THE INFLUENCE OF

INSULIN-LIKE GROWTH FACTOR I AND ITS ANALOGUES

ON FIBROBLASTS AND DERMAL WOUND HEALING

NICHOLAS JOHN MARSHALL M.B.B.S. (ADEL)

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DEDICATION

This thesis is dedicated to my wife, Caroline, my children, Henry and Georgina; Caroline for her enduring love, encouragement, support and patience. To Henry and Georgina who provide so much wonderment and joy to me every day of my life.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any 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.

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

Nicholas Marshall

1998

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SYNOPSIS

A comprehension of the wound repair process is central to all surgical practice. The science of wound healing has progressed dramatically in recent years to facilitate this understanding, particularly in the area of growth factor biology and offers exciting possibilities for the manipulation of this process.

One cytokine that has shown particular promise has been insulin-like growth factor-I (IGF-I). As the second messenger for growth hormone (GH), it is postulated to mediate the majority of anabolic functions associated with GH. Of clinical importance is the associated reversal of previously depressed IGF-I levels and reduced protein catabolism when GH is administered to patients with severe burns. The potential role of IGF-I is further supported by the finding that IGF-I is found locally at the wound site as well as in measurable concentrations systemically. It appears, also, to have important vulnerary effects upon fibroblasts and keratinocytes including cell migration and protein synthesis. Six carrier proteins, known as IGF binding proteins (IGFBPs), have been identified. Although little is known of how they modulate IGF biology within a wound it appears that IGFBP-1 and -3 can potentiate IGF-I activity.

With the development of recombinant DNA techniques, a range of IGF variants (analogues) are now commercially available. The value of these peptides lies in their apparent increased biological activity *in vitro* and to a more limited degree *in vivo*. Primarily, the greater potency of the analogues is related to decreased affinity for IGFBPs and consequently increased IGF bioavailability. Therefore they can be used to examine indirectly the influence that IGFBPs have in wound repair.

In light of this information, the objectives of this study were to investigate:

- the levels of IGFs and the presence of binding proteins in human wound fluid;

- the potency of IGF-I and two analogues in several *in vitro* models of fibroblast activity;

- the effect of IGF-I and one of the analogues upon healing in both normal and diabetic rodent wounds.

Characterisation of IGFs and IGFBPs in human wound fluid and plasma was achieved by obtaining time matched wound fluid samples (from split skin graft donor sites) and plasma. These were subjected to acid gel permeation chromatography (acid gel HPLC) or acid-ethanol extraction to separate the binding proteins from the IGFs and then performing RIAs to quantify the IGFs. The identity of IGFBPs found in the samples was determined using Western ligand and immunoblotting techniques.

Established models of cell growth (methylene blue dye absorbance by cultured monolayers and radiolabelled thymidine incorporation) were used to examine the potency of a variety of growth factors. Specifically these included recombinant human IGF-I (rhIGF-I); two IGF-I analogues (des(1-3) IGF-I and long [Arg^3] IGF-I (LR^3 IGF-I)), IGF-II, GH and transforming growth factor β (TGFβ). Experiments examining protein synthesis (proline incorporation) and the functional activity of fibroblasts (fibroblast populated collagen lattice contraction, FPCL) were also preformed.

The efficacies of IGF-I, IGFBP-2, IGF-II, GH and the analogue, LR^3 IGF-I were tested using a rodent incisional model of wound repair. Compromised repair was examined using streptozotocin-induced diabetes in the same model.

Healing by secondary intention was examined using an established model of excisional wound repair modified to explore an IGF-I analogue's ability to augment repair in both normal and diabetic wounds.

Several important findings were documented. These were:

- Western ligand and immunoblots of human wound fluid samples revealed the presence of IGFBPs -2, -3 and -4. The intensity of these bands was less than those obtained for time matched samples of plasma. Specifically, wound fluid contained low molecular weight IGFBP-3 fragments.

- IGF-I and IGF-II concentrations as determined by RIA following either acid gel HPLC or acid-ethanol extraction were correlated for each procedure using linear regression. Good correlation for the extraction techniques was observed for IGF-I analysis, in contrast poor correlation was seen for IGF-II. This was particularly evident in the wound fluid samples.

- FPCL assays and cell growth experiments demonstrated a clearly greater response to IGF-I compared to GH and importantly a lack of synergism for a combination of GH and IGF-I. In addition, greater potency was demonstrated by the analogues, LR^3 IGF-I and des (1-3) IGF-I, when applied to the FPCL model. The studies of protein synthesis (^{3}H-proline incorporation) confirmed the relative potency of the IGF-I analogues compared to rhIGF-I as well as IGF-II.

- Topically applied GH, IGF-I or the two in combination (concentration of 100μg/ml), failed to produce significant increases in strength in rodent wounds as compared to the vehicle treated control wounds. However when this was increased to 1mg/ml of peptide, IGF-I treated wounds were up to 40% stronger than their paired vehicle treated wounds (p=0.008).

- Diabetic animals demonstrated weaker wounds than the normal animals and this reduced wound strength was not restored by rhIGF-I nor LR^3 IGF-I although there appeared to be a trend in favour of LR^3 IGF-I

Having identified that IGFBP-2 is found in human wound fluid, this binding protein was applied in combination with IGF-I to incisional wounds using our rodent model. The results obtained in this model indicated that it did not enhance the effect of rhIGF.

Wound strength data gathered from the excisional wound study showed that wounds on normal animals treated with 10μg of LR^3 IGF-1 per treatment were significantly weaker than control wounds however rhIGF-I and LR^3 IGF-I at a dose of 100μg did not alter strength significantly. In conjunction, no treatment altered the rate of wound contraction however diabetes slowed the rate of wound closure.

Although IGFs are well documented as mitogens for cell lines involved in wound repair, little success has been reported demonstrating a similar potency using animal models except where the peptide is coupled with an IGFBP or in studies of compromised wound healing. The human wound fluid study confirmed the existence of IGFBPs and the presence of IGFBP fragments in the acute wound environment.

Despite clearly demonstrated increases in activity of the analogues using tissue culture models, this potency could not be translated to enhancement of dermal wound repair. The failure of *in vitro* success to translate to animal models raises clinically relevant points. Products that may be suitable as topical wound healing agent should be tested *in vivo* as well as *in vitro*. Secondly these studies suggest that a single agent is unlikely to be effective when used alone. The timing of treatment application and a myriad of other factors within the wound, including IGFBPs may be important variables in determining the role of IGF-I in wound repair.

In conclusion, the studies outlined in this thesis provide evidence that:

- IGF-I, IGF-II and their IGFBPs are present in exudate produced by a partial thickness cutaneous wound.

- IGFBPs negatively modulate the activity of IGFs *in vitro*.

- In contrast IGFs do not necessarily exhibit enhanced activity *in vivo* at the wound site if their IGFBP affinity is decreased. Possible roles of IGFBPs in wound repair are discussed.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

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1.1 Introduction: - Historical Perspective

"Rumour, myth and unsubstantiated opinion have guided wound management since our
forebears achieved upright ambulation....."

Reiter, D. 1994 (1)

An understanding of wound repair and the restoration of tissue integrity is fundamental to successful surgical practice. Without attention to the science as well as the art of wound closure, surgical procedures are likely to be fraught with complications.

Historically, wound healing and its management have undergone various stages of 'revolution' (2). The ancient civilisations of Egypt, Greece, India and Europe appreciated the necessity for wound debridement and closure, as well as protection of the injured parts with clean dressings and the avoidance of corrosive agents. A more aggressive attitude was adopted throughout the Middle Ages and treatments such as burning or boiling oil were used 'to help the wound to heal'. The caretaker attitude to wound healing was revitalised serendipitously by the Frenchman, Ambroise Pare. He found that healing of amputation sites occurred with fewer complications if milder treatments were used. Thus the pendulum had begun to swing towards the more modern concept of gentle wound care where the body's response drives the repair process.

