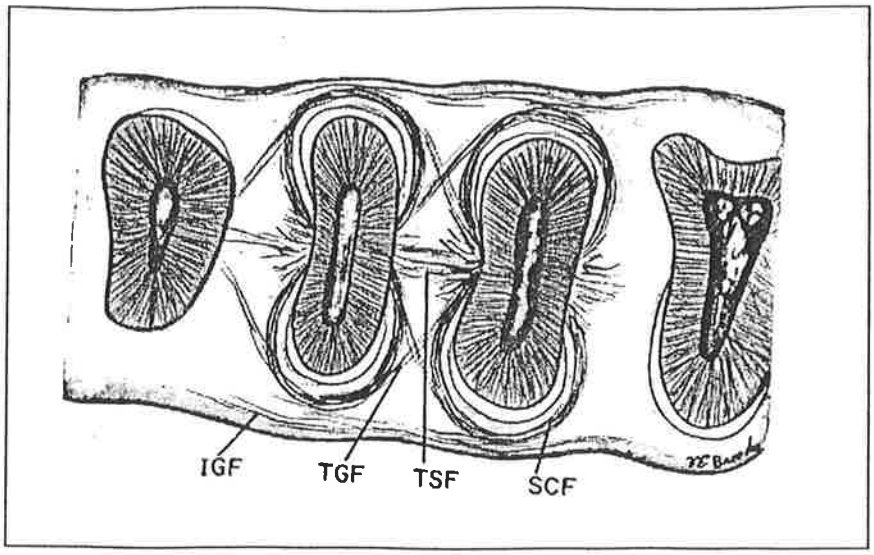


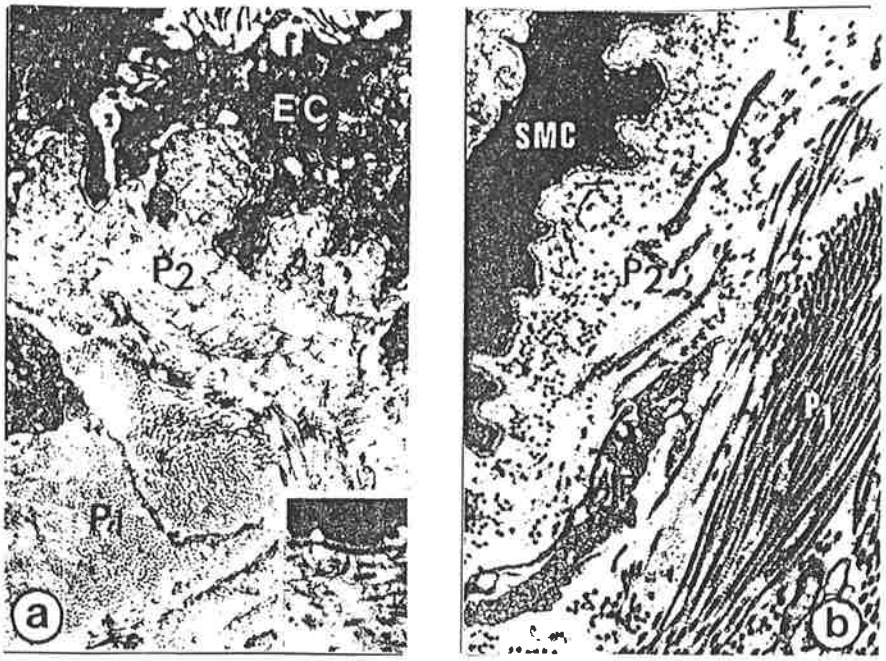
Fig 6-7 Collagen fiber groups in gingiva showing the origin, traverse, and insertion of intergingival fibers (IGF), transgingival fibers (TGF), transseptal fibers (TSF), and semicircular fibers (SCF). (From Schluger et al.[1] Reproduced with permission.)

tum. The distribution and organization of matrix constituents in these structures are outlined in this section.

Gingiva. In the gingiva, collagen fibers form various groups that are classified by location, origin, and insertion.[1] The *dentogingival* fibers arise from the cementum immediately apical to the base of epithelial attachment and splay out into gingiva, while the *dentoperiosteal* fibers bend apically over the alveolar crest and insert into the buccal and lingual periosteum. The *alveologingival* fibers originate from the alveolar crest, course coronally, and terminate in the free and papillary gingiva, whereas the *circular* fiber group passes circumferentially around the cervical region of teeth in the free gingiva. The *semicircular* fibers traverse from the cementum at the proximal root surface, extend into the free marginal gingiva, and insert into a corresponding position on the opposite side of the tooth. The *transgingival* fibers traverse between the cemento-enamel junction to the free marginal gingiva of the adjacent tooth, and the *intergingival* fibers extend along facial and lingual marginal gingiva from tooth to tooth. *Transseptal* fibers arise from the cemental surface just apical to the base of epithelial attachment, traverse the interdental bone, and insert into a comparable position on the opposite tooth. These various fiber groups are interdependent for function, and their anatomical relationship is believed to determine the pattern of spread of inflammatory periodontal diseases.[1] The organization of some of these fiber groups is schematically illustrated in Fig 6-7.

Like other connective tissues, the gingiva contains a heterotypic mixture of collagen types, with type I being the major species.[6] The ultrastructural distribution of collagen types has been studied using collagen-type–specific antibodies and by electron microscopy. Type I collagen is the main collagen species in all layers of gingival corium.[107-109] In the gingiva, collagen fibers are arranged in two patterns of organization; one consists of large, dense bundles of thick fibers, and the other is a loose pattern of short, thin fibers mixed with a fine reticular network[110] (Fig 6-8). These fibers contain both type I and III collagens, and the type I is preferentially organized into denser fibrils in the lamina propria. Although it is not restricted to any particular region, the type III appears to be localized mostly as thinner fibers in a reticular pattern near the basement membrane at the epithelial junction[109,111] (Fig 6-9a). The type III is a component of Sharpey's fibers (Fig 6-9b).

Immunostaining data have revealed that type V collagen has a parallel filamentous pattern, and it appears to coat dense fibers composed of type I and III collagens.[109,112] The gingival connective tissue also contains type VI collagen, which is present as diffuse microfibrils in lamina propria, around blood vessels and near epithelial basement membrane and nerves.[112] In the gingiva, basement membrane is present at the epithelial junction, rete pegs, nerves, and around blood vessels and, like other basement-membrane structures, contains type IV collagen, laminin, and heparan sulfate proteoglycan.[109,110,112] The collagen-type composition of

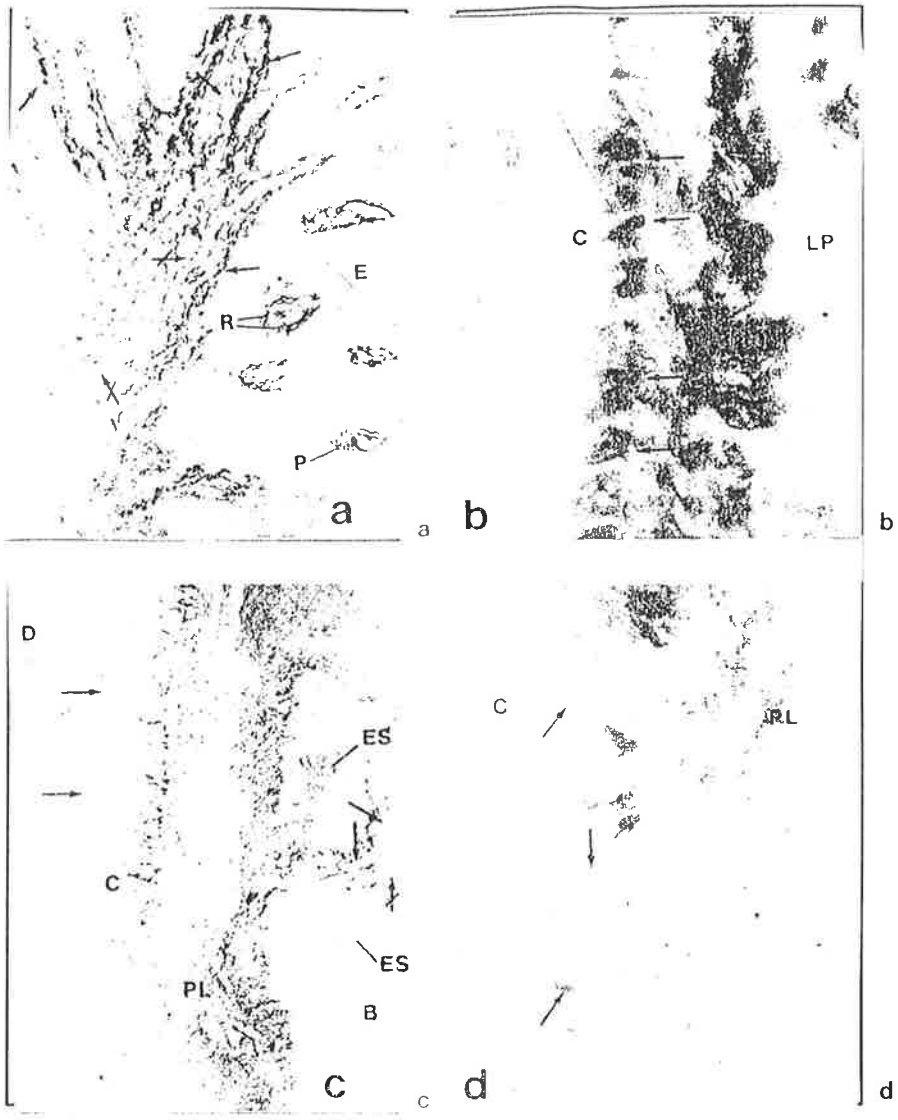


Figs 6-8a and b Distribution of dense (60- to 70-nM, P1) and thinner (40- to 60-nM, P2) collagen fibers at gingival lamina propria (a) and near a blood vessel (b), respectively, viewed by electron microscopy (× 10,000). The P2, which is a loose pattern of organization mixed with nonstriated fibrillar material, is found near basement membranes. (EC) epithelial cell; (SMC) smooth muscle cell; (F) fibroblast. The inset in "a" shows P2 magnified 20,000 times. (From Chavrier et al.[110] Reproduced with permission.)

Table 6-7 *Distribution of Collagen Types in the Periodontium*

Tissue	Collagen Type*	Location
Healthy gingiva	I	Lamina propria
	III	Lamina propria
	IV	Basement membranes
	V	Collagen fibers, blood vessels
	VI	Microfibrils
Periodontal ligament	I	Principal, secondary fibers
	III	Same as type I
	V	Collagen fibers
Cementum	I	Sharpey's fibers, fibrillar cementum
	III	Sharpey's fibers
	V	Sharpey's fibers
Alveolar bone	I	Bone matrix, Sharpey's fibers
	III	Sharpey's fibers
Inflamed gingiva	I	Same as healthy gingiva
	III	Same as healthy gingiva
	V	Same as healthy gingiva
	IV,V,VI	Same as healthy gingiva
	$[\alpha 1(I)_3]$	Lamina propria
Edentulous ridge	I	Same as healthy gingiva
	III	Same as healthy gingiva
	V	Same as healthy gingiva

* In all structures, type I collagen is the major species accounting for 80%–85% in the gingiva to 99% in bone. Type III is the second most predominant collagen in gingiva and periodontal ligament, forming ~15% of the total. In alveolar bone and cementum, the type III is restricted to Sharpey's fibers. The content of all other collagens together in healthy tissues is <1%.

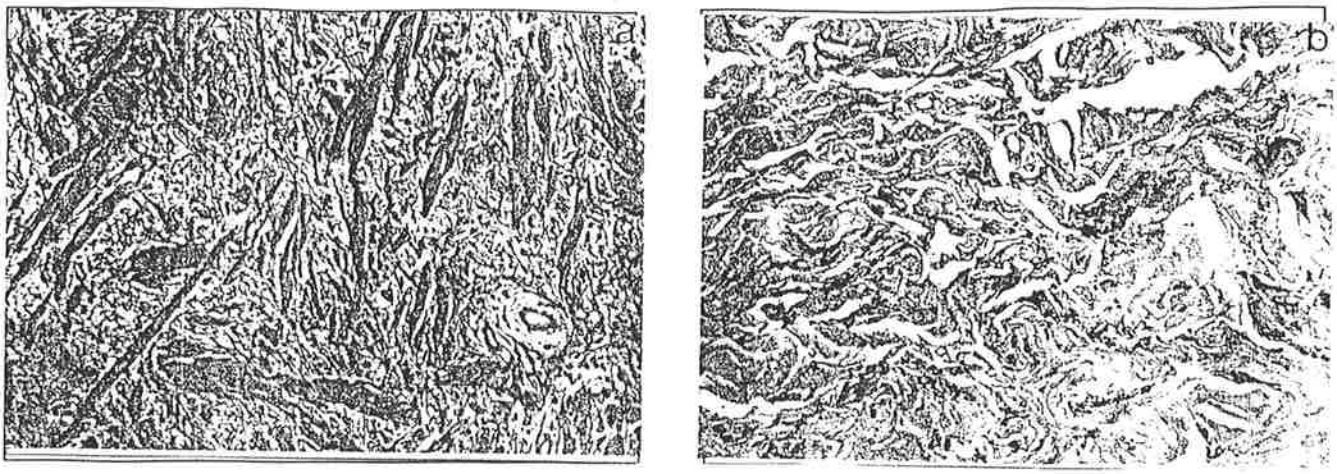


Figs 6-9a to d Distribution of type III collagen in the periodontium as revealed by an anti-type III collagen antibody: (a) section of gingival papilla in which the antibody strongly stains areas adjacent to epithelium (arrows) and rete peg junctions, and blood vessel wall (crossed arrows). (E) epithelium that is unstained; (P) papilla; (R) reticular pattern of staining. (b) Junction between cementum (C) and lamina propria (LP) of gingiva; cementum is unstained, and strongly staining material (arrows) is Sharpey's fibers. (c) Alveolar bone and periodontal ligament. (B) alveolar bone; (PL) periodontal ligament; (C) cementum; (D) dentin; (ES) endosteal spaces. Cementum and alveolar bone are largely unstained except at Sharpey's fibers (arrows). (d) Higher magnification of Sharpey's fibers (arrows). (From Wang et al.[111] Reproduced with permission.)

gingiva and other periodontal structures is summarized in Table 6-7.

