SYNDECAN-1 EXPRESSION DURING POSTNATAL TOOTH AND ORAL MUCOSA DEVELOPMENT IN 2 DAY TO 6 WEEK OLD RATS

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DANIEL DE ANGELIS
B.D.S. B.Sc. (Adel.)

ORTHODONTIC UNIT

SCHOOL OF DENTISTRY

FACULTY OF HEALTH SCIENCES

THE UNIVERSITY OF ADELAIDE

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2 ABSTRACT

The syndecans are a family of heparan sulphate proteoglycans that regulate cell/matrix interactions which influence cell growth, proliferation and morphology. The expression of syndecan-1 during mouse molar crown development appears to be stage-regulated by epithelial-mesenchymal interactions. The presence of syndecan-1 in the epithelium of the rat oral mucosa, and in immature dental epithelium (Hertwig's epithelial root sheath) during root development is a possibility. Further syndecan-1 expression might be detected in remnant embryonic dental epithelium (epithelial cell rests of Malassez). The aim of this study was to observe changes in the expression of syndecan-1 in both the developing epithelium of the rat oral mucosa, and in the epithelial cell rests of Malassez in the developing periodontium of normal rat molars, from late crown development through to early eruption. Immunohistochemistry (Syndecan-1 N-18) and histochemistry (Alcec Blue) was used to observe changes in the expression of syndecan-1 in 2 day to 6 week old rats. Results indicated that during normal tooth development in the rat, labelling or staining of variable intensity for syndecan-1 was demonstrated in the stratified oral epithelium above the stratum basale in rat tongue and palate, and in ameloblasts of the developing molar in 2 day and 2 week old rats. Histochemical staining of the pre-dentine and dentine layers was consistent in all specimens. Labelling or staining for syndecan-1 was negative in the rat periodontal ligament which might suggest that either syndecan-1 was not expressed during normal molar root development or that continued work is required for identification of a suitable label in rats.

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

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Daniel D. De Angelis

B.D.S. B.Sc. (Adel.)

4 INTRODUCTION AND AIM

4.1 Introduction

The cellular microenvironment, including the immediate pericellular matrix, is constantly changing and cells must maintain the ability to monitor molecular information from their external environment in order to maintain normal development and function. The syndecans have been described as a family of transmembrane proteoglycans that regulate cell/matrix interactions which influence cell growth, differentiation, proliferation, adhesion and shape (Bernfield et al., 1992). The role of syndecans during tooth formation in mice has been described. Matrix components such as collagens I, III and V, fibronectin and tenascin, bind to syndecans initiating changes in cell behaviour which is important in mesenchymal cell condensation and differentiation (Salmivirta et al., 1991; Thesleff et al., 1996).

The expression of syndecan-1 during crown formation in mouse molars has been well documented and is understood to be stage-regulated by epithelial-mesenchymal interactions (Thesleff et al., 1995; Thesleff et al., 1996). During crown formation syndecan-1 is initially expressed by embryonic dental epithelium at the bud stage. With continued tooth development syndecan-1 is lost from the dental epithelium and becomes expressed in the dental mesenchyme. Syndecan-1 is then re-expressed by dental epithelium during the bell stage (Thesleff et al., 1995; Thesleff et al., 1996). Based on this model of syndecan-1 expression by epithelium during mouse molar crown development, the presence of syndecan-1 in the epithelium of the rat oral mucosa, and in immature dental epithelium (Hertwig's epithelial root sheath) during root development is a possibility. Further syndecan-1 expression might be identified in remnant embryonic dental epithelium, specifically, in the epithelial cell rests of Malassez of the developing periodontium.

Apart from extensive studies documenting syndecan-1 expression in developing mouse oral tissues (Vainio et al., 1989; Thesleff at al., 1991; Thesleff et al., 1995; Thesleff et al., 1996), studies available describing syndecan-1 expression in developing rat oral tissues are limited.

4.2 Aim

The aim of this study was to observe changes in the expression of syndecan-1 in both the developing stratified epithelium of the rat oral mucosa, and in the epithelial cell rests of Malassez in the developing periodontium of normal rat molars, from late crown development through to early eruption.

5 LITERATURE REVIEW

5.1 The basement membrane

The basement membrane primarily originates from epithelial tissue including the enamel organ. It is located between the dental papilla mesenchyme and the inner enamel epithelium during early tooth development (Sawada, 1995) where it is directly associated with differentiation of mesenchymal cells into odontoblasts (Thesleff et al., 1979). The basement membrane comprises intrinsic components (type IV collagen, laminin and heparan sulphate proteoglycans) as well as extrinsic components (fibronectin, entactin, and collagen type I, III V) (Lesot et al., 1981; Thesleff et al., 1981; Ten Cate, 1994; Flug et al., 1995). Heparan sulphate is an important glycosaminoglycan of the mature epithelial cell basement membrane and is involved in cellular organization, including tooth formation and transmembrane permeability-significant in metastasis and tumour invasion (Jiang et al.,1996).

5.2 Structure and distribution of the syndecans

The syndecans represent a group of four integral membrane proteoglycans (syndecan-1,-2,-3 and -4) containing a core protein and a heterogeneous heparan sulphate glycosaminoglycan side chain (Gallagher et al., 1989; Bernfield et al., 1992; Jalkanen et al., 1992; Carey 1997) at the surface of adherent cells. The syndecan core protein comprises three domains, a highly conserved (C-terminal) endodomain, a transmembrane-domain; and, a matrix-communicating ectodomain possibly containing both heparan and/or chondroitin sulphate (Figure 1) (Bartold & Narayanan, (1998). The ectodomain exhibits structural diversity and it might be suggested that the syndecans have similar chemical properties only differing in their reactions due to a difference in the ectodomain (Carey 1997; Mali et al., 1990; Jalkanen et al., 1991).

The mechanisms controlling the syndecan glycosaminoglycan chain type, number, size and structure are unclear, however, the variety in glycosylation does appear to alter the properties of the proteoglycan to bind matrix molecules (Carey 1997). For instance, syndecan from mouse mammary cells binds tenascin with a low affinity in contrast to the high affinity syndecan has for tenascin in tooth development. Therefore, syndecans in different tissues exhibit different binding properties and possibly different functions.

The structure of the core protein and not the glycosaminoglycan side chains might make heparan sulphate proteoglycans such as syndecan site specific. This was supported by a

immunohistochemical study directed against the core proteins of cell surface heparan sulphate proteoglycans of normal murine mammary gland cells. In this study,

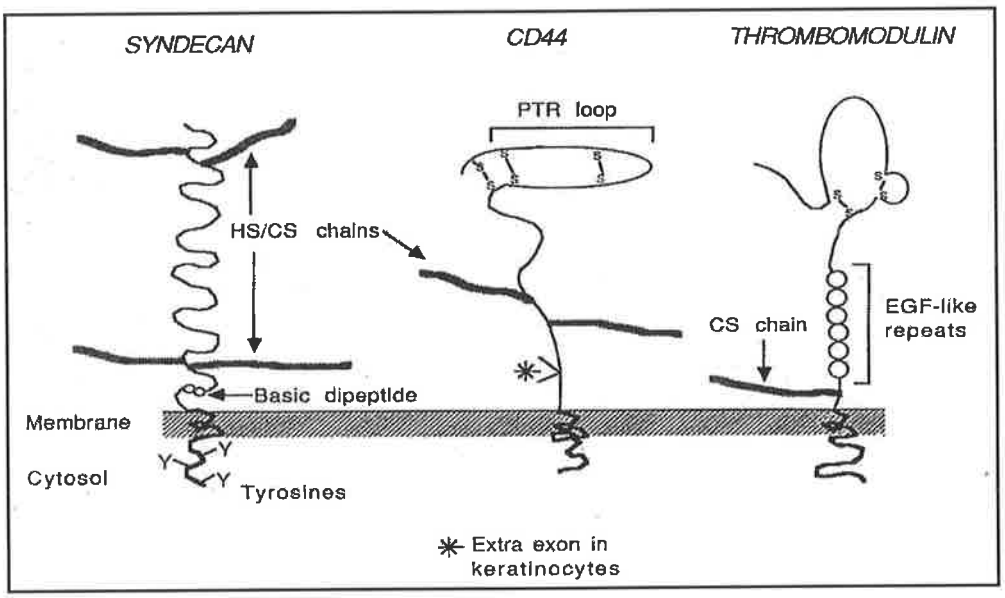


Figure 1. Schematic diagram of the structure of the cell surface syndecan displaying its three domains, a matrix communicating ectodomain, the transmembrane domain and the cytoplasmic endodomain. HS, heparan sulphate; CS, chondroitin sulphate (From Hardingham & Fosang, 1992, p. 866).

an antibody raised against the cell surface proteoglycan of normal murine mammary gland cells recognized the cell surface proteoglycan, but failed to detect the basement membrane proteoglycan. These findings suggest that although heparan sulphate-rich proteoglycans may posses similar glycosaminoglycan chains they may be sorted by epithelial cells to different sites based on the structure of their core proteins (Jalkanen et al., 1991).

In mature animal, and human tissues, syndecans are usually confined to epithelial cells including the surfaces of stratified epithelial cells. The syndecans are also localized to embryonic mesenchyme during organogenesis, including that of the tooth (Thesleff et al., 1988; Lcppa ct al., 1991; Inki et al., 1994). Syndecans are expressed by progenitor B cells in bone marrow and plasma cells suggesting a role in B cell functional development (Carey 1997; Mali et al., 1990; Vainio et al., 1991). Heparan sulphate proteoglycans have also been observed in animal cells: (1) extracellularly, in the fibroblast pericellular matrix; (2) within parenchymal cells of the basement membrane; (3) on the cell surface in mesenchyme; and, (4) intracellularly, within secretory vesicles and the endoplasmic reticulum (Jalkanen et al., 1988).

Specifically, immunohistochemical studies performed in mice show syndecans in mammary epithelia are polarized at the basolateral surfaces connecting the cellular cytoskeleton to the extracellular matrix (Mali et al., 1990). In particular, syndecan-1 is specific to the basolateral surface of epithelia in the trachea and in other epithelial cell varieties it is present over the entire cell surface such as the squamous epithelia of the tongue (Carey, 1997). Syndecans have also been cloned from human lung fibroblasts (Hardingham & Fosang, 1992).

5.3 Expression of the syndecans

During organogenesis the embryo is influenced by reciprocal interactions between mesenchyme and epithelial products (of the ectodermal and endodermal tissues) where syndecan production is stage-specific (Thesleff et al., 1991; Vainio et al., 1991; Bai et al., 1994). Studies of embryogenesis in mice show syndecan-1 is detected at the 4-cell stage. It is initially present within cells then appears at the surfaces of cells at sites of cell contact during the 8-cell stage and then the 16-32 cell stages. As the blastocyst develops syndecan-1 expression occurs at the boundary between the primitive ectoderm and endoderm, consistent with the role of syndecan-1 as an organizer of epithelia and as a matrix receptor. As development proceeds, syndecan-1 is produced at the basolateral surface of the embryonic ectoderm cells (Bernfield et al., 1992).

In the mesoderm, expression of syndecan-1 shifts from the anterior to the lateral plate mesoderm. Alternatively, syndecan expression is reduced in epithelial somites when they spread into sclerotome, myotome, and dermatome components. Subsequent tissue interactions then determine expression of syndecan-1. For instance, during tooth formation epithelial tissues express syndecan-1 during early bud stage and the mesenchyme does not. As development proceeds mesenchyme expresses cell surface syndecan-1 and proliferates to condense around the epithelial bud. The epithelial cells experience a change in shape, temporarily losing their cell surface syndecan-1 re-expressing it in the bell stage and in proliferating pre-ameloblasts (Bernfield et al., 1992). Therefore, it appears cell surface syndecan-1 is re-produced by epithelium as it becomes morphologically stable and is down-regulated in the mesenchymal component as the cells undergo differentiation at their terminal aspects, for example, differentiation of mesenchymal cells into odontoblasts. With continued development terminally differentiating epithelia, such as differentiating ameloblasts from enamel epithelium

during advanced bell stage, reduce their expression of syndecan-1 (Bernfield et al., 1992).

5.4 Regulation of the syndecans

In tooth development syndecan expression is highly regulated by dental epithelium and embryonic dental mesenchyme. During wound repair, such as damaged periodontium incident to tooth movement, induction of syndecan-1 mRNA occurs. In addition, condensation of embryonic cells in tooth formation may provide evidence for post-transcriptional control of syndecan-1 (Thesleff et al., 1991).

In *situ* hybridization experiments involving cultured explants of recombined dental epithelium and mesenchyme (Thesleff et al., 1995), show that growth factors may act as epithelial signals inducing (cell-specific) syndecan-1 expression during organogenesis. It has been proposed that the variation in distribution of these factors during tooth development may relate to altered patterns of syndecan expression in the bud, cap and bell stages (Salmivirta et al., 1991). For example, fibroblast growth factor stimulates the proliferation of dental mesenchyme during the cap stage subsequent to induction of syndecan. Furthermore, transforming growth factor-beta, which is situated in the dental epithelium during initial tooth development, induces syndecan production in cultured cells (Thesleff et al., 1995). Epidermal growth factor is also an important signal in tooth development (Bai et al., 1994)

The factors underlying syndecan selection and membrane adherence are unknown. It is possible that extracellular dependent cross-linking of cell surface syndecan molecules may trigger syndecan binding to the actin-cytoskeleton. The cytoplasmic component of the syndecan-1 molecule may then function in targeting of the proteoglycan in polarized epithelial cells. For instance, expression of syndecan-1 in transfected Madin-Darby kidney cells has resulted in targeting of syndecan-1 to the basolateral surface of epithelia (Carey, 1997). However, modification of the cytoplasmic domain resulted in syndecan-1 being present in both the basolateral and apical regions of the plasma membrane.

In mature tissues, the location of syndecans at the surface of cells might regulate its expression. Spontaneous shedding of the syndecan ectodomain and the associated glycosaminoglycan from the surface of cells results in the loss of syndecan expression by endocytosis or by degradation in lysosomes (Bernfield et al., 1992; Carey 1997).

Finally, pathological states could alter syndecan expression. During cutaneous wound healing, and possibly periodontal healing after destruction, syndecan-1 and -4 expression is increased by cells participating in wound repair. Expression is reduced at the leading edge of wounds by keratinocytes while expression is increased by fibroblasts and endothelial cells during organization of granulation tissue. In addition, analyses of irradiation induced malignancies of mouse epidermal tissues shows reduced amounts of syndecan-1 expression on the surfaces of these cells on account of an increase in tumour necrosis factor-alpha (Inki et al., 1994; Carey 1997).

5.5 Functions of the syndecans

Syndecans act as cell surface receptors for many extracellular matrix molecules. As co-receptors, syndecans bind ligands in conjunction with other cell surface molecules and mediate their action. For example: (1) cells bind to fibronectin through both its heparan-binding domain (using syndecan-1) and its integrin domain (using a beta-1 integrin); binding to both domains is required for normal actin filament organization which may be involved in determining cell shape and organization of epithelia;

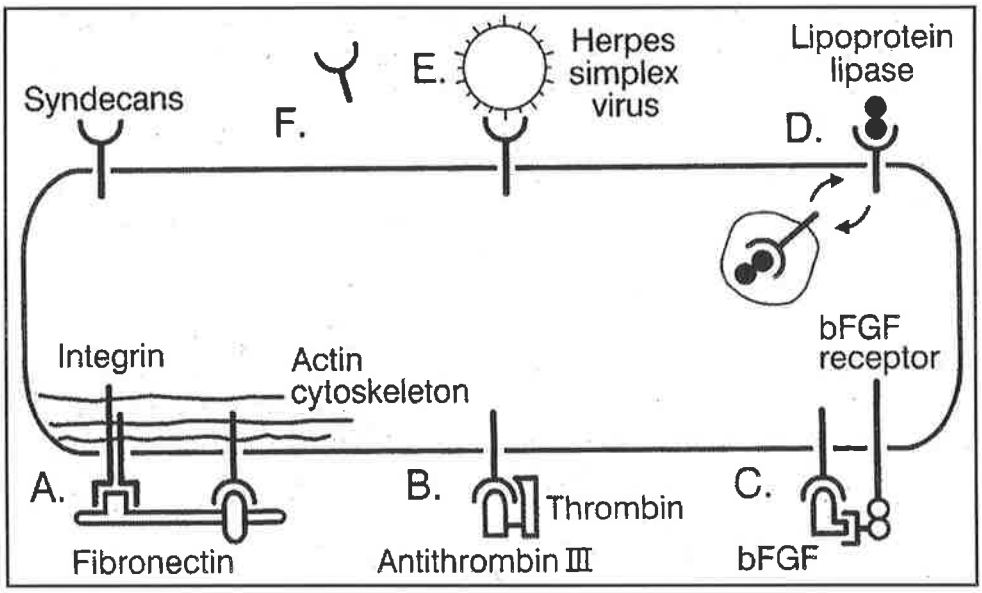


Figure 2. Schematic diagram showing the functions of cell surface syndecan as it binds to a variety of components of the extracellular matrix. FGF; fibroblastic growth factor (From Bernfield et al., 1992, p. 381).

(2) lipoprotein lipase adheres to the cell surface heparan sulphate proteoglycan on which it cycles to a non-degradative intracellular compartment; (3) cleavage of the syndecan extracellular domain at a protease susceptible site near the plasma membrane releases bound ligands from the cell surface providing competitive binding sites in the extracellular matrix; and (4) fibroblastic growth factor adheres to cell surface syndecan-1, which requires an interaction with cell surface heparan sulphate proteoglycan, as does the induction of a biological response through the fibroblastic growth factor receptor (Figure 2) (Bernfield et al., 1992).

The capability of syndecan-1 to bind extracellular matrix molecules, such as fibroblastic growth factor, epidermal growth factor granulocyte, macrophage colony-stimulating factor, interleukin-1 and -3 and interferon gamma, shows its contribution to the regulation of cell proliferation and behaviour. In addition, the ability of syndecans to bind ligands allows communication between the ectodomain, membrane-domain and endodomain. This communication results in the transfer of matrix organization into cellular organization by an idiopathic mechanism (Carey 1997; Mali et al., 1990) controlling cell behaviour such as epithelial differentiation during tooth development (Salmivirta et al., 1991).

5.6 Expression of the syndecans during tooth organogenesis

Tooth development in the mouse and rat appears to serve as a comparable model for studies determining the factors regulating organogenesis, morphogenesis and differentiation in humans. This is because events such as the initiation of tooth development, including the morphological changes in cell shape and the differentiation of both odontoblasts and ameloblasts, have been extensively studied (Thesleff et al., 1996).

Structural similarities between rats and humans have been recognized for some time. These similarities include the histological development of the rat molar (Table 1), rat tooth form and function and similarities in the rat periodontium (Schour & Massler, 1967). Furthermore, the transmembrane and cytoplasmic domains of the syndecans are 96% identical between mice and humans (Thesleff et al. (1990). Therefore, experiments performed on mice documenting the characteristics of the syndecans provide a useful guide for human study.

The significance of the syndecans in tooth development are characterized by the following events: (1) interstitial matrix components (collagens I, III and V, fibronectin, and tenascin) bind to the ectodomain which is important in mesenchymal cell condensation and differentiation during the bud, cap and bell stages;

Table 1. A summary of the chronology of the development of the rat dentition (Modified from Farris & Griffith, 1967, p.106).

Developmental process	Incisors	Molar, M-1	Molar, M-2	Molar, M-3.
Initiation: 1st appearance of dental lamina	14th day in utero	13th day in utero	14-15th day in utero	20th day in utero
Commencement of ameloblast histo-differentiation	18-19th day in utero	20th day in utero	21-22nd day in utero	8-10th day post natal
Commencement of dentine apposition	20-21st day in utero	20-21st day in utero	1-2nd day postnatal	13-14th day postnatal
Crown completion	Growth continuous	11th day postnatal	13th day postnatal	21st day postnatal
Commencement of root bifurcation	None present	14-16th day postnatal	16-18th day postnatal	24th day postnatal
First appearance in oral cavity	Approximately the 10th day postnatal	19th day postnatal	22nd day postnatal	35th day postnatal

NB: Maxillary teeth are chronologically about 24 hours behind the mandibular teeth. Amelogenesis commences approximately 12-24 hours following apposition of dentine.

(2) syndecan is localized to cell-matrix interfaces suggesting its role in the regulation of cell proliferation; (3) syndecan expression is controlled by secondary inductive reactions between epithelia and mesenchyme; and (4) the close association between cell proliferation and syndecan expression in proliferating mesenchymal cells indicates syndecans have an important role in cell growth. This comes from evidence that syndecan-1 binds fibroblast growth factor and may present it to its specific receptor (Salmivirta, et al., 1991; Thesleff et al., 1996).

5.6.1 Tooth crown formation

Tooth development involves a series of epithelial-mesenchymal interactions in which syndecan expression is stage-specific (Table 2) (Thesleff et al., 1991; Vainio et al., 1991; Bai et al., 1994). Prior to the bud stage, dental epithelium directs tooth development and later induces mesenchyme to acquire odontogenic potential (Thesleff et al., 1995).