Medical practice, in an era of aging populations, has become dominated by the management of chronic and debilitating disease. Despite appropriate mechanical debridement as well as antibiotic therapy for prevention and treatment of infection, successful uncomplicated healing frequently does not occur. There are a number of disease processes that impact upon wound healing in modern western society. This may be directly related to their pathology as with paraplegia (3), chronic venous stasis (4), vasculitic disorders, peripheral vascular disease and diabetes mellitus (5). However prescribed treatments also contribute to wound morbidity. Examples include steroids in chronic airway disease and the effects chemotherapeutic drugs (6) for the management of malignancies. Consequently, pursuit of an understanding of the mechanisms involved in wound healing has formed the basis for intense scientific investigation of the biochemical and cellular pathways associated with the wound healing response. Further impetus has been provided by recognition at a cellular level that similarities exist between foetal growth and tumour proliferation (7).

1.2 OVERVIEW OF WOUND REPAIR

Regeneration and repair with scar formation are the means by which animals replace lost tissue. Phylogenetically regeneration is the more primitive mechanism although it is well recognised that deer antlers repair in this manner. In humans, damaged skin is repaired principally by formation of scar covered by a layer of epithelium (8).

The physiological process of wound repair may be divided temporally into three phases as described by Kanzler et al (9). Kanzler suggests that three broad phases can be identified namely *substrate phase, proliferative phase* and *remodelling phase*. Other authors including

Schilling (10) have provided more elaborate and detailed divisions of the healing sequence. While these phases are clearly evident within any wound they do overlap and are subject to extrinsic and intrinsic influences.

Peptides known as cytokines, mitogens or growth factors modulate much of the cellular activity in the wound milieu. However much of their physiology is yet to be elucidated, particularly those mechanisms activated when administered topically or systemically.

Cytokines regulate wound repair by a variety of mechanisms. Some have effects on their cells of origin (autocrine), some influence neighbouring cells (paracrine) and others target tissues distant from their tissue of origin (endocrine). In the healing wound, these peptides may originate essentially from four cell lines. These are endothelial, stromal (namely fibroblasts), epithelial (keratinocytes) and inflammatory cells especially macrophages. During wound healing, insulin-like growth factor I (IGF-I) is one of many polypeptides to appear in the wound milieu early and act by such pathways (11-13).

The Substrate Phase

During the substrate phase, complex cellular and cytokine interactions provide the foundation for subsequent repair. Initially it is characterised by haemostasis and an inflammatory response (14). Platelet aggregation during haemostasis is associated with the degranulation of α granules and the subsequent release of important cytokines such as transforming growth factor beta, platelet derived growth factor and insulin like growth factors (15). As demonstrated by Kuwano, inhibition of platelet degranulation can impair wound healing (16) thereby underpinning the importance of platelet function to initiate the cytokine cascade in wound repair. Other cellular elements include monocytes/ tissue

macrophages, neutrophils and, later, reparative cells such as fibroblasts and endothelial cells. The latter cells already preside within the wound. In response to chemoattractants released by macrophages they migrate through the lattice created by the fibrin plug to eventually restore skin integrity. Conversion of fibrinogen to fibrin and the deposition of plasma fibronectin provide a provisional matrix over which cell migration can occur (17). Along with thrombin these proteins stimulate fibronectin production by macrophages to create a structure later supplemented by collagen fibrils deposited by fibroblasts positioned near the wound edge (18).

The inflammatory response is well summarised by Wahl & Wahl (14). Migration of neutrophils into the wound is initiated by the release of 12-hydroperoxyeicosatranoic acid (12-HPETE) and leukotriene B4 (LTB_4). The primary role of neutrophils is to debride the wound, thereby removing proteinaceous debris including bacteria and damaged cells. Then follows the appearance of monocyte/macrophages recruited to the wound in response to specific chemoattractants that include collagen (19), elastin (20), fibronectin (21), activated thrombin (22) and transforming growth factor β (TGFβ) (23). Macrophages appear to orchestrate the healing response by releasing chemoattractants and mitogenic substances such as growth factors and members of the interleukin family (24-27) while also performing phagocytic activities. Therefore their contribution is regarded as essential for effective wound repair (28) unlike that of neutrophils. Hunt (29) suggested that macrophages also mediate collagen metabolism and wound vascularisation through cytokines released in response to tissue hypoxia.

The Proliferative Phase

From the substrate phase the wound progresses to the proliferative phase. This is a process characterised by angiogenesis, fibroplasia and collagen synthesis as well as epithelialisation (29). Neovascularisation of the wound begins 3 to 5 days after wounding, and enables delivery of nutrients to the wound (30). Injured tissue is relatively ischaemic and consequently hypoxic. This low oxygen tension and the associated lactate accumulation promote capillary bud formation. Angiogenesis can be induced by many factors besides hypoxia including fibronectin, basement membrane fragments and the various growth factors that are released by activated macrophages. These include acidic and basic fibroblast growth factor (FGF) (31), platelet-derived growth factor (PDGF) (32) and heparin. The endothelial bud is comprised histologically of a leading tip of migrating endothelial cells originating from pre-existing post-capillary venules and other endothelial cells that demonstrate proliferation behind this bud. Controlled release of proteolytic enzymes from advancing endothelial cells allows their migration through the wound by degrading the basement membrane (33-35). A lumen develops as differentiation proceeds although control of this process is poorly understood (36).

Migration and proliferation of fibroblasts begin 48 to 72 hours after injury. Collagen production by these cells restores the mesenchymal matrix. This activity also is closely modulated by chemoattractants and mitogens produced primarily by macrophages but also by other fibroblasts (29). The work of Levene et al (37) suggests that fibroblasts require stimuli to proliferate that are separate to those which induce collagen synthesise. Macrophage-derived lactate appears to be the most significant factor in promoting the latter process, while growth factors in particular PDGF (38-40) and EGF (41) can stimulate

fibroblast migration and proliferation. Accumulated evidence exists showing promotion of fibroblast activity by PDGF both alone and in combination with other growth factors such as TGF-α (42), TGFβ (43,44) FGF (45), insulin or insulin-like growth factors (46). However the stimuli required to proliferate are probably separate to those which induce collagen synthesise.

Wound contraction

Fibroblasts contribute to wound closure by mediating wound contraction as well as producing the collagen required to restore dermal strength. There still exists substantial debate as to the mechanism of wound contraction. Collagen fibres at the wound site were once regarded as the source of the effective force. However contraction will still occur where collagen synthesis is prevented or its normal polymerisation is impaired for example in larthyrism (47,48). Billingham and Medawar have postulated that the reparative cells within the wound and not extracellular matrix (ECM) were responsible for the contractile forces generated (49).

Gabbiani characterised the myofibroblast in 1971 (50,51). This cell was described as having features common to both smooth muscle cells and fibroblasts. These included microfilaments that were similar in structure to smooth muscle cell actomyosin (α smooth-muscle actin), and which enlarged as the granulation tissue matured. Rudolph et al (52) observed that these microfilaments extended beyond the cell and formed a fibronexus. They suggested that this may be a method by which cells attached to collagen and to other cells to form a transmembranous assembly that allowed the construction of multicellular strands.

Therefore the coordinated contractile activity by these collections of cells produces wound contraction. Other features that these cells have in common with both smooth muscle and fibroblasts include:

1) the presence of cytoplasmic longitudinal bundles of microfilaments (stress fibres);

2) dense bodies scattered along the stress fibres;

3) abundant rough endoplasmic reticulum;

4) multiple nuclear membrane folds (53).

Alignment of these cells and their associated filaments may provide the mechanism for a synchronous contractile response and may therefore be responsible for wound contraction. However doubt exists about the origin of the myofibroblast and whether this specialised cell is necessary for wound contraction. Ehrlich et al (54) questioned the existence of myofibroblasts as a discrete cell population. He proposed that wound contraction resulted from individual fibroblasts moving through the matrix. This migration led to rearrangement of the connective tissue matrix. Harris et al demonstrated locomotion in their experiments using silicone sheeting (55). Their observation was that wrinkling of the silicone developed beneath the fibroblast as the cells attempted to move. Therefore fibroblasts, working as individual units to re-organise their surrounding matrix, may generate forces of contraction through their own locomotion and reorganisation of collagen fibrils (56). These forces are presumably generated by the sliding of cytoplasmic actin-myosin filaments in response to adenosine triphosphatase (ATPase) activity (54). Ehrlich also argues that the myofibroblast is a transitional state for fibroblasts found in granulation tissue at the periphery of the

wound. The fibroblasts assume this form as they prepare to migrate from the healing wound during resolution of fibroplasia (55). However contraction can occur in collagen gel lattices with cells not identifiable morphologically as myofibroblasts (57).