The gingiva also contains fibronectin, which is localized over collagen fibers,[109,113] osteonectin,[114] tenascin,[115] and elastin.[116] Tenascin is present diffusely in the gingival connective tissue, and prominently near subepithelial basement membrane in the upper connective tissue and capillary blood vessels.[115,117] Although elastin is a minor constituent of gingival connective tissue, it is relatively more prominent in the submucosal tissues of the more movable and flexible alveolar mucosa[116] (Fig 6-10).

The uronic acid content of the gingiva is approximately 0.3% of total dry weight. Dermatan sulfate is the major GAG in gingival connective tissue, accounting for 60% of total GAGs, and heparan sulfate forms 5%. Heparan sulfate is the predominant species in gingival epithelium. Hyaluronic acid and chondroitin sulfate are other GAG species in these structures. The molecular size of sulfated gingival GAGs range from 15,000 for heparan sulfate to 27,000 for dermatan sulfate, while hyaluronan is the largest with molecular weight of 340,000.[118,119] Gingival proteoglycans have been identified as decorin, biglycan, versican, and syndecan. Immunohistochemical studies have shown that dermatan sulfate GAG and decorin proteoglycan are present within the gingival tissues closely associated with collagen fibers, especially in the subepithelial region (Fig 6-11). Biglycan is a relatively minor constituent of the gingiva, but it appears to be localized in the matrix near the oral epithelium.[120-122] In the gingiva, the GAGs are largely made by fibroblasts, which may synthesize up to six different proteoglycans, including decorin,



Figs 6-10a and b Elastin in human oral tissues. (a) attached gingiva; (b) alveolar mucosa. Note heavy deposits in the alveolar mucosa between collagen fibers and its virtual absence in attached gingival connective tissue. The tissue was treated with an antibody to tropoelastin and visualized by silver-intensified protein A-gold immunohistochemistry. (× 100) (Figs 6-10 and 6-11 from Bartold.[116] Reproduced with permission.)

Fig 6-11 Distribution of dermatan sulfate proteoglycans in human gingival tissue. The tissue was reacted with a monoclonal antibody to dermatan sulfate and visualized by immunoperoxide procedure. Note intense staining in the immediate subepithelial connective tissue. (E) epithelium; (CT) connective tissue. (× 75)

biglycan, versican, and syndecan.[123-125] The spectrum of the proteoglycan molecules synthesized by gingival fibroblasts resembles those identified in gingival tissues.

Periodontal ligament. In mature periodontal ligament, collagen fibers are distinguished as *principal fibers* and *secondary fibers*. The principal fibers are dense bundles that traverse the periodontal space obliquely and insert into the cementum and alveolar bone as Sharpey's fibers. In zones where extensive mesiodistal tooth movement has occurred, the Sharpey's fibers may continue from one tooth to another through interproximal bone. The secondary fibers are randomly oriented fibrils located between the principal fibers.

The principal collagen species in Sharpey's and other collagen fibers of the periodontal ligament is type I, and this collagen also constitutes the fibrous component of endosteal spaces.[108] Type III collagen appears to coat Sharpey's fibers (see Figs 6-9b to 6-9d). These two collagen types are co-distributed with types V and XII,[126] and fibronectin. Blood vessels contain type I, III, IV, and V collagens. The periodontal ligament also contains small amounts of elastin and tenascin, which is present in connective tissue and in zones along cementum and bone.[117]

The distribution of proteoglycans in periodontal ligament is similar to that in gingival tissue. GAG components present are hyaluronate, heparan sulfate, dermatan sulfate, and chondroitin sulfate, of which dermatan sulfate is the principal species.[121,127] The finding that dermatan sulfate is the principal GAG is consistent with the highly collagenous nature of the periodontal ligament. Periodontal ligament GAGs have a molecular size in the order of 18,000 to 20,000, making them slightly smaller than their counterparts in the gingival connective tissue. Two principal proteoglycans in the periodontal ligament are versican and decorin.

Table 6-8 *Glycosaminoglycans and Proteoglycans in the Periodontium*

Tissue	GAG*	Proteoglycans†
Gingiva	DS‡, HA, CS, HS§	CS-PG, DS-PG, decorin, biglycan, versican, CD44
Periodontal ligament	DS‡, CS, HA, HS	CS-PG, DS-PG
Alveolar bone	CS‡, DS, HA, HS	CS-PG
Cementum	CS‡, DS, HA	CS-PG

* GAG, glycosaminoglycans; DS, dermatan sulfate; CS, chondroitin sulfate; HS, heparan sulfate; PG, proteoglycan.

† The localization of proteoglycans is as follows: CS-PG, general matrix in soft tissues and matrix and lacunae of bone and cementum; DS-PG and decorin, subepithelial matrix in the gingiva; decorin, predominantly at the subepithelial matrix; HA, mostly in the epithelium; CD44, on epithelial cells. See text for references.

‡ Major species. In the gingiva DS is the major species in connective tissue.

§ HS is the major GAG species in the gingival epithelium.

Cementum. Collagen fibers are present as fine, randomly oriented fibrils embedded in granular matrix in primary cementum, which is devoid of cells. Secondary cementum contains cells, coarse collagen fibrils oriented parallel to the root surface, and Sharpey's fibers at right angles. Recently, the presence and organization of collagen fibers have formed the basis for new classification of cementum.[126,129] Thus, the *acellular afibrillar* cementum located at the dentinoenamel junction does not contain collagen fibers or cells. *Acellular extrinsic fiber* cementum also does not have cells, but it contains large numbers of Sharpey's fibers. It is present in cervical to midroot areas and anchors teeth. *Cellular* cementum located at apical and interradicular root surfaces contains both extrinsic (Sharpey's) and intrinsic collagen fibers, while *repair* cementum has only the intrinsic fiber system. Intrinsic and extrinsic fibers differ in their orientation, the former occurring randomly and parallel to the root surface, the latter embedded at right angles. The Sharpey's fibers of cementum and alveolar bone are largely responsible for tooth anchorage.

Approximately 50% of the inorganic matrix in the cementum is hydroxyapatite, whereas the organic matrix is composed of predominantly type I and III collagens.[130] The type III is associated with Sharpey's fibers (see Fig 6-9b). A variety of nonfibrous proteins are also present in cementum; these include BSP-II, osteopontin, tenascin, fibronectin, osteonectin, and proteoglycans.[131-133] Cementum contains hyaluronate, dermatan sulfate, and chondroitin sulfate, and chondroitin sulfate is the predominant GAG. These GAGs, closely associated with cementoblasts, are lightly distributed throughout the matrix.[134,135] A variety of biologically active polypeptides are also present in cementum as minor biochemical components, and these are discussed later.

Alveolar bone. Collagens are major constituents of the alveolar bone matrix.[108,111] The bone is attached to principal fibers of periodontal ligament through Sharpey's fibers. As in the cementum, chondroitin sulfate is the major GAG species in the alveolar bone, and it is present along with heparan sulfate, dermatan sulfate, and hyaluronate.[136] Immunohistochemical localization studies have shown that these molecules are distributed on cells in their lacunae and in the mineralized matrix. Analysis of alveolar bone proteoglycans have identified a chondroitin-sulfate-rich proteoglycan as the major species,[136,137] which may be a mixture of decorin and biglycan.

For a summary of the constituent collagen and proteoglycan types and their locations in various periodontal components, see Tables 6-7 and 6-8.

Connective Tissue in Periodontal Disease

The periodontium is the primary location of several diseases, and many systemic and drug-induced diseases have manifestations in the periodontium, especially in the gingiva.[1] Gingivitis is one of the most common human afflictions. In this lesion, gingival connective tissues are destroyed within three to four days after

plaque accumulation, and this is associated with the migration of PMNs into junctional epithelium and gingival sulcus. The destruction begins at perivascular collagen bundles. Approximately 70% of collagen within the foci of inflammation is lost, mostly due to the activity of PMN. This lesion may remain established for years or decades and may be reversible.[138] Gingivitis is a frequent occurrence during pregnancy because sex hormones affect the inflammatory response and cellular metabolism during wound healing.

Chronic periodontitis leads to extensive destruction of gingival connective tissue, periodontal ligament, and alveolar bone, and it sometimes affects the root surfaces. Although plasma cells and lymphocytes are present in this lesion, macrophages and PMN are the major destructive cells. Large numbers of PMN may cause recurring destruction during times of cyclic disease. The collagen level is reduced, and fibrosis and scarring of the gingival tissue may also occur at foci of inflammation. As the disease progresses, destruction may expand to alveolar bone housing and tooth roots; this causes the teeth to become loose and may lead to tooth loss. Gingival fibrosis, manifested by scarring of gingival tissues, is seen in slowly progressive human periodontitis and it is common in baboons and chimpanzees; however, this is not seen in dogs, rodents, minks, and marmosets.[1,139] These changes are described in detail in Chapter 4.

Quantitative and qualitative changes occur in the gingival collagens of patients with the above diseases.[6] In the gingiva, collagen becomes more soluble, indicating active synthesis of new collagen and/or impaired crosslinking. The ratios of collagen types are altered; the amount of type V collagen increases and may exceed type III, and a new collagen, type I trimer, may appear[6,109,140] (Fig 6-12). Type I trimer is a homotrimer of $\alpha 1$(I) chains, which accumulates in certain hereditary collagen molecular diseases and in embryonic tissues and tumors. The amount of type V collagen, which coats collagen fibrils composed of type I and III collagens, increases, and this collagen may be present in greater proportion than type III.[109,140] Changes also occur in noncollagenous gingival constituents; in beagle dogs, noncollagenous proteins are lost from diseased gingiva.[6]

Changes in the gingival proteoglycans are fewer than those noted for collagens. Matrix proteoglycans are lost from the center of inflammatory foci but appear to be present in higher concentrations around the periphery. In inflamed gingival tissues, the amount of dermatan sulfate decreases while the content of chondroitin sulfate increases. Degradation of both proteoglycan core proteins and hyaluronic acid are characteristic features of inflamed gingival connective tissues.[141] The principal proteoglycan synthesized by inflammatory cells is chondroitin sulfate[142]; therefore, in inflamed tissues chondroitin-sulfate levels increase at the expense of dermatan sulfate, which is lost along with collagenous components.