Table 2. Expression and distribution of syndecan and associated extracellular matrix components and cell surface effector molecules during tooth organogenesis (Modified from Thesleff, et al, 1991, p.235). IEE, inner enamel epithelium; OEE, outer enamel epithelium.

Development stage	Fibronectin	Collagen III	Tenascin	Syndecan	EGF binding
Bud					
Dental epithelium	-	-	-	++	+++
Oral epithelium	-	-	-	+++	++
Dental mesenchyme	++	++	+++	+++	+
Mesenchyme	++	++	+	-	++
Cap					
IEE	-	-	-	-	-
OEE	-	-	-	+	+
Oral epithelium	-	-	-	+++	++
Dental mesenchyme	++	+	+	+++	+++
Surrounding mesenchyme	++	++	++	-	++
Early bell					
Dental epithelium	-	-	-	-	-
Oral epithelium	-	-	-	+++	++
Dental papilla	++	+	+	+	-
Dental follicle	+	+	-	+	++
Dental basement membrane	+++	+	+++	-	-
Late bell					
Ameloblasts	-	-	-	-	-
Oral epithelium	-	-	-	+++	++
Odontoblasts	-	-	-	-	-
Dental papilla (pulp)	+++	+++	+++	-	-
Dental follicle	++	+++	-	+	+++
Predentine	++	+	-	-	-
Dentin	-	-	-	-	-

Studies involving tooth germs in mouse and rat embryos, indicate tooth development begins around day 10 of gestation (Thesleff et al., 1991) commencing from the oral ectoderm as a thickening of the oral epithelium lining the facial processes at the dental arches (Figure 3).

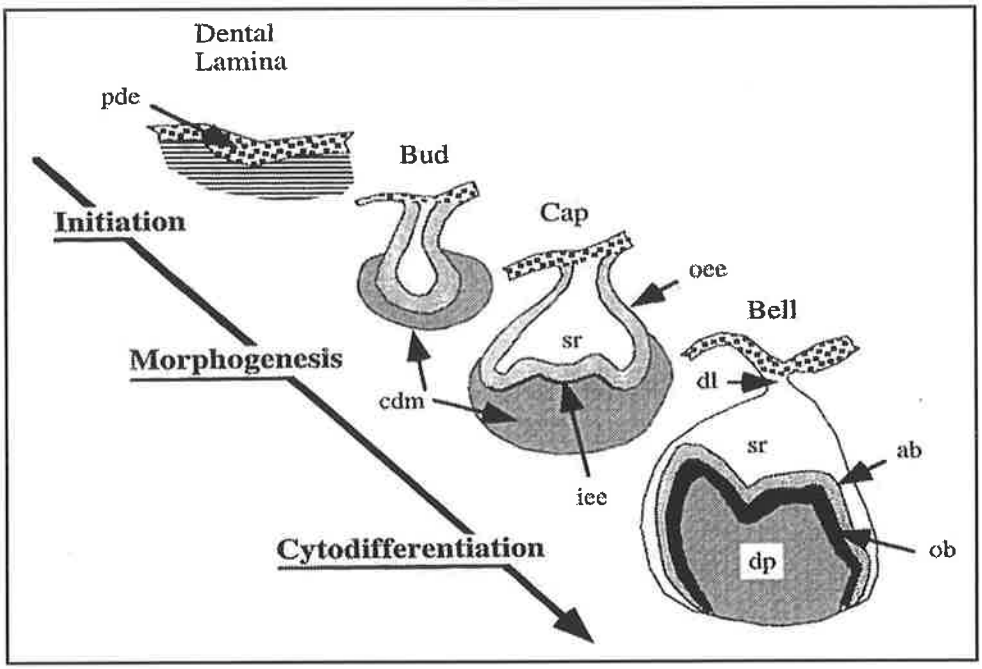


Figure 3. Schematic diagram of the progressive stages of tooth organogenesis; ab, ameloblasts; cdm, condensed dental mesenchyme; dl, dental lamina; dp, dental papilla; iee, inner enamel epithelium; oee, outer enamel epithelium; ob, osteoblasts; pde presumptive dental epithelium; sr, stellate reticulum (From Bartold & Narayanan, 1998 p. 165).

Immunohistochemical studies report the presence of syndecan in the 11 day mouse embryo under the thickened epithelium. The epithelium invaginates into the underlying jaw mesenchyme and condenses around the epithelial bud following induction probably from the interaction between syndecan-1 and tenascin. Radioactive labelling of syndecan-1 in the condensed dental mesenchyme of mouse embryos shows syndecan-1 binds tenascin. The simultaneous early appearance of these two molecules suggests their ligand-receptor complex may regulate adhesion of proliferative cells and influence cell morphology. Accordingly, cell-matrix interactions between these two molecules in dental mesenchyme may direct mesenchymal cell condensation around the epithelial bud (Salmivirta et al., 1991; Thesleff et al., 1996; Thesleff et al., 1990).

The epithelium maintains the capacity to initiate odontogenesis, however, during bud stage (day 13-14) (Thesleff et al., 1991) epithelial-mesenchymal interactions result in tooth forming ability shifting to the condensed dental mesenchyme which continues to regulate development until bell stage. The down-regulation of syndecan production in dental epithelium may be attributed to changes in the syndecan core protein structure (Jalkanen et al., 1988).

The following stages are characterized by rapid cell proliferation and differentiation, with both epithelial and mesenchymal cells differentiating into specialized cell types, producing the organic matrices of the mouse molar tooth germ (Thesleff et al., 1996). During the cap stage (16 day-old mouse embryos) (Thesleff et al., 1979), the dental epithelium invaginates at its undersurface and the mesenchyme commences differentiation into dental papilla mesenchyme. The dental epithelium gives rise to inner and outer enamel epithelium and cells of the stellate reticulum. Epithelial-mesenchymal interactions continue to control organogenesis until bell stage, as directed by the dental papilla mesenchyme, which continues to express syndecan. Under the basement membrane of the inner enamel epithelium cellular differentiation begins. Mesenchymal cell reactions with the epithelial basement membrane and the extracellular matrix result in the differentiation of mesenchymal cells into odontoblasts. With further development fibronectin and collagen type III continue to be expressed. Syndecan expression is down-regulated in the dental papilla mesenchyme but is still present in the dental sac surrounding the mouse molar tooth germ.

Syndecan expression has been found in the stratum intermedium and the stellate reticulum, both derived form the inner enamel epithelium of the enamel organ, in the bell stage (17 day-old mouse embryos) (Thesleff et al., 1979). Crown development is essentially complete during the late bell stage by terminal differentiation of cells. Pre-dentine matrix produced from odontoblasts (19 day-old mouse embryos) (Thesleff et al., 1979) formed from the dental papilla and sac is mineralized. Mineralization and enamel secretion by ameloblasts is observed in the 21 day-old mouse embryo. Terminal differentiation of odontoblasts, occurring in 18 day-old mouse embryos (Thesleff et al., 1979), is believed to be instructed by the epithelial basement membrane and triggered by dental epithelium during early bell stage. The inner enamel epithelium in the cuspal areas stop proliferating and become polarized and columnar pre-ameloblast cells are present (Thesleff & Aberg, 1997).

In relation to ameloblast development, Vainio et al., (1991) has found syndecan to be briefly produced in epithelial pre-ameloblasts before fully differentiating. During advanced bell stage, the constituents of the pre-dentine matrix (Thesleff et al., 1979), as well as reciprocal signals from odontoblasts, determine differentiation of ameloblasts from enamel epithelium and the enamel matrix is secreted. Syndecan-1 is absent in terminally differentiating odontoblasts and with the onset of ameloblast differentiation (Bai, et al ., 1994). The dental papilla and sac give rise to the cementoblasts,

periodontal ligament and alveolar bone. Finally the terminal differentiation of ameloblasts, like that of odontoblasts, is induced by epithelial-mesenchyme interactions (Thesleff et al., 1995; Thesleff et al., 1996). Therefore, syndecan expression in dental mesenchyme does not parallel its expression in dental epithelium.

The syndecans expressed by dental epithelium may play a different role to syndecans expressed in the dental mesenchyme as determined by their glycosaminoglycan. In the epithelium, syndecan appears to control cell adhesions by a mechanism of cell aggregation, whereas syndecan in mesenchyme may function in cell-matrix interactions and regulation of growth (Vainio et al., 1991). Immunohistochemical studies using a heparan sulphate antibody found surface heparan sulphate proteoglycans in the enamel organ throughout tooth development. This suggests two possibilities. Firstly, at different periods of tooth development syndecan-1 could have different molecular structures. This is consistent with the fact that syndecan-1 exhibits polymorphic isoforms (with respect to its acidic domains and sulphated sequences) dependent on the epithelial cell type and stage of differentiation. Alternatively, heparan sulphate proteoglycans different from syndecan-1 may be expressed during tooth development (Bai et al., 1994).

5.6.2 Tooth root formation

Following amelogenesis and dentinogenesis adjacent to the proposed site of the cementoenamel junction, root development begins. Cells of the cervical loop of the enamel organ, a region where cells of the inner and outer enamel epithelium are contiguous, proliferate downward forming Hertwig's epithelial root sheath (Figure 4).

Cellular division of Hertwig's epithelial root sheath cells results in apical migration of sheath cells through underlying dental ectomesenchyme dividing these tissues into a dental papilla (the future dental pulp) and the dental follicle (the future periodontal ligament). With continued development Hertwig's epithelial root sheath cells, specifically inner enamel epithelium (Bartold et al., 1988), induce adjacent ectomesenchymal cells of the dental papilla to differentiate into odontoblasts laying down dentine matrix. A portion of cells from Hertwig's epithelial root sheath then fragment while others migrate away from the outer surface of the recently formed dentine root surface into dental follicular tissue. Root surface dentine then becomes exposed to cementum-forming (dental follicle) cells which attach and assemble upon a matrix coating the dentine surface. Dental follicle cells differentiate into cementoblasts,

the major source of cementum during development and later posteruption function forming the cementum covering the root surface (MacNeil & Somerman, 1993; Harrison & Roda, 1995; Alatli et al., 1996; Bartold & Narayanan, 1998). The disintegrated epithelial cells of Hertwig's epithelial root sheath never entirely disappear from the periodontium and remain as small strands termed epithelial cell rests of Malassez (Spouge, 1980; Harrison & Roda, 1995; Bartold & Narayanan, 1998).

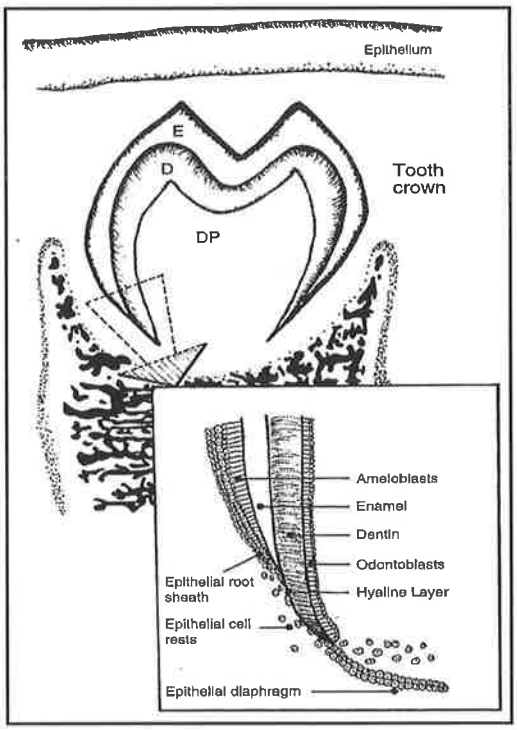


Figure 4. Schematic diagram of root and cementum formation following crown completion. Hertwig's epithelial root sheath comprises cells derived from the enamel epithelium responsible for forming the precursor matrix required for root formation via dentine and cementum deposition. Enamel, E; Dentine, D; and dental papilla, DP (Modified from Bartold & Narayanan, 1998, p. 155).

5.6.3 Cementum and dentine: glycosaminoglycan composition and distribution in the tooth root

Cementum may be very similar in composition to dentine and bone both histologically and in terms of its glycosaminoglycan content. Using immunohistochemistry the primary glycosaminoglycans present within human cementum have been isolated. This was performed using proteolytic digestion of guanidine/ethylenediaminetetra-acetic acid (EDTA) and collagenase extracts of cementum with analysis by membrane electrophoresis. The glycosaminoglycans isolated were hyaluronic acid, chondroitin

sulphate (the major species present) and dermatan sulphate (Bartold 1987; Bartold et al., 1988). Heparan sulphate or keratan sulphate were not detected. However, keratan sulphate-containing proteoglycans (lumican and fibroglycan) have been described within bovine pre-cementum and pre-cementocyte lacunae (Cheng et al., 1996). These observations may implicate the importance of proteoglycans in the development of tooth cementum (Bartold & Narayanan, 1998).

The predominant glycosaminoglycan in hard and mineralized dental tissues, including mineralized periodontal connective tissues, cementum, dentine and bone, is chondroitin sulphate. This is different to that of soft dental tissues, namely gingivae and periodontal ligament, where dermatan sulphate is the major glycosaminoglycan, according to Kirkham et al., (1995).

5.6.4 Glycoconjugates: potential roles during root development

The literature describing the functional role(s) of glycosaminoglycans during root formation is limited. However, the distribution of glycoconjugates including glycosaminoglycans, glycolipids, glycoproteins and sialoproteins, has been studied in mouse root development (Sasano et al., 1992). In a study designed to localize glycoconjugates during mouse molar root development, peanut agglutin and Maclura pomifera lectins, which bind strongly to D-galactose-N-acetyl-D-galactosamine residues on glycoconjugates, were applied to serial sections of first molar teeth using lectin histochemistry. Maclura pomifera was found to stain the outer most layer of root dentine, developing cellular cementum, alveolar bone and Hertwig's epithelial root sheath. Peanut agglutin stained Hertwig's epithelial root sheath and the associated basement membrane during epithelial-mesenchymal interactions, initial root formation and initial mineralization. These findings might suggest glycoconjugates serve important roles in cementogenesis and root development (Sasano et al., 1992).

The glycoproteins laminin and tenascin are described as attachment proteins which are involved in cell-matrix reactions during dentinogenesis and could also have active roles in root organogenesis and cementogenesis. Laminin, occurs on the root surface before cementogenesis and is produced during Hertwig's epithelial root sheath-root formation. Tenascin is present during cementogenesis (the pre-cementum layer) and in mature cementum. The presence of tenascin in conjunction with fibronectin during early cementogenesis provides evidence of their influence in cementum differentiation (MacNeil & Somerman, 1993).

The role of cellular division and apical migration of Hertwig's epithelial root sheath cells during root development has been described (MacNeil & Somerman, 1993; Harrison & Roda, 1995; Bartold & Narayanan, 1998). The chemical signals responsible for root development may be derived from epithelial-mesenchymal reactions between Hertwig's epithelial root sheath and adjacent mesenchymal tissue (MacNeil & Somerman, 1993). For example, migration of pre-cementoblast cells may be stimulated by chemoattractant chemicals situated in the root-associated basement membrane, Cho & Garant 1988 (Cited in MacNeil & Somerman, 1993, p.553). Hertwig's epithelial root sheath may also manufacture epithelial product(s) in its basement membrane prior to cellular fragmentation. Immunohistochemical studies have shown the chemotactic glycoproteins, fibronectin and laminin, to have been produced on the root portion of Hertwig's epithelial root sheath during tooth development, according to Thomas & Kollar 1989 (Cited in MacNeil & Somerman, 1993, p.553). Following migration of cells to their destined sites they become anchored by such attachment molecules. In particular, fibronectin has been isolated in regions of pre-cementum production during root development suggesting its functional role in attachment and migration of dental ectomesenchymal cells on the early matrix of root dentine (MacNeil & Somerman, 1993). Fibronectin is found in high concentrations in the human periodontium and cellular cementum and binds to a number of molecules, including proteoglycans, heparin and collagen.

5.6.5 Glycosaminoglycans: potential roles in the mineralization process

Kirkham et al., (1995) have explored the ability of certain glycosaminoglycans to affect the potential of periodontal ligament collagen to mineralize in the region of Sharpey's fibres. It has been suggested that the juxtaposition of certain proteoglycans with type I collagen may prevent calcification of soft connective tissues. Moreover, Kirkham et al., (1995) examined the glycosaminoglycan content and the effect on mineralization of sheep periodontal ligament in vitro, using enzyme digests to remove periodontal ligament glycosaminoglycans. These researchers found dermatan sulphate to be the major glycosaminoglycan of the periodontal ligament, with chondroitin-4-sulphate and heparan sulphate present in reduced amounts (Kirkham et al., 1995). In contrast, the glycosaminoglycan of the mineralized periodontal tissues was chondroitin sulphate, predominantly chondroitin-4-sulphate, as it was for cementum (containing greater than 60% chondroitin-4-sulphate) and alveolar bone (containing 80% chondroitin sulphate). It was concluded that the potential functions of glycosaminoglycans such as dermatan

sulphate can affect the capacity of periodontal ligament collagen to mineralize by inhibiting hydroxyapatite nucleation and slowing crystal growth.

Embery et al., (1998) have examined the interaction of a small chondroitin sulphate proteoglycan and the glycosaminoglycans of chondroitin-4-sulphate, dermatan sulphate and heparan sulphate, with hydroxyapatite. Following incubation of these respective glycosaminoglycans with hydroxyapatite, and the generation of glycosaminoglycan-hydroxyapatite complexes, the influence of chondroitin-4-sulphate and dermatan sulphate on hydroxyapatite-induced crystal growth was examined. It was found that chondroitin-4-sulphate had a higher binding capacity for calcium ions than dermatan sulphate. This might have accounted for the role of chondroitin sulphate-proteoglycan and dermatan sulphate in mineralized tissues and in non-mineralized tissues respectively.

5.6.6 Epithelial cell rests of Malassez: postulated functions

Epithelial cell rests of Malassez are believed to manifests several functional roles, these include: (1) protection of the root from resorption; (2) prevention of ankylosis; (3) maintenance of the periodontal ligament integrity according to Lindskog, et al., 1988 (Cited in Wesselink & Beertsen,1993, p.399); and, (4) initiation of cementogenesis. The latter role is consistent with the observations of Brice et al., (1991). These researchers set out to identify epithelial cells in the periodontal ligament by examining the buccal root surfaces of premolars extracted for orthodontic purposes in adolescent patients undergoing rapid maxillary expansion. Brice et al., (1991), found that epithelial cell clusters, which are ultrastructurally very similar to the inner layer of epithelial cells in Hertwig's epithelial root sheath and the epithelial cell rests of Malassez, are capable of initiating cementogenesis. This proceeded the work of Gurling & Sampson (1985) who described how epithelial cell rests might initiate cytodifferentiation of follicular cells into cementoblasts resulting in cementum repair following damage to acellular cementum.

The potential role of epithelial cell rests in cyst formation has been described by Thesleff (1987). During pathological conditions (e.g. generalized inflammation and periapical cyst formation) there is an increase in the synthesis of epidermal growth factor increasing binding and activation of epithelial cell rests of Malassez (reported to contain an abundance of epidermal growth factor receptors). This process may then initiate a chain of biochemical events characterized by translation, activation of protein

kinases, phosphorylation of regulatory proteins, transcription and translation. The latter culminates in epithelial cell proliferation and periapical cyst formation which appears to be related to the degree of inflammation.

Inflammatory mediators and cytokines produced locally may also influence epithelial cell rest proliferation (Thesleff, 1987). Lin et al., (1996) has reported that it is possible for inflammatory mediators such as prostaglandins (e.g. prostaglandin E2) and cytokines (interleukin-1, interleukin-6) to regulate epidermal growth factor receptor activity during inflammation. Evidence of this notion comes from studies describing the work of Brunette (1984) who showed that upregulation of cyclic AMP, caused by prostaglandins (e.g. prostaglandin E2), stimulates growth of epithelial cell rests of Malassez; while interlukin-1 and -6 stimulate epithelial cell mitosis.

5.7 Functions of the syndecans during orthodontic tooth movement

5.7.1 The mechanism of bone resorption

Bone comprises type I collagen, a prime target for degradative activity during resorption and bone glycoprotein-produced by osteoblasts and activates osteoclast differentiation. Bone resorption in reaction to a mechanical stress, such as an orthodontic force, stimulates osteoblasts which produce pro-collagenase (the precursor to collagenase). This results in collagenolysis and demineralization ensues culminating in bone resorption and tooth movement (Figure 5) (Miyajima et al., 1992).

In studies carried out on five day old foetal rat long bone organ cultures and stimulating bone resorption, an interaction was found between interleukin-1 with parathyroid hormone, prostaglandin E, tumour necrosis factor and epidermal growth factor (Dewhirst, & Stashenko, 1992). Importantly, type I collagen, tumour necrosis factor and epidermal growth factor all bind to syndecan-1. Perhaps, therefore, an interaction between syndecan-1 and these effector molecules might occur during bone resorption. Syndecan-1 could bind or present one or all of these ligands to their primary receptors during the resorptive process.