Several investigators have shown that 80-90% of the fibroblasts, observed in the region of a wound at the time of greatest contractile activity, have features typical of the myofibroblast (50,51). These cells do not exist solely at the edge of the wound but may be found throughout the granulation tissue appearing initially at one week and persisting to at least the fourth week. Their distribution appears to change as they form localised aggregates that become more evenly distributed throughout the wound by the fourth week. Bouisson et al (58) used electron microscopy to show that activated fibroblasts and myofibroblasts arise from resting fibroblasts at the wound edge. These cells will transiently express alpha smooth muscle actin (59) and may support the wound against surrounding tissue tension. Garana et al (60) suggest that fibroblasts may not be the only cell type that can exhibit myofibroblast features, as corneal keratinocytes may do likewise.

Collagen gel lattices provide an *in vitro* model of wound contraction. Fibroblasts, keratinocytes and endothelial cells (61,62) can all be used to seed a mix of collagen and tissue culture medium (57). Besides cell type, gel contraction also depends upon other factors particularly cell number, collagen concentration and collagen type (56,57,61).

Regardless of the cell phenotype responsible for contraction, growth factor and cytokine research has shown that control of this process is complex. Changes during wound healing occur in the fibroblast/myofibroblast that alter responses to particular cytokines. These responses relate to the stage of repair from which the cells have been harvested and whether the fibroblasts used are normal quiescent dermal cells (63) or derived from hypertrophic

scar or keloid tissue (64,65). An example of this is the observation that pre-treatment of a fibroblast populated collagen lattice with b-FGF down regulates the TGF-β responses if fibroblasts are derived from the early stages of wound repair. However these two growth factors are synergistic if the cells are derived from older wounds (66). It may ultimately prove more beneficial to retard or manipulate than to facilitate fibroblast contractile activity in healing or healed wounds as significant scarring or contracture can be the ultimate clinical outcome.

Epithelialisation

Mammalian epidermis is separated from the underlying musculoskeletal structures by two distinct layers. These are the subcutaneous adipose tissue and the dermis. By division of keratinocytes in the basal layer at a constant rate the epidermis can balance the loss of cells through desquamation (67). The cycle involves division of the basal cells, migration of the daughter cells to the granular layer over approximately 14 days and then a further 14 days before they desquamate. Keratin accumulation which first appears around the nuclei of cells in stratum spinosum (prickle layer), accompanies the changes in cell morphology that characterise this process.

Restoration of epithelial integrity during wound repair is accomplished by both keratinocyte proliferation and migration. Although these mechanisms would seem to be related they have been shown by Sarret et al (68) to be independent using computer-assisted image analysis. In this study the authors demonstrated that cell migration can continue despite inhibition of cellular proliferation. Exposure of keratinocytes to ECM proteins influences the migratory response. For example, fibrin and fibronectin will stimulate cell movement but the appearance of laminin, a glycoprotein found in the basement membrane of the dermal-

epidermal junction towards the end of keratinocyte migration suggests that laminin alone may inhibit migration (69). Woodley et al have postulated therefore that this molecule may be the 'stop' signal to re-epithelialisation (70).

Keratinocyte migration and proliferation in tissue culture, are regulated partly by growth factors. In particular epidermal growth factor (EGF), IGF-I and heparin binding epidermal growth factor (HB-EGF) (71-73) promote keratinocyte activity. A molecular mechanism substantiating EGF's ability to promote migration is provided by the observation that EGF promotes $\beta 1$ integrin subunit expression by fibroblasts and in conjunction with the $\alpha 2$ and $\alpha 5$ subunits it appears necessary for keratinocyte migration over fibronectin and type IV collagen (71,74). Sarret et al (68) dispute this by concluding from their own study that EGF did not influence keratinocyte migration. Ando et al (71) highlight the difference in the composition of the media used in these two studies. In particular, their media contained a carrier protein for EGF and IGF-I to minimise non-specific losses to test-tube walls, culture dishes and other laboratory equipment. In addition, TGF$\beta 1$ can retard re-epithelialisation partly by reducing the normal hyperproliferative response (75,76) although Sarret (68) has also shown that migration is promoted by TGFβ.

While *in vitro* keratinocyte activity and epidermal outgrowth in skin explants (77) are clearly influenced by EGF, its use in both human and animal studies to promote re-epithelialisation (78) has largely been unsuccessful. Thornton (79), using a porcine model, was unable to accelerate healing with EGF alone or in combination with silver sulphadiazine (SSD). This was in contradiction to the work of Brown et al (80). In addition, Arturson (81) could not improve epithelialisation in epidermal and scald wounds using a rat

model, although like other investigators (82) he has shown a significant improvement in the rate of re-epithelialisation of corneal wounds.

Clinical studies involving the treatment of wounds with EGF have also produced conflicting results. Brown et al examined both acute partial thickness wounds (83) and chronic wounds (84) and demonstrated accelerated healing using EGF. However in a randomised trial comparing SSD to a mixture of SSD and EGF applied to partial thickness wounds in normal volunteers, Cohen et al observed no significant difference (78).

These results highlight some of the difficulties in applying growth factors to wound healing models. These relate principally to the nature of the vehicles and models used in these experiments as well as incomplete understanding of the biochemical pathways and sites through which chemical messengers such as EGF exert their effects. Therefore caution is required when extrapolating *in vitro* results to animal and human studies.

Remodelling

The final defined phase is that of remodelling. The dermal response to injury is characterised by both transient and permanent changes in tissue architecture. Once wound integrity has been restored collagen content will continue to increase, reaching a maximum 2 to 3 weeks after injury (9) and it is then rearranged to improve the tensile strength of the wound. This is characterised by modifications to the calibre of collagen fibres and to their alignment and degree of crosslinking. The activity directed at the crosslinking and reorganisation of collagen fibrils along lines of tension represents a balance of collagen and matrix protein synthesis and lysis (18). Based upon data from animal studies, wound

strength is restored by the conversion of Type III collagen (immature) to type I collagen (85,86). This is accomplished by catabolism by collagenases of old collagen and synthesis of new protein. Bacterial collagenase, lysosomal protease and tissue collagenase can only act upon exposed collagen fibrils therefore other proteases and hyaluronidase work upon noncollagen material to enable collagen digestion to proceed. However this maturation process has yet to be defined in human healing.

While remodelling continues indefinitely, the scar never returns to the tensile strength of uninjured tissue. Levenson (87) showed that even after 12 months the wound does not appear to regain either the architecture or the tensile strength of normal skin, maximum strength being 80% of the unwounded tissue. These authors also reported that hydroxyproline content within the wound strips to correlated well with the tensile strength results. Levenson concluded that remodelling probably begins when the first new collagen is laid down in the wound.

Of the cytokines that are involved in this phase of healing, TGF-β appears to be the most influential. It has been implicated in a multitude of fibrotic disease processes as summarised by Border and Noble (88). Studies performed by Shah et al (72) indicated that remodelling of the dermal matrix can be manipulated using antibodies to TGFβ isoforms. Their results demonstrated that the abnormal architecture associated with adult scar in the neodermis was eliminated when neutralising anti-TGFβ antibodies administered in the first week after wounding without any effect upon healing time or tensile strength. Therefore as with epitheliasation and wound contraction, manipulation of the growth factor profiles in a healing wound may also influence the long term quality of the ECM and hence the wound.