Excessive accumulation of connective-tissue elements is a feature of drug-induced gingival hyperplasia and gingival fibromatosis. Gingival hyperplasia is a common complication in some patients using diphenylhydantoin (phenytoin), cyclosporin, nifedepine, and other drugs.[143] But ingestion of drugs alone is insufficient for evolution of this lesion, as it requires a genetic component, age, and inflammation as additional factors. In these lesions, the gingival margin and interdental papillae overgrow; the overgrowth may become so extensive that teeth are displaced and their crowns covered with overgrown gingival tissues. Clinically these lesions have a cauliflower-like appearance with enlarged epithelium and foci of infiltrating leukocytes. Although during early stages these lesions are highly cellular, in mature lesions the matrix-to-cell ratio is close to normal.[143,144]

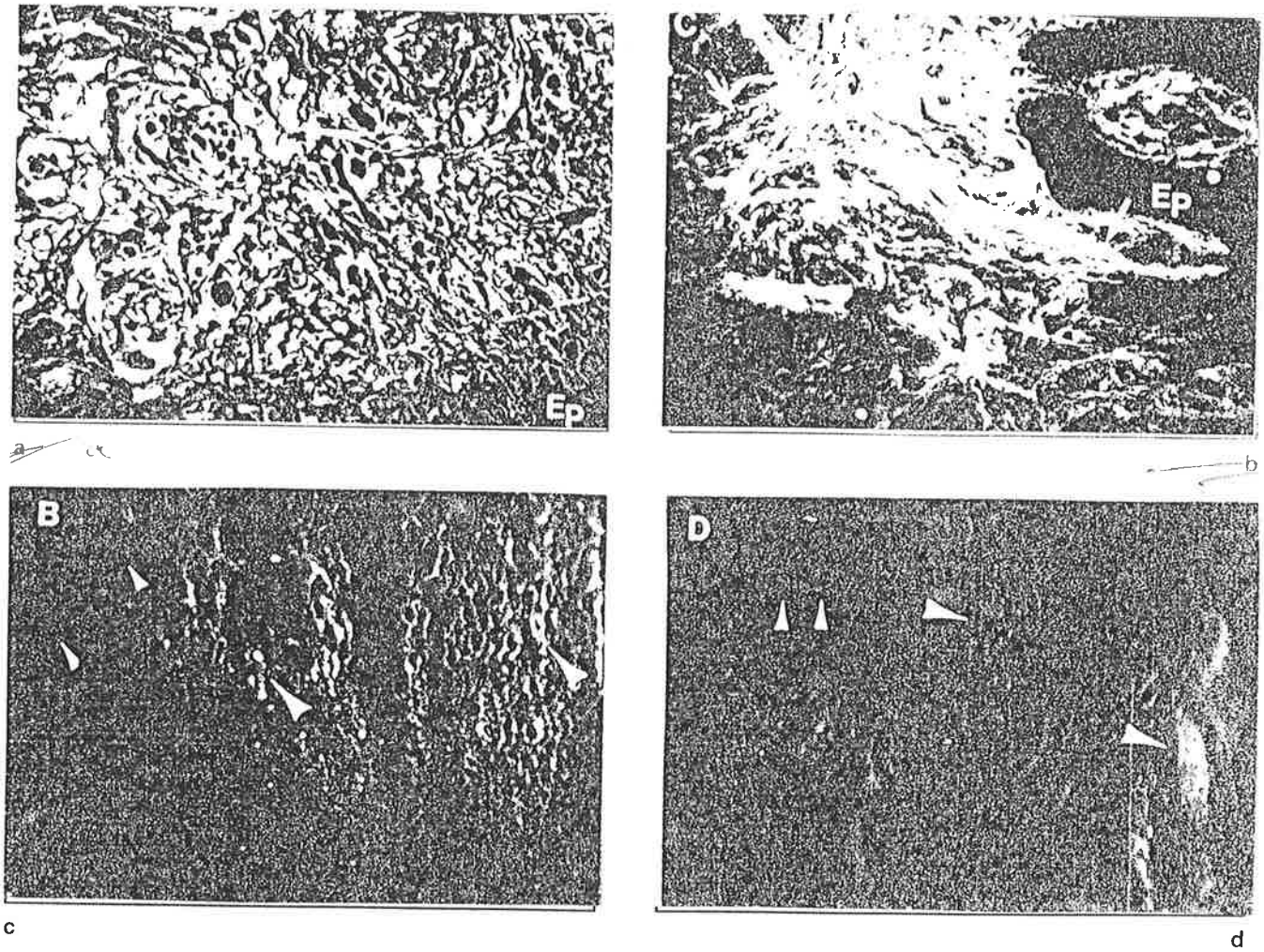
The amount of noncollagenous proteins increases in phenytoin-induced gingival hyperplasia.[145] Collagen content increases, and the type I/III ratio changes with loss of type I and increased type III.[146] Interestingly, the collagen composition of edentulous ridge resembles skin more than gingiva, in collagen type ratios and in hydroxy-amino-acid content.[6]

Gingival fibromatosis is an idiopathic, progressive, fibrous gingival enlargement, which is inherited as autosomal dominant disease. It may be focal or generalized and, unlike drug-induced gingival hyperplasia, does not have an inflammatory component.

Attempts have been made to utilize connective-tissue alterations as indicators of periodontal disease. The gingival crevicular fluid (GCF) contains many breakdown products arising from inflammation, therefore studies have focused on measuring the levels of plasma proteins, bacterial and host enzymes, and collagen degradation products such as hydroxyproline and $\alpha 1$(I)-N-propeptide in the GCF.[147] However, differences between healthy and diseased tissues are subtle and do not appear to correlate well with the severity of disease.

Biochemistry of tissue alterations. The biochemical composition of diseased connective tissues is determined by various degradation and synthesis processes associated with inflammation and healing response. The hallmark of inflammatory periodontal disease is connective-tissue destruction. During inflammation, PMN and macrophages bring forth matrix degradation, and the degradation can occur either through phagocy-

Figs 6-12a to d Distribution of type I and III collagens in normal and inflamed human gingiva. The collagens were visualized by indirect immunofluorescence using antibodies to these collagens. (a) normal gingiva, anti-type I collagen antibody; (b) inflamed gingiva, anti-type I collagen antibody; (c) normal gingiva, anti-type III collagen antibody; (d) inflamed gingiva, anti-type III antibody; (Ep) epithelium. Note staining is considerably less in (b) and (d) indicating loss of collagens. (From Narayanan et al.[109] Reproduced with permission.)



tosis or by MMP released by these cells. (Enzymes involved in these processes are discussed on pages 67 and 68.) Interstitial collagenases MMP-1 and MMP-8 and gelatinases MMP-2 and MMP-9 are important in this regard.[146] The PMN is capable of secreting large quantities of these enzymes from granules during acute inflammation, presumably causing extensive destruction in a short time. Differences in susceptibility of different collagen types may be one reason that differences occur in collagen type ratios in diseased gingiva. For example, greater susceptibility of type III and resistance of type V and type I trimer to proteinases may help explain why the amounts of these collagens vary in inflamed tissues.[6]

Collagenase and gelatinase activities have been identified in GCF and saliva of patients with natural and experimental periodontitis.[149,150] The major enzyme species present in the GCF are MMP-8 and MMP-9; however, neither 72-kDa gelatinase nor stromelysin-1 has been identified. The activities of MMPs in GCF are inhibited by tetracycline, indicating that they are derived from PMN and leukocytes, and not from fibroblasts. Fibroblast enzymes are not inhibited by this drug.[151,152] The collagenases of GCF from patients with

adult- and diabetes-associated periodontitis are susceptible to tetracycline, while those from localized juvenile periodontitis are relatively more resistant.[152] Attempts have been made to correlate GCF-collagenase activities to disease severity. However, although the overall activity is higher in periodontitis and it decreases with treatment, there appears to be no clear correlation with individual sites, disease severity, or bone loss.[148,153-156]

Collagenases in inflamed gingiva may also be derived from fibroblasts and epithelial cells.[148] Immunolocalization studies have shown that these cells express collagenases; however these appear to be involved in remodeling rather than disease.[156] Nevertheless, in the context of periodontal disease, enzyme production by these cells could be activated by inflammatory mediators and products of plaque bacteria (see Birkedal-Hansen[148] for a review). MMP expression is induced by IL-1, TNF-α, con A, cyclic AMP, and prostaglandin E_2 (PGE_2), whereas it is repressed by TGF-β, steroids, and IFN-γ. These mediators can affect MMP activity through TIMPs as well; for example, TGF-β and IL-1 induce TIMP-1 expression, while TGF-β suppresses TIMP-2.[148,157] Osteoclasts do not express MMP, and during bone resorption these cells appear to utilize acid hydrolases (cathepsins) for matrix degradation.

Many other hydrolytic enzymes have been identified in inflamed periodontal tissues. The most notable among these enzymes are β-glucuronidase, aryl sulfatase, and hyaluronidase.[158-160] The substrates for such enzymes would clearly be the carbohydrate components of the proteoglycans as well as hyaluronate. However, in light of current biochemical analysis of inflamed tissues, there is little evidence to support a primary role for these enzymes in matrix degradation. More likely, these enzymes are involved in a secondary capacity, in the breakdown of GAG chains after initial proteolytic cleavage of proteoglycans.

Another important source of matrix-degrading enzymes in inflamed gingiva is the microbial plaque. In particular, the black-pigmented *Bacteroides* species synthesize numerous proteases capable of disrupting periodontal extracellular matrix.[161,162] For example, *Porphyromonas gingivalis, Clostridium histolyticus* and some facultative *Bacillus* species from dental plaque secrete collagen-degrading enzymes.[163,164] Other enzymes with trypsin-like activities have been described in *Porphyromonas gingivalis, Treponema denticola, Bacteroides forsythus;* these enzymes degrade type I collagen and fibronectin.[165,166] Bacterial enzymes could also facilitate MMP activity by activating their inactive precursors and by degrading MMP-inhibitors. Alternatively, they can act as antigens stimulating the cytokine production by host inflammatory cells. Although these mechanisms are a possibility, bacterial enzymes have not been detected in the GCF.[147]

The bacterial enzymes can also degrade proteoglycans. Oral bacteria synthesize hyaluronidase, neutral proteinases, heparinase, chondrosulfatase, and chondroitinase.[167-169] All of these enzymes have the potential to degrade periodontal proteoglycans and to influence the matrix indirectly through activation of interleukins and by affecting fibroblast function. For instance, the enzyme released by *P. gingivalis* has been found to have a significant effect on proteoglycan synthesis by periodontal ligament fibroblasts.[166]

Although they have not been investigated actively, products of normal cell metabolism could also be agents of tissue destruction.[170,171] Oxygen-derived free radicals such as hydroxyl- and superoxide radicals are integral reaction products of normal cellular metabolism, but these are active in cells undergoing respiratory bursts at inflammatory sites. These highly reactive molecules can damage bacteria, degrade macromolecules such as collagen, proteoglycans, and hyaluronic acid, promote degradation of polyunsaturated fatty acids, and ultimately cause damage of structural membranes.[171] In addition, free radicals can also inhibit the action of anti-proteases, and stimulate the production from the plasma of a factor chemotactic for neutrophils and prostaglandin synthesis. The role of these free radicals in periodontal tissue destruction has been largely overlooked in favor of the more commonly cited enzymatic degradation. However, given their highly reactive nature and abundance in inflamed periodontal tissues, their role in inflammation-mediated tissue destruction should not be discounted. Studies on the effect of oxygen-derived free radicals on gingival proteoglycans and hyaluronate have demonstrated a susceptibility of these molecules to depolymerization by such reactive molecular species in vitro.[172]

Interaction between connective-tissue cells and mediators. Fibroblasts play a major role in normal connective-tissue turnover and during wound repair and regeneration. In inflamed gingival tissues, fibroblasts interact with numerous cytokines and growth factors derived from inflammatory cells, plasma serum, and local environment.[6] These factors can affect the growth and synthesis activities of resident cells, thereby affecting the quantity and type of molecules produced in diseased gingiva.[6,173] The effect may be on all cells or only on one or more subpopulations. In the latter case, growth and products of a subpopulation of cells are affected.[6] This appears to be one mechanism contributing to enhanced matrix accumulation in drug-induced gingival hyperplasia.[6]

Repair and regeneration. Repair of damaged tissues is a major biological response of all animals. However, the nature of the repair process may often lead to compromised function. In this respect the periodontium is no exception. Tissues affected by gingivitis usually regenerate to their complete form and function; however, this may not be the case with periodontitis. Once the destructive phase reaches the deeper periodontal structures, regeneration is less likely to happen on a clinically predictable basis. The major goal of periodontal therapy is to re-establish soft-tissue attachment and to restore lost bone. Accomplishment of this goal requires matrix synthesis and regeneration of gingival connective tissues, formation of new cementum and bone, and attachment of connective-tissue fibers to diseased root surfaces.[129,174,175]

Experiments in wound models of the periodontium and other tissues indicate that inflammation is the key component of healing response. Typically, a wound-healing response is initiated by inflammation when PMNs migrate into the wound site filled with fibrin clot. The PMNs, and later monocyte/macrophages, remove damaged tissue and foreign matter; this "demolition" phase is carried out through phagocytosis and by enzymes secreted by these cells. "Organization" of granulation tissue follows when endothelial cells actively divide, forming capillaries and myofibroblasts, and fibroblasts begin to synthesize matrix components. The granulation tissue is eventually remodeled and replaced by a permanent repair tissue.

These processes require the participation of a variety of cell types and molecules. PMNs are the major cell type present during the acute inflammation phase, while monocyte-macrophages predominate during the later stages. In soft tissues, fibroblasts are largely responsible for the synthesis of collagen and other connective-tissue matrix constituents. During healing and repair, matrix synthesis begins when fibroblasts move into the wound site; the synthesis becomes significant at approximately 7 days, peaks at 3 weeks, and may continue for months until tensile strength of the tissue is restored. Healing takes place through a similar sequence of events in bone, although in this tissue fibrous and bony calluses form the intermediate stage instead of a granulation tissue.