Interleukin-1 activated bone resorption is reported to be the prime factor responsible for osteoclast activity; and it is believed to stimulate bone resorption indirectly through the stimulation of osteoblasts by an idiopathic mechanism. Interleukin-1 could act in two ways: (1) as a secondary messenger pathway involving the hydrolysis of phosphatidylcholine; or (2) through activation of cyclic AMP. In fact, interleukin-1 has

been found to interact with parathyroid hormone and prostaglandin E to activate cyclic AMP in osteoblasts (Dewhirst & Stashenko, 1992).

Gingival crevicular fluid has been used as a diagnostic indication of tissue changes and chemical components within the periodontium. Heparan sulphate proteoglycans have been found to be the major component in gingival epithelium while being a minor component in the periodontal tissues and ligament (Purvis et al., 1984; Last et al., 1988). The major proteoglycans isolated from the periodontal ligament are small dermatan sulphate proteoglycans and smaller traces of heparan sulphate proteoglycans. Important constituents of gingival crevicular fluid have been proteoglycans associated with tissue destruction. Chondroitin sulphate has been predominantly observed in gingival crevicular fluid in sites associated with destruction of deep periodontal tissues, including resorption of alveolar bone, periodontitis and in dentoalveolar structures undergoing increased loads (i.e. orthodontic tooth movement and occlusal trauma) according to Waddington et al., (1994). Merrilees et al., (1983), have attempted to examine the origin of the glycosaminoglycans described above by two cell types occurring in porcine periodontal ligament: fibroblast-like cells and cells of the epithelial cell rests of Malassez). Results showed that hyaluronic acid was the main glycosaminoglycan in the epithelial cell rests of Malassez. Fibroblast-like cells gave rise to chondroitin sulphate with smaller amounts of hyaluronic acid, dermatan sulphate and heparan sulphate.

Waddington et al., (1994) have also isolated proteoglycan metabolites from gingival crevicular fluid extracted from a human teeth undergoing remodelling during bodily orthodontic tooth movement. Chondroitin-sulphate was reported as the principal sulphated proteoglycan together with heparan sulphate, which had only been previously reported in gingival crevicular fluid isolated from teeth in retention following orthodontic tooth movement (Waddington et al., 1994). It was concluded that the appearance of heparan sulphate proteoglycan metabolites in gingival crevicular fluid might be the result of modified cell participation during increased bone remodelling, limited degradation of extracellular matrix components, or the synthesis of proteoglycans by the periodontium during repair in response to bone remodelling (Bartold 1987; Waddington et al., 1994; Wijdenes et al., 1996).

Kirkham et al., (1992) have also assessed the association between cell surface heparan sulphates, such as syndecans, and the dentoalveolar process in diseased sheep

periodontium. Although these studies have been conducted by examining the glycosaminoglycan distribution in periodontal disease some important points might be associated with inflammation occurring during tooth movement. For instance,

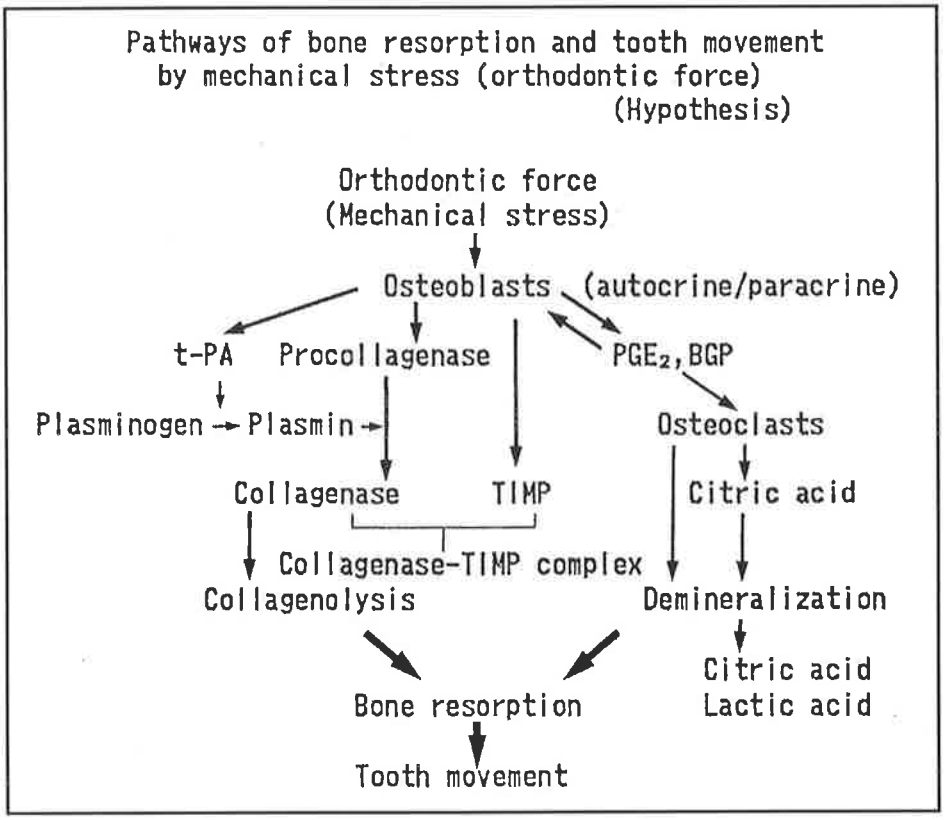


Figure 5. Schematic representation of bone resorption and tooth movement incident to orthodontic force. Plasminogen activator, t-PA ; bone Gla protein, BGP (From Miyajima et al., 1992, p. 312).

protein and total sulphated glycosaminoglycan content might be elevated through increases in cellularity, vascularity or inflammation, which could be associated with increases in cell-surface proteoglycan. An increase in chondroitin-4-sulphate proteoglycan has been observed in tissue adjacent to alveolar bone associated with increased bone resorption (Kirkham et al., 1992). Alternatively, biochemical studies of mechanical loading in connective tissues demonstrate that increased loading is associated with reduced glycosaminoglycan content.

5.8 Expression of the syndecans in malignant neoplasia

Tooth development requires the collaboration between a number of cell types and their regulation in normal tooth development. However, when these molecular events

become excessive unrestricted tumourigenesis may occur (Kainulainen et al., 1996). This is characterized by basement membrane modifications, uncontrolled cell proliferation, minimal cell differentiation, altered cell morphology and decreased cellular adhesion (Parish et al., 1987; Inki, et al., 1994; Nakanishi et al., 1994).

Syndecan-1 expression is associated with normal (non-malignant) epithelial cell organization, proliferation, differentiation and shape and the main difference between normal and malignant cell types has been found to be the reduction in syndecan expression. Loss of syndecan-1 expression might be the mechanism by which malignant cells loosen their adhesions to each other and the extracellular matrix and fail to respond to the normal regulatory controls of their environment (Inki et al., 1994). In addition, chemical changes have been reported in the syndecan-1 glycosaminoglycan structure in tumourous tissue with more chondroitin sulphate proteoglycans and fewer heparan sulphate proteoglycans found to be present (Esko et al., 1988).

The difference in syndecan-1 expression between normal and malignant cells is supported by in vitro studies of a mouse mammary tumour cell line (S115 cells) and mouse epidermis cells with squamous cell carcinomas induced by ultraviolet radiation. When S115 cells are transfected with syndecan-1 they are prevented from developing an abnormal characteristic of tumour cells (Jalkanen et al., 1991). It is believed that the syndecan-1 ectodomain is sufficient for normal behaviour in S115 cells (Mali et al., 1994).

Fibroblastic growth factors function biologically in angiogenesis, induction reactions (e.g. tooth development), periodontal repair and neoplastic transformation (Bernfield et al., 1992). The ability of syndecan-1 to bind fibroblastic growth factor, therefore, might suggests it may have a role in the regulation of angiogenesis and possibly neovascularization during malignant change (Inki et al., 1994). In particular, interference of fibroblastic growth factor eight, which is responsible for malignant transformation, by syndecan-1 inhibits tumour cell growth (Zang et al., 1994).

The effect of growth factors and cytokines have been observed in relation to syndecan-1 regulation in human keratinocytes and endothelial cells during tumourigenesis. Specifically, tumour necrosis factor-alpha, (a pro-inflammatory cytokine produced by macrophages) has been found to inhibit syndecan-1 production in vivo and in vitro in endothelial cells. This suggests that tumour necrosis factor-alpha may be a regulator of syndecan-1 production during organogenesis and carcinogenesis. Osteotropic cytokines

are important local factors regulating bone cell function and remodelling and in non-malignant tissues immune cells of the bone marrow might synthesize these cytokines. However, in malignant tissues tumour cells are the significant producers of the cytokines (Hirgara et al., 1995).

The spread of tumour cells into tissues is dependent on the ability of these cells to bypass the cell membrane. The mechanism by which this occurs remains unclear, however, tumour cells may express endoglycosidases cleaving heparan sulphate proteoglycan side chains in the extracellular matrix enabling tumour cell passage (Gallagher et al., 1986). Therefore, the ability of heparan sulphate proteoglycans to inhibit endoglycosidases is an important mechanism in tumour cell inhibition (Parish et al., 1987).

6 MATERIALS AND METHODS

6.1 Trial study

6.1.1 Experimental animals and other material

Animals comprised 1 mature (12 month old) Sprague-Dawley rat; and 10 Sprague-Dawley rat pups. Two rats were killed at each of the following time intervals: 2 days, 1 week, 2 weeks, 4 weeks and 6 weeks. Rats were supplied by the Waite Agricultural Research Institute, Adelaide, South Australia and housed in the Central Animal House, The University of Adelaide. All animals were killed by carbon dioxide asphyxiation.

Formalin fixed and paraffin embedded sections of human oral squamous cell carcinomas (provided by Associate Professor David Wilson, Faculty of Health Sciences, University of Adelaide) were also used in the trial study.

6.1.2 Tissue retrieval and fixation

Purpose - determination of suitable fixation protocols for: (1) routine staining; (2) histochemistry; and, (3) immunohistochemistry.

The rat maxilla and mandible were dissected out, halved and fixed in either 10% neutral buffered formalin (Appendix 10.4) for 12 hours (Culling, 1974); Zamboni's solution (Zamboni & De Martino, 1967) for 12 hours; Carnoy's solution for 12 hours (Culling, 1974); or 80% ethanol (-70°C) for 3 hours. Frozen tissues were left to thaw prior to processing (Wiebkin, 1996) by dehydration and embedding.

6.1.3 Decalcification, dehydration, embedding and sectioning

After fixation specimens were placed into labelled uni-cassettes (Tissue Tek), which were immersed in a beaker containing continuously agitated 4% ethylenediaminetetra-acetic acid, EDTA, solution (in a phosphate buffer, pH 6) (Appendix 10.5). The EDTA solution was changed twice weekly.

Decalcification was tested weekly by periodic dental radiography (0.12 seconds, 65kV EktaSpeed film).

Tissues were then dehydrated, embedded (Appendix 10.15), sectioned and mounted by routine methods onto glass slides. All glass slides were pre-coated with 3-amino propyltriethoxysaline (Appendix 10.6) to promote adhesion of tissue sections.

6.1.4 Staining and labelling

6.1.4.1 Routine haematoxylin and eosin

Haematoxylin and eosin (Appendix 10.1) was applied to transverse and longitudinal sections of rat maxilla and mandible to determine the best orientation of tissue sections and to observe the timing of rat tooth development.

6.1.4.2 Immunohistochemistry

6.1.4.2.1 Rabbit anti-laminin

This was a rabbit polyclonal antibody against rat laminin (EY Laboratories, #AT-2404-1, Appendix 10.10.1) used for determination of a suitable tissue fixation technique for the main study. The secondary antibody was a biotinylated anti-rabbit immunoglobulin (EY Laboratories, #BA-1000, Appendix 10.11.1).

6.1.4.2.2 Cytokeratin AE1-AE3

This was a mouse monoclonal antibody against rat cytokeratins (Cell Marque, #CMC532, Appendix 10.10.5) used for positive reference identification of epithelial cell rests of Malassez (Leedham, 1992) in the rat periodontium. For this study access was available to cytokeratin AE1-AE3 labelled serial sections, corresponding to each section labelled with the anti-syndecan-1 antibodies used for the trial and main studies.

6.1.4.2.3 Alpha syndecan-1

This was a mouse monoclonal antibody against rat syndecan-1 (Appendix 10.10.2) donated by Dr Ole Wiebkin, Department of Medicine, Royal Adelaide Hospital, South Australia. The secondary antibody was a biotinylated anti-mouse immunoglobulin (Vector Laboratories Incorporated #BA-2000) (Appendix 10.11.2).

In view of the limited stock of Alpha syndecan-1 it was ultimately not possible to use it in the main study. Therefore, two further antibodies (Syndecan-1 B-B4 and Syndecan-1 N-18) were tested to identify a suitable immunohistochemical marker for rat syndecan-1 to be used in the main study.

6.1.4.2.4 Syndecan-1 B-B4

This was a mouse monoclonal antibody against human syndecan-1 (Serotec Limited, #MCA 681, Appendix 10.10.3). The secondary antibody was a biotinylated anti-mouse

immunoglobulin (Vector Laboratories Incorporated, #BA-2000) (Appendix 10.11.2). This antibody was deleted from the main study.

6.1.4.2.5 Syndecan-1 N-18

This was a goat polyclonal antibody against mouse syndecan-1, specific for syndecan-1 of mouse, rat and human origin (Santa Cruz Biotechnology, Incorporated # sc-7100, Appendix 10.10.4). The secondary antibody was a biotinylated anti-goat immunoglobulin (Vector Laboratories, Incorporated # BA-5000) (Appendix 10.11.3).

The avidin-biotin complex peroxidase technique (Appendix 10.7) was used for all immunolabelling procedures. Tissue sections were treated with hydrogen peroxide/methanol blocking solution. After washing in phosphate buffered saline sections underwent antigen unmasking (i.e. trypsin and citrate) where required (Appendix: primary antibodies). Sections then were washed and incubated with 3% normal horse serum for 30 minutes to block non-specific binding of the secondary antibody. Sections were then incubated overnight with the primary antibody. After washing, sections were then incubated with a secondary biotinylated antibody for 30 minutes and then streptavidin peroxidase (Pierce, USA, Appendix 10.12) for 1 hour, before application of diaminobenzidine peroxidase substrate solution (Pierce, USA, Appendix 10.13). Sections were then lightly counterstained with Mayer's haematoxylin (Appendix 10.2) prior to mounting on glass slides.

6.1.4.3 Histochemistry

6.1.4.3.1 Alcec Blue

Alcec Blue (Aldrich, Incorporated, Appendix 10.3) at pH 5.8 and increasing magnesium chloride electrolyte concentrations allows for differential binding of available charged groups (e.g. sulphates) on proteoglycans (Wiebkin, 1996; Scott 1967).

Sections of rat maxilla and mandible were stained with Alcec Blue, for detection of heparan sulphate glycosaminoglycans, including syndecan-1, at increasing electrolyte concentrations (0.36 mol/L - 0.78 mol/L magnesium chloride). Briefly, sections were de-waxed, re-hydrated and immersed in Alcec Blue solution for 60 minutes (Appendix 10.3.1) as described by Wiebkin (1996). Sections were then dehydrated, cleared and mounted.

6.1.5 Controls

Sections of rat tongue exposed to 4% EDTA served as a positive staining-control tissue. Sections of rat tongue exposed to 4% EDTA, to which the primary antibody was not added, served as a negative staining-control tissue. Control tissues were stained or labelled concurrently with all experimental sections (rat maxilla and mandible) to determine the degree of baseline staining or labelling.

Rat tongue was prepared in the same manner as the experimental sections to examine the potential influence of fixation and decalcification on histochemistry and immunohistochemistry.

6.2 Main study

6.2.1 Aim

To observe changes in the expression of syndecan-1 in both the developing epithelium of the rat oral mucosa, and in the epithelial cell rests of Malassez in the developing periodontium of normal rat molars from late crown development through to early eruption.

6.2.2 Ethics approval

Permission for the use of rats in the study was obtained from the Animal Ethics Committee of the University of Adelaide. Approval number S/51/98.

6.2.3 Experimental animals and tissue retrieval

Rat pups were produced by 2 female Sprague-Dawley rats at an estimated 2 weeks gestation, supplied by the Waite Agricultural Research Institute, South Australia.

Maxillae were dissected from 24 rats. Four were killed at each of the following time intervals: 2 days, 1 week, 2 weeks, 3 weeks, 4 weeks and 6 weeks. Rats ranging in age from 0-13 days postpartum, were killed by decapitation without prior anaesthesia, and pups 14 days and older by asphyxiation in a carbon dioxide chamber.

6.2.4 Fixation and decalcification

Rat maxillae were fixed for 12 hours (10% neutral buffered formalin) and then placed into labelled uni-cassettes which were immersed in a beaker containing continuously agitated 4% EDTA in a phosphate buffer, pH 6. Decalcification of specimens was carefully monitored by periodic dental radiography at 1 week intervals. At the

completion of decalcification rat maxillae were washed (24 hours) in running tap water for removal of residual salts. As for the trial study, rat tongue served as control tissue and underwent the same tissue processing protocol.

6.2.5 Dehydration and embedding

Rat maxillae and tongue specimens were then dehydrated (Shandon Citadel 2000 automatic processor, Appendix 10.15) and placed into Reichert specimen block holders for paraffin embedding (Reichert Jung embedding machine). Specimen blocks were then frozen until required for sectioning (Leitz Wetzlar 1512 microtome).

For orientation purposes, all tissue blocks were trimmed and embedded in a consistent manner as follows: (1) *longitudinally*, rat maxillae were cut into halves and the cut surface placed flat against the base of the wax holder to align the molars longitudinally with the base of the cassette; and, (2) *transversely,* entire maxillae were placed mucosal palatal surface down against the base of the cassette enabling transverse sections of the rat maxillary first molar (M-1) to be cut.

6.2.6 Sectioning

Specimens were sectioned at 5 micrometers and ribbons floated on a temperature controlled DHA Anax water bath (240 volts, 45°C) prior to mounting on glass slides pre-coated with 3-amino propyltriethoxysaline. Each slide contained a ribbon of 3 sections. Slides were then placed in a 1974 Anax Labmaster oven (60°C), for flattening of sections prior to staining or labelling.

Haematoxylin and eosin staining was routinely performed on sections 150 micrometers apart to determine: (1) in *longitudinal* sections, where the periodontal ligament was located and when it started to appear; and, (2) in *transverse* sections, when the centre of the crown started to be observed.

In *longitudinal* sections, cutting started from the palatal aspect of the periodontal ligament up to and beyond the periodontal ligament of the buccal aspect of the mesiobuccal root. This ensured the entire width of the rat maxillary first molar (M-1). root was sectioned. For *transverse* sections, cutting commenced from the centre of the rat maxillary first molar (M-1) crown to the point beyond the root apices where only periodontal ligament was present, ensuring the entire length of the mesiobuccal root was sectioned (Figure 6). Levels 0-4 comprised the cervical root third, 5-10 the middle root third, and levels 11-15 the apical root third. The mesiobuccal root of M-1 was

selected for examination because it was the largest root and, therefore, would provide the largest number of sections.

6.2.7 Staining and labelling

6.2.7.1 Haematoxylin and Eosin

Haematoxylin staining was routinely performed on sections 150 micrometers apart as a guide to identification of the morphology of the mesiobuccal root of M-1 and the surrounding periodontium, and to determine zero points. Zero points were the points at which recording of syndecan-1 expression or Alcec Blue staining was to begin and end, in the developing root of the rat molar (refer to section 6.2.9).

6.2.7.2 Histochemistry

Dewaxed and rehydrated sections were immersed for 60 minutes in Alcec Blue solution (pH 5.8, 0.42mol/L magnesium chloride). Sections were then dehydrated, cleared and mounted. Histochemical detection of heparan sulphate glycosaminoglycan side chains was demonstrated by pale blue staining.

6.2.7.3 Immunohistochemistry

After dewaxing, sections were treated with hydrogen peroxide/methanol blocking solution. Sections were washed in phosphate buffered saline and then incubated with 3% normal horse serum for 30 minutes, before being incubated overnight with Syndecan-1 N-18 (1:600). Sections were washed and then incubated with a biotinylated rabbit anti-goat immunoglobulin for 30 minutes, and then streptavidin peroxidase for 1 hour, prior to application of diaminobenzidine peroxidase substrate solution. Sections were then lightly counterstained with Mayer's haematoxylin prior to mounting. Positive reference identification of epithelial cell rests of Malassez was provided by staining adjacent sections with cytokeratin AE1-AE3.

Specimens were sampled at each 150 micrometer level and 3 sequential sections stained in the following serial order: haematoxylin and eosin, Alcec Blue, then Syndecan-1 N-18.