1.3. GROWTH HORMONE (GH) AND IGF-I AXIS IN WOUND HEALING

Growth Hormone

Physiologically GH is primarily responsible for linear body growth and increase in organ size in children until puberty. However its role thereafter is less clear. A direct role for GH in wound healing is debatable as GH deficient individuals heal normally (89). There is little doubt that GH does influence dermal and epidermal growth and that its receptor and binding protein are expressed by skin fibroblasts (90). Immunoreactivity for GH can also be identified in skin appendages (91). Several experimental studies have demonstrated beneficial effects of GH upon collagen deposition (92,93) and other healing parameters including improved abdominal wound bursting strength (94). Zaizen et al demonstrated both dose dependent and time dependent effects of GH in malnourished animals after laparotomies compared to normal animals. Unfortunately a normally nourished group that received GH was not included in the experimental design. Rasmussen et al (95) also concluded that improved granulation tissue formation and collagen deposition in wound chambers in response to GH were dose dependent and that GH did not appear to produce excessive collagen deposition in granulation tissue. Garrel et al (89) provided further support with their examination of the effect of growth hormone releasing factor (GRF) upon wound healing indices in rodent incisional wounds and implanted polyvinyl sponges. Their results revealed elevated GH levels in wound biopsy specimens and thus implicated GH as an important influence in wound healing physiology. Granulation tissue production can also be stimulated directly using subcutaneous GH as demonstrated by Steenfos and Jansson (96).

Growth hormone is able to stabilise fluid distribution in critically ill patients (97) and reverse the catabolic state associated with severe paediatric thermal injury (98). Subjects undergoing major abdominal surgery and receiving intravenous parenteral nutrition post-operatively also demonstrated improved nitrogen balance with biosynthetic human GH (99). In normal volunteers, protein net balance can also be increased by subcutaneous rh-GH (100). However hyperglycaemia can be a significant clinical complication following GH supplementation. To oppose this hyperglycaemic response GH can be administered with insulin to produce a greater anabolic effect than that seen with insulin alone (101). Besides its effects upon protein metabolism, GH influences carbohydrate and fat utilisation within the body by encouraging the mobilisation of fat stores and enhancing conversion of fatty acids to acetyl coenzyme-A. It also reduces glucose utilisation and promotes glycogen deposition (102).

Herndon's study (98) demonstrates the link between GH, IGF-I levels and the catabolic state of paediatric patients with total body surface area (TBSA) burns greater than 50%. This study demonstrated that GH administration alleviates depressed IGF-I levels and that GH promoted earlier healing of skin graft donor sites. This benefit contributed to a reduction in hospital bed days. The work of Müller et al (103) considered the IGF-I levels in adult burn patients and confirmed a decline of IGF-I levels in this catabolic state. The studies by Herndon et al (98) and Gatzen et al (97) have demonstrated recovery of depressed IGF-I levels following subcutaneous or intramuscular GH thereby suggesting an important association between reversal of catabolic states, GH administration and IGF-I levels. However these studies did not confirm IGF-I as the mediator for the improvement in the clinical states of these patients nor as the mediator of GH activity. To date GH remains the only growth factor with accepted efficacy in the management of cutaneous thermal injuries

however becaplermin (recombinant human PDGF-BB) has been approved for use in the United States for the treatment of diabetic ulcers (104).

Insulin-like Growth Factor I

Salmon and Daughaday in 1957 postulated that a 'sulphation factor' was largely responsible for the growth-related effects of GH. These initial observations were based upon studies of sulphate incorporation of chondroitin sulphate by chondrocytes (105). This relationship between GH and IGF-I was formalised into the Somatomedin hypothesis in 1972 and the label somatomedin C was given to this 'sulfation factor' (106). The discovery of IGFs arose from analyses that suggested the existence of three apparently distinct factors. These factors firstly mediated GH effects on chondrogenesis (somatomedin); secondly appeared responsible for non-suppressible insulin-like activity (NSILAs) in serum and thirdly had multiplication stimulating activity (MSA).

The NSILAs were factors that continued to promote glucose uptake by adipocytes despite anti-insulin sera, while the MSA, produced in serum-conditioned media, promoted the proliferation of cultured cells (107). These factors collectively became known as insulin-like growth factors I and II and account for all the somatomedin/NSILA/MSA activity in plasma (108). Correlation of changes in serum IGF-I levels with GH levels and the growth rates in humans provided support for the somatomedin hypothesis in respect to human physiology (109).

Several extensive reviews have detailed the physiology of systemic IGFs and their effects on local tissue and their activity in wound healing (109-113). The IGF family consists of the two peptides, IGF-I and II, their carrier proteins, insulin-like factor binding protein (IGFBP) -1 to -6 and the IGF receptors. Rinderknecht and Humbel (114) were the first to characterise IGF-I and II as polypeptides of 70 and 67 amino acids respectively that share ~80% structural homology with the insulin precursor proinsulin. IGF-I is a basic protein of 7.5 kDa that is better known for its place as the second messenger for GH. It circulates in plasma in significant concentrations and is also released by platelet degranulation macrophages and into the wound (15). Its levels within the wound are augmented by fibroblast and keratinocyte synthesis. IGF-II (7.0kDa) is a neutral peptide and is present in serum at higher concentrations than IGF-I.

Two specific IGF receptors have been identified, types I and II. The activity of IGF-I at the target tissue level is predominantly mediated through the type I receptor that resides in the cell membrane. It comprises paired linked cysteine extracellular α subunits linked to paired transmembranous β subunits that are associated with a tyrosine kinase intracellular domain (108). While IGF-I binds preferentially to this receptor, it does exhibit low affinity with both the insulin receptor and the IGF-type II receptor. The type II receptor also binds mannose-6-phosphate and appears to activate a GTP-binding protein via a G-protein activating sequence in the cytoplasmic domain of the receptor (108). The function of this second receptor is essentially unknown and consequently IGF-II's physiological role remains unclear.

The IGFBPs are important to IGF physiology. These six proteins modulate IGFs both at a local tissue level and also within plasma (115,116). The IGFBPs have molecular weights in

the range 23 to 46 kDa with considerable conservation of primary structure. They appear to be expressed diversely in those tissues that respond to IGF-I stimulation and which produce IGFs. IGFBP-3 is the main carrier of IGFs in the circulation. Its production is partly under GH control (117,118) while the others are expressed predominantly at specific developmental stages. For example, IGFBP-2 appears predominantly in foetal life and in particular tissues as illustrated by human skin fibroblasts that secrete IGFBP-3, -4, -5 and -6 (116). This dynamic balance possibly influences significantly the activity of IGFs at the wound site.

1.4. BIOLOGICAL ACTIVITY OF IGF-I

IGF-I is a potent anabolic agent to chondrocytes as well as osteoblasts. It promotes glycogen synthesis, cell proliferation and differentiation in cell culture while subcutaneous infusions cause dose related increases in body weight, tibial epiphyseal plate width and thymidine incorporation in hypophysectomised animals (109,113). However bolus injections of IGF-I or IGF-II did not significantly alter blood glucose levels in these animals. The IGFs appear to be important in foetal growth (IGF-II) and growth up to puberty but under normal circumstances they do not regulate glucose metabolism (109). After puberty their levels plateau then decrease with age.

The role of IGF-I in the wound healing process is less distinct. *In vivo* and cell culture studies show that IGF-I is known to influence the activity of cell types active in wound repair including macrophages (119), keratinocytes (71), fibroblasts (120) and endothelial

cells (112). It is also chemotactic for endothelial cells (121,122). Gillery et al (123) demonstrated increased fibroblast activity in a gel lattice. Contraction of the gels was modestly increased following incubation to IGF-I mixed with low concentrations of foetal bovine serum.

Cook and co-workers (124), in a series of fibroblast culture assays provided evidence that GH stimulates IGF-I expression. This local production of IGF-I appeared to induce growth of the fibroblasts in the presence of a low concentration of GH-deficient serum. Their conclusions supported the premise that IGF-I provides paracrine stimulation to its target cells as well having an endocrine mode of activity. Expression of IGF-I protein, mRNA and receptors have also been confirmed in experimental wounds (96,125,126) and human skin (90).

Few experimental studies have demonstrated efficacy of IGF-I in animal models despite promising results in tissue culture models and obvious expression of this peptide in wounds and wound fluid (112). Evidence exists to support its application as a single agent in compromised states of healing. Using the Hunt-Schilling chamber model, Suh et al (127) demonstrated that IGF-I infusion (15µg per day) elevated protein and DNA synthesis as well as hydroxyproline content within granulation tissue in steroid treated animals.