The course of wound-healing events is regulated by soluble mediators present at the site of injury. These molecules include degradation products of fibrin clot and host tissue, and growth factors, cytokines, and lymphokines secreted by platelets, macrophages, and other cells.[25] Prominent among these are PDGF, TGF-β, IL-1, IFN-γ, and TNF-α. A family of at least eight polypeptides, collectively called *bone morphogenetic proteins* (BMPs) or *osteogenic proteins* (OPs), participate in the healing of bone; these molecules are stored in the extracellular matrix of bone along with several growth factors, and are released when the matrix is degraded during inflammation.[176,177]

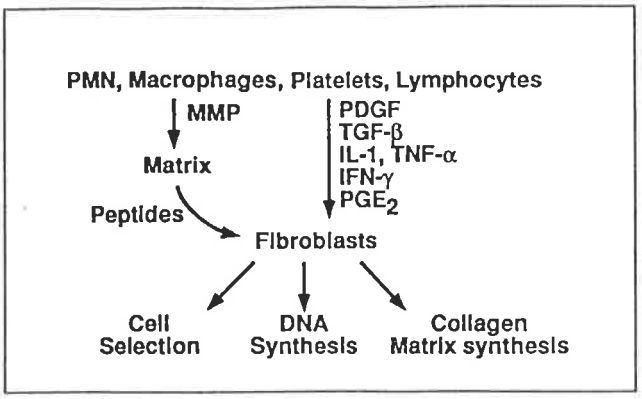


Fig 6-13 A scheme showing possible interactions between polypeptide mediators and resident cells during inflammation and wound repair. MMP and other hydrolytic enzymes released by inflammatory cells, and presumably by fibroblasts and epithelial cells through activation by bacterial substances, degrade the matrix. Cytokines, growth factors, and other molecules secreted by inflammatory cells and matrix degradation products affect the growth and synthesis activities of resident cells. These substances influence all the resident cells, or they may interact with and select one or more subpopulations and enrich these cells. Interference with any of these processes is likely to lead to aberrant healing or pathological alterations.[6]

Inflammatory mediators affect cells in a number of ways. These molecules influence cell migration, attachment, and growth, and their synthesis activities.[25,178] PDGF, which may be a homo- or heterodimer of A- and B-chain subunits, is the major mitogen for mesenchymal cells. TGF-β activates the synthesis of collagen and other matrix components, while IFN-γ and TNF-α have the opposite effect. IL-1, as described previously, mediates matrix degradation through activation MMP synthesis (Fig 6-13; see also Table 6-2). In contrast to these molecules, the BMPs are unique in that they trigger inflammatory and wound-healing responses in injured bone and accomplish total healing of the injury.[176,177]

The effect of these molecules may be broad and directed to all connective-tissue cells, or they may be specific to certain cell types or subtypes. The latter type of interaction can result in selective proliferation and enrichment of certain cell populations (see Fig 6-13). By these activities, chemical mediators are believed to dictate the type and sequence of healing responses, and aberrations in any of these processes are likely to lead to pathological connective-tissue alterations.[2]

Whether healing occurs by regeneration (new tissue is identical to old tissue) or repair (wound is replaced by a scar) depends mostly on the participating cell type, and to a lesser extent on the matrix scaffolding remaining after injury. Regeneration occurs when the *labile* cells carry out the healing. These are undifferentiated uni- or pluripotent cells, which have unlimited division potential. In contrast, repair occurs when fully differentiated *permanent* cells with no division capacity are to be replaced. Repair is carried out by *stable* cells. Mesenchymal cells such as fibroblasts, osteoblasts, and glial cells form the stable cell pool; these cells have a limited division potential, they normally remain quiescent, and they divide on demand in response to mediators such as PDGF.

Presumably, several cell types are necessary for periodontal regeneration. These include fibroblasts for soft connective tissues, cementoblasts for cementogenesis, osteoblasts for bone, and endothelial cells for angiogenesis.[179] Where the regenerating cells are located is not clear, and this is especially true for the cementoblasts. Experiments in mice models indicate these cells may be derived from precursor cells located paravascularly in periodontal ligament and in endosteal spaces of alveolar bone.[180–183] Recent studies using cultured fibroblasts have indicated that in humans such cells may be present as fibroblast subpopulations in the periodontal ligament and gingiva.[179,184–186] Irrespective of their location, for regeneration to occur the cells responsible must participate in the right location and temporal sequence. More importantly, for regeneration to succeed, certain cells, especially the epithelial cells, have to be excluded from the healing site.

Recently, attempts have been made to exploit some of these principles in periodontal therapy. In one approach, conditions were created to select the cells responsible for regeneration. Early attempts utilized *root-surface conditioning* either by demineralization or by coating with chemical agents such as fibronectin, or both. Demineralization was intended to neutralize periodontitis-induced hypermineralization, and to expose collagen fibers. Exposed collagen fibers were believed to discourage the attachment of unwanted epithelial cells, but to favor fibroblasts to attach and help "splice" new with old collagen fibers. However, this procedure did not yield predictable regeneration, and may cause ankylosis and root resorption as side effects.[174,175,187,188] The advantage of using fibronectin root surface coating[189] was also uncertain because serum contains high fibronectin levels and providing additional protein is unlikely to have a beneficial effect.

More recently, a novel procedure has been utilized in which a physical barrier was introduced by surgically placing a membrane between connective tissue of periodontal flap and curetted root surface. The membrane was expected to prevent apical migration of gingival epithelial cells onto the root surface, exclude unwanted gingival connective tissues, and facilitate the repopulation of the wound site with periodontal ligament cells.[190–192] This *guided tissue regeneration* was the first procedure to demonstrate good potential for regeneration of root cementum, alveolar bone, and periodontal ligament, and for increasing the incidence of new attachment formation. Although the clinical results of this procedure are still variable, it has gained wide acceptance,[174,175,192] and resorbable membranes are currently being evaluated as physical barriers.

In another approach, polypeptide growth factors have been locally applied to facilitate the cascade of wound-healing events that lead to new cementum and connective-tissue formation. Among the myriad growth factors currently characterized and available, epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), PDGF, and TGFs have been proposed for their regulatory effects on immune function, epithelium, bone, and soft connective tissues. Two of these growth factors, PDGF and IGF, have recently been shown to enhance regeneration in beagle dogs with artificially induced periodontal disease.[193] More recently, a combination of PDGF and dexamethasone has also been shown to enhance periodontal regeneration in monkeys,[194] and it is expected that BMPs will also have potent effects.[195]

Role of cementum. An essential requirement for periodontal regeneration is the formation of new cementum into which new periodontal ligament fibers are to be inserted.[129] This is necessary to replace the diseased root, which is contaminated with bacterial endotoxins (the endotoxins inhibit attachment and growth of periodontal cells[196]), and which is removed during therapy.[197] Although some information is available on the biology of cementogenesis in mice during development,[198,199] how cementum formation is regulated in adult humans is not clear. Recent studies indicate that the connective-tissue matrix of cementum contains fibroblast growth factor and a battery of other growth factors, osteopontin, BSP-II, and an as yet unidentified polypeptide, which mediate cell adhesion and spreading.[131–133,200–203] These molecules affect the migration, attachment, and proliferation of periodontal cells and their matrix synthesis[132,204] and, more importantly, they manifest cell specificity and tissue specificity among the same cell type.[205–207] In addition to these soluble polypeptides, the extracellular matrix of cementum can also regulate the differentiation of precursor cells into

cementoblasts. Thus, cementum appears capable of providing informational signals for the recruitment, proliferation, and differentiation of periodontal cells, and for regulating the regeneration of cementum as well as adjacent periodontal components.

New perspectives. Significant information has been gathered on the biochemistry of normal and diseased periodontal structures. These studies are being pursued and they continue to provide a more complete picture of the periodontium at the ultrastructural level. However, sufficient information on cementogenesis and the biochemical constituents of cementum is lacking. Efforts to characterize cementum components at protein and gene levels have just begun; these efforts are likely to open new avenues and make it possible to apply modern biotechnological approaches to future periodontal research. So far, satisfactory in vitro systems have not been available to study cementogenesis and to evaluate the function of cementum components; the availability of cultured cells from cementum tumors,[208] although they are far from ideal, may be useful in this regard.

Although wound-healing models for other organs provide a wealth of information, the intricacies of periodontium may be unique in having to coordinate and integrate the processes involved in soft- and hard-tissue healing. Specifically, the cell types and subtypes necessary for the regeneration of periodontal components need to be identified.[179]

Along with tissue regeneration, the control of tissue destruction is an important therapeutic goal in periodontitis. Whether degradation of periodontal tissues in advancing or aggressive periodontal diseases is caused by accelerated breakdown or a failure in normal regulation remains to be established. Recently, advances have been made in developing agents to block the activities of enzymes, cytokines, and other inflammatory agents such as prostaglandins.[209-211] These advances have significant clinical ramifications.

To understand the rational basis for therapeutic procedures, information is needed on the variety of molecular and cellular processes associated with the formation of each periodontal component. Molecules participating in these processes must be identified, and the determination made how they interact with target cells and what signals are needed to trigger their biological actions.

The Physiology of Bone

Bone Structure and Remodeling

Bone is a metabolically active organ, composed of both mineral and organic phases. It is exquisitely designed for its role as the load-bearing structure of the body. To accomplish its task, it is formed from a combination of dense, compact bone and cancellous (trabecular) bone that is reinforced at points of stress. The mineral phase of the skeleton contributes about two thirds of its weight, while the remaining one third is organic matrix primarily consisting of collagen and small amounts of proteoglycan, lipid, and several noncollagenous proteins, such as osteopontin, osteonectin, osteocalcin (bone gla-protein), and matrix gla-protein.

Two major cell types are found in bone. The first is the osteoblast, whose function is to synthesize the organic matrix components and direct the events resulting in mineralization. The second cell type is the osteoclast, whose function is to resorb both the mineral and organic phases of the bone. In concert, osteoclast and osteoblast, in a process known as *coupling*, remodel and maintain the skeleton throughout the life of the organism.

The bone consists of two macroscopically different envelopes: cortical bone, which is predominantly found in the long bones of the extremities, and cancellous bone, which is predominantly found in the vertebral column and the pelvis. Both types of bone are found in the maxilla and the mandible, although cortical bone is more prominent in the mandible. Cortical bone makes up 80% of the bone in the body and cancellous bone 20%. However, because cancellous bone is metabolically more active, skeletal metabolism is approximately equal between the two envelopes. The two envelopes are regulated and respond differently to different hormones, factors, and treatment modalities.

When studying bone under polarized light, a clear lamellar pattern is visible in both cortical and cancellous bone. However, if bone turnover is very high or disturbed—or during healing when primary bone is formed—the lamellar pattern disappears and *woven bone* is formed.

Cortical bone is made up of the Haversian system (cortical osteon), which is found around central blood vessels and which may branch within the cortex of the bone. Spatially, the cells in the Haversian system cover a relatively small surface area, while cells in cancellous bone occupy a large portion of the surface. This observation may explain why cortical bone exhibits lower metabolic activity than cancellous bone.[212]

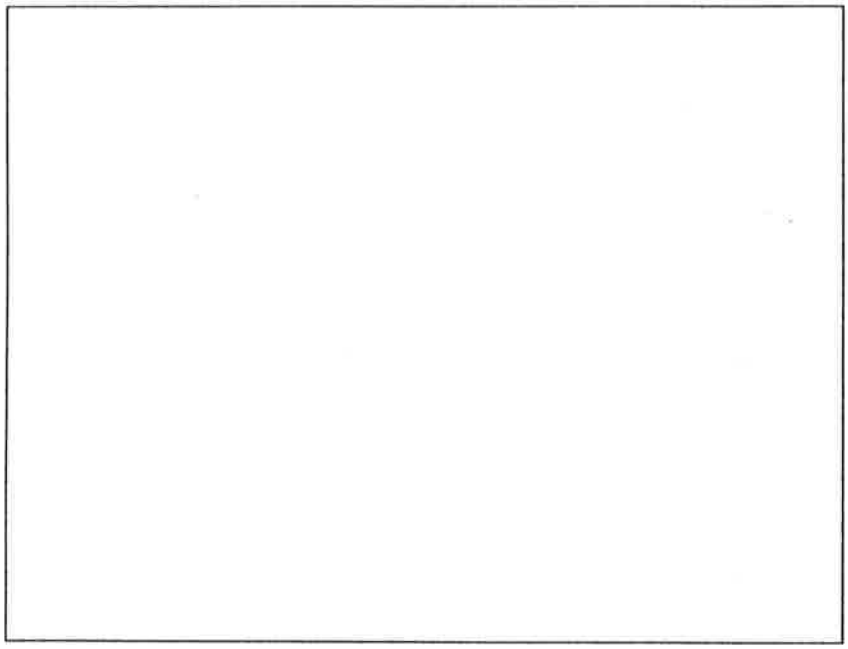


Fig 6-14 Modeling of bone by the concerted action of osteoclasts and osteoblasts. Bone is removed by osteoclasts and new bone synthesized by osteoblasts. The process is delicately balanced and regulated by local factors and hormones that act on the cells to assure that bone resorption and formation are coupled with each other.

Cortical bone is delimited by the *periosteum* on the outside and the *endosteum* on the inside. The inner cortical bone, the endosteal surface, exhibits pronounced osteoclastic and osteoblastic activity. The periosteum is important during growth, fracture repair, and healing around implants. During growth, the periosteum is important for bone *modeling*. Modeling is the process by which bone reshapes itself to create an organ with maximal compressive strength. Usually at the periosteal surface, bone formation exceeds bone resorption, creating a net increase in the outer diameter of bone with age. However, at the endosteum, modeling as well as remodeling occurs, and resorption generally exceeds bone formation, resulting in a net expansion of marrow capacity with age. Importantly, this endocortical formation may become especially pronounced during states of high turnover, such as thyrotoxicosis and post-menopausal syndrome.