6.2.8 Analysis

Sections were examined using conventional light microscopy (Olympus CH microscope) at magnifications of x40, x100, x200, and x400. Sections were assessed for the presence or absence and intensity of syndecan-1 expression and Alcec Blue staining

in the oral epithelium of the rat tongue and palate, and in the periodontium (for epithelial cell rests of Malassez), surrounding the mesiobuccal root of the rat maxillary first molar (M-1), from late crown formation through to early eruption. The intensity of syndecan-1 expression and Alcec Blue staining in the stratified epithelium of the oral mucosa, and in the periodontium, was subjectively analysed.

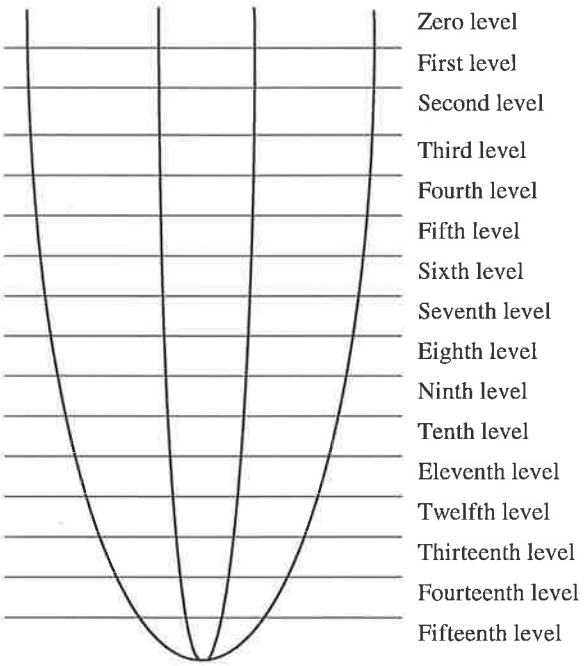


Figure 6. Schematic diagram of the mesiobuccal root (M-1) illustrating the levels at which transverse sections were stained using histochemistry and immunohistochemistry (Modified from Leedham, 1992, p.44)

The location of staining in epithelial cell rests relative to the tooth root were made using an 8 point registration system adapted from Andreasen (1987) and Andersson et al., (1987) as follows:

(1) In *transverse* sections, a cross-hair microscope eye-piece comprising 4 equal octants was superimposed over the centre of the mesio-buccal root so that one line bisected the buccal-lingual aspect and another the mesial-distal root aspect of the rat maxillary first molar (M-1). The tooth root was then subdivided into 8 regions (Figure 7), for the purpose of locating and recording labelling/staining of epithelial cell rests of Malassez in the periodontium on a data recording sheet (Appendix 10.16).

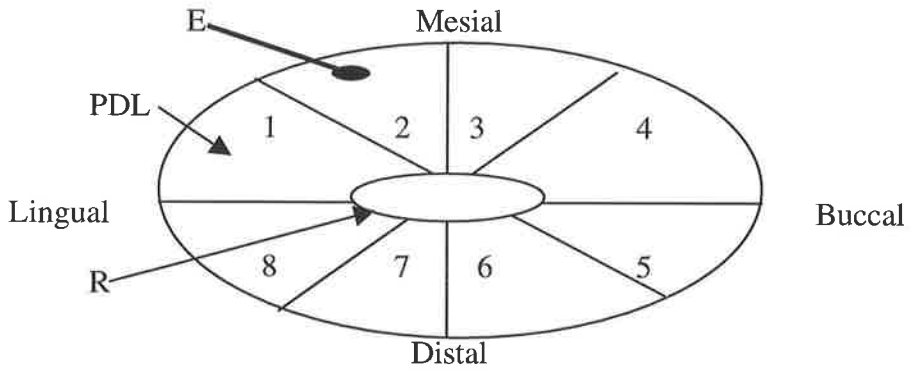


Figure 7. Schematic diagram of a transverse root section displaying the octant system and the 8 registration regions used to record labelling/staining of epithelial cell rests of Malassez at each root level. Mesiobuccal root, R; periodontal ligament, PDL; epithelial cell rests of Malassez, E. (Modified from Leedham, 1992, p.45).

(2) In *longitudinal* sections, the *mesial* aspect of the mesiobuccal root was divided into zones as follows: the cervical root third, the middle root third and the apical root third. The *furcal* aspect of the mesiobuccal root was divided into an apical half and a furcal half. The location of epithelial cell rests of Malassez within these regions was then observed and recorded.

6.2.9 Zero points

Establishment of the "zero and end" points, or the point at which syndecan counting was to begin and end, was determined by assessing sequential haematoxylin and eosin stained sections. In *transverse* sections, the zero point began at the point of first appearance of the maxillary molar M-1 tooth cusp tip at the approximate centre of the crown. The end point was represented by the apical periodontal ligament. In *longitudinal* sections, the zero point began at the point of first appearance of the palatal aspect of the mesiobuccal root of M-1 and the disappearance of the buccal aspect of the mesiobuccal root and commencement of the periodontal ligament.

7 RESULTS

7.1 Trial study

7.1.1 Fixation

Observation of sections labelled with rabbit anti-rat laminin showed that formalin fixation (10% neutral buffered formalin, 12 hours) of Sprague-Dawley rat tissues appeared to produce superior fixation in contrast to Zamboni's solution, Carnoy's solution and ethanol. This conclusion was made on the observation that formalin fixed tissues produced well defined labelling of the basement membrane between the stratum basale (of the stratified oral epithelium) and the lamina propria of the rat oral mucosa (Figures 8A-8C). In addition, formalin fixation produced superior architectural and cytological preservation of the stratified oral epithelium of the oral mucosa, and the tooth and periodontium compared to the other fixation techniques.

7.1.2 Labelling and staining

7.1.2.1 Immunohistochemistry

7.1.2.1.1 Alpha syndecan-1

Labelling with Alpha syndecan-1 in the stratified oral epithelium of rat tongue exposed to EDTA was negative in the stratum corneum and the stratum basale, light to moderate in intensity in the stratum granulosum and of high intensity in the stratum spinosum (Figure 9A). Labelling in the stratum granulosum and stratum spinosum occurred on the cell membranes of the epithelial cells.

Labelling was negative in the stratified oral epithelium of rat tongue and palate, exposed to EDTA, (Figure 9B); and was negative in the rat periodontium, including the epithelial cell rests of Malassez, despite positive reference identification labelling (cytokeratin AE1-AE3) of epithelial cell rests of Malassez in adjacent tissue sections, (Figures 9C, 9D).

7.1.2.1.2 Syndecan-1 B-B4

Labelling with Syndecan-1 B-B4 was negative in the stratified oral epithelium of the rat tongue (Figure 10A). Labelling was also negative in the stratified oral epithelium of the rat palate (Figure 10B) and the rat periodontium (including the epithelial cell rests of Malassez).

Examination of labelling in positive-control tissues (human oral carcinomas) revealed labelling of high intensity (Syndecan-1 B-B4) in the cell membranes in all cell layers of normal human stratified oral epithelium (Figure 10C). In areas of squamous cell carcinoma labelling with Syndecan-1 B-B4 was of light intensity in moderately differentiated cancerous cells and very light intensity in poorly differentiated cancerous cells (Figure 10D).

7.1.2.1.3 Syndecan-1 N-18

Cytoplasmic labelling with Syndecan-1 N-18, in the stratified oral epithelium of rat tongue (exposed to EDTA), was of high intensity in the stratum corneum, moderate to high in the stratum granulosum and the stratum spinosum, and negative in the stratum basale at all ages examined: 2 days to 6 weeks (Figure 11A).

Cytoplasmic labelling with Syndecan-1 N-18 in the stratified oral epithelium of the rat palate was of high intensity in the stratum corneum and the stratum spinosum, and moderate in intensity in the stratum granulosum (Figure 11B) at all ages examined. Labelling was negative in the stratum basale. Labelling with Syndecan-1 N-18 of light to moderate intensity was observed in the cytoplasm of ameloblasts in sections of 2 day and 2 week rats (Figure 11B). Labelling was negative for the rat periodontium, including the epithelial cell rests of Malassez, despite positive reference identification labelling (cytokeratin AE1-AE3) of epithelial cell rests of Malassez in adjacent tissue sections (Figures 11C,11D).

7.1.2.2 Histochemistry

7.1.2.2.1 Alcec Blue

Cytoplasmic staining with Alcec Blue in the stratified oral epithelium of the rat palate was of moderate to high intensity in the stratum corneum, light in intensity in the stratum granulosum and the stratum spinosum; and negative in the stratum basale, for all ages examined, 2 days to 6 weeks (Figures 12A,12B).

Cytoplasmic staining with Alcec Blue in the developing rat molar, from 2 days to 6 weeks, was of moderate to high intensity in the pre-dentine and dentine (Figures 12A-12C); and of light intensity in ameloblasts of sections from 2 week old rats. Staining was negative for the rat periodontium (Figure 12C).

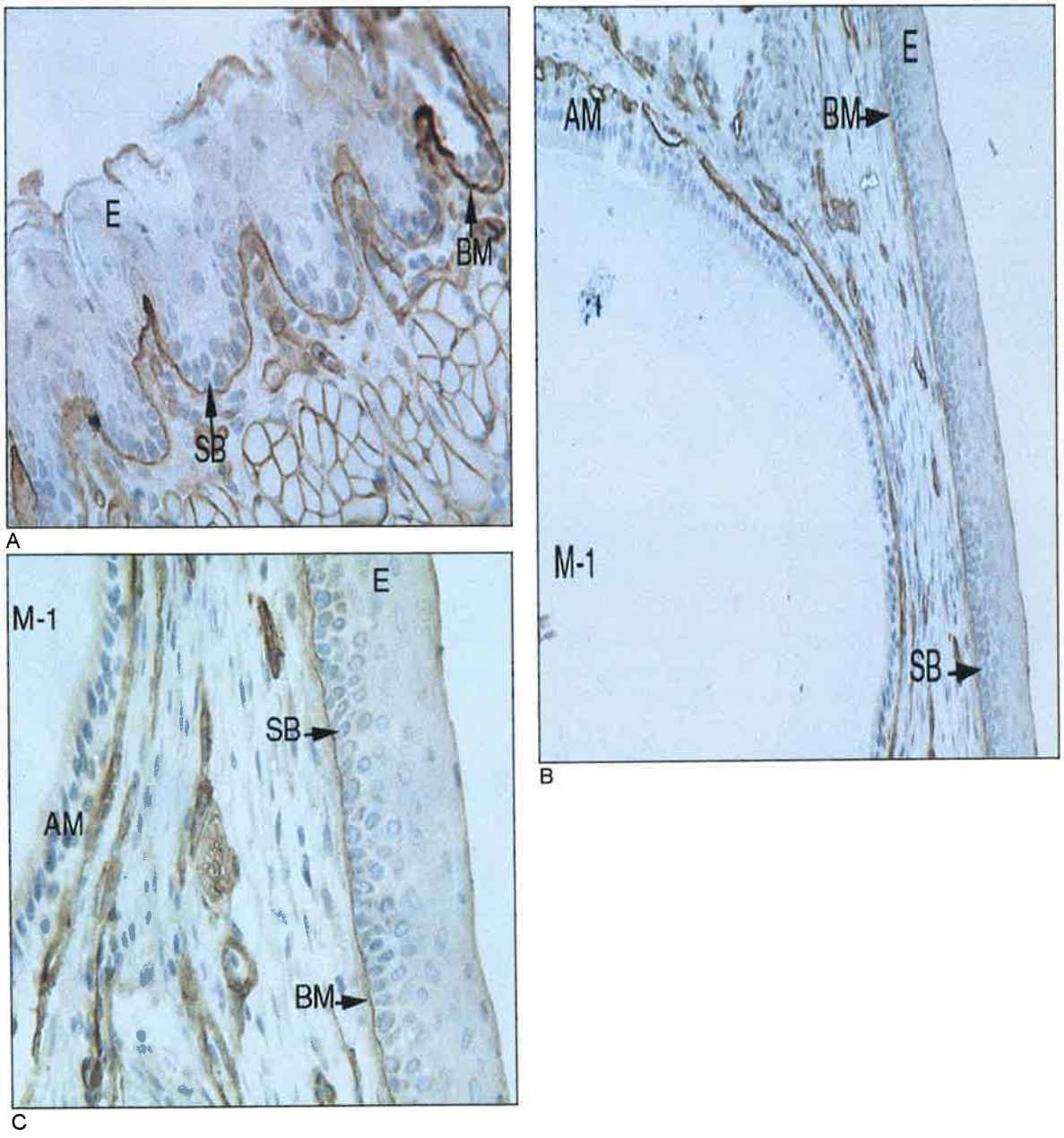


Figure 8. Trial study: Rabbit anti-laminin labelling (1:2000) of 10% neutral buffered formalin fixed rat tissue.

A. Photomicrograph of rat tongue mucosa showing positive labelling of the basement membrane (BM) of the oral epithelium (E) as well as that of small blood vessels and skeletal muscle fibres. Stratum basale (SB). Rat tongue specimen (original magnification x 200).

B. Photomicrograph of rat palatal mucosa and the developing rat maxillary first molar (M-1), showing positive labelling of the basement membrane (BM) and positive labelling of blood vessel basement membranes. Oral epithelium (E); stratum basale (SB); ameloblasts (AM). Rat maxillary specimen, longitudinal section (original magnification x 100).

C. High power photomicrograph of rat palatal mucosa and the developing rat maxillary first molar (M-1), showing the oral epithelium (E), with positive labelling of the basement membrane (BM) as well as positive labelling of blood vessel basement membranes. Stratum basale (SB); ameloblasts (AM). Rat maxillary specimen, longitudinal section (original magnification x 200).

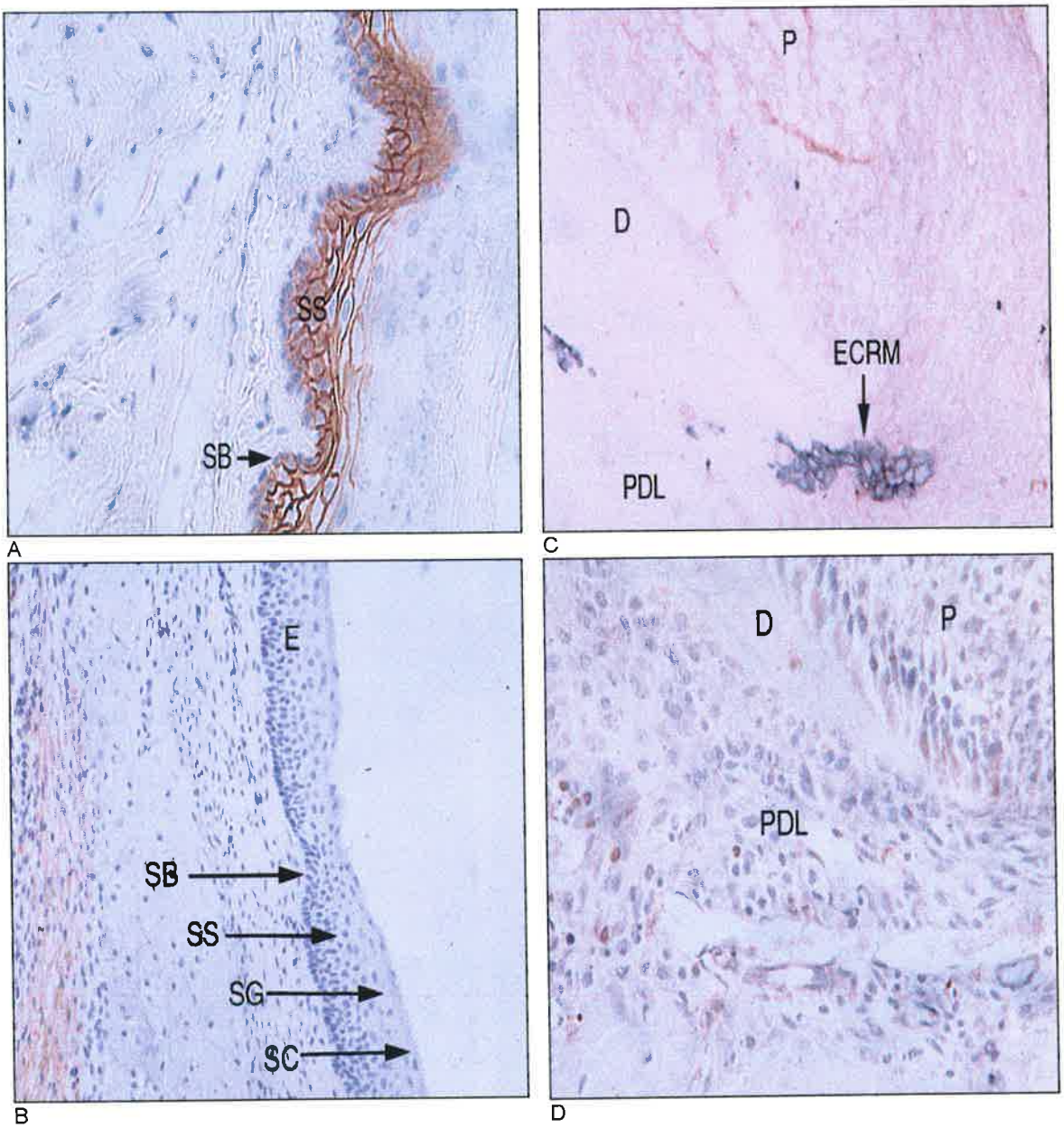


Figure 9. Trial study: Alpha syndecan-1 (1:200) labelling (Figures A,B,D) and cytokeratin AE1-AE3 (1:15,000) labelling (Figure C) of 10% neutral buffered formalin fixed rat tissue.

A Photomicrograph of rat tongue mucosa showing positive labelling of the oral epithelium in cells comprising the stratum spinosum (SS). Stratum basale (SB). Rat tongue specimen (original magnification x 200).

B. Lower power photomicrograph of rat palatal mucosa showing negative labelling of the oral stratified epithelium (E). Stratum corneum (SC); stratum granulosum (SG); stratum spinosum (SS); stratum basale (SB). Rat maxillary specimen exposed to EDTA (original magnification x 100).

C. Photomicrograph of the apical periodontal ligament (PDL) third of the first rat maxillary molar (M-1), showing positive labelling of epithelial cell rests of Malassez (ECRM) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Rat maxillary specimen, longitudinal section (original magnification x 400).

D. Photomicrograph of an adjacent tissue section, to that shown in Figure C, showing negative labelling of epithelial cell rests of Malassez in the periodontal ligament (PDL) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Rat maxillary specimen, longitudinal section (original magnification x 400).

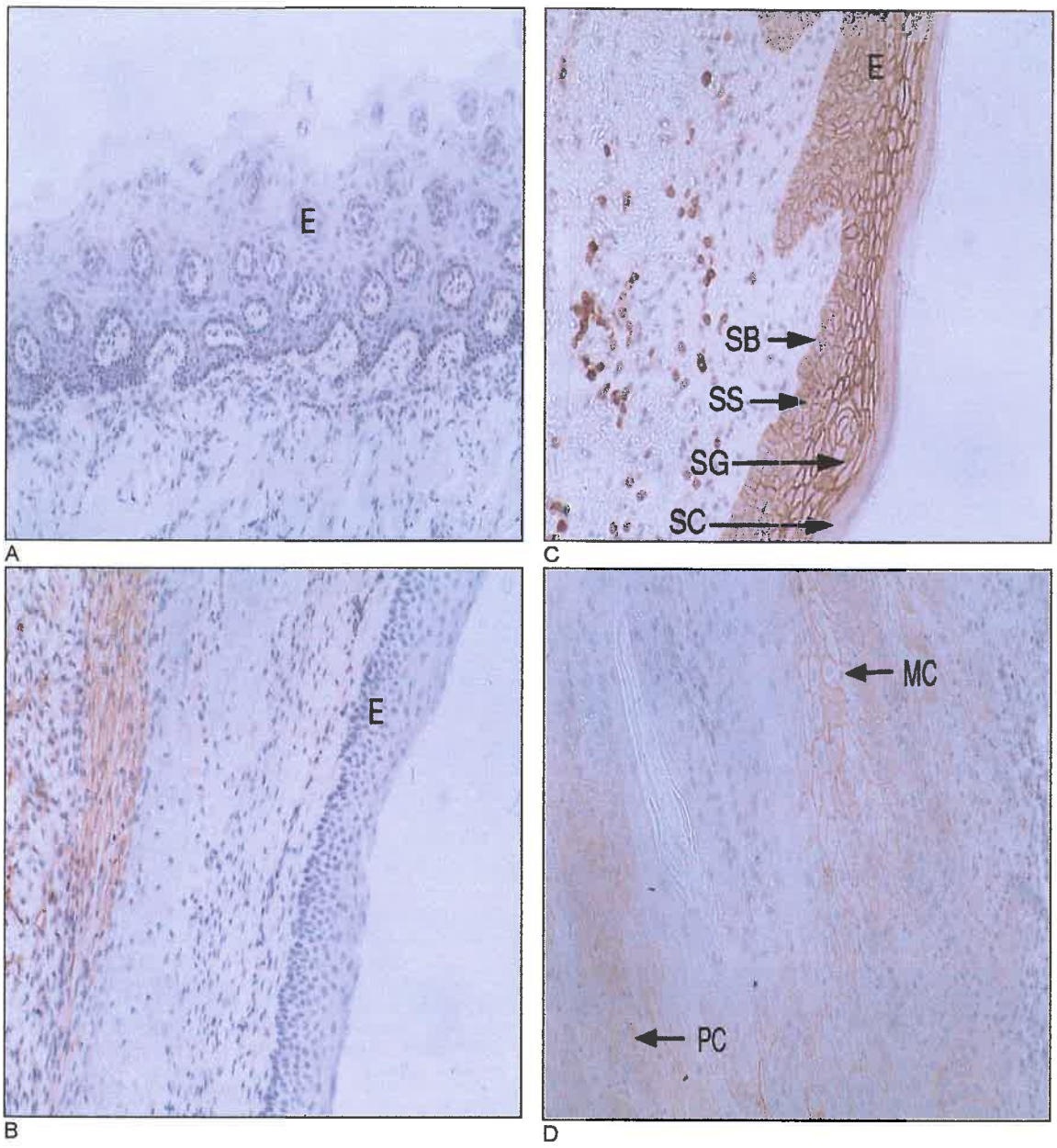


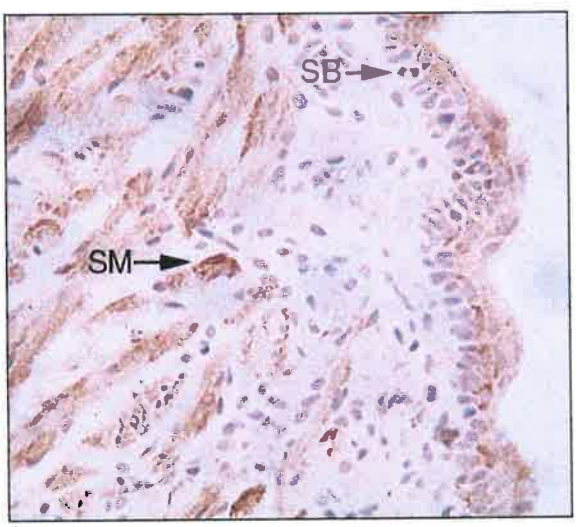
Figure 10. Trial study: Syndecan-1 B-B4 labelling (1:1000) of 10% neutral buffered formalin fixed rat tissue.