Although appearing relatively inactive in animals with normal healing capabilities when applied alone, IGF-I is effective when combined with other growth factors particularly PDGF or with one of the IGFBPs. Certainly the activity of IGF-I in periodontal wounds is enhanced in the presence of PDGF (128-130). A similar response can be demonstrated for the closure of excisional wounds in diabetic mice (46,131). The binding proteins may also positively influence IGF-I activity even though they reduce its free concentration. When

applied in combination with IGFBP-1, IGF-1 can increase wound strength up to 33% (132) and granulation tissue production by 52% in normal animals. A similar increase above control levels was obtained for ischaemic wounds in a rabbit ear model as demonstrated by Jennische et al (133). Spencer et al (134) claimed increases in wound healing parameters of 180% using an IGF-I/IGFBP-3 combination. Tsuboi et al (135) examined histological parameters of wound repair and found that the combination of IGF-I and IGFBP-1 produced more rapid epithelialisation and granulation tissue formation compared to either proteins alone or their vehicle. They concluded that this combination may have clinical relevance to the treatment of patients with diabetes and difficult wounds. As hypophysectomised animals receiving appropriate thyroxine and glucocorticoid replacement exhibit impaired ability to produce granulation tissue (136) it would seem that IGF-I should facilitate healing if administered topically or parenterally.

No human studies currently exist to indicate efficacy or safety of IGF-I as a wound healing agent. However IGF-I has been used in clinical trials as an agent to augment the treatment of insulin dependent diabetes mellitus (IDDM) (137-139). Unfortunately Jabri and coworkers documented several adverse effects of the systemically administered IGF-I including hypoglycaemia, unreliable reduction and control of hyperglycaemia, arthralgia and peripheral oedema. While no conclusive data exists regarding absorbency and the influence of factors such as tissue pH, *in vivo* results suggest that topical application may be an efficacious technique that circumvents these systemic side effects.

The presence and secretion of IGFBPs in tissue culture models can complicate interpretation of the results of experiments employing IGFs. Recently analogues or variants of the human IGF-I peptide have been isolated, designed and engineered using recombinant DNA

technology (140,141). In particular two that have been extensively tested include a truncated variant found naturally in the brain known as des(1-3)IGF-I and a fusion peptide known as long [Arg^3] IGF-I (LR^{3}IGF-I). The latter has an amino acid substitution at position 3, an arginine residue replacing a glutamine residue in addition to an eleven amino acid N-terminal extension peptide with a bridging Val-Asn dipeptide. These peptides exhibit increased *in vitro* and *in vivo* activity compared to the authentic recombinant human IGF-I (rhIGF-I) peptide despite reduced affinity for the type I receptor. It is their reduced binding to the IGFBPs that allows greater bioavailability (140,142). Further *in vivo* studies have shown these analogues to be equally, or more, potent than rhIGF-I in both normal (143) and compromised metabolic rodent models especially in the presence of diabetes and steroid treatment or malnutrition (144-146). IGF analogues produced a net gain in muscle protein in steroid treated animals by both retarding proteolysis and increasing protein synthesis (145). They can also restore growth in animals with streptozotocin induced diabetes (146). Other consequences of these structural changes in compromised metabolic states include increased clearance from plasma (147,148) and accelerated gut growth (149).

Therefore these peptides are particularly suited to the study of IGF-I in the wound environment as they can allow examination of IGF effects with minimal interference by the the IGFBPs. Further potential may best be demonstrated in a diabetic model given the beneficial effect of IGF-I and its analogues in diabetic animals as shown by Tomas et al (146,150). These authors showed that the analogues, as well as rhIGF-I, should prove to be useful adjuncts to routine insulin therapy in diabetic patients particularly those that are insulin-resistant.

1.5. DIABETES AND WOUND HEALING

Diabetes mellitus is the most common endocrine disorder found in modern western society. It afflicts approximately 15% of the population over the age of 65 (151) and is defined by hyperglycaemia associated with glycosuria. It presents in several forms but may be categorised primarily as being early onset (insulin-dependent) or mature-onset (non-insulin dependent), type 1 and type 2 respectively.

The complications of diabetes reported include ischaemic heart disease, cerebro-vascular disease, diabetic nephropathy, accelerated atherosclerosis as well as retinopathy, cataracts and limb threatening ulceration (152). However hyperglycaemia also predisposes affected individuals to an increased incidence of infections such as urinary tract infection, cellulitis and post-operative infections. Diabetics undergoing clean surgical procedures are five times more likely to develop wound infections than nondiabetics (10.7% compared to 1.8%) (5). Neuropathies affecting peripheral nerves and the autonomic nervous system are more commonly seen in diabetics (153). These two factors together contribute to the development of ulceration in diabetics. Chronic ulceration of the lower extremity and other aberrations of wound healing are the most common reasons for hospital admission in Australia for diabetics. Thirty-five to 40% of patients undergoing lower limb amputation will be diabetic (154-156). The cumulative risk over 25 years of any diabetic having an amputation is approximately 11% (157) and of those with chronic leg ulcers up to 25% will also have diabetes (158). Of importance, even if the ulcers do heal, new ulcers will develop in up to 70% within 5 years (159). Up to 20% of hospitalised diabetics will have a lower limb ulcer (160). In the United Kingdom, the economic cost alone is considerable with diabetic foot

problems accounting for 1.25 million hospital bed-days worth £220 million annually (156). These data demonstrate the significance of diabetes in the management of chronic wounds.

Several studies have examined the effect of diabetes in wound repair. Growth factor production is retarded (161) and wound strength is decreased (162) in both endogenous and toxin induced models of type 1 diabetes. These changes are accompanied by a decrease in total collagen content within the skin. The mechanism for these alterations is not fully understood but it may be related to microvascular ischaemia, impaired inflammatory response, reduced collagen deposition (163) or generalised increased proteolytic activity (164).

The application of IGF-I to the management of diabetes has been limited to the treatment of hyperglycaemia. No studies have demonstrated its efficacy in the treatment of human diabetic wounds using either topical or systemic administration. While studies such as that of Morrow et al (138) clearly showed the benefits afforded by rhIGF-I in the management of insulin resistant syndromes, others raise concerns about its long term systemic effects. These workers have suggested that IGF-I could worsen diabetic retinopathy and nephropathy by promoting the proliferation of vascular smooth muscle cells and glomerular mesangial cells (137,165). Several tumours also express and show *in vitro* growth in response to IGF-I or IGF-II (166,167). In addition clinically relevant systemic doses of IGF-I can produce significant adverse effects including myalgias, tachycardia, dyspnoea, oedema of the hands and face, trismus and facial nerve palsy (139).

The presence of IGF-I receptors in dermis and epidermal cells, the peptide's documented *in vitro* mitogenic effects and the risks of systemic adverse effects with large subcutaneous

doses or intravenous infusions would suggest that topical applications of IGF-I may provide safer and more logical means of delivery of this agent to enhance diabetic wound healing.

1.6. STATEMENT OF OBJECTIVES

The literature underpins the potential of IGF-I to augment the wound repair process however its activity *in vitro* is complex and animal experiments demonstrate only limited efficacy of IGF-I alone. The conditions of its use invariably determine its potency to improve wound strength, increase collagen deposition or accelerate wound closure.

This study aims to test several hypotheses.:

- 1) IGF-I and IGF-II as well as their binding proteins are identifiable in significant concentrations in human wound fluid obtained from acute partial thickness dermal wounds.

- 2) IGF-I activity *in vivo* and *in vitro* is augmented by GH.

- 3) Reduction of binding protein affinity confers greater potency to the IGF-I molecule in models of wound healing.

- 4) Compromised wound repair as illustrated by a model of type I diabetes mellitus can be improved with the topical application of IGF-I.

The objectives are to demonstrate:

- the levels of IGFs and the existence of their binding proteins in acute wound fluid;

- the effects of IGF analogues long $[Arg^3]$ IGF-I (LR^3IGF-I) and des(1-3)IGF-I compared to native recombinant human IGF-I (IGF-I) in various fibroblast culture models;

- the effect of IGF-I and LR^3IGF-I upon wound healing in normal and diabetic animals.