Cancellous bone consists of trabeculae with thicknesses ranging from 50 to 400 μm. The trabeculae are interconnected in a honeycomb pattern, maximizing the mechanical properties of the bone.

In bone, there is a constant process of modeling and remodeling. In this process, a constant resorption of the bone occurs on a particular bony surface, followed by a phase of bone formation (Fig 6-14). In normal adults, there is a balance between the amount of bone resorbed by osteoclasts and the amount of bone formed by osteoblasts.[213] Bone remodeling must be distinguished from bone modeling, which is the process associated with the formation and growth of bones in childhood and adolescence.[213] Bone modeling consists primarily of processes at the periosteum and endosteum, leading to changes in the shape of growing bone. During modeling, resorption and formation are spatially related and sometimes proceed in an uncoupled fashion. Moreover, modeling is continuous and covers a large surface, while remodeling is cyclical and usually only covers a small area.[212]

The current concept of bone remodeling is based on the hypothesis that osteoclastic precursors become activated and differentiate into osteoclasts, which begin the process of bone resorption. This phase is followed by a bone-formation phase. The number of sites entering the bone-formation phase, or *activation frequency*, together with the individual rates of the two processes, determine the rate of tissue turnover.[214,215] While resorption depth and mean wall thickness may vary by only 10% to 20% of normal in different diseases, activation frequency may vary by 50% to 100%. Thus, in most diseases, the activation frequency is the most important regulator of bone turnover and changes in bone mass.[214]

The termination of bone resorption and the initiation of bone formation in the resorption lacuna occurs through a coupling mechanism.[216] The coupling process ensures that the amount of bone removed is similar to the bone laid down during the subsequent bone-formation phase. The detailed nature of the activation and coupling mechanism is still unknown, although some growth factors, such as various lymphokines, fibroblast

growth factor (FGF), transforming growth factor–beta (TGF-β), and prostaglandins, have been proposed.[217] Whether the activation of osteoblasts begins simultaneously with osteoclastic recruitment or at some later stage during lacunar development is still unsettled.

During aging, bone undergoes changes in its three-dimensional structure that have a profound impact on its physical characteristics. This process starts at approximately 25 to 30 years of age, when maximal bone formation is achieved. From that age, a steady decline in bone mass begins around 30 years of age for both men and women.[218,219] The decrease in bone mass leads to thinning of cortical bone due to tunneling, or trabeculation, of the endosteal cortical envelope, with expansion of the marrow cavity accompanied by some gain in bone diameter.[220]

Changes in cortical bone mass are sex-dependent. Men exhibit an age-dependent increase in resorptive activity, leading to increased osteon diameter. Mean thickness of the bone formed in the osteon, however, remains constant with age. Thus, a trend toward a more negative balance between resorption and formation is reflected in increased Haversian canal diameter.[221] In contrast, women generally show reduced resorptive activity. This is reflected in decreases in osteon diameter with age, but unlike men, mean thickness of bone decreases with age, leading to a pronounced negative balance and increase in Haversian canal diameter.[221]

In cancellous bone of 20- to 80-year-old individuals, there is a decrease of about 45% in the fractional volume of trabecular bone; this is accompanied by a decrease in mean thickness of the horizontal trabeculae with no significant change in mean thickness of the vertically oriented trabeculae.[222] Furthermore, an increase in the distance between horizontal trabeculae during aging has been observed for both men and women, but the change is slightly greater for women. Histomorphometry of histologic sections of vertebra has shown an age-related decrease in mean trabecular bone thickness, with a decrease in trabecular volume. Moreover, a pronounced age-related increase in marrow space volume was also demonstrated, which was significantly greater for women than men.[223]

The Osteoblast

Cells of the osteoblast lineage occupy a central position in bone metabolism. Osteoblasts are cells with a number of functions. They are well-known for synthesizing the organic matrix of bone and participating in its mineralization. In addition, they respond to circulating hormones, growth factors, and cytokines produced by themselves or other cells of the marrow, which play a major role in cell-to-cell communication and maintenance of bone. The formation of a structurally sound skeleton, with its strength and integrity conserved by constant remodeling, is the result of many direct and indirect influences on the osteoblast.

Types and functions. The word *osteoblast* has been traditionally used to describe those cells in bone responsible for bone formation. Now, however, it is recognized that cells of the osteoblast lineage are also involved in a much larger number of functions. These include playing a role in the production of paracrine and autocrine factors (cytokines, growth factors), which profoundly influence bone resorption as well as bone formation. Osteoblasts also produce proteases, which are involved in matrix degradation and matrix maturation.

It is possible to separate mature osteoblasts into several main subpopulations: those that synthesize bone matrix; those that line trabeculae and endosteal surfaces; those that are called osteocytes and are buried in their lacunae, communicating with other osteocytes; and those cells on the surface of the bone.

Osteoblasts have been found to have gap junctions that connect them with neighboring osteoblasts and adjacent bone-lining cells,[224] providing an important mechanism for intercellular communication. Osteoblasts also communicate with osteocytes, below the bone surface, through a network of canalicular connections.

The osteoblast is an active cell, with a very prominent Golgi apparatus and extensive endoplasmic reticulum, reflecting its capacity for protein synthesis. It produces a bone-matrix-containing type I collagen as well as noncollagenous proteins, such as osteonectin, osteopontin, osteocalcin, and various proteoglycans. Osteoblasts control the process of bone mineralization at three levels: 1) in its initial phase, by production of an extracellular organelle called the matrix vesicle, which has a major role in primary calcification; 2) at a later stage, by controlling the ongoing process of mineralization by modifying the matrix through the release of different enzymes; and 3) by regulating the amount of ions available for mineral deposition in the matrix.

Primary mineralization of bone occurs through a complex series of synthetic and regulatory events under the control of osteoblasts. This type of mineralized tissue is formed during embryonic and fetal bone development, and postfetal bone growth, as well as during bone repair and induction, either orthotopically or heterotopically. Matrix vesicles, organelles produced by osteoblasts and located in the extracellular matrix, are

active in the initial phases of primary mineralization. This observation has been demonstrated repeatedly in developing tissue, as well as in repair tissue, and in pathological processes. It is important to note, however, that calcification via matrix vesicles is not the exclusive mechanism responsible for crystal formation in bone. The strategies used by cells to regulate events in the matrix are diverse and complex. However, careful regulation of the initial events helps to ensure that calcification is under cellular control. While it is accepted that mineral crystals are first seen in matrix vesicles, bulk-phase mineral deposition may not require matrix vesicles or their constituents.[225]

Because of their multiple functions, osteoblasts are under tight control by hormones such as parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3, estrogen, growth hormone, and thyroxin. They also respond to a number of growth factors and cytokines. In addition, some growth factors, such as TGF-β and insulin-like growth factor–1 (IGF-1), not only are produced by osteoblasts but directly affect them, indicating there exists both a paracrine and autocrine role for these factors in bone.

Osteoblasts are thought to be derived from pluripotential stem cells present in the stromal fibroblastic system of bone marrow and other connective tissues, such as periosteum.[226] The stem cell from which osteoblasts arise is distinct from that giving rise to osteoclasts, which originate from the hematopoietic system.[227] Nevertheless, it is possible to draw analogies between the two pathways, since in both systems, stem cells are able to differentiate into cells of several lineages.[228] The osteoblast, at different stages of differentiation, will possess different properties, depending on its location in bone and other local and humoral influences.

The entire developmental sequence of the osteoblast can be divided into three distinct phases.[229] Using a cell culture model, it has been shown that the initial, proliferative phase (days 0 to 15) is characterized by the synthesis of an organized, bone-specific extracellular matrix. After proliferation ceases, a second phase, characterized by matrix maturation (days 16 to 20), begins. This renders the matrix competent for the final phase of mineralization during days 20 to 25.

Osteoblasts synthesize a collagen-rich matrix, called *osteoid*, which mineralizes to form mature bone. Approximately 90% of the organic matrix of bone is collagen, and most of this is type I collagen. The correct transcription and translation of type I collagen by osteoblasts is necessary for normal bone formation, and, indeed, most of the genetic mutations identified in osteogenesis imperfecta are located on one of the two structural genes for type I collagen.[230] In bone matrix, there are also many noncollagenous proteins produced by osteoblasts. Several of these proteins have been recently characterized and cloned.[231] Table 6-9 shows a summary of our current knowledge of the major noncollagenous proteins in bone, their function, and what factors or hormones regulate their production.

The matrix produced by the osteoblast performs a number of functions, including structural support, orientation, and polarization of cells, binding of latent or active cytokines and growth factors, and regulation of adjacent cells. The matrix also participates in the mineralization process by providing the proper structure and regulatory signals.

Hormones and coupling. With the exception of calcitonin, all of the hormones, cytokines, and growth factors that act upon bone, as an organ, mediate their activity through osteoblasts.[232,233] Parathyroid hormone, as well as PTH-related peptides, have specific receptors on the osteoblast, and their effects are mediated by cyclic AMP at the post-receptor level. A similar effect has been observed with prostaglandin E_2 (PGE_2). 1,25-dihydroxyvitamin D_3 [$1,25\text{-}(OH)_2D_3$] works through a specific nuclear receptor and affects osteoblast differentiation and matrix production. Estrogen and growth hormone also work through a receptor in osteoblasts, although part of the effect observed with these hormones is mediated by the production of IGF-1, which by itself, has a direct effect on osteoblasts. Several peptide growth factors also evoke responses from osteoblasts through receptors, such as epidermal growth factor (EGF), TGF-α, TGF-β, and platelet-derived growth factor (PDGF). A family of cytokines, that were initially thought to only act on the immune system has been found to contain several members that are not only produced by osteoblasts but also have substantial action on bone. This group includes interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factors (TNFs) α and β.

For many years, the activation of resorption was thought to be mediated by PTH, PGE_2, $1,25\text{-}(OH)_2D_3$, or IL-1 via direct action of these mediators on existing osteoclasts, or on osteoclast precursors. Since the 1970s, however, a new concept has surfaced. According to this view, resorbing hormones act directly on osteoblasts, which then produce other factors that regulate osteoclast activity. This results in both bone formation and bone resorption being *coupled*. The coupling theory is based on the observation that once resorption occurs, osteoblasts respond by making more bone matrix. That is, any change in resorption or formation results in a change in the other. A hypothetical mechanism for explaining the coupling phenomenon is that

Table 6-9 *Some Noncollagenous Proteins in Bone Matrix*

Protein	Known Function	Regulation of Production by Osteoblasts
Osteocalcin	Inhibit mineralization, recruit bone-cell precursors	1,25-$(OH)_2D_3$, PTH, glucocorticoids
Osteonectin	Facilitate type I collagen mineralization, suppress rate of hydroxyapatite crystal growth, modulate cell attachment and detachment	Glucocorticoids, TGF-β, IGF-1
Osteopontin	Cell-binding activity, osteoclast-anchoring activity, mineral-binding activity	1,25-$(OH)_2D_3$, TGF-β, retinoic acid, glucocorticoids, PTH
Bone sialoprotein	Cell-binding activity	Glucocorticoids, 1,25-$(OH)_2D_3$
Bone proteoglycan (biglycan)	Function unclear	Not well characterized
Bone proteoglycan II (decorin)	Bind to collagen fibers, regulate fiber growth, bind/present growth factors in matrix	Not well characterized
Thrombospondin	Bind and organize matrix, cell attachment	TGF-β
Matrix gla-protein	Prevent growth plate mineralization	Retinoic acid, 1,25-$(OH)_2D_3$

resorbing bone produces a factor(s) that influences the rate and/or extent of osteoblastic activity.[234] It now seems likely that a discrete coupling factor does not exist, but that the coupling process may be mediated by a number of factors.

Recently, TGF-β has been considered as one of the possible factors active in this process. Latent TGF-β is produced by osteoblasts and deposited in the extracellular matrix. As resorption takes place, latent TGF-β is released from the matrix. Concomitantly, PTH and other bone-resorbing hormones increase production and release of enzymes such as plasminogen activator by osteoblasts, which activate latent TGF-β. Alternatively, proteinases released by the osteoclast during resorption may also activate latent TGF-β. In either case, active TGF-β then enhances osteoblast differentiation and matrix formation and inhibits osteoclast activity. A scheme illustrating how TGF-β may regulate bone resorption and formation is shown in Figure 6-15.

Proteolysis and increased cellular activity are found at sites of normal and pathological tissue remodeling. There is considerable evidence that osteoblasts are responsible for the production of proteinases, enzymes capable of mediating the localized turnover of both unmineralized and demineralized bone matrix. These enzymes are different from the enzymes produced by osteoclasts, which are responsible for direct action on bone matrix at acid pH. The proteinases that osteoblasts produce are active at neutral pH and include collagenase and plasminogen activator. Some of the proteinases produced by osteoblasts are present in extracellular matrix vesicles. Their release during maturation of osteoid may be important for mineralization of the matrix, as well as for latent growth factor activation.