A. Photomicrograph of rat tongue mucosa showing negative labelling of the oral epithelium (E). Rat tongue specimen (original magnification x 200).

B. Lower power photomicrograph of rat palatal mucosa showing negative labelling of the oral epithelium (E). Rat maxillary specimen exposed to EDTA (original magnification x 100).

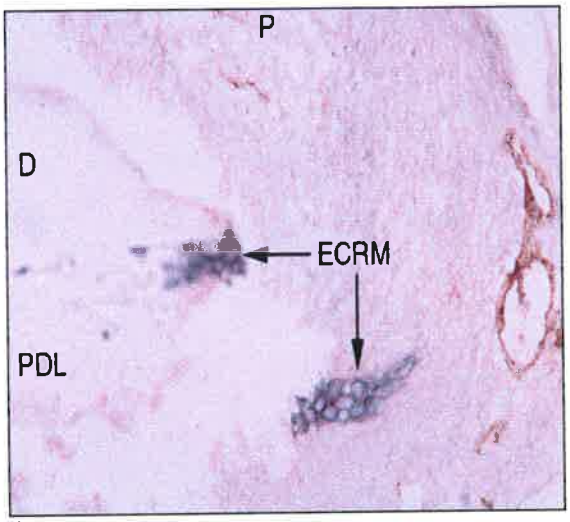
C. Photomicrograph of human oral mucosa showing positive labelling of plasma cells and labelling in the cell membranes of cells of normal stratified oral epithelium (E), adjacent to an area of carcinoma. Stratum corneum (SC); stratum granulosum (SG); stratum spinosum (SS); stratum basale (SB). Specimen of human oral mucosa (original magnification x 100).

D. High power photomicrograph of human oral carcinoma showing light positive labelling in the cell membranes of moderately differentiated cancerous cells (MC) and poorly differentiated cancerous cell (PC). Human oral squamous cell carcinoma specimen (original magnification x 400).

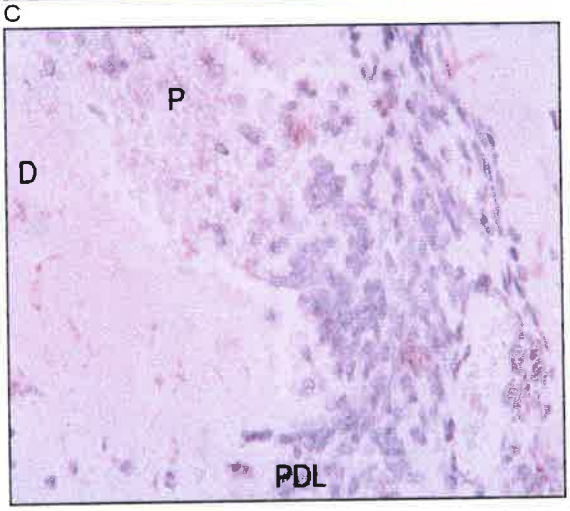


A

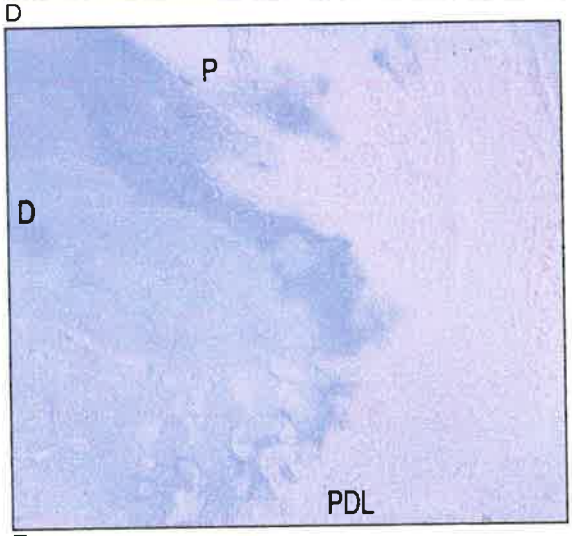
B



C



D



E

Figure 11. Trial study: Syndecan-1 N-18 (1:600) labelling (Figures A,B,D); cytokeratin AE1-AE3 (1:15,000) labelling (Figure C); and Alcec Blue (0.42mol/L) staining (Figure E) of 10% neutral buffered formalin fixed rat tissue.

A. Photomicrograph of rat tongue mucosa showing positive labelling of the oral epithelium above the stratum basale (SB), and positive labelling of skeletal muscle fibres (SM). Rat tongue specimen (original magnification x 100).

B. Photomicrograph of the rat palatal mucosa and the developing rat maxillary first molar (M-1), showing positive labelling of the oral epithelium above the stratum basale (SB), and positive labelling in ameloblasts (AM). Rat maxillary longitudinal section from a 2 day old rat (original magnification x 40).

C. Photomicrograph of the apical periodontal ligament (PDL) third of the first rat maxillary molar (M-1), showing positive labelling of epithelial cell rests of Malassez (ECRM) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Rat maxillary longitudinal section from a 4 week old rat (original magnification x 400).

D. Photomicrograph of an adjacent tissue section, to that shown in Figure C, showing negative labelling of epithelial cell rests of Malassez in the periodontal ligament (PDL) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Rat maxillary longitudinal section from a 4 week old rat (original magnification x 400).

E. Photomicrograph of an adjacent tissue section, to that shown in Figures C and D, showing negative staining of epithelial cell rests in the periodontal ligament (PDL) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Rat maxillary longitudinal section from a 4 week old rat (original magnification x 400).

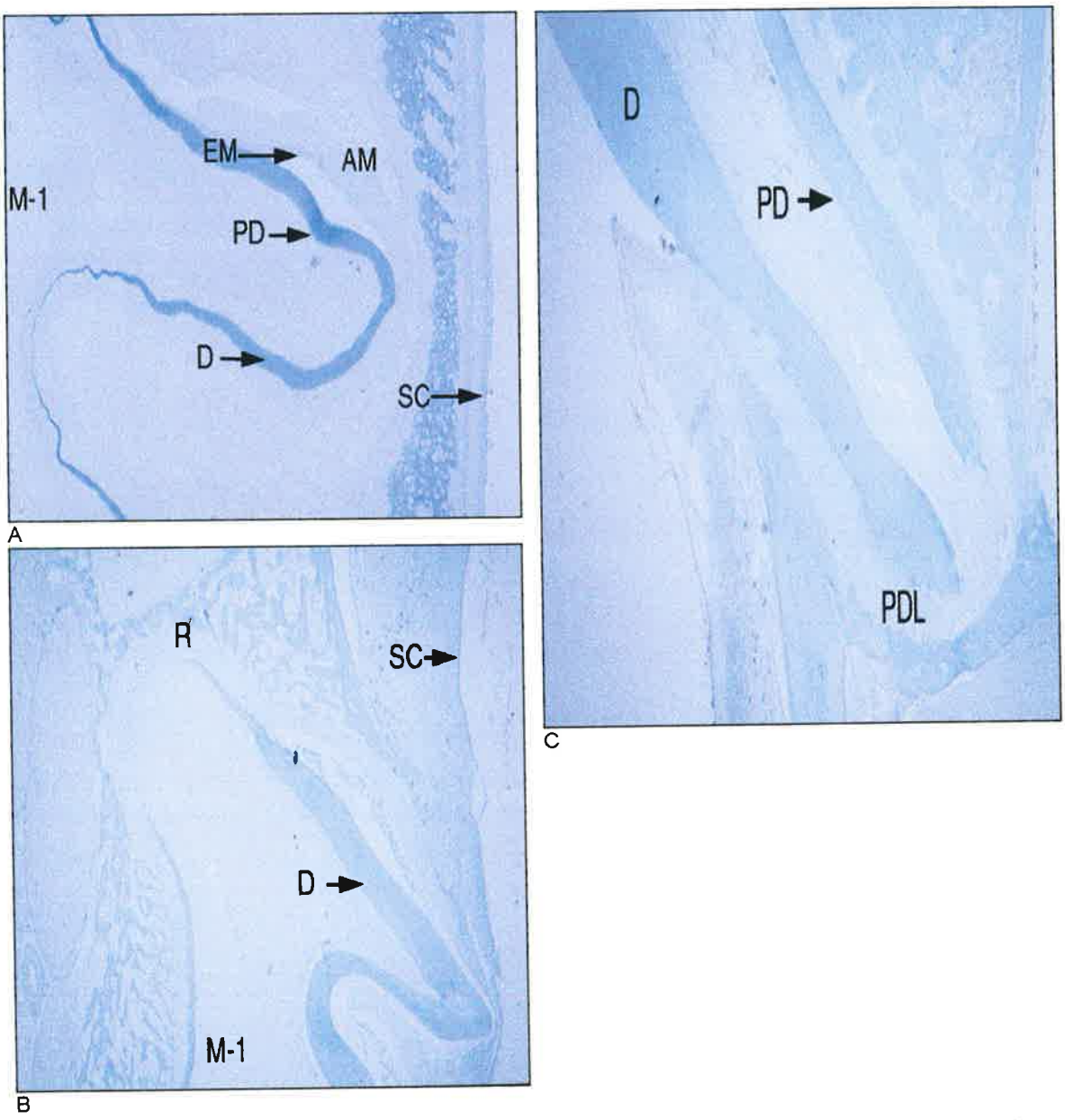


Figure 12. Trial study: Alcec Blue (0.42mol/L) staining of 10% neutral buffered formalin fixed rat tissue.

A. Photomicrograph of rat palatal mucosa and the developing rat maxillary first molar (M-1), showing positive staining of the stratum corneum (SC) of the oral epithelium. There is also positive staining of the pre-dentine (PD) and dentine (D), and light blue staining of the enamel matrix (EM). Ameloblasts (AM). Rat maxillary longitudinal section of a 2 day old rat (original magnification x 40).

B. Photomicrograph of rat palatal mucosa and the developing rat maxillary first molar (M-1), showing positive staining of the stratum corneum (SC) of the oral epithelium. There is also positive staining of coronal dentine (D) and root dentine (R). Rat maxillary longitudinal section of a 2 week old rat (original magnification x40).

C. Photomicrograph of rat palatal mucosa and an erupting rat maxillary first molar (M-1), showing positive staining of pre-dentine (PD) and dentine (D) of the mesiobuccal root. There is no staining of the periodontal ligament (PDL) surrounding the mesiobuccal root and the apex has not yet completed development. Rat maxillary longitudinal section of a 6 week old rat (original magnification x100).

7.2 Main study

7.2.1 Immunohistochemistry (Syndecan-1 N-18)

Based on analysis of the anti-syndecan-1 antibodies used in the trial study and the lack of availability of other more suitable markers, Syndecan-1 N-18 was chosen as the immunohistochemical marker for the main study.

7.2.1.1 Oral epithelium-stratum corneum

Cytoplasmic labelling in the stratum corneum of the stratified oral epithelium of the rat tongue and palate was of high intensity in sections from 2 day (Figures 13A,13B) and 3 week old rats (Figures 16A, 16B); generally high intensity in sections from 6 week old rats (Figures 18A, 18B); moderate to high intensity in sections from 1 week (Figures 14A, 14B) and 4 week old rats (Figures 17A, 17B); and generally moderate to high intensity in sections from 2 week old rats (Figures 15A, 15B).

7.2.1.2 Oral epithelium-stratum granulosum

Cytoplasmic labelling of the stratum granulosum of the stratified oral epithelium of the rat tongue and palate was generally of high intensity in sections from 3 week old rats (Figures 16A, 16B); moderate to high intensity in sections from 1 week (Figures 14A, 14B) and 6 week old rats (Figures 18A,18B); moderate in intensity in sections from 4 week old rats (Figures 17A, 17B); generally moderate intensity in sections from 2 day old rats (Figures 13A, 13B); and, light to moderate in intensity in sections from 2 week old rats (Figures 15A, 15B).

7.2.1.3 Oral epithelium-stratum spinosum

Cytoplasmic labelling of the stratum spinosum in the stratified oral epithelium of the rat tongue and palate was of high intensity in sections from 2 day (Figures 13A, 13B) and 1 week old rats (Figures 14A, 14B); generally moderate to high in intensity in sections from 3 week old rats (Figures 16A, 16B); moderate to high in intensity in sections from 6 week old rats (Figures 18A, 18B); light to moderate in intensity in sections from 2 week old rats (Figures 15A, 15B); and of light intensity in sections from 4 week old rats (Figures 17A, 17B). Staining was negative in the stratum basale in sections for all ages examined.

7.2.1.4 Ameloblasts

Labelling of ameloblasts in the developing rat molar was patchy in distribution, and of light intensity, in sections of all rats examined from the 2 day and 2 week time intervals (Figures 13B &15B).

7.2.1.5 Periodontium and epithelial cells

Hertwig's epithelial root sheath, positively identified by cytokeratin AE1-AE3, did not fragment into epithelial cell rest clusters/strands until observation of sections from the 3 week old rat pups (Figure 16C). Labelling (Syndecan-1 N-18) and staining (Alcec Blue) of the rat periodontium, including epithelial cell rests of Malassez, was negative for all ages examined, despite reference identification of epithelial cell rests of Malassez with cytokeratin AE1-AE3 (Figures 16C-16E, 17C-17E & 18C-18E).

7.2.2 Histochemistry (Alcec Blue)

7.2.2.1 Oral epithelium-stratum corneum

Cytoplasmic staining of the stratum corneum of the stratified oral epithelium of the rat tongue and palate was of moderate to high intensity in sections from 2 day (Figures 13C, 13D), 1 week (Figures 14C, 14D), 4 week and 6 week old rats; moderate in intensity in sections from 2 week old rats (Figures 15C, 15D); and, light to moderate intensity in sections from 3 week old rats.

7.2.2.2 Oral epithelium-stratum granulosum

Cytoplasmic staining of the stratum granulosum of the stratified oral epithelium of the rat tongue and palate was generally of moderate intensity in sections from 2 day old rats (Figures 13C, 13D); light to moderate in sections from 1 week (Figures 14C, 14D), 4 week and 6 week old rats; generally light intensity in sections from 2 week old rats (Figures 15C, 15D); and, of light intensity in sections from 3 week old rats.

7.2.2.3 Oral epithelium-stratum spinosum

Cytoplasmic staining of the stratum spinosum of the stratified oral epithelium of the rat tongue and palate was of light intensity in sections from 2 day (Figures 13C,13D), 3 week, 4 week and 6 week old rats; light to moderate intensity in sections from 1 week old rats (Figure 14C, 14D); and, generally light intensity in sections from 2 week old

rats (Figure 15C,15D). Staining of the stratum basale was negative for all ages examined.

7.2.2.4 Ameloblasts and enamel matrix

Staining of the enamel matrix of the developing rat molar was of moderate intensity in sections from 1 week old rats (Figure 14C, 14D). Staining of ameloblasts was of light intensity in sections from 2 week old rats (Figure 15C).

7.2.2.5 Pre-dentine and dentine

Staining of the pre-dentine and dentine of the developing rat molar was of high intensity in sections from 2 day old (Figure 13C, 13D), and 3 week to 6 week old rats (Figures 16E, 17E & 18E); moderate in sections from 1 week old rats (Figure 14C, 14D); and, generally moderate in sections from 2 week old rats (Figures 15C, 15D).

Table 3. Table showing results of semiquantitative analysis of immunohistochemical (Syndecan-1 N-18) and Alcec Blue stained sections of 2 day to 2 week old rat tissues (stratified oral epithelium of the rat tongue and palate, and the developing periodontium around M-1). Staining was of variable intensity in rats for all ages examined.

		TONGUE EPITHELIUM SYNDECAN-1 N-18			PALATAL EPITHELIUM SYNDECAN-1 N-18			M-1 (N-18)		PALATE AND TONGUE EPITHELIUM (ALCEC BLUE)				RAT MAXILLARY FIRST MOLAR M-1 (ALCEC BLUE)			
Age	Rat	SC	SG	SS	SC	SG	SS	SB	AM	SC	SG	SS	SB	EM	AM	PD	D
2 day	1	+++++	+++	++++	+++++	+++	+++++	0	+	++++	+++	+	0	0	0	+++++	+++++
	2	+++++	+++	++++	+++++	+++	+++++	0	+	++++	+++	+	0	0	0	+++++	+++++
	3	+++++	+++	++++	+++++	+++	+++++	0	+	+++	++	+	0	0	0	+++++	+++++
	4	+++++	++	++++	+++++	++	+++++	0	+	+++	+++	+	0	0	0	+++++	+++++
1 week	1	++++	++++	+++++	++++	++++	+++++	0	0	++++	+	++	0	+++	0	+++	+++
	2	++++	++++	+++++	++++	++++	+++++	0	0	++++	+	++	0	+++	0	+++	+++
	3	+++++	++++	+++++	+++++	++++	+++++	0	0	++++	+	++	0	+++	0	+++	+++
	4	+++++	++++	+++++	+++++	++++	+++++	0	0	++++	+	++	0	+++	0	+++	+++
2 week	1	+++++	+	+	+++++	+	+	0	+	+++	+	+	0	0	+	+++	+++
	2	++++	++	++	++++	++	++	0	+	+++	+	+	0	0	+	+++	+++
	3	++++	+	+	++++	+	+	0	+	+++	+	+	0	0	+	+++	+++
	4	++++	++	++	++++	++	++	0	+	+++	+++	++	0	0	+	++++	++++

Stratified oral epithelium: SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale; AM, ameloblasts; EM, enamel matrix; PD, pre-dentine; D, dentine; N-18, Syndecan-1 N-18; M-1, rat maxillary first molar. Staining intensity: 0, negative; +, light; ++, light-moderate; +++, moderate; ++++, moderate-high; +++++, high.

Table 4. Table showing results of semiquantitative analysis of immunohistochemical (Syndecan-1 N-18) and Alcec Blue stained sections of 3 week to 6 week old rat tissues (stratified oral epithelium of the rat tongue and palate, and the developing periodontium around M-1). Staining was of variable intensity in rats for all ages examined.