CHAPTER 2

A STUDY OF IGFS IN HUMAN WOUND FLUID AND PLASMA

2.1. Introduction

2.2 Materials

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2.4.4. WLB and Immunoblot Analysis of Whole Human Wound Fluid and Plasma

2.5. Discussion

2.1. INTRODUCTION

IGF-I and IGF-II levels are readily measured in serum of both humans and experimental animals. Declining concentrations can be demonstrated with advancing age (168,169) and malnutrition (170-172) but may be increased by greater physical activity (168). Furthermore lower systemic IGF-I levels are associated with other abnormalities of metabolism such as osteoporosis (173), Laron syndrome (174), diabetes mellitus (161) and chronic renal failure (175).

The impact of the IGF system upon the recovery of critically ill patients has generated much interest in the literature since Herndon et al (98) demonstrated that exogenous GH reversed the catabolic state associated with severe burns. These burnt children exhibited accelerated donor site healing and increased serum IGF-I levels. Reduction of circulating IGF-I concentrations and alterations in IGFBPs profiles have been associated with the catabolic effects of severe burns (176), major elective abdominal surgery (177) and critically ill patients (178). Chronic states such as malnutrition (172), cirrhosis (179) and diabetes mellitus (180,181) tend to decrease IGF-I serum concentrations. Concomitantly IGFBP-1 and IGFBP-2 levels can increase in plasma (180,181). However during pregnancy IGF-I levels are elevated while at the same time IGFBP-3 may be rendered less stable (182).

M ller et al (103) demonstrated that burns of increasing severity were associated with lower IGF-I levels during the early post-burn period. Increased protein loss from the wounds possibly contributed to falls in IGF-I as this correlated with decreasing albumin concentrations. However altered IGFBP profiles associated with acute metabolic stresses will also significantly influence IGF bioavailability. This may be more relevant in explaining such changes to IGF-I activity. Surgery influences binding proteins in a variable

way as IGFBP-3 appears to increase during the early post-operative phase following cardiac surgery (183) and after major abdominal surgery it falls (177,184). IGFBP-1 increases in response to fasting or critical illness (177,185), but this recedes with adequate nutritional support. However it is difficult to accurately compare the reported IGF levels in these studies as the procedures to extract IGFs from their IGFBPs are so varied. Similarly this applies also to the measurement of IGFs in these samples after dissociation of IGFs from their binding proteins.

Drain fluid from mastectomy and abdominoplasty wounds or wound fluid from pressure sores have been used as models for collection human 'wound fluid' (173,186). Spencer et al (112) demonstrated the presence of IGFs in mastectomy wound fluid and quantitated IGF-I by RIA after acid-ethanol extraction of the samples. Another model available is that of a suction induced blister model described by Xu et al (187). This was employed to determine the concentration of IGF-I and the IGFBPs in interstitial wound fluid (187). However comparison of values obtained in these studies is not possible due to the varying techniques used to measure IGFs. To address this issue, the 3rd International Symposium on Insulin-like Growth Factor published guidelines detailing detailed procedures to validate these technique (188). These included:

- Parallelism between the IGF reference curve and dilution curves of IGF extracts of representative samples. This is a requirement but does not exclude interference by IGFBPs.

- Acidification of sample extracts at pH <2.8 followed by acid size exclusion chromatography with IGF measurement performed by actual RIA of neutralised

fractions. Interference of residual IGFBPs is detected as apparent immunoactivity in the elution volume corresponding to IGFBPs.

- Recovery of unlabelled IGF pre-incubated with representative samples before separation and IGF RIA. The separation technique must produce low variability and high recovery of tracer.

- Comparison of measurements in representative samples by the IGF assay to be validated with measurements after complete separation of IGFs from IGFBPs by acid size chromatography using the same RIA (samples should represent the expected extremes of IGF to IGFBP ratios where problems are to be expected).

- It may also be necessary to directly assay the extracted sample for residual IGFBPs by RIA or Western ligand blotting or immunoblotting. However these techniques may not comprehensively identify all IGFBPs or their fragments.

Further support is gained from animal models for the premise that IGF-I plays an important role in wound repair as well as providing important systemic anabolic effects. These have been used to demonstrate significant concentrations of IGF-I in wound fluid (96,112,133) as well as detect mRNA and receptor expression in cells involved in healing (96,125,189).

This study was designed to document and describe the changes of IGF concentrations and IGFBP expression within the wound fluid exudate produced from a healing acute wound.

Aims:

The aims of this series of experiments were to:

- demonstrate the presence of IGFs at the wound site by assessing their concentrations in wound fluid over the time course of healing of partial thickness wounds and compare these values to time matched plasma samples;

- compare the values of IGF-I and IGF-II obtained using RIA following treatment of the samples by two methods of extraction of IGFs from IGFBPs;

- detect and identify IGFBPs within wound fluid using Western ligand and immunoblotting techniques.

2.2 MATERIALS

Recombinant human IGF-I (10ng/ml) and rhIGF-II (10ng/ml) standards were obtained from GroPep Pty Ltd. Ovine anti rabbit IgG was purchased from Silenus Laboratory. (Hawthorn, VIC, Australia). Rabbit IgG, rabbit anti-human IGF-I antibody and rabbit anti human IGF-II polyclonal antibody were obtained from GroPep Pty Ltd, (Adelaide, SA, Australia). Rabbit polyclonal antibodies to IGFBPs -1 to -3 and IGFBP -5 were purchased from Upstate Biotechnology Incorporated (Lake Placid, NY, USA). Rabbit anti-sheep IGFBP-4 was a gift from Dr. J. Owens (Dept of Obstetrics and Gynaecology, University of Adelaide, SA). Horseradish peroxidase conjugated with goat anti-rabbit IgG was obtained from Dakopath A/S Dako corporation, Denmark. Reagents for HPLC mobile phase and RIA buffer were purchased from AnalaR Chemicals. RIA buffer was made in 4 litre quantities and contained 800 mg protamine sulphate, 14.89 g EDTA, 18.72 g sodium dihydrogen phosphate, 2.0 ml Tween 20, 1.0 g sodium azide which was adjusted to pH 7.5 with NaOH. High performance liquid chromatography (HPLC) was performed using a Protein-Pak 125 gel permeation column (Waters, Sydney, NSW, Australia) mounted on a HPLC pump (GBC).

Recombinant human IGF-I and rhIGF-II obtained from GroPep Pty Ltd (Adelaide, SA, Australia) were iodinated with ^{125}I to a specific activity of approximately 50-80 μCi/μg using the Chloramine-T reaction as described by Van Obberghen-Schilling and Poussegur (190). TEMED (N,N,N',N'-Tetramethylethylenediamine) and 40% Bis/acrylamide were purchased from BioRad Laboratories (Hercules, CA, USA) while ammonium persulphate was obtained from Eastman Kodak Co (Rochester, NY, USA). Opsite™ was donated by Smith & Nephew Pty Ltd (Hertfordshire, UK). Bovine serum albumin was obtained from

Sigma. Western saline was prepared in 2 litre volumes containing 0.01M NaCl and 0.015M

Tris, solution pH 7.4.

2.3 METHODS

2.3.1. Protocol for fluid collection

Approval for this study was given by the Human Ethics Committees of the Queen Elizabeth Hospital (Woodville, SA) and the University of Adelaide (Adelaide, SA). All patients were enlisted into this project after providing informed consent.

Listed below are the inclusion and exclusion criteria for participation in the study:

Inclusion Criteria

   Aged 20-80 years

   Procedure requiring the harvesting of a split skin graft

   Able to provide informed consent

Exclusion Criteria

   Deemed not suitable by treating physician

   Refusal to sign consent or unable to provide informed consent

   Sensitivity to Opsite™

Withdrawal Criteria

   Excessive leakage from beneath dressing

   Wound infection

   Patient non-compliance

   Discretion of treating physician or desire of patient to withdraw
   from trial

   Post-operative complications

Patients presenting for skin grafting to the Plastic Surgery service at the Queen Elizabeth Hospital were approached for enlistment into the study. Informed consent was obtained and data were collected concerning medical history, medications and illnesses that may influence wound repair or affect plasma IGF-I levels (see appendix A).