The Osteoclast

The osteoclast is the cell responsible for resorption of the extracellular bone matrix. Under normal conditions, bone resorption plays a major role in the homeostasis of both the skeleton and serum calcium level. Further, bone resorption is also essential for the proper growth and remodeling of bone and is tightly coupled to the process of bone formation by osteoblasts. The correct functioning of this coupled process leads to the maintenance of the skeleton. When the balance between bone resorption and formation is disturbed during growth, repair after trauma, or regeneration during periodontal treatment, a change in size and shape of the individual bone will occur.

Changes in the coupling balance also result in various pathologic conditions. For example, osteopetrosis and osteosclerosis are characterized by dense bone and

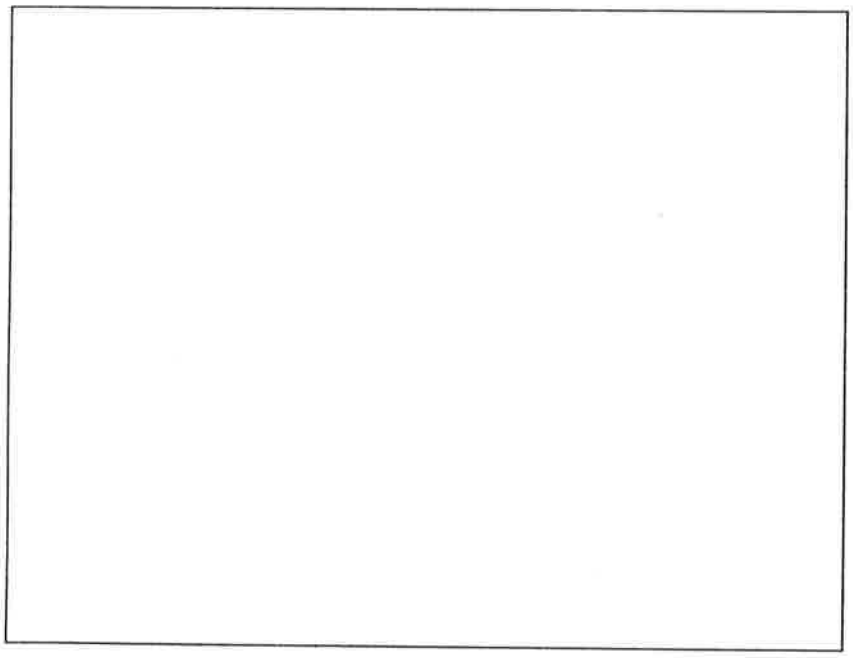


Fig 6-15 Osteoclasts secrete proteolytic enzymes, such as plasminogen activator, or acids that activate latent TGF-β in the extracellular matrix. Active TGF-β may then stimulate osteoblast differentiation and matrix formation and inhibit osteoclast activity. This model explains how TGF-β may act as a coupling factor to regulate osteoclast and osteoblast activities in bone modeling (see Fig 6-14).

osteoporosis by porous bone. Faulty coupling can also result in high resorption, as seen in high bone-turnover diseases like hyperparathyroidism and Paget's disease.

Characteristics. Osteoclasts have proven difficult to characterize. They are relatively rare in bone and cover only 1% of the bone surface; they are terminally differentiated and do not proliferate; they are attached to the mineralized matrix; and they are fragile due to their large size. Only recently have reliable methods been developed for the isolation and culture of these cells from bone[235,236] or bone-marrow cultures.[237]

The osteoclast is a highly motile cell that attaches to, and migrates along, the interface between bone and the bone marrow (endosteum). It is generally a multinucleated cell (although mononuclear osteoclasts are also encountered), formed by the asynchronous fusion of mononuclear precursors derived from the bone marrow and differentiating within the granulocyte-macrophage lineage. When activated, the osteoclast attaches to the mineralized bone matrix by forming a tight, ring-like zone of adhesion called the sealing zone, involving a specific interaction between the cell membrane and specific bone matrix proteins (Fig 6-16).

The space contained inside this ring of attachment and between the osteoclast and the bone matrix constitutes the bone-resorbing compartment. The osteoclast synthesizes several proteolytic enzymes, which are then vectorially transported and secreted into this extracellular bone-resorbing compartment. Simultaneously, the osteoclast lowers the pH of this compartment by extruding protons across its apical membrane (facing the bone matrix). The concerted action of the enzymes and the low pH in the bone-resorbing compartment lead to the dissolution of the mineral phase and digestion of the organic phase of the bone extracellular matrix. After resorbing to a certain depth, determined by mechanisms that remain to be elucidated, the osteoclast detaches and moves along the bone surface before reattaching and forming another resorption pit.

From this brief description, it is apparent that the activated osteoclast is a morphologically and functionally polarized cell, with one pole facing the bone matrix, toward which most of the secretion is targeted (the apical pole), and a pole facing the soft tissues in the local microenvironment (bone marrow or periosteum), which provides mostly regulatory functions (the basolateral pole).

The osteoclast is usually found singly or in low numbers at any one given time and site, characteristically at the interface between soft and calcified tissues, in an area where the bone matrix is fully mineralized. It is usually found on the periosteal surface, although most of the remodeling occurs along the endosteum. The osteoclast is easily characterized by its size, 50 to 100 μm, its multinucleation (usually 2 to 10 nuclei), and its presence within resorption lacuna at the calcified matrix–bone marrow interface. The apical area of the cell, closest to the matrix, is characterized by a densely stained attachment apparatus and a lightly stained,

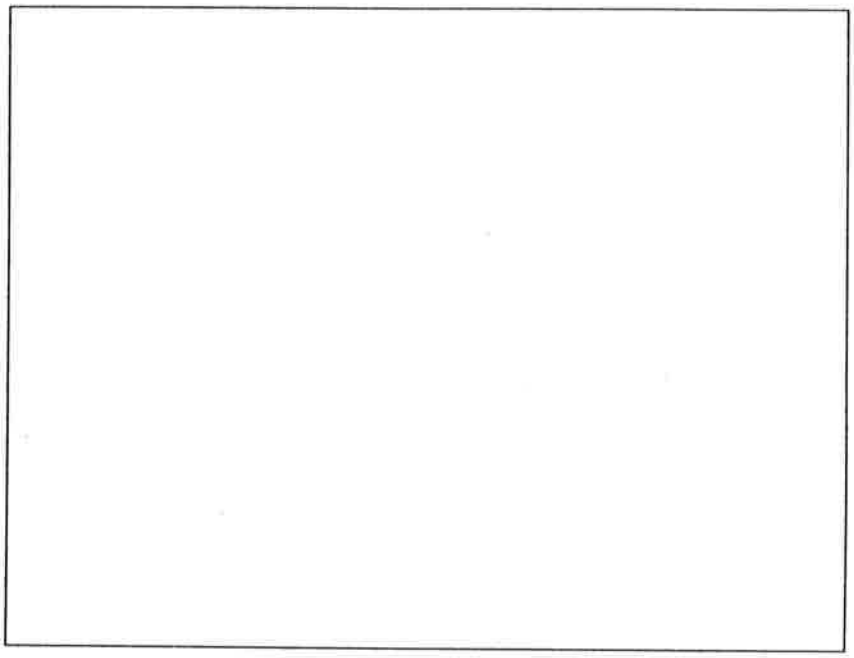


Fig 6-16 Longitudinal section through an activated osteoclast. Note the sealing zone, which delimits the bone-resorbing compartment along the apical membrane.

highly vacuolated, and striated central area called the ruffled border. At the ultrastructural level, morphological polarity of the cell is evident[238,239] (see Fig 6-16). The peripheral region of the apical membrane is tightly juxtaposed to the matrix and is called the sealing zone.[240] In the adjacent cytoplasm, an organelle-free area is found, called the clear zone, which is characteristically enriched in contractile protein. The osteoclast has an enlarged, well-developed Golgi apparatus that is actively engaged in biosynthesis and secretion of proteins.[239]

Activity. The structural features of the osteoclast that have been described above are each reflections of a specific function. The clear zone and the sealing zone are responsible for the attachment of the osteoclast to the bone matrix; the ruffled border corresponds to the area of ion transport and protein secretion; and the basolateral membrane is a major site for receipt and integration of regulatory signals.

Inside the osteoclast, an extensive cytoskeleton, composed of actin filaments and various actin-binding proteins, can be observed. This is involved in osteoclast migration and adhesion to bone and other surfaces. It has been reported that osteoclasts migrating across a surface (bone or glass) display a pattern characteristic of actin filaments.[241]

The cytoskeletal complex of the clear zone is necessary for anchoring and stabilizing the osteoclast on the bone surface. However, because the cytoskeletal complex is inside the cells, this complex is not directly responsible for that interaction. This role is filled by integral membrane proteins whose cytoplasmic domains interact with the cytoskeleton and whose extracellular domains interact with the appropriate bone-matrix proteins. These transmembrane proteins are members of the integrin family of adhesion molecules, which mediate cell-substratum and cell-cell interactions.[242] When osteoclasts are either activated or inhibited, rapid and dramatic changes occur in the cytoskeleton and attachment structures, further demonstrating the functional importance of these structures in bone resorption. Moreover, some of these changes might be associated with the regulation of the osteoclast's intracellular calcium level and/or pH.[243] Hence, the cytoskeleton and integral membrane receptors of the integrin family play essential roles in osteoclast motility, in specific attachment to the bone surface, in establishing the seal at the periphery of the extracellular bone-resorbing compartment, and in regulating the activity of the osteoclast.

The osteoclast is actively engaged in the synthesis of lysosomal enzymes that proceed through the Golgi and are transported from the trans-Golgi region to the ruffled-border apical membrane in coated transport vesicles. These transport vesicles then fuse exclusively with the ruffled border's plasma membrane and release their contents into the bone-resorbing compartment.[238,239] The enzymes secreted by the osteoclast into the bone-resorbing compartment participate in the degradation of the extracellular matrix and include acid phosphatase,

aryl-sulfatase, β–glucuronidase, and several cysteine proteinases such as cathepsins B and L.[244] The major importance of these proteolytic enzymes is that they are capable of degrading helical collagen in an acidic environment.[245] Recently, it has also been found that tissue-plasminogen activator,[246] as well as collagenase (matrix metalloproteinase-1),[247] are produced by osteoclasts, suggesting a role for these two proteinases in bone resorption. In addition to the secretion of enzymes and protons, osteoclasts, like other cells in the monocyte-macrophage lineage, synthesize and secrete lysozyme.[248]

Acidification of the extracellular bone-resorbing compartment has emerged over the last few years as one of the most important features of osteoclast biology.[249,250] The proton pumps in the osteoclast allow for acidification of extracellular fluids. During bone resorption, osteoclast-mediated acidification is required for the dissolution of the mineral phase and the enzymatic degradation of the organic phase of the extracellular matrix.[238,251] The acidification process directly involves two components: the apical electrogenic proton pump itself and carbonic anhydrase II. Briefly, the protons transported by the H^+ ATPase across the apical membrane are generated in the cytoplasm by the reversible hydration of CO_2 to produce carbonic acid, which ionizes to form protons and HCO_3.[252] In conclusion, bone resorption includes an initial phase characterized by acidification, which is required for the dissolution of the mineral phase, followed by a degradation phase involving cysteine proteinases, which degrade the bulk of the collagen present in the acidified bone-resorbing compartment.

In contrast, collagenase and metalloproteinase action occurs at a more neutral pH. Consequently, if these enzymes are involved in bone resorption, it is likely that they are activated by enzymes released by the osteoclast or by the low pH in the resorption compartment. Alternatively, they may function after displacement of the cell from its previous resorbing site and subsequent neutralization of pH.

There are several obvious reasons why calcium is of major importance in the regulation of osteoclast function. First, the cell's activity is directly and indirectly regulated by several calciotropic hormones that function in calcium homeostasis. Second, the osteoclast is probably exposed to the highest local calcium concentrations of any cell in the body due to the dissolution of hydroxyapatite crystals in the acidic microenvironment of the bone-resorbing compartment. Third, bone resorption by the osteoclast is the major mechanism whereby calcium is mobilized from the skeleton, maintaining the correct calcium concentration in the extracellular fluids of the body.

In recent years, it has been suggested that hormones and/or local factors activate osteoclasts to start bone resorption. When the calcium concentration in the bone-resorbing compartment reaches a threshold level, calcium sensors open a novel type of calcium channel.[253,254] The increase in intracellular Ca^{+2} causes the osteoclasts to become inactivated, leading to osteoclast detachment. This allows the mobilized extracellular calcium to diffuse into the extracellular fluids. Later, the osteoclasts reattach and go through a second cycle of resorbing activity. This scenario provides a logical explanation for the cyclic activity of the osteoclast, as well as the multilacunar nature of resorption sites seen in vivo and in vitro.