		TONGUE EPITHELIUM SYNDECAN-1 N-18			PALATAL EPITHELIUM SYNDECAN-1 N-18				M-1 (N-18)		PALATE AND TONGUE EPITHELIUM (ALCEC BLUE)				RAT MAXILLARY MOLAR M-1 (ALCEC BLUE)			
Age	Rat	SC	SG	SS	SC	SG	SS	SB	PDL	ECRM	SC	SG	SS	SB	EM	PD	D	PDL
3 week	1	++++	++++	++	+++++	+++++	++	0	0	0	+	+	+	0	0	+++++	+++++	0
	2	++++	++++	++++	+++++	+++++	++++	0	0	0	+	+	+	0	0	++++	++++	0
	3	++++	++++	++++	+++++	++++	++++	0	0	0	+++	+	+	0	0	+++++	+++++	0
	4	++++	++++	++++	+++++	+++++	++++	0	0	0	+++	+	+	0	0	+++++	+++++	0
4 week	1	+++++	++++	+	+++++	++++	+	0	0	0	+++++	+	+	0	0	+++++	+++++	0
	2	+++++	+++	+	+++++	+++	+	0	0	0	++++	++	+	0	0	++++	++++	0
	3	++++	+++	++	++++	+++	++	0	0	0	+++	++	+	0	0	+++++	+++++	0
	4	+++++	++	+	++++	++	+	0	0	0	+++	+	+	0	0	+++++	+++++	0
6 week	1	+++++	+++++	++++	+++++	+++++	++++	0	0	0	++++	+	+	0	0	+++++	+++++	0
	2	+++++	++++	+++	+++++	++++	+++	0	0	0	+++++	+	+	0	0	+++++	+++++	0
	3	+++++	++++	+++	+++++	++++	+++	0	0	0	++++	++	+	0	0	+++++	+++++	0
	4	+++++	+++++	++++	++++	+++++	++++	0	0	0	++++	++	+	0	0	+++++	+++++	0

Stratified oral epithelium: SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale; PDL, periodontal ligament; ECRM, epithelial cell rests of Malassez; EM, enamel matrix, PD, pre-dentine; D, dentine; N-18, Syndecan-1 N-18; M-1, rat maxillary first molar. Staining intensity: 0, negative; +, light; ++, light-moderate; +++, moderate; ++++, moderate-high; +++++, high.

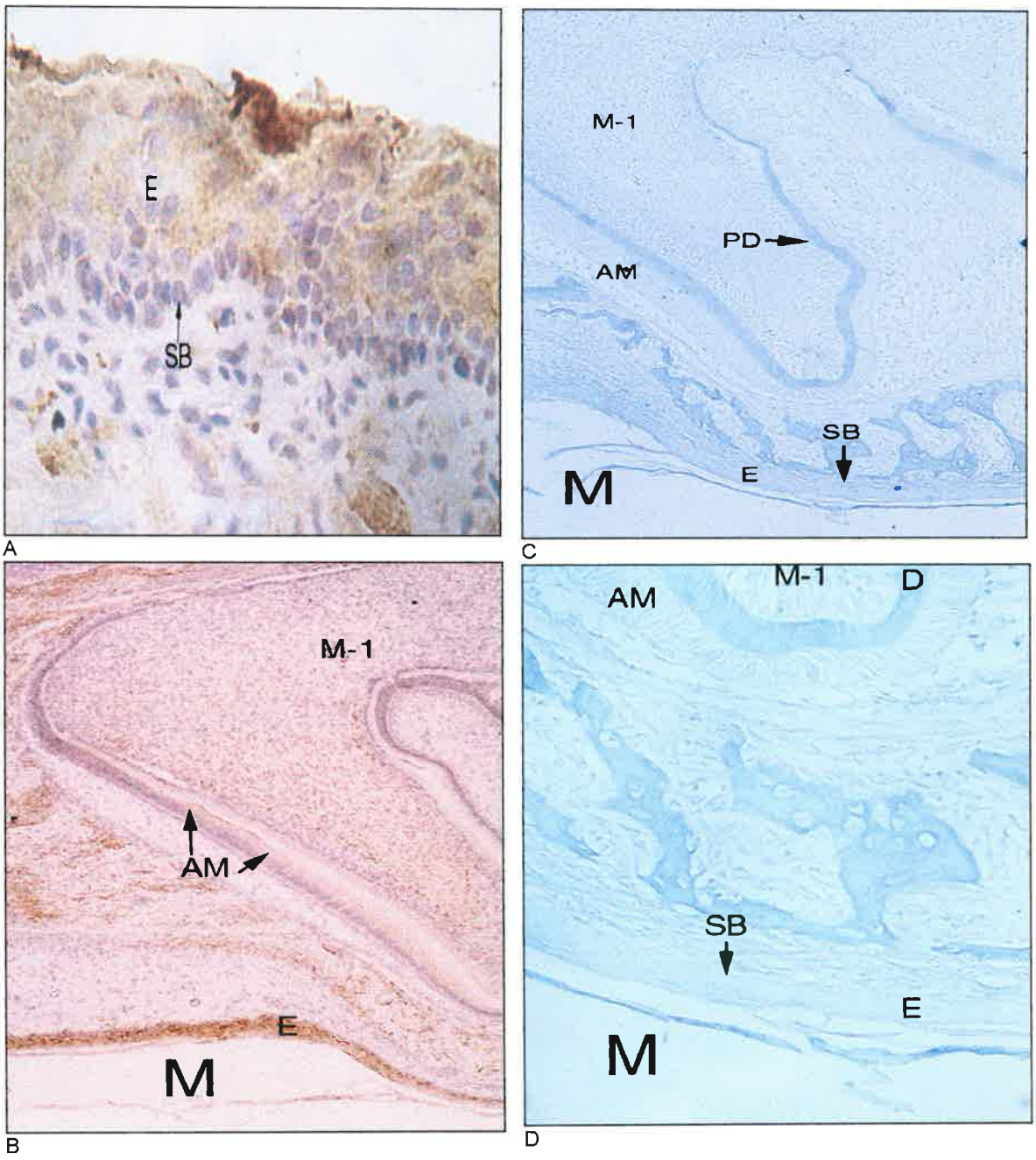


Figure 13. Main study: Syndecan-1 N-18 (1:600) labelling (Figures A & B) and Alcec Blue (0.42 mol/L) staining (Figures C & D) of 10 % neutral buffered formalin fixed rat tissue (section from a 2 day old rat).

A. Photomicrograph of rat tongue mucosa showing positive labelling of the oral epithelium (E) above the stratum basale (SB). Rat tongue specimen, longitudinal section (original magnification x 200).

B. Low power photomicrograph of rat palatal mucosa (M) and the developing rat maxillary first molar (M-1), showing positive labelling of the oral epithelium (E), and light positive labelling of ameloblasts (AM). Rat maxillary specimen, longitudinal section (original magnification x 40).

C. Low power photomicrograph of the rat palatal mucosa (M) and the developing rat maxillary first molar (M-1), showing staining of the oral epithelium (E) in particular the stratum corneum. There is also staining of the pre-dentine (PD) and dentine. Stratum basale (SB); ameloblasts (AM). Rat maxillary specimen, longitudinal section (original magnification x 40).

D. High power photomicrograph of rat palatal mucosa (M) and the rat maxillary first molar (M-1), showing staining of the oral epithelium (E), as well as dentine (D). Stratum basale (SB); ameloblasts (AM). Rat maxillary specimen, longitudinal section (original magnification x 200).

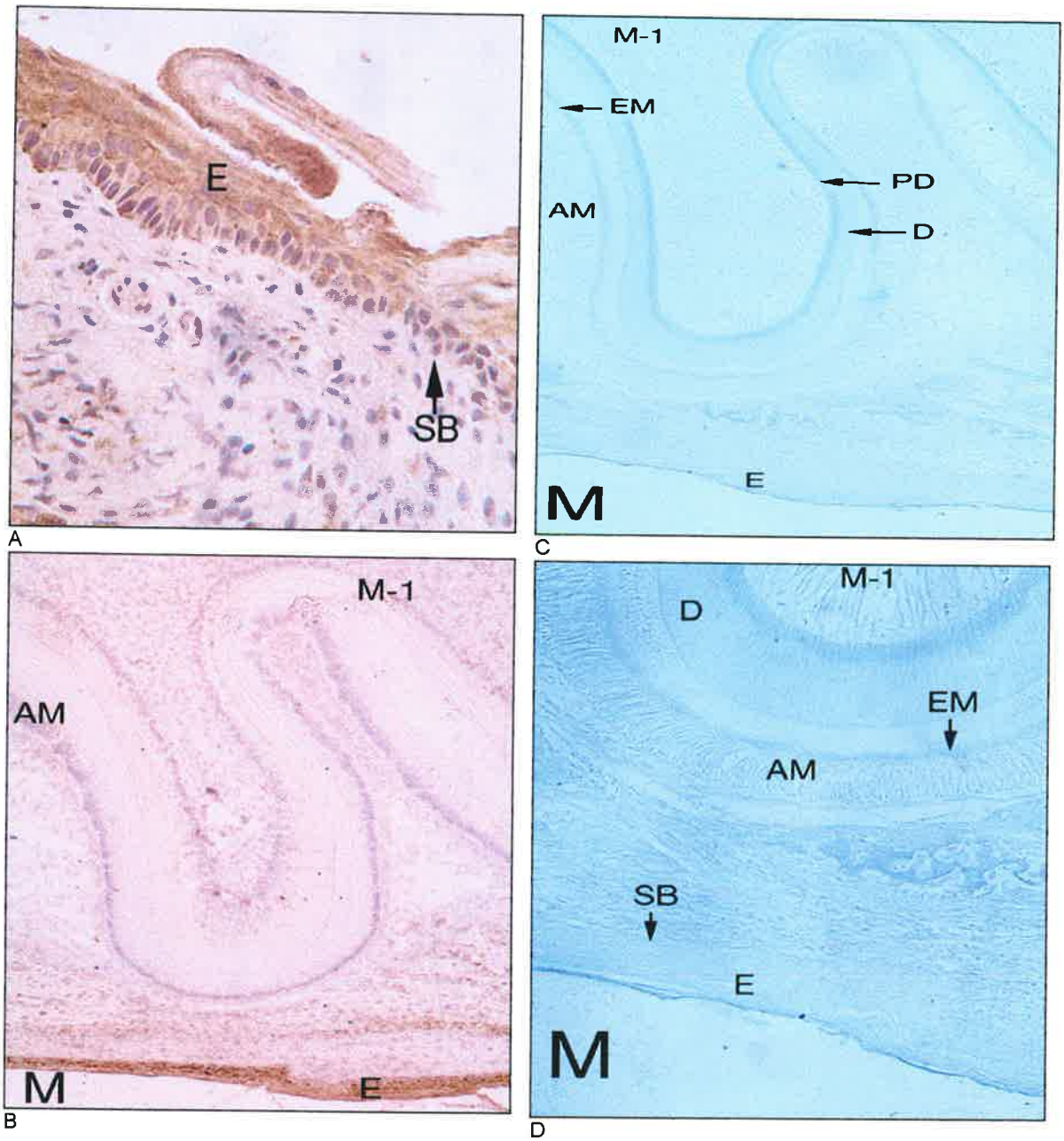


Figure 14. Main study: Syndecan-1 N-18 (1:600) labelling (Figures A & B) and Alcec Blue (0.42 mol/L) staining (Figures C & D) of 10 % neutral buffered formalin fixed rat tissue (section from a 1 week old rat).

A. Photomicrograph of rat tongue mucosa showing positive labelling of the oral epithelium (E) above the stratum basale (SB). Rat tongue specimen (original magnification x 200).

B. Photomicrograph of rat palatal mucosa (M) and the developing rat maxillary first molar (M-1), showing positive labelling of the oral epithelium (E), and negative labelling of ameloblasts (AM). Rat maxillary specimen, longitudinal section (original magnification x 40).

C. Photomicrograph of rat palatal mucosa (M) and the developing rat maxillary first molar (M-1), showing staining of the oral epithelium (E), and staining of the pre-dentine (PD), dentine (D) and the enamel matrix (EM). Ameloblasts (AM). Rat maxillary specimen, longitudinal section (original magnification x 40).

D. High power photomicrograph of rat palatal mucosa (M) and the rat maxillary first molar (M-1), showing staining of the oral epithelium (E), the enamel matrix (EM), and dentine (D). Stratum basale (SB); ameloblasts (AM). Rat maxillary specimen, longitudinal section (original magnification x 200).

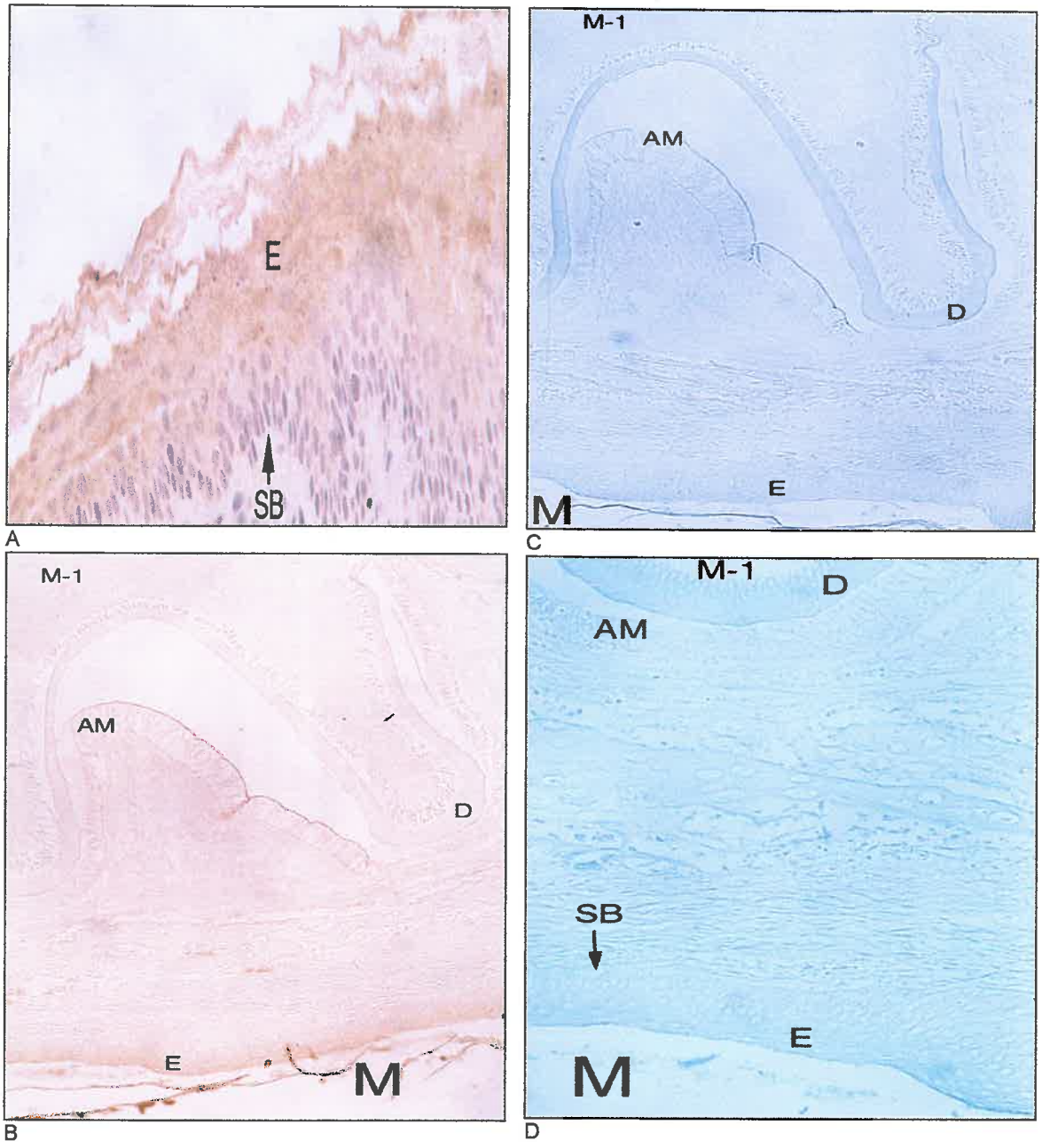


Figure 15. Main study: Syndecan-1 N-18 (1:600) labelling (Figures A & B) and Alcec Blue (0.42 mol/L) staining (Figure C &D) of 10 % neutral buffered formalin fixed rat tissue (section from a 2 week old rat).

A. Photomicrograph of rat tongue mucosa showing positive labelling of the oral epithelium (E) above the stratum basale (SB). Rat tongue specimen (original magnification x 200).

B. Photomicrograph of rat palatal mucosa (M) and the developing rat maxillary first molar (M-1), showing positive labelling of the oral epithelium (E) and ameloblasts (AM). Dentine (D). Rat maxillary specimen, longitudinal section (magnification x 40).

C. Photomicrograph of rat palatal mucosa (M) and the developing rat maxillary first molar (M-1), showing staining of the oral epithelium (E), ameloblasts (AM) and dentine (D). Rat maxillary specimen, longitudinal section (original magnification x 40).

D. High power photomicrograph of rat palatal mucosa (M) and the rat maxillary first molar (M-1), showing staining of the oral epithelium (E), and dentine (D). Stratum basale (SB); ameloblasts (AM). Rat maxillary specimen, longitudinal section (original magnification x 200).

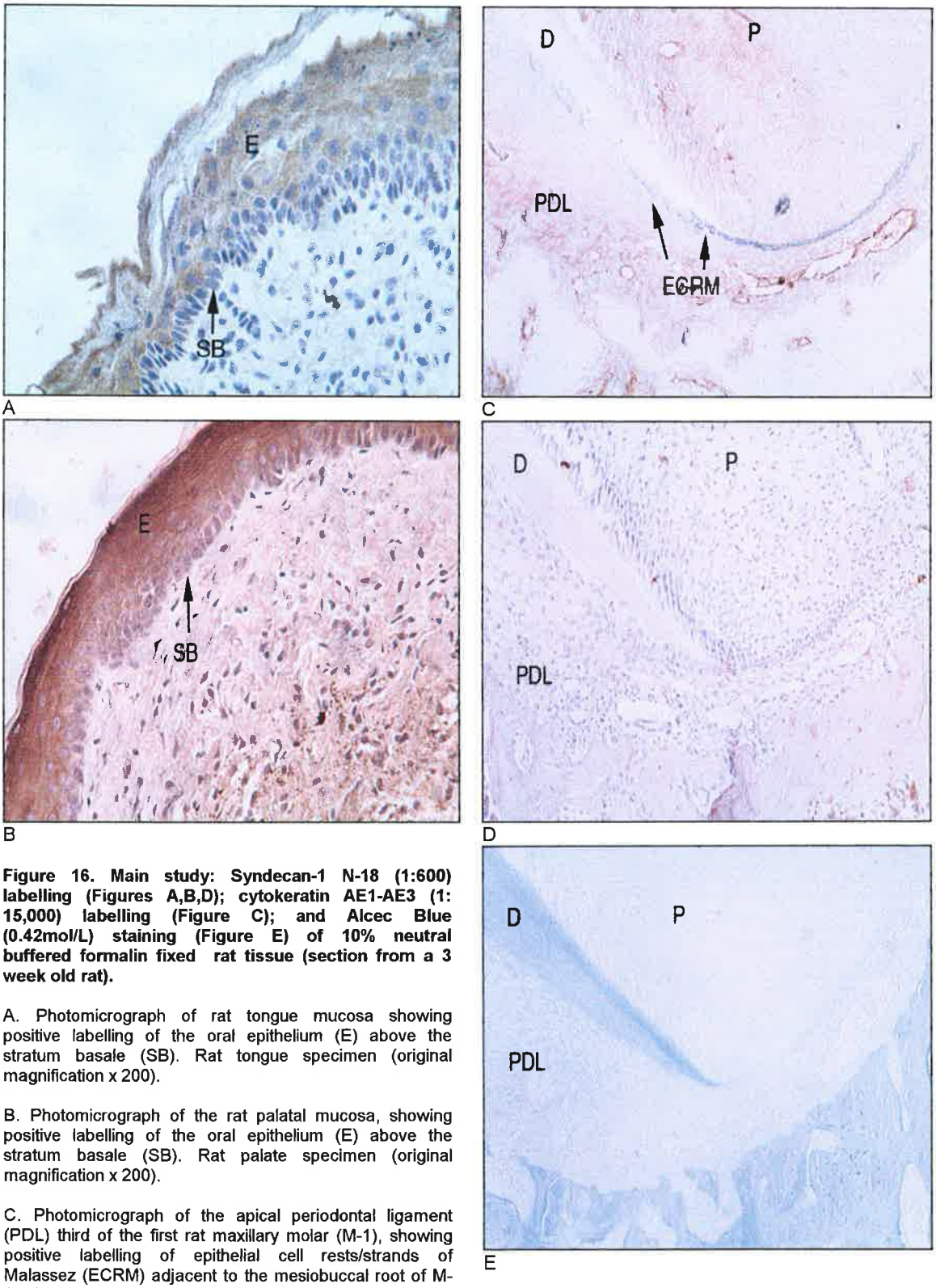


Figure 16. Main study: Syndecan-1 N-18 (1:600) labelling (Figures A,B,D); cytokeratin AE1-AE3 (1: 15,000) labelling (Figure C); and Alcec Blue (0.42mol/L) staining (Figure E) of 10% neutral buffered formalin fixed rat tissue (section from a 3 week old rat).

A. Photomicrograph of rat tongue mucosa showing positive labelling of the oral epithelium (E) above the stratum basale (SB). Rat tongue specimen (original magnification x 200).

B. Photomicrograph of the rat palatal mucosa, showing positive labelling of the oral epithelium (E) above the stratum basale (SB). Rat palate specimen (original magnification x 200).