Twenty-one patients were recruited to the study with five patients completing the time course of sampling. Patients were withdrawn for several reasons including leakage of wound fluid from beneath the dressing, pain or post-operative complications. One elderly patient was withdrawn from the study after he experienced a myocardial infarction 24 hours after his surgery. This poor recruitment to completion of study ratio reflected a number of issues relating to study design as well as patient compliance. Opsite, once punctured can be difficult to reseal adequately; nursing care of the dressings was variable, some patients found the dressing too uncomfortable and withdrew from the study. In six cases their was insufficient wound fluid at day 5 to permit sampling. These were therefore withdrawn.

All patients were female aged 32 to 76 years with no important co-morbidities except one who was a non-insulin dependent diabetic. Wound fluid was collected post-operatively with time matched samples of plasma on day 1, 3 and 5. A baseline plasma specimen was obtained pre-operatively on the day of surgery. Once collected all samples were placed in a heparinised 125 IU/10ml vial and stored at 4°C until centrifuged and aliquoted into 50 μl volumes for storage at -20°C.

Samples were labelled in numerical order with the prefix 'Q'. They also received the codes 'WF' for wound fluid samples or 'Pl' for plasma samples. The third part of the code

denoted the day of sampling from the time of the operation for example a sample taken on day 1 after surgery were given the code 'd1'.

2.3.2. Procedure for Acid Gel Permeation Chromatography (AGPC)

Samples were prepared by adding 40 μl of wound fluid or plasma to 260 μl of deionised water and 100 μl of 4 x mobile phase (200mM acetic acid and 50mM triethylamine, 0.05% Tween-20, pH 2.5). Delipidation of each sample solution was performed using a Freon extraction method (191). A Protein-Pak gel permeation column was equilibrated in the mobile phase and 300 μl of each fraction was loaded and chromatographed at 0.25 ml/min as described by Owens et al (192) A Protein-Pak gel permeation column was equilibrated in the mobile phase and 300 μl of each fraction was loaded and chromatographed at 0.25 ml/min as described by Owens et al (192) at pH 2.5. Tracer recovery runs were performed before and after each extraction procedure and ranged from 89% to 95%. Fractions were then pooled and IGFs quantitated by radioimmunoassay.

2.3.3. Acid -Ethanol (AE) Extraction

Wound fluid and plasma samples were also treated using the acid-ethanol extraction technique as originally described by Daughaday et al (193) to extract IGFs from the IGFBPs.

Briefly, sample volumes of 40 μl were mixed with 160 μl of acid-ethanol (87.5% ethanol and 12.5% 2N HCl). The solutions were mixed thoroughly and allowed to stand at room

temperature for 30 minutes before being centrifuged at 1800 x g at 4°C for another 30 min. The supernatants (100 μl) were transferred to polypropylene tubes and 40 μl 0.855M Tris base was added. This was then diluted ten fold with RIA buffer.

2.3.4. Radioimmunoassay

Measurement of IGF-I by RIA was performed by addition of 100 μl of HPLC fractions, mobile phase alone or standards in mobile phase to 60 μl 0.4 M Tris followed by 200 μl RIA buffer, 50 μl anti IGF-I antibody (final dilution 1:80000), 50 μl [I^{125}]IGF-I tracer (25 x 10^3 to 30 x 10^3 cpm) to produce a 460 μl solution. These were then incubated overnight at 4°C before adding ovine anti rabbit IgG and rabbit IgG. After incubating another 30 min at 4°C, 1 ml of 0.9% polyethylene glycol (PEG) was added. The samples were finally centrifuged at 4000 x g for 20 min at 4°C before aspirating the supernatant and measuring radioactivity of the pellet using a gamma counter (Wallace LKB 1261 Multigamma). All standards and controls were tested in triplicate for each extract.

IGF-II levels were measured using this same technique with the following alterations:-

- assay volume was 380 μl (50 μl of HPLC fraction, 30 μl of 0.4 mol/l Tris);

- anti IGF-II polyclonal serum replaced anti IGF-I antibody (1:7500 dilution);

- [I^{125}]IGF-II (20x 10^3 to 25 x 10^3 cpm) was used as the tracer.

Following AE extraction, 100 µl of extract sample, 100 µl of RIA buffer, 50 µl of either anti-IGF-I antibody (1/80000 dilution) or 50 µl of anti IGF-II polyclonal serum (1/7500 dilution) and 50 µl of the appropriate tracer ([I^{125}]-rhIGF-I or [I^{125}]-rhIGF-II) were mixed to provide the RIA incubation solution.

2.3.5. Western Ligand Blot (WLB) and Immunoblot Analysis

The use of WLB to document IGFBP profiles is well described (194,195). The samples used in these experiments were diluted in non-reducing sample buffer. This dilution was 1:4 and the plasma 1:19 for the wound fluid samples. After incubating at 65°C for 15 min the samples along with [^{14}C]-labelled molecular weight markers (Amersham Rainbow Markers, Amersham International, Aylesbury, U.K.) were electrophoresed on a 12.5% wt/vol SDS-polyacrylamide slab gel at a constant current. A selection of acid-ethanol extracts was diluted 1:1 with loading buffer before also being separated on a 12.5% polyacrylamide gel.

Electrotransfer to nitrocellulose membranes (0.45µm; Scleicher & Schuell, Dassel, Germany) was performed using a semi-dry blotter (Multiphor II Novablot, Pharmacia) for 1 hour. The membranes were probed with [^{125}I]-IGF-II (1x10^6 cpm) for two hours after being blocked with 3% BSA/western saline overnight,. The IGF/IGFBP complexes were visualised autoradiographically (RX medical film, Fuji Photo Film Co., Tokyo, Japan). The resultant IGFBP bands were identified using western immunoblots. The membranes were probed with monoclonal anti-IGFBP-1, -2, -3, -4 and -5 antibodies (1 in 5000 dilution). The separated complexes were visualised using the enhanced chemiluminescence detection

system (Amersham International, Aylesbury, U.K.) and by exposing the treated nitrocellulose membranes to radiographic film.

2.3.6. Statistics

The results for AE and AGPC methods were compared using linear regression analysis. As the data were not normally distributed the Spearman correlation coefficient was calculated to determine the significance of the relationship between the two techniques for IGF-I and IGF-II levels in both wound fluid and plasma. A p-value less than 0.05 was deemed significant. The statistical software package Sigmastat version 1.0 (Jandel Corporation, San Ramon, CA., USA) was used for all analyses.

2.4. RESULTS

2.4.1. Acid gel permeation chromatography

AGPC which was used to separate IGFs from the IGFBPs was validated using WF and plasma samples from patient 16 taken on the fifth day after operation. IGF-I and IGF-II immunoactivity in the fractions recovered after AGPC are shown in figures 2.1 and 2.2 respectively. They demonstrate that baseline separation in wound fluid was achieved for both peptides. Serum IGFBP and IGF-I separation had previously been demonstrated in our laboratory using human samples (data not published). Each sample was subsequently fractionated into four pools at a rate of 0.25 ml/min : *pool 1* (2 mls collected at 6.5 min to 8.5 min) containing the IGFBP peak; *pool 2* (0.5 ml at 8.5 to 9.0 min); *pool 3* (2 mls at 9 min to 11 min) containing IGF-I and IGF-II and *pool 4* (0.5 ml at pool at 11 min to 11.5 min).

Figure 2.3 illustrates IGF-I accumulation in wound fluid samples collected during the first 5 days of healing. The values obtained were generally lower than those obtained for plasma samples and lay between 50 and 100 ng/ml. Matched *plasma* IGF-I levels did not change dramatically over the sampling period while IGF-I levels in the wound fluid rose marginally during the same period.