Factors Regulating Bone Formation

The formation of new bone consists of two major steps that include the production of a new organic matrix by the osteoblasts and the mineralization of that matrix. Agents that regulate bone formation act on the osteoblast to either increase or decrease replication of cells in the osteoblastic lineage or to modify the differentiated function of the osteoblast. As outlined above, bone formation is controlled by systemic hormones and local factors. For the most part, the local regulators of bone formation are growth factors that act directly on cells of the osteoblastic lineage. These local factors may affect their cell of origin or different cells, and thus act as either autocrine or paracrine factors, respectively.[255]

While systemic hormones are likely to have direct effects on the bone-forming cells, they frequently act by stimulating the production of local growth factors. Systemic hormones can regulate growth-factor activity by one or more of four different mechanisms: 1) by regulating factor synthesis, as well as release from cells; 2) if the factor is released from the cell in a latent form, its physiologic effect can be regulated by controlling when the factor becomes activated; 3) by regulating receptor binding; and 4) by regulating the production of a binding protein that stabilizes the factor and promotes its binding to the receptor. Since the production of a particular growth factor is not unique to any specific tissue, it has been postulated that systemic hormones may provide target tissue specificity for a growth factor.

Growth factors that regulate bone formation have some common features: they are polypeptides; they exert their activity by binding to specific receptors on the cell surface; they primarily act locally; they are natural products of cells; and they are multifunctional in that they can stimulate a wide variety of cellular activities. In cases such as periodontal regeneration, the

combined effect of many growth factors is involved.[256]

This section addresses the more "classic" growth factors synthesized by connective-tissue cells and includes platelet-derived growth factor, insulin-like growth factors I and II, transforming growth factors β1 and β2, fibroblast growth factors, heparin-binding growth factors 1 and 2, and bone morphogenetic proteins.

Platelet-derived growth factor (PDGF). PDGF is a cationic, heparin-binding polypeptide with a molecular weight of approximately 30,000 daltons. The active growth factor consists of a disulfide-linked dimer, which is the product of two distinct genes, PDGF-A and PDGF-B.[257] Three isoforms exist: PDGF-AA, PDGF-AB, and PDGF-BB. The most biologically active form in skeletal tissues is PDGF-BB. PDGF is produced by osteoblasts,[258,259] but much of the PDGF found in bone is probably derived from serum and platelets.

PDGF stimulates DNA synthesis and cell replication in osteoblasts,[255] as well as increases bone-collagen synthesis and the rate of bone-matrix apposition.[260] In addition to its effect on bone formation, it has also been reported to increase bone resorption and collagen degradation,[255] although the mechanism for these effects is not clear.

A PDGF receptor containing two subunits has been found. PDGF binds to one or both subunits and forms a complex as part of the receptor-activation process. Only recently has it been found that a PDGF receptor is present in osteoblasts.[261] While little is known about the regulation of PDGF-AA synthesis, there is some evidence suggesting that PDGF-AA activity is regulated at the level of receptor binding. PDGF-BB might be critical in wound healing or fracture repair, since it is released after platelet aggregation. It may also play an entirely different role in bone-cell physiology than that of PDGF-AA.

Heparin-binding growth factors (HBGFs). HBGFs are members of a family of seven related heparin-binding proteins.[262] Acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) are the two better known forms of the HBGFs and were the first to be purified, sequenced, and cloned.

Bone matrix is a rich source of FGFs, and bovine skeletal cells, expressing the osteoblastic phenotype, secrete basic, as well as acidic, FGF.[263] Both FGFs have been shown to be mitogenic for bone cells and to enhance collagen and noncollagen protein synthesis in bone culture. In bone cells, FGF interacts with heparin, which enhances the effect of FGF on bone-cell replication. Therefore, FGFs, and HBGFs generally, have significant effects on bone-cell replication, but their specific function in bone-cell biology and their role as therapeutic agents needs further study.

Insulin-like growth factors (IGFs). IGFs are non-glycosylated polypeptides with molecular weights of approximately 7,500 daltons. There are two forms of IGF: IGF-I, initially termed somatomedin C, and IGF-II, initially termed multiplication-stimulating activity. IGF-I appears to be the principal growth regulator in bone and cartilage. The liver is the major source of circulating IGF-I and is the target tissue for growth hormone that regulates IGF-I production. The role of systemic IGF-I is not entirely clear, since most tissues synthesize small amounts of this growth factor. In connective tissues, IGF-I and IGF-II are among the most abundant growth factors present[264] and are synthesized by most cell types present in skeletal tissue, including bone fibroblasts and osteoblasts.[265] Therefore, IGFs probably act as either paracrine or autocrine regulators of bone formation. Production of IGFs is increased by hormones such as growth hormone, estradiol, and local factors like PGE_2,[264] whereas production is inhibited by cortisol. Both forms of IGF increase preosteoblastic cell replication and have a stimulatory effect on osteoblastic collagen synthesis and bone-matrix apposition,[266] and decrease the degradation of collagen. IGFs play a major role in the maintenance of bone mass. Osteoblasts express both the IGF-type 1 and -type 2 receptors,[267] and it has been suggested that the type 1 receptor mediates the effect of IGF on bone formation. IGF is probably one of the most important regulators of bone mass because it is synthesized by bone cells and is present in substantial concentrations in the bone.

Transforming growth factor–beta (TGF-β). Transforming growth factors are polypeptides that have been isolated from a variety of normal and malignant tissues. They were initially identified by their ability to stimulate in non-neoplastic cells what appears to be a growth habit characteristic of malignant cells. TGF-α is not synthesized by bone cells, but because it stimulates bone resorption, it may play a role in the development of hypercalcemia in some forms of malignancy. TGF-β is a polypeptide with an approximate molecular weight of 25,000 daltons that is synthesized by skeletal cells. It is one of the most abundant growth factors in bone matrix.[268] At present, at least five isoforms have been identified, as well as a number of polypeptides, that show significant homology with TGF-β and form the TGF-β superfamily of polypeptides. Among them are the bone morphogenetic proteins (BMPs), the activins, and the inhibins. Activin and TGF-β have similar stimulatory effects on bone formation.[269] TGF-β is synthesized

by osteoblasts in inactive (latent) form. A major step regulating TGF-β activity involves its conversion to a biologically active peptide; this can be accomplished in vivo and in vitro by brief acidification or proteolytic cleavage. TGF-β has been shown to stimulate pre-osteoblastic cell replication, osteoblastic collagen synthesis,[270] and bone-matrix apposition.

Bone cells have three discrete TGF-β receptors. The effects of TGF-β on bone-cell function can be regulated by altering the binding of the growth factor to its receptors.[269] Treatment with glucocorticoids decreases the effect of TGF-β on DNA and collagen synthesis in bone cells. The mechanism of this action seems to be related to shifting of TGF-β binding away from active receptors and toward the complex.[269]

In addition to its direct effect on bone-cell function, TGF-β has important interactions with other growth factors. TGF-β mRNA levels are increased by FGF, whereas TGF-β decreases PDGF-AA binding to its bone-cell receptor. TGF-β has been shown to initiate and regulate critical events during fracture repair.[271] Furthermore, cells within the fracture callus have been shown to express TGF-β mRNA, and this factor may be induced and act at the local level during fracture healing.

Bone-morphogenetic proteins (BMPs). During embryogenesis, new bone formation occurs through a complex series of cellular interactions. Most bones of the body initially go through a phase of cartilage formation and calcification, and then replacement by bone in a process known as endochondral bone formation. Interestingly, bone repair in adults is very similar to this process. During bone repair and bone-matrix destruction, many growth factors are released that affect the healing process. One of them is a unique factor with osteoinductive activity, BMP. Since its original discovery, it has been found that what was originally believed to be one protein actually belongs to a family of proteins consisting of at least seven different members (BMP1 through BMP7).

The implantation of BMP in ectopic sites, as well as in bone, induces the production of new bone through an endochondral pathway. BMP has a direct effect on osteoblasts by stimulating the differentiation of osteoblast precursor cells into more mature osteoblasts. Further, BMP has also been shown to stimulate collagen production by mature osteoblasts. In the endochondral pathway, BMP induces chondrocyte differentiation and matrix mineralization.

Factors Regulating Bone Resorption

In recent years, it has become increasingly clear that many of the cellular events involved in bone resorption are modulated by a group of local factors (osteotropic cytokines), which have extremely potent effects on bone cells in both in vitro and in vivo systems. Many of the effects of local factors on bone resorption appear to be overlapping and are seemingly redundant; for example, interleukin-1 (IL-1), tumor necrosis factor–alpha (TNF-α), and lymphotoxin appear to affect bone resorption similarly. As we learn more about the mechanism of bone resorption, the role of each factor in the process will become clearer.

Cytokine regulation is likely to be more important for trabecular bone than for cortical bone because trabecular bone is closer to the marrow, which is a rich source of cytokines. Many of the potent osteotropic cytokines, such as IL-1, IL-6, TNF-α, and TGF-β, mediate a multiplicity of effects in the body in addition to their effects on bone cells. However, there are other cytokines, such as BMP, that have relatively specific effects on bone cells. Most of the osteotropic cytokines, such as IL-1, TNF, lymphotoxin, and IL-6, are clearly products of immune cells that are present in regions where bone modeling is actively occurring. However, stromal cells, as well as bone cells, also produce these factors.[272] The production of cytokines by osteoblasts is regulated by bacteria, lipopolysaccharide, other cytokines, and different hormones (estrogen and steroids). It has also been observed that cytokines and hormones can have synergistic effects on bone resorption. For example, the effect of IL-1 together with PTH on bone resorption is greater than the additive effects of either IL-1 or PTH alone.[273]

The hypothesis that local factors play a major role in regulating bone remodeling has been strengthened by recent data that directly implicate at least one of these cytokines in normal osteoclastic bone resorption. In mice with the op/op variant of osteopetrosis, there is a defect in the region coding for colony-stimulating factor (CSF) of the macrophage series (CSF-M), resulting in decreased CSF-M production. In this variant, the mice do not form functional osteoclasts. As a result, they do not form marrow cavities properly, and they develop osteopetrosis. If the mice are treated with CSF-M, the disease is reversed.[274]

Cytokines appear to play a major role in bone pathology. Solid tumors and carcinomas have been shown to produce IL-1.[275] Cytokines have also been associated with the bone destruction seen in chronic inflammatory conditions, such as rheumatoid arthritis and periodontal disease. The observed increase in cytokines during these

diseases, has been suggested as the cause of increased localized osteolytic bone destruction.

Interleukin-1 (IL-1). IL-1 is a powerful and potent bone-resorbing cytokine.[276] It has been found that IL-1α and IL-1β are equally potent in stimulating bone resorption and probably exert their effects on bone-resorbing cells in several ways. They stimulate proliferation of precursor cells, but also probably act indirectly on mature cells to stimulate bone resorption.[277] The effects of IL-1 probably occur by two mechanisms. One mechanism is the stimulation of the production and release of PGE_2, which in turn stimulates bone resorption. The second mechanism involves the direct action of IL-1 on the osteoclast, which is independent of prostaglandin synthesis, through an 80,000-dalton receptor.

IL-1 has complex and apparently paradoxical effects on bone formation. The continued presence of IL-1 inhibits bone formation in vivo and in vitro.[278,279] IL-1 appears to stimulate proliferation of cells at early stages of differentiation in the osteoblast lineage, but inhibits functions characteristic of the fully differentiated state. In contrast, transient exposure to IL-1 has been shown to stimulate bone formation by osteoblasts.

Interleukin-6 (IL-6). In some experimental models, IL-6 appears to have no effects on bone resorption. However, in others,[280] it stimulates bone resorption. IL-6 is also responsible for the formation of cells with an osteoclastic phenotype. Bone cells also have the ability to produce IL-6, which seems to be greater when the stimulus is by another cytokine.

Tumor necrosis factor (TNF) and lymphotoxin. Lymphotoxin and TNF are two closely related cytokines that have equivalent effects on bone cells. They are both multifunctional cytokines produced by activated lymphocytes, and they share the same receptor. Their major effect on bone is to stimulate osteoclastic bone resorption.[281] It has been suggested that part of the effect of TNF is mediated by PGE_2, as well as by IL-6. TNF also affects cells with osteoblast phenotypes and inhibits differentiated function and stimulates cell proliferation. Production of TNF in some tumors, like squamous cell carcinomas, may be responsible for paraneoplastic syndromes.

Gamma interferon (IFN-γ). Gamma interferon is a multifunctional cytokine, which in most biological systems has effects similar to TNF or IL-1. However, it has an effect on bone resorption that is opposite that of IL-1 and TNF. Gamma interferon is more effective in inhibiting IL-1– or TNF-induced bone resorption than systemic hormones like PTH or $1,25\text{-}(OH)_2D_3$. Further, it has been found in long-term marrow cell cultures that gamma interferon inhibits the formation of cells with the osteoclast phenotype.

Colony-stimulating factors (CSFs). CSF has the ability to stimulate differentiation of osteoclast precursors into mature osteoclasts. Recently, it was found that there are a number of human and animal tumors associated with granulocytosis in which increased production of CSFs are involved. In many of these tumors, hypercalcemia is associated with increased bone resorption.

It is possible that CSFs mediate their effects on osteoclast formation indirectly. For example, early studies showed that CSF stimulates IL-1 production, which stimulates prostaglandin synthesis.