C. Photomicrograph of the apical periodontal ligament (PDL) third of the first rat maxillary molar (M-1), showing positive labelling of epithelial cell rests/strands of Malassez (ECRM) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Rat maxillary specimen, longitudinal section (original magnification x 200).

D. Photomicrograph of an adjacent tissue section, to that shown in Figure C, showing negative labelling of epithelial cell rests of Malassez in the apical periodontal ligament (PDL), adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Rat maxillary specimen, longitudinal section (original magnification x 200).

E. Photomicrograph of an adjacent tissue section, to that shown in Figures C and D, showing negative staining of epithelial cell rests of Malassez in the apical periodontal ligament (PDL) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Rat maxillary specimen, longitudinal section (original magnification x 200).

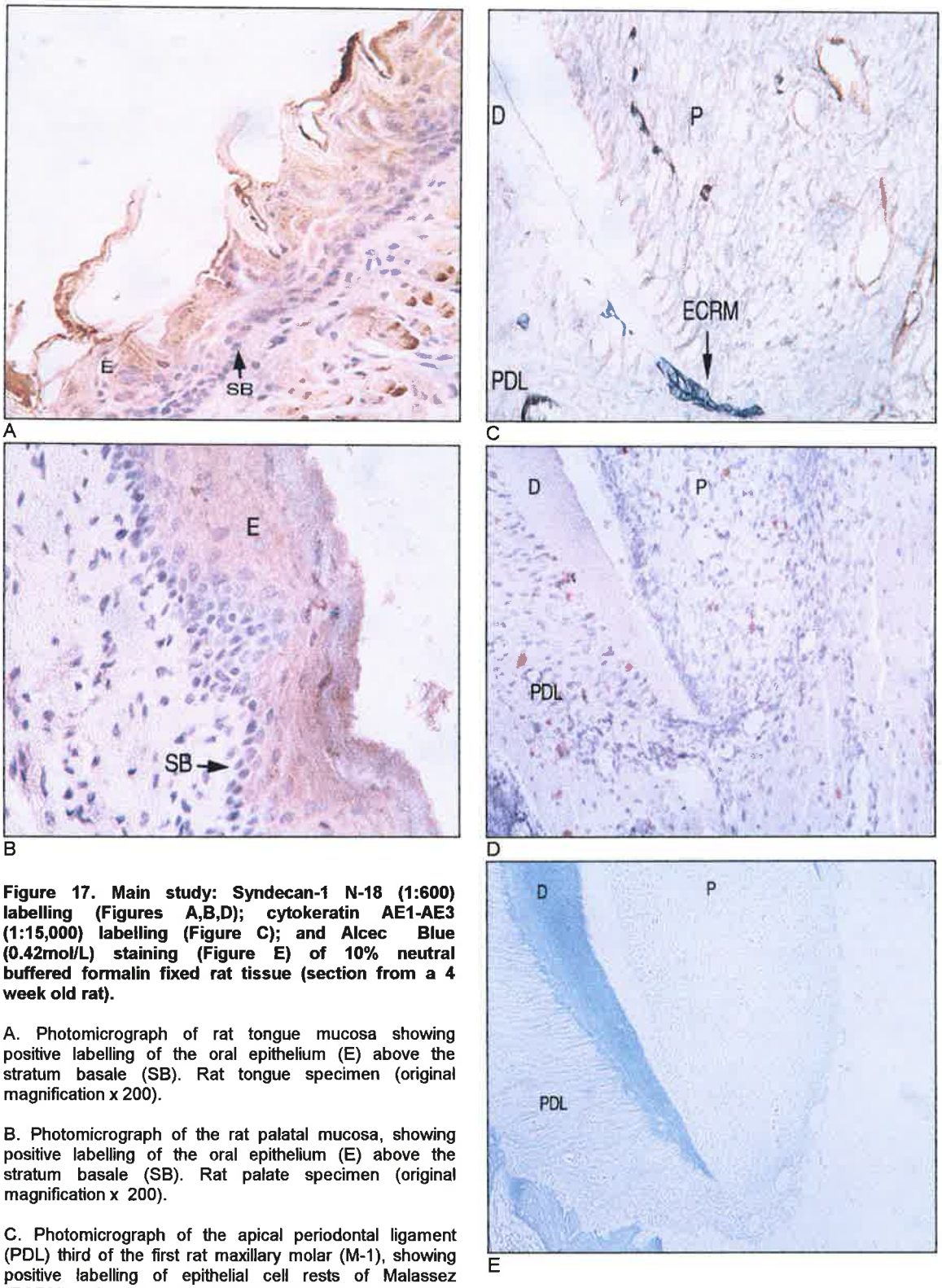


Figure 17. Main study: Syndecan-1 N-18 (1:600) labelling (Figures A,B,D); cytokeratin AE1-AE3 (1:15,000) labelling (Figure C); and Alcec Blue (0.42mol/L) staining (Figure E) of 10% neutral buffered formalin fixed rat tissue (section from a 4 week old rat).

A. Photomicrograph of rat tongue mucosa showing positive labelling of the oral epithelium (E) above the stratum basale (SB). Rat tongue specimen (original magnification x 200).

B. Photomicrograph of the rat palatal mucosa, showing positive labelling of the oral epithelium (E) above the stratum basale (SB). Rat palate specimen (original magnification x 200).

C. Photomicrograph of the apical periodontal ligament (PDL) third of the first rat maxillary molar (M-1), showing positive labelling of epithelial cell rests of Malassez (ECRM) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Rat maxillary specimen, longitudinal section (original magnification x 200).

D. Photomicrograph of an adjacent tissue section, to that shown in Figure C, showing negative labelling of epithelial cell rests of Malassez in the apical periodontal ligament (PDL) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Rat maxillary specimen, longitudinal section (original magnification x 200).

E. Photomicrograph of an adjacent tissue section, to that shown in Figures C and D, showing negative staining of epithelial cell rests of Malassez in the apical periodontal ligament (PDL) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Rat maxillary specimen, longitudinal section (original magnification x 200).

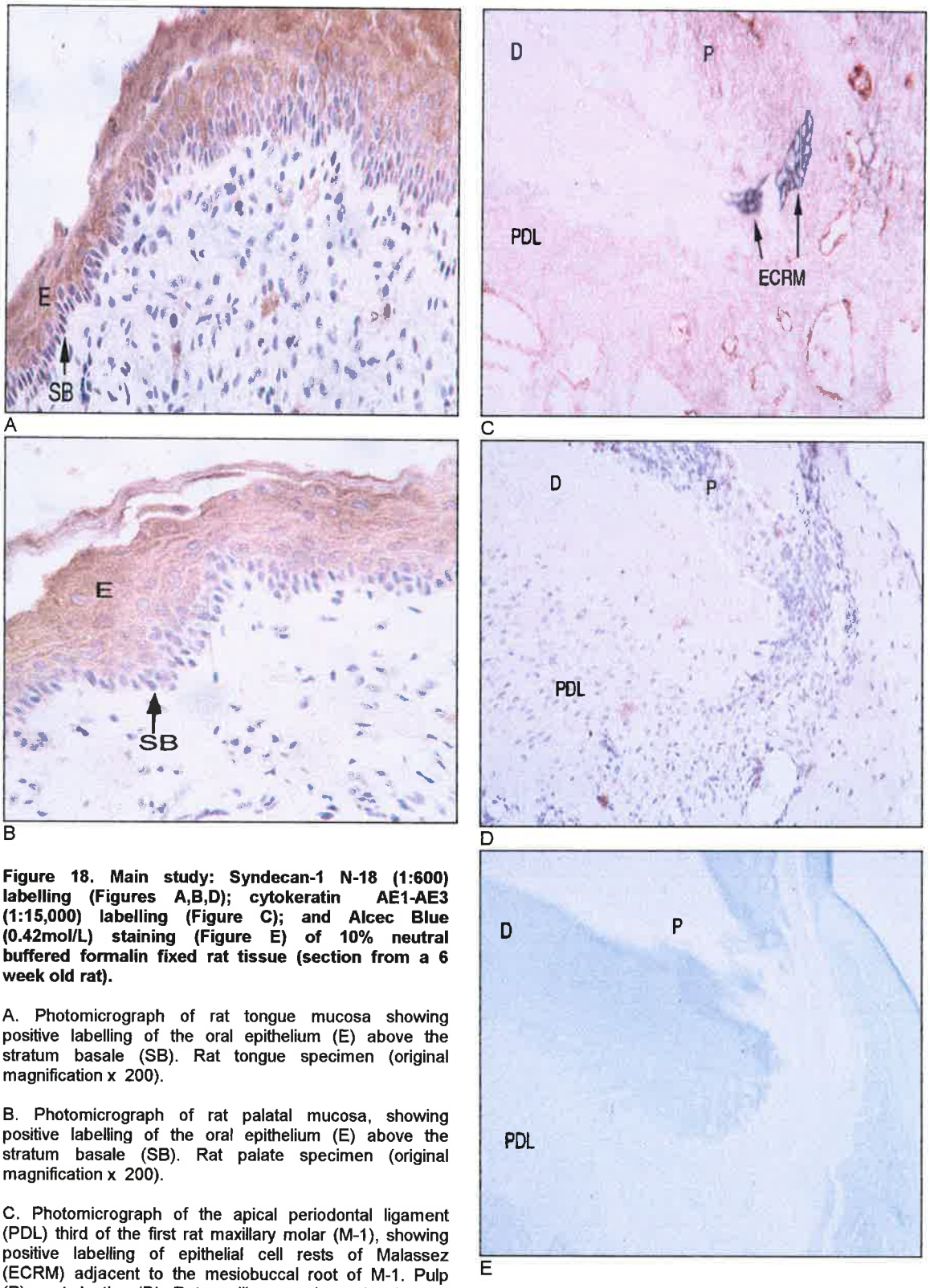


Figure 18. Main study: Syndecan-1 N-18 (1:600) labelling (Figures A,B,D); cytokeratin AE1-AE3 (1:15,000) labelling (Figure C); and Alcec Blue (0.42mol/L) staining (Figure E) of 10% neutral buffered formalin fixed rat tissue (section from a 6 week old rat).

A. Photomicrograph of rat tongue mucosa showing positive labelling of the oral epithelium (E) above the stratum basale (SB). Rat tongue specimen (original magnification x 200).

B. Photomicrograph of rat palatal mucosa, showing positive labelling of the oral epithelium (E) above the stratum basale (SB). Rat palate specimen (original magnification x 200).

C. Photomicrograph of the apical periodontal ligament (PDL) third of the first rat maxillary molar (M-1), showing positive labelling of epithelial cell rests of Malassez (ECRM) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Rat maxillary specimen, longitudinal section (original magnification x 200).

D. Photomicrograph of an adjacent tissue section, to that shown in Figure C, showing negative labelling of epithelial cell rests of Malassez in the apical periodontal ligament (PDL) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Rat maxillary specimen, longitudinal section (original magnification x 200).

E. Photomicrograph of an adjacent tissue section, to that shown in Figures C and D, showing negative staining of epithelial cell rests of Malassez in the apical periodontal ligament (PDL) adjacent to the mesiobuccal root of M-1. Pulp (P); root dentine (D). Rat maxillary specimen, longitudinal section (original magnification x 200).

8 DISCUSSION

8.1 Trial study

8.1.1 Immunohistochemistry

8.1.1.1 Alpha syndecan-1

The high intensity of labelling produced by Alpha syndecan-1 in the stratum spinosum of the stratified oral epithelium of rat tongue, not exposed to EDTA, suggested the antibody detected syndecan-1 expression on the cell membranes of epithelial cells in this cell layer. Negative labelling in the stratified oral epithelium of rat and palate tongue, exposed to EDTA, might suggest that EDTA affected labelling with Alpha syndecan-1 in the stratum spinosum. Labelling by Alpha syndecan-1 was negative in the rat oral mucosa and in the epithelial cell rests of Malassez in the periodontium of the developing rat molar. Possible reasons for this negative staining result are discussed in section 8.3.

8.1.1.2 Syndecan-1 B-B4

Labelling with Syndecan-1 B-B4 was negative in the stratified oral epithelium of rat tongue and palate (exposed to EDTA). According to Jalkanen (1999, personal communication), Syndecan-1 B-B4 is species-specific and recognizes only a limited epitope of syndecan-1 in human epithelial cells. This fact was consistent with the results of the present study. Application of Syndecan-1 B-B4 to sections of human oral squamous cell carcinomas produced high intensity labelling of syndecan-1 in the cell membranes in all cell layers of normal human oral epithelium. This labelling intensity was reduced in moderate and poorly differentiated cancerous cells. This finding was consistent with immunohistochemical studies performed by Anttonen et al., (1999) who used Syndecan-1 B-B4 to detect syndecan-1 in human head and neck squamous cell carcinomas. These researchers concluded that the reduction in Syndecan-1 B-B4 labelling intensity was due to a decrease in the expression of syndecan-1 associated with poorly or undifferentiated carcinomas.

8.1.1.3 Syndecan-1 N-18

The high intensity of labelling with Syndecan-1 N-18, in the stratified epithelium of rat tongue and palatal mucosa, is consistent with reports describing syndecan-1 expression being greatest in the stratum corneum layer of stratified epithelium (Anttonen et al.,

1999). Cytoplasmic labelling observed in ameloblasts of sections from 2 day and 2 week old rats might have been due to the detection of syndecan-1 within intracellular components or compartments (Bernfield et al., 1992). Labelling with Syndecan-1 N-18 was negative in the epithelial cell rests of Malassez in the periodontium of the developing rat molar. Possible reasons for this negative staining result are discussed in section 8.3.

8.1.2 Histochemistry

8.1.2.1 Alcec Blue

The high intensity of Alcec Blue staining observed in the stratum corneum of the stratified oral epithelium of rat palatal mucosa is also consistent with the notion that syndecan-1 expression is greatest in the stratum corneum (Anttonen et al., 1999). In addition, staining of ameloblasts could have been due to the detection of heparan sulphate glycosaminoglycans within intracellular components. The appearance of consistent staining in the pre-dentine and dentine might suggest that syndecan-1 was distributed within these tooth layers during development of the rat molar.

8.2 Main study

8.2.1 Oral epithelium-stratum corneum

Syndecan-1 expression (Syndecan-1 N-18) and Alcec Blue staining in the stratum corneum of the stratified epithelium in the rat tongue and palatal mucosa, was of a higher intensity compared to the stratum granulosum and spinosum. This finding was consistent with reports describing syndecan-1 expression being highest in the stratified epithelium cell-cell regions of keratinocytes and lower in deeper epithelial layers. This result might suggest a role of syndecan-1 in intracellular adhesion (Anttonen et al., 1999) or cell differentiation (Inki et al., 1994) of keratinocytes.

8.2.2 Oral epithelium-stratum granulosum and stratum spinosum

The stratum granulosum and spinosum of the stratified epithelium in the rat tongue and palatal mucosa, displayed a reduced staining or labelling intensity for syndecan-1 compared with the stratum corneum. This finding might be explained by considering the developmental sequence of cells within the stratified epithelium (Ten Cate, 1989).

In the basal layer, epithelial cells are produced and then undergo proliferation. As cells from the basal layer undergo morphological changes and mature (i.e. keratinize) they

form the suprabasal layers (the stratum spinosum and granulosum) eventually migrating to the surface (stratum corneum) of the stratified epithelium (Ten Cate, 1989). In view of this, Bernfield et al., (1992) have described how syndecan-1 expression correlates with the stability and maturity of epithelia, while being down-regulated during epithelial proliferation and migration. In the present study, it might be suggested that epithelial cells of the stratum corneum displayed higher intensity staining having obtained morphological stability following maturation from the deeper epithelial cell layers.

8.2.3 Oral epithelium-stratum basale and other developing tooth components

The stratum basale of the stratified oral epithelium of the tongue and palate and other developing tooth components (including odontoblasts, dentine, the cervical loop, the root sheath, pulp cells and the periodontal ligament) failed to display Alcec Blue staining or labelling (Syndecan-1 N-18) in all ages examined. Negative staining or labelling of the stratum basale might further support the notion of a reduction in the expression of syndecan-1 in epithelial cells actively undergoing proliferation and migration (Bernfield et al., 1992).

8.2.4 Ameloblasts

Staining or labelling of ameloblasts, which develop embryologically from the inner enamel epithelium (as reviewed by Bartold & Narayanan, 1998) could have been attributed to the presence of cytoplasmic components expressing syndecan-1. This suggestion follows reports describing the distribution of syndecan-1 intracellularly within storage and secretory vesicles (Sawada, 1995) of various secretory cells, the endoplasmic reticulum (Hayashi et al., 1987; Jalkanen et al., 1988; Gallagher et al., 1986) and possibly the cell nucleus (Bernfield et al., 1992). The stage-wise appearance of ameloblast labelling observed in 2 day and 2 week old sections may support the notion that syndecan-1 expression is dependent upon the stability of these epithelially derived cells.

Calcification of the enamel matrix is rarely homogenous (Schour & Massler, 1967) and there are daily rhythmic changes in the process of enamel formation. It might be suggested that the enamel matrix secreted in sections from 1 week old rats was associated with a change in ameloblast behaviour and morphology; ameloblasts become tall and columnar and develop Tomes' processes during the secretory stage (Mjor & Fejerskov, 1986). This concept has been observed during normal crown development

(Thesleff et al., 1996). When epithelial cells undergo changes in morphology and behaviour, a reduction in syndecan-1 expression is an important mechanism enabling changes in shape, behaviour and function of these cells during normal organogenesis (Bernfield et al., 1992).

8.2.5 Epithelial cell rests and the rat periodontium

The absence of syndecan-1 expression (Syndecan-1 N-18) and Alcec Blue staining in the epithelial cell rests of Malassez in the periodontium of the rat molar for all ages examined, might again, be explained by considering the developmental fate of embryonic syndecan-1 as it is modified by epithelial-mesenchymal interactions (Bernfield et al., 1992). As epithelial cells change shape, subsequent proteolysis of syndecan during normal development is necessary to allow the rapid and controlled release of cells from their immediate pericellular matrix, facilitating cellular movements and changes in cellular behaviour (Bernfield et al., 1992).

This has been shown in epithelial-mesenchymal interactions during tooth development where syndecan-1 expression is down-regulated in dental epithelium after early bud stage, corresponding to a period of rapid epithelial proliferation and differentiation (Thesleff et al., 1996), but up-regulated during late bell stage. Based on this model of syndecan-1 expression during tooth crown development, it may be suggested that the absence of syndecan-1 staining or labelling observed in epithelial cell rests of Malassez in this study, might have resulted from a reduction in syndecan-1 expression following proteolysis conferred by instability (i.e. shape changes, proliferation or differentiation) in epithelial cell rests (Jalkanen et al., 1987). The possibility of a reduction in syndecan-1 expression associated with the proliferation of epithelial cells has been suggested by Hayashi et al., (1987), in which they describe the syndecan-1 antigen as being lost or masked when epithelial cells actively proliferate. This notion may also be consistent with the events surrounding malignant change (Gallagher et al., 1986), where proteolysis of syndecan-1 promotes tumourigenic growth by reducing the attachment of cells to the normal regulatory mechanisms of the immediate pericellular matrix (Esko et al., 1988).

8.2.6 Pre-dentine and dentine

Alcec Blue staining of high intensity was consistently demonstrated within the pre-dentine and dentine layers of the developing rat molar for all ages examined. Given the proteoglycan content of dentine comprises mainly chondroitin sulphate (Kirkham et al.,

1995) the results of the present study might suggest that the magnesium chloride electrolyte concentration of the Alcec Blue solution used was too low. Alternatively, it could be suggested that heparan sulphate proteoglycans, in particular syndecan-1, were distributed within the pre-dentine and dentine layers of the rat molar during normal development.

8.3 Technical factors contributing to immunohistochemical and histochemical staining

In addition to possible histological and biochemical factors describing the labelling or staining results observed in the present study there are technical factors associated with the labelling or staining techniques to be considered.

1. Fixation. Formalin fixation may displace antigens (Larsson, 1988) reducing staining or labelling intensity for syndecan-1. According to Wiebkin (1996), the optimal method of tissue fixation is immersion in pre-cooled (<-50°C degrees) ethanol and then allowing frozen section to thaw. Alcohol tissue fixation maximizes retention of glycogen which is soluble, easily diffusible (Wiebkin, 1996) and will more readily degrade in other fixation processes.

2. Decalcification. This may dissolve sugars acting to protect mucosubstances/ glycosaminoglycans from denaturing (Wiebkin, 1996). This might have explained the negative labelling result observed with Alpha syndecan-1 in the stratified epithelium of rat palate and periodontium exposed to EDTA.

3. Antigen masking of syndecan-1 epitopes by formalin fixation. This was unlikely in the present study given unmasking techniques to expose potential syndecan-1 epitopes did not improve labelling in the stratified epithelium of rat tongue and palate, and in the rat periodontium.

4. Staining or labelling reliability. The author was not aware of any literature regarding prior use, successful reactivity, or specificity, of the antibody other than the claims of the manufacturer (Santa Cruz Biotechnology, Inc.). The specificity of Syndecan-1 N-18 was of particular concern given its polyclonal nature potentially increasing its cross-reactivity with similar epitopes (Mighell et al., 1998). Alternatively, the Alcec Blue solution had to be continuously agitated to prevent magnesium chloride from precipitating out, therefore, casting doubt on the degree of saturation and electrolyte concentration of the staining solution.

5. Limitations of histochemical identification of proteoglycans. Scott (1967) described that cationic dyes, including Alcec Blue, may have difficulty penetrating tissues and binding to polyanionic sites given their bulky size. He has suggested, therefore, that detection of tissue polyanions using critical electrolyte concentration alone is an inadequate technique.

6. Negative labelling or staining of epithelial cell rests of Malassez in the periodontium. Despite positive reference identification of epithelial cells by cytokeratin AE1-AE3 in sections from 3 week to 6 week old rats, labelling (Syndecan-1 N-18) and Alcec Blue staining was negative in the present study. This finding might suggest that the observational period examined was too early for detection of syndecan-1 and that periodontal epithelial cells might have been in a migratory or proliferative phase even at 6 weeks. In addition, the mesiobuccal root of the rat maxillary first molar (M-1) had not completed development (as observed in sections by open root apices), and the tooth was still erupting.

Alternatively, epithelial cell rests may have expressed different isoforms of syndecan-1, or expressed syndecan-1 at different times or different concentrations during rat tooth development (Wilson, D. 1999, personal communication).

7. Different staining recognition or binding sites. Syndecan-1 N-18 recognizes the amino terminal of syndecan-1 according to the manufacturer's claims. This is different to Alcec Blue which detects heparan sulphate glycosaminoglycans (Wiebkin, 1996). This may explain the differences in staining or labelling patterns observed in the stratified epithelium of the oral mucosa, and in the pre-dentine and dentine of the developing rat molar in the present study.

8. Heparan sulphate is a variable molecule. Heparan sulphate displays a wide variation in its polyanionic charge and one might be concerned whether its presence could be confidently discerned by critical electrolyte concentration (Bartold, M., 2000, personal communication). This concern is shared by Scott (1967) who reports that the critical electrolyte concentration principle alone does not allow the identification of polyanion substrates and that definite numbers for substances cannot be attributed to specific ranges of critical electrolyte concentration values.

Furthermore, chondroitin sulphate will often bind more strongly than heparan sulphate in ion exchange columns where retention is a function of overall charge. If this is an indication of the variable polyanionic nature exhibited by heparan sulphate

proteoglycans, then it may be suggested that the critical electrolyte concentration for heparan sulphate would not be that much different from chondroitin sulphate (Rapraeger, A., 2000, personal communication). This also supports the possibility that the critical electrolyte concentration (0.42mol/Lm magnesium chloride) of Alcec Blue in the present study was not too low and this may explain the pattern of high intensity staining observed in the present study in the pre-dentine and dentine of the developing rat molar.

8.4 Future research

In the present study, the absence of labelling (Syndecan-1 N-18) and Alcec Blue staining in the rat periodontium, from late crown formation, to root formation, apexification, and early eruption, indicates the need for further investigation into detection of syndecan-1 in the developing periodontium of normal rat molars.

The absence of labelling for syndecan-1 in the rat periodontium, despite positive reference identification (cytokeratin AE1-AE3) of epithelial cell rests of Malassez, might suggest that continued work is required for identification of a suitable label in rats. The specificity of Syndecan-1 N-18 was an ongoing concern given only the manufacturer's claims that the antibody detects syndecan-1. Furthermore, its polyclonal nature potentially increased the cross-reactivity of the antibody with similar epitopes where labelling was observed. There were also concerns regarding the magnesium chloride electrolyte concentration of the Alcec Blue dye given the staining solution had to be continuously agitated to prevent the magnesium chloride precipitating out of solution.

The examination period of the rats used in the present study could have been to early for detection of syndecan-1 and epithelial cells might have been in a migratory or proliferative phase even in sections from 6 week old rats. Therefore, further work is required in studying older periodontal tissues with the aim of identifying syndecan-1 expression in resting epithelial cells. This might ultimately lead to studies looking at syndecan-1 expression incident to orthodontic tooth movement in the rat allowing an analysis of the potential role of syndecan-1 in inflammation and repair. This follows reports of the collection of heparan sulphate proteoglycans from human gingival crevicular isolated from teeth held in retention following orthodontic tooth movement (Waddington et al., 1994). These researchers speculated the release of heparan sulphate

proteoglycans into crevicular fluid to be produced by the periodontium in the process of repair.

9 CONCLUSIONS

1. Syndecan-1 N-18 produced labelling of variable intensity for syndecan-1 within the stratified epithelium of rat tongue and palatal mucosa above the stratum basale, in rats for all ages examined. Light labelling was also observed in ameloblasts during normal molar crown development in sections from 2 day and 2 week old rats, but was negative in sections from 1 week old rats. Labelling with Syndecan-1 N-18 was negative in the stratum basale, developing tooth components, including odontoblasts, dentine, pulp cells and the developing root sheath; and in epithelial cell rests of Malassez in the rat periodontium during the examination period.

2. Alcec Blue produced staining of variable intensity, for heparan sulphate proteoglycans within the stratified epithelium of the rat tongue and palatal mucosa above the stratum basale in rats for all ages examined. Staining was also observed in the enamel matrix in sections from 1 week old rats and in ameloblasts of sections from 2 week old rats, during normal molar crown development. Staining was consistently observed in the pre-dentine and dentine layers of the rat molar. Staining was negative in the stratum basale, other developing tooth components including: odontoblasts, dentine, pulp cells and the developing root sheath; and in epithelial cell rests of Malassez in the rat periodontium.

3. The absence of labelling (Syndecan-1 N-18) and Alcec Blue staining in epithelial cell rests of Malassez observed during postnatal formation of the rat periodontium might suggest that either syndecan-1 was not detected, was down-regulated, or was not expressed, during the stages of rat tooth development examined. This might be consistent with the notion of proliferating and migrating epithelial cells, and perhaps, reflects these events during the development of Hertwig's epithelial root sheath and the epithelial cell rests of Malassez. Therefore, further research is required in both studying older periodontal ligament tissues with the aim of identifying resting (i.e. non-proliferating or non-migrating) epithelial cells.

4. Alternatively, the absence of labelling or staining for syndecan-1 in epithelial cell rests of Malassez observed during postnatal formation of the rat periodontium could indicate that continued work is required for identification of a suitable label in rats.

10 APPENDICES

10.1 MAYER LILLIE HAEMATOXYLIN AND EOSIN STAINING SOLUTION

10.1.1 HAEMATOXYLIN

Use

Staining in light microscopy in conjunction with eosin; or for immunohistochemical counterstaining.

Preparation

5g haematoxylin (Mayer Lillie Haematoxylin, Lynch et al., (1969); 500ml distilled water; 300ml glycerol; 50g ammonium alum (mordant); 0.2-0.4 sodium iodate; 20ml acetic acid.

10.1.2 EOSIN

Use

Contrast light microscopic cytoplasmic dye for nuclear stains such as haematoxylin.

Preparation

1% stock solution Eosin Y (Lynch et al., 1969); 0.25ml of 40% formaldehyde solution per 100ml to prevent bacterial growth; and 2ml acetic acid/100ml.

10.1.3 HAEMATOXYLIN AND EOSIN STAINING PROTOCOL

(1) Removal of wax: xylol 2x2 minutes. Absolute alcohol 2x2 minutes. (2) Check for incomplete removal of wax and xylol: 1 dip in tap water. (3) Staining: haematoxylin 10 minutes, and remove excess stain by washing in running tap water (1 minute). (4) Differentiation: one dip, 0.5% hydrochloric acid. (5) Blue: running water for 10 minutes and dilute alkali, 2 dips. (6) Differentiation: 70% alcohol for 3 dips. (7) Dehydrate, clear and mount: absolute alcohol 2x2 minutes; xylol 2x2 minutes.

10.2 MAYER LILLIE HAEMATOXYLIN COUNTERSTAINING

Protocol

Stain in haematoxylin (4 dips); wash in running tap water for 2 minutes; place in acid alcohol (4 dips); wash in running tap water for 2 minutes and then place in 1% aqueous lithium carbonate (10 seconds).

10.3 ALCEC BLUE STAINING SOLUTION

Use

The inorganic cation, magnesium, Mg^{2+}, exhibits preferential binding to carboxyl and phosphate groups over sulphate compounds and acts to displace the cationic charge of Alcec Blue from anionic sites. However, electrostatic binding of the Alcec Blue cationic charge to anionic sites, in the presence of inorganic electrolytes (i.e. Mg^{2+}) can be reversed. Competition will occur for the highly anionic sites (i.e. sulphates) on glycoconjugates, such as heparan sulphate proteoglycans, between the positive charges in both the competing inorganic cation (i.e. Mg^{2+}) and the cationic charge of Alcec Blue. It follows that the use of Alcec Blue at increasing electrolyte concentrations of magnesium chloride, gives a method for differentially identifying glycosaminoglycans in tissue (Wiebkin, 1996).

Preparation

Alcec blue 0.05g (Aldrich Inc, Wisconsin); 100ml distilled water; 0.205g sodium acetate: 1.218g magnesium chloride, pH 5.8.

10.3.1 ALCEC BLUE STAINING PROTOCOL

Flood section with Alcec Blue solution: for 60 minutes, then wash in tap water to remove excess stain. Dehydrate, clear and mount: absolute alcohol 2 minutes, xylol 2 minutes.

10.4 NEUTRAL BUFFERED FORMALIN SOLUTION

Use

Tissue fixation (4% buffered formosaline)

Preparation

900ml distilled water; 6.5g anhydrous sodium phosphate dibasic; 3.5g anhydrous sodium phosphate monobasic; 100ml of 40% stock formaldehyde solution mixed together.

10.5 ETHYLENEDIAMINETETRA-ACETIC ACID (EDTA)

Use

Decalcification of hard tissues.

Preparation

Dissolve 40g EDTA in 100ml distilled water; add 24.2g sodium cacodylate and sodium hydroxide to bring pH to 7.3 at 4^{0}C.

10.6 SLIDE COATING PROCEDURE

(1) Soak slides: overnight in detergent. (2) Wash slides: in several changes of running water for at least 3 hours, drain but do not dry. (3) Rinse slides: 30 seconds, in 2 consecutive containers of absolute alcohol. (4) Dip slides in 3-amino propyltriethoxysaline (APT): 2 % solution made up with absolute alcohol. (5) Rinse slides: 30 seconds in 2 consecutive containers of absolute alcohol. (6). Rinse slides: in a large clean container of distilled water. (7) Dry slides, 37^{0}C oven overnight.

10.7 IMMUNOHISTOCHEMICAL LABELLING PROTOCOL: AVIDIN-BIOTIN PEROXIDASE COMPLEX TECHNIQUE

(1) Deparaffinize sections: xylene 2x2 minutes, alcohol 2x2 minutes.

(2) Block endogenous peroxidase activity: 0.5% H_2O_2 in 95% methanol for 30 minutes.

(3) Rinse in PBS buffer (pH 7.35): 2 x 3 minute washes.

(4) Apply pre-treatment for primary antibody if required to unmask antigens in specimens masked by formalin-fixation and paraffin embedding.

Antigen retrieval/enzyme digestion: incubate slides for 3 minutes in 0.0625 g Trypsin type II enzyme (Sigma catalogue number # T-8128) dissolved in 250mls phosphate buffered saline (pre-warmed in a 37° water bath).

Microwave treatment: place slides into plastic slide holders and load into 250mls of citrate (Institute of Medical and Veterinary Science store) or target retrieval solution (Dako, catalogue number #s-1699) in a microwave pot. Place slides in a microwave oven (Sharp 700W) and set for 2.15 minutes on medium high and 3 minutes on low. Remove pots when time is completes and allow to cool for 50°C.

(5) Incubate sections with 3% Normal horse serum (NHS): 30 minutes.

(6) Drain NHS and incubate with primary antibody overnight.

(7) Rinse in phosphate buffered saline (PBS) buffer (pH 7.35): 2 x 3 minute washes.

(8) Incubate with biotinylated secondary antibody:30 minutes.

(8) Rinse in PBS buffer (pH 7.35): 2 x 3 minute washes.

(9) Incubate with streptavidin perioxidase tertiary complex: 60 minutes;

(10) Rinse in PBS buffer (pH 7.35): 2 x 3 minute washes.

(11) Apply perioxidase substrate solution (DAB): 7 minutes.

(12) Rinse in PBS buffer (pH 7.35): 2 x 3 minute washes.

(13) Counterstain with Mayer's Haematoxylin

(14) Dehydrate clear and mount.

10.8 PHOSPHATE BUFFERED SALINE (PBS)

Preparation

Dissolve 27.6g of monobasic sodium phosphate in 1 litre of 0.9% saline solution (solution A). Next mix 28.39g of dibasic sodium phosphate in 1 litre of 0.9% saline solution (solution B). Add 16ml of solution A to 84ml of solution B and dilute to 200ml to achieve a final pH of 7.5, giving 10mM sodium phosphate, pH 1.7, 0.9% saline.

10.9 METHANOL/HYDROGEN PEROXIDE BLOCKING SOLUTION

Use

To reduce background immunohistochemical labelling by blocking endogenous peroxidase in tissue specimens.

Preparation

3.32ml hydrogen peroxide (100vol 30%w/v) (BDH Laboratories, catalogue number #10366) in 200ml methanol, giving 0.5% H_2O_2 in 95% methanol.

10.10 PRIMARY ANTIBODIES

10.10.1 Rabbit anti-laminin

Use

Recognizes an intrinsic component of the cellular basement membrane known as laminin (from EY Laboratories, catalogue number #AT-2404-1).

Preparation

2ml vial, concentration 6mg/ml. This product requires protein digestion pre-treatment of paraffin sections (e.g. trypsin).

10.10.2 Alpha syndecan-1

Use

Alpha syndecan-1, a mouse monoclonal antibody against rat syndecan-1 , recognizes a heparan sulphate rich membrane glycoprotein of 200kD known as Syndecan-1 (donated by Dr Ole Wiebkin, Department of Medicine, University of Adelaide, South Australia, Australia).

Preparation

600µl of solution at 1/50 dilution. This product does not require protein digestion pre-treatment of paraffin sections (e.g. trypsin).

10.10.3 Syndecan-1 B-B4

Use

Syndecan-1 B-B4, a mouse monoclonal antibody against human syndecan-1, recognizes a heparan sulphate-rich membrane glycoprotein, syndecan-1. It stains all plasma cells, plasma cell lines and epithelial and endothelial cells (from Serotec Limited, catalogue number # MCA681).

Preparation

2ml vial, purified immunoglobulin concentration 0.1mg/ml. This product does not require protein digestion pre-treatment of paraffin sections (e.g. trypsin).

10.10.4 Syndecan-1 N-18

Use

An affinity purified goat polyclonal antibody, raised against a peptide corresponding to an amino acid sequence mapping at the amino terminus of the precursor form of syndecan-1 of mouse origin. Specific for syndecan-1 of mouse, rat and human origin (Santa Cruz Biotechnology, catalogue number # SC-7100).

Preparation

200 µg immunoglobulin in 1ml phosphate buffered saline. Tissue specimens must be formalin-fixed and embedded in paraffin blocks. Antigen unmasking may be performed by protein digestion pre-treatment of paraffin sections (e.g. trypsin) (specification data sheets, Santa Cruz Biotechnology, Incorporated)

10.10.5 Cytokeratin AE1-AE3

Use

A cocktail of mouse monoclonal antibody, it stains cytokeratins present in normal and abnormal tissues, in particular, the antibody displays high sensitivity and specificity in recognizing epithelial cells by immunohistological methods (Cell Marque).

Preparation

1ml vial of concentrated antibody. This product requires protein digestion pre-treatment of paraffin sections (e.g. trypsin).

10.11 SECONDARY ANTIBODIES

10.11.1 Anti-rabbit immunoglobulin

Use

Biotinylated secondary antibody, anti-rabbit immunoglobulin, to Rabbit anti-laminin (EY Laboratories Incorporated, catalogue number #BA-1000)

Preparation

Reconstitute 1.5mg of biotinylated anti-rabbit immunoglobulin precipitate by adding 1ml of water to produce a recommended concentration range of 2-10μg/ml.

10.11.2 Anti-mouse immunoglobulin

Use

Biotinylated secondary antibody, anti-mouse immunoglobulin (Vector Laboratories, catalogue number #BA-2000) to Alpha Syndecan-1 and Syndecan-1 B-B4.

Preparation

Reconstitute 1.5mg of biotinylated anti-mouse immunoglobulin precipitate by adding 1ml of water to produce a recommended concentration range of 2-10μg/ml.

10.11.3 Anti-goat immunoglobulin

Use

Biotinylated secondary antibody, anti-goat immunoglobulin (Vector Laboratories, catalogue number #BA-5000) to Syndecan-1 N-18.

Preparation

Reconstitute 1.5mg of biotinylated anti-goat immunoglobulin precipitate by adding 1ml of water to produce a recommended concentration range of 2-10μg/ml.

10.12 STREPTAVIDIN HORSERADISH PERIOXIDASE

Use

ImmunoPure Streptavidin, Horse Radish Peroxidase-conjugated antibody (Pierce, catalogue number #21126).

Preparation

The concentrate is prepared by dissolving the lyophilised powder in distilled water (1mg / 0.5 ml). The concentrate is then aliquoted in 0.5ml lots and stored. Working solution: 1ul SPC concentrate / ml normal horse serum.

10.13 PERIOXIDASE SUBSTRATE SOLUTION

Use

Preformed avidin-biotin complex 3,3'-diaminobenzidine, enables binding of avidin to the biotin of the secondary antibody, producing a reddish brown stain.

Preparation

DAB Substrate Kit For Perioxidase, comprising dropper bottles (Vector Laboratories, catalogue number # SK-4100).

To 5ml distilled water, add 2 drops of buffer stock solution and mix well. Add 4 drops of DAB stock solution and mix well. Add 2 drops of the hydrogen peroxide solution and mix well. Incubate the tissue sections at room temperature with the substrate solution until stable suitable staining develops, as determined by the investigator. Four minutes provided optimal staining intensity in the present study.

10.14 NORMAL HORSE SERUM

Use

To reduce the amount of cross-reaction between the biotinylated secondary antibodies and endogenous tissue immunoglobulins.

Preparation

1ml aliquots of Donor Horse Serum (CSL Biosciences, catalogue number #09531901) were prepared from a stock solution of 100mls, and added to 29 ml phosphate buffered saline.

10.15 TISSUE PROCESSING

Tissues were placed in a Shandon Citadel 2000 automatic processor and left for 24 hours according to the following protocol:

Seventy percent alcohol 1 hour. Seventy percent alcohol 1 hour. Eighty percent alcohol 2 hours. Ninety percent alcohol 2 hours. One hundred percent alcohol for 2 hours. One hundred percent alcohol for 2 hours. One hundred percent alcohol for 2 hours. Clearene for 2 hours. Clearene for 2 hours. Clearene for 2 hours. Wax for 2 hours. Wax for 3 hours.

10.16 DATA RECORDING SHEETS FOR LABELLING (SYNDECAN-1 N-18) AND ALCEC BLUE STAINING (PAGES 66 AND 67)

APPENDIX 10.16

Data recording sheet: **longitudinal** sections

Syndecan-1 N-18 /Alcec Blue

			Cervical 1/3 *Mesial*					Middle 1/3 *Mesial*					Apical 1/3 *Mesial*					Apical 1/2					Furcal 1/2					Oral mucosa epithelium						
MB root	**ECRM region**		1	2	3	4		1	2	3	4		1	2	3	4		1	2	3	4			1	2	3	4		SC	SG	SS	AM	EM	PD	D
Slide	Level*																																		
	1																																		
	2																																		
	3																																		
	4																																		
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	14																																		
	15																																		

ECRM, epithelial cell rests of Malassez; MB, mesiobuccal root rat maxillary first molar. ECRM labelling/staining regions: 1. region facing the tooth root; 2. region facing the tooth crown; 3. region facing the tooth periodontal ligament; 4. region facing the tooth apex. Stratified oral epithelium: SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; AM, ameloblasts; EM, enamel matrix; PD, pre-dentine; D, dentine. * All levels equal 150 micrometers.

APPENDIX 10.16

Data recording sheet: **transverse** sections

Syndecan-1 N-18 /Alcec Blue

		Cervical 1/3									Middle 1/3									Apical 1/3									Oral mucosa epithelium						
MB root	ECRM region	1	2	3	4	5	6	7	8		1	2	3	4	5	6	7	8		1	2	3	4	5	6	7	8		SC	SG	SS	AM	EM	PD	D
Slide	Level *																																		
	1																																		
	2																																		
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ECRM, epithelial cell rests of Malassez; MB, mesiobuccal root rat maxillary first molar. ECRM labelling/staining regions (1-8) apply to those regions described in Figure 7 (Materials & Methods). Stratified oral epithelium: SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; AM, ameloblasts; EM, enamel matrix; PD, pre-dentine; D, dentine. * All levels equal 150 micrometers.

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