Prostaglandins and other arachidonic-acid metabolites. A number of arachidonic-acid metabolites act as modulators of bone-cell function. These factors are produced by immune, marrow, and bone cells. Prostaglandins of the E series were some of the first-described and best-tested stimulators of osteoclastic bone resorption. PGEs are slow-acting, but powerful, mediators of bone resorption and affect both active mature osteoclasts, as well as differentiated osteoclast precursors. The effect of PGE is local and has been shown to mediate the effects of other factors like epidermal growth factor (EGF) and transforming growth factor–β (TGF-β). PGE is produced by osteoblasts and has effects not just on bone resorption, but on bone formation as well. In vitro it has been found that high doses of PGE are inhibitory, while low doses stimulate bone formation. However, in vivo it appears that the effect of PGE is clearly associated with an increase in periosteal bone formation.[282] There have also been recent reports that other arachidonic-acid metabolites stimulate bone resorption. Arachidonic acid can be metabolized by an alternative enzyme system, 5-lipoxygenase, which also produces metabolites capable of stimulating bone resorption.

Regulation of Bone by Systemic Hormones

Parathyroid hormone (PTH). It has been known for 70 years that PTH affects bone-cell function, may alter bone remodeling, and causes bone loss. It is now apparent that PTH acts on both bone-resorbing cells and bone-forming cells. The net effect of the hormone depends on whether it is administered continuously or intermittently. When administered continuously, it

increases osteoclastic bone resorption and suppresses bone formation. However, when administered in low doses intermittently, its major effect is to stimulate bone formation, a response that has been called the anabolic effect of PTH.

PTH stimulates osteoclasts to resorb bone. In patients with primary hyperparathyroidism, there is a profound loss of bone associated with increased osteoclastic bone resorption (a disease referred to as *osteitis fibrosa cystica)*. In organ cultures, PTH increases osteoclast activity, with resultant degradation of bone matrix and release of bone mineral.[283] In culture, parathyroid hormone produces a prolonged period of bone resorption after only four to six hours of treatment, while other agents, such as PGE_2 or 1,25-$(OH)_2D_3$, require a longer period of incubation. These phenomena can be explained by the fact that PTH activates mature osteoclasts to resorb bone, whereas other agents exert their effects by increasing the formation of new osteoclasts.

In the in vivo situation, bone cells are never exposed to PTH alone, and other local factors and hormones are always present and interact with PTH. PTH probably acts at multiple points in the osteoclast lineage. It clearly stimulates mature, multinucleated osteoclasts to form ruffled borders and resorb bone. In addition, it has effects on cells earlier in the osteoclast lineage. However, the precise molecular mechanisms by which PTH exerts its effects on these cells is still not known. The effect of PTH on osteoclast precursors is combined with direct effects on the cell itself, with an indirect effect of regulating other cells to produce local factors that influence the osteoclast precursor cells, such as regulating cells of the granulocyte-macrophage type, to produce granulocyte-monocyte–colony-stimulating factor (GM-CSF).

The effect of PTH on mature osteoclasts is also indirect because osteoclasts will not resorb bone unless osteoblasts are present, suggesting that PTH may stimulate osteoclastic bone resorption by interacting with cells in the osteoblast lineage.

PTH responsiveness is used as one of the criteria for characterizing cells of the osteoblast lineage. The response of osteoblasts to PTH includes an increase in adenylate cyclase activity, changes in proliferation, alkaline phosphatase activity, and production of type I collagen. The effect of PTH depends on its concentration. At low concentrations, the effect is anabolic, and at high concentrations, the effect is catabolic.[284] One mechanism by which PTH enhances bone turnover or remodeling in vivo is by promoting the production of coupling factors.[234]

The mechanisms used by cells of the osteoblast phenotype to communicate with osteoclasts are still not known, but may involve the production of soluble mediators. It has been suggested that osteoblasts may prepare the bone surface for osteoclastic bone resorption by producing proteolytic enzymes. However, this theory is still questionable.

In summary, the effects of PTH on bone-forming and bone-resorbing cells are complex. Although much information is known regarding the expression of PTH and regulation of PTH synthesis and secretion in the parathyroid cell, there is still much to be learned about the mechanisms by which PTH stimulates osteoclasts and osteoblasts and the relationship between these effects and maintenance of normal bone volume and control of calcium homeostasis.

1,25-Dihydroxyvitamin D_3 (1,25). The active metabolites of vitamin D_3 have complex effects on calcium homeostasis and bone regulation. 1,25 and 24,25-$(OH)_2D_3$ (24,25) are active metabolites of vitamin D that have been shown to directly affect bone-cell function. 1,25 has a catabolic effect on bone, while 24,25 has an anabolic effect on bone. Both hormones promote the absorption of calcium and phosphate from the gut. 1,25 also stimulates osteoclastic bone resorption in vitro and in vivo. In the absence of vitamin D, there is a failure of mineralization. This leads to rickets in children and osteomalacia in adults. A receptor for 1,25 has been identified and characterized.[285]

The effect of 1,25 on osteoclastic bone resorption is different from that of other well-known bone-resorbing factors. 1,25 has a very slow onset of action, with a shallow dose-response curve. 1,25 increases both osteoclast number and activity, with an increase in ruffled-order size and clear-zone volume. Mature osteoclasts do not have receptors for 1,25; thus, the effects of this hormone on mature osteoclasts are most likely mediated indirectly through other cells.[286] The major effect of 1,25 on osteoclastic bone resorption may be to stimulate the fusion or differentiation of committed osteoclast progenitors to form mature cells. Use of 1,25 to treat of infants with malignant osteopetrosis demonstrates that failure to form competent osteoclasts can be successfully treated. 1,25 also has other effects on bone cells that may be indirect.[287] For example, 1,25 influences and modulates cytokine production by immune cells. Given the potency of many cytokines as regulators of osteoclast function, the importance of 1,25 for osteoclastic bone resorption is probably great.

Calcitonin. Although calcitonin has been known for 30 years, there is little insight into its physiological role in calcium homeostasis or its importance in the bone remodeling process. Friedman et al[288] demonstrated that

calcitonin was able to inhibit osteoclastic bone resorption. The effect of calcitonin on osteoclasts is mediated through cyclic AMP. Calcitonin decreases osteoclast activity, as can be seen within minutes after treatment by decreases in ruffled-border size and clear zone.[289] Other studies show that calcitonin inhibits the formation of new osteoclasts and causes the dedifferentiation of osteoclasts into mononuclear cells.

The effects of calcitonin on bone resorption are short-lived, however. Osteoclasts eventually lose their responsiveness to calcitonin after continuous exposure, a phenomenon referred to as *escape.*[290] The molecular mechanism for escape has never been clearly demonstrated. One explanation for this phenomenon may involve a decrease in receptor number after long periods of exposure. Another possible explanation is that a second population of osteoclasts, which is not responsive to calcitonin, emerges.[291] Since calcitonin has only a transient effect on bone resorption, there has been considerable speculation as to whether it has an important role in calcium homeostasis. Today, it is believed that it inhibits bone resorption transiently when bone turnover is not needed for calcium homeostasis (as after a meal rich with calcium). A receptor for calcitonin has been identified in osteoclasts and shown to directly mediate the effect of calcitonin on these cells.[292]

Estrogens. Estrogen clearly inhibits the increase in bone resorption associated with menopause. Following estrogen withdrawal, an initial increase in bone turnover can be observed. Later, bone resorption occurs faster than bone formation, with a net effect of bone loss. Treatment with estrogen prevents these effects.[293,294]

The effects of estrogen are mediated by a combination of direct and indirect effects. The direct effect is mediated by specific receptors found in cells of the osteoblast[295] and osteoclast[296] lineages. The effects of estrogen on osteoclasts are in part direct and in part mediated through osteoblasts. Some indirect effects of estrogen result from its enhancing the expression of growth factors like IGF-1 and TGF-β, and of cytokines, others from its inhibition of prostaglandin production by bone cells.

It is also possible that estrogen has a prolonged effect in stimulating bone formation. Low doses of estrogen increase skeletal growth in children, and it is clear that there is a marked increase in endosteal bone formation in rodents and birds treated with estrogens. Estrogen has now been shown to enhance the expression of TGF-β and IGF-1 in cells with the osteoblast phenotype.[295,297] Thus, the major effect of estrogen may be to inhibit osteoclastic bone resorption, but it may also have the additional effect of stimulating bone formation.

Androgen's effect on osteoclastic bone resorption is probably similar to estrogen's. Hypogonadal or castrated males have decreased bone mass associated with increased bone resorption.

Summary

Bone is a very active organ. In the adult, it is continuously renewed and capable of complete repair after injury. Both of these processes involve opposing events —the dissolution of existing mineral content with resorption of the extracellular matrix, and the formation of a new matrix. Distinct cell lineages are responsible for bone resorption and formation. The osteoclast lineage is of hematopoietic origin and includes the differentiated multinucleated osteoclast, which is the primary bone-resorbing cell. The osteoblast, which forms bone matrix, appears to originate from a stromal cell within the bone-marrow cavity. Mechanisms that couple osteoclastic and osteoblastic activities must exist within the bone. The process of bone resorption and bone formation are very carefully regulated by both systemic hormones and local factors. Changes in the normal balance of bone modeling and remodeling will cause diseases such as periodontal disease. In periodontal disease, the response of bone to local factors, produced by the inflammatory process, changes the bone-remodeling balance, with a net effect of bone resorption and loss of attachment.

Periodontal tissue can also repair and regenerate itself. Periodontal regeneration involves the reconstruction of lost supporting tissues, including alveolar bone, cementum, periodontal ligament, and gingival attachment. This process is regulated by the local production of growth factors. These factors stimulate the cellular events of regeneration, which potentially consist of cell chemotaxis, proliferation, differentiation, and formation of extracellular matrix, and will lead to new bone formation and new attachment.

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X. CONCLUSIONS

Studies on the proteoglycans of the periodontium first began in the 1950's with early histochemical identification in gingival biopsies of material identified as "mucopolysacchardes". It is indeed interesting to note that one of the early pioneers in this field was Dr Margaret Dewar, working from the Institute for Dental Research in Sydney, who published a paper titled *Observations on the composition and metabolism of normal and inflamed gingivae* in 1955. The "Australian Connection" with periodontal connective tissue research continued with the seminal work of Professor John Thonard during the 1960' in which special histochemical techniques were used to locate various mucopolysaccharides to both the gingival connective tissues and gingival epithelium. By the 1970's, biochemical techniques had been developed and refined which allowed the definitive identification of glycosaminoglycans in tissue extracts of human gingival and periodontal ligament. It was at this time that I commenced my PhD studies and pioneered the biochemical isolation, identification and characterization of proteoglycans in human gingival epithelium and connective tissue. During the 1980's the application of cell culture became very useful for the determination of cell synthesis of proteoglycans, and the study of these molecules at the cellular and molecular level became possible. Antibodies (both polyclonal and monoclonal) also became available which enabled precise immunolocalizations to be made within the tissues. In addition, these antibodies improved the means of isolating and identifying particular proteoglycan molecular species. The development of molecular biology, and the availability of specific molecular probes, has further refined the definitive analyses of proteoglycan synthesis and its regulation at the genetic level. By using these techniques, the effects of inflammation in the periodontium on the proteoglycan content, structure and distribution have been determined. At the present time, much of the ground work has been covered, and the stage is now set for exciting new investigations into the biological roles that these molecules play in the periodontium with respect to tissue repair and regeneration.

The papers presented in this compilation were, at the time of their publication, the first documented biochemical analyses of proteoglycans in human gingival epithelium and connective tissues (both hard and soft). In terms of contribution to the field of periodontal research, these studies have provided the basis for a more comprehensive understanding of the biochemical composition of the periodontal tissues and what happens to them following inflammatory episodes. These studies have advanced dental knowledge in the following areas:

1. The proteoglycan content and composition of the periodontal tissues have been confidently established.

2. The cellular source and types of proteoglycans made by cells of the periodontium have been established.

3. The effects of inflammation on periodontal tissue proteoglycans have been established at both the tissue and cellular level.

4. Several key elements in periodontal inflammation have been demonstrated to have a direct influence on the proteoglycans associated with the periodontal tissues.

5. Proteoglycans have been suggested to be critical determinants in the early processes associated with the regulation of periodontal regeneration by growth factors.

An expanded understanding of the molecular composition of the periodontal connective tissues is important for a number of reasons. The periodontal tissues are amongst the most metabolically active tissues in the entire human body. Given their strategic location and exposure to a wide variety of chemical and mechanical stress, it is remarkable how well these tissues remain intact. Nonetheless, disease is still common in the periodontal tissues, with all the classic signs and symptoms of chronic inflammatory disease. The delicate balance between tissue damage and tissue destruction is well recognized. Indeed, all reactions within the periodontium, whether they be pathological, reparative or regenerative, occur within a complex environment which is exquisitely sensitive to disruption as a result of matrix destruction or interference of fibroblast metabolic activity. By studying both tissue destruction, as well as the reparative processes, we are assured of developing a deeper understanding of the periodontal tissues which will ultimately lead to improved therapeutic treatments based on a sound knowledge of tissue destruction and repair. Thus the fundamental outcomes of periodontal therapy rely on a sound knowledge of these responses which enable us to recognize what constitutes an acceptable clinical result.