The IGF-II levels were between 5 and 7 fold higher than those observed for IGF-I by RIA. These also showed no consistent pattern except that in 4 of the 5 patients the final plasma levels were as high as or higher than those detected at day 0. This contrasted with the wound fluid concentrations in that IGF-II levels declined between day 1 and day 5 samples. In 3 out

of 5 patients IGF-I levels showed no consistent time related pattern in wound fluid and again the plasma levels generally changed very little between day 0 and day 5.
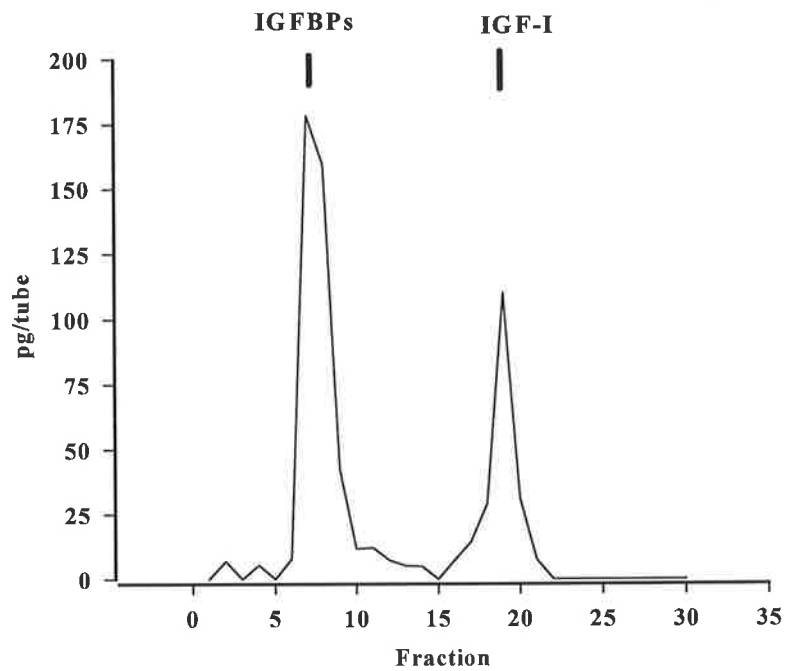
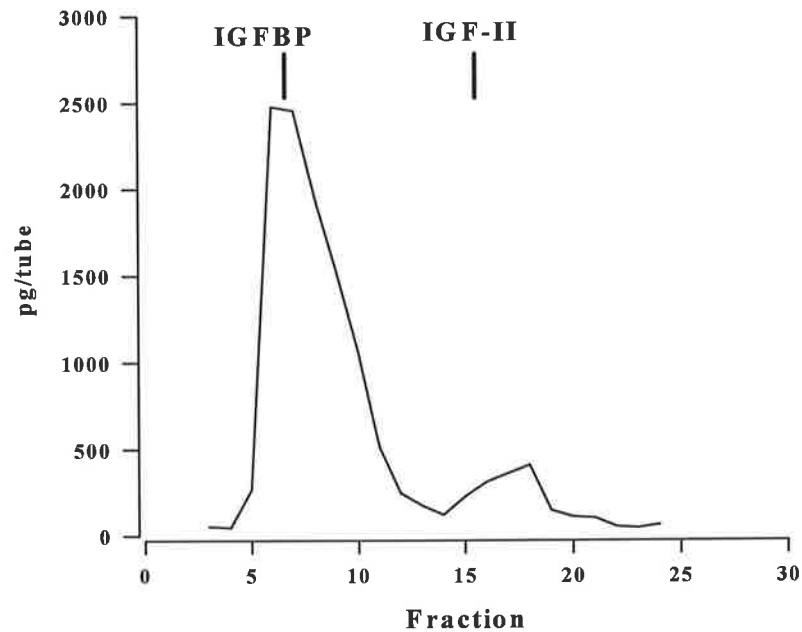**FIGURE 2.1.** IGF-I concentrations in human wound fluid. Elution profile of wound fluid samples following AGPC at pH 2.5.

A representative sample of wound fluid was diluted in HPLC running buffer before loading onto the Protein Pak column and eluted at 0.25 ml/min. Fractions (0.25 ml) were collected and subjected to IGF-I RIA as described in Materials and Methods 2.3.2 and 2.3.4. Baseline separation of the IGF-I peak and the IGFBPs was achieved.

FIGURE 2.2. IGF-II in human wound fluid. Elution profiles of human wound fluid samples following gel permeation HPLC at pH 2.5.

A representative sample of wound fluid was diluted in HPLC running buffer before loading onto the Protein Pak column and eluted at 0.25 ml/min. Fractions (0.25 ml) were collected and subjected to IGF-II RIA as described in Materials and Methods 2.3.2 and 2.3.4. Baseline separation of the IGF-II peak and the IGFBPs was achieved.
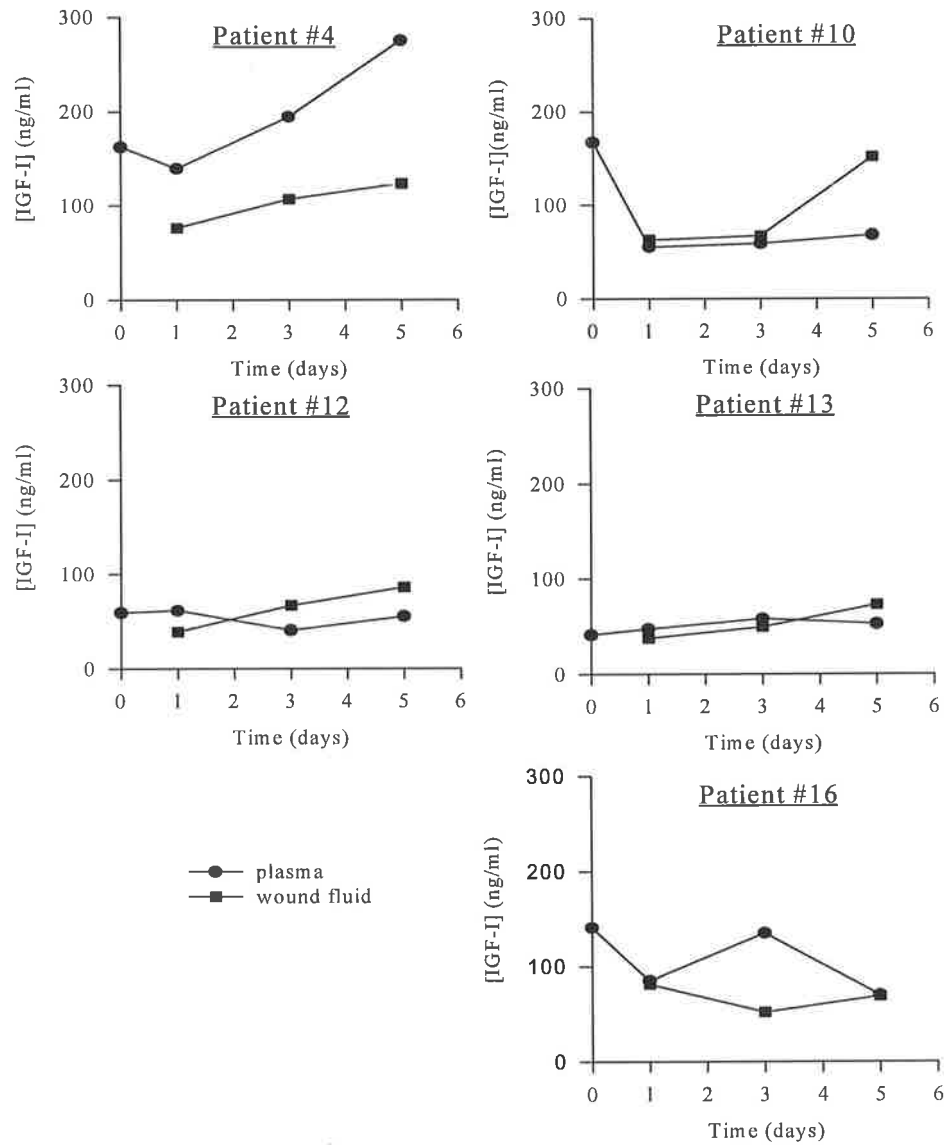
FIGURE 2.3. Comparison of IGF-I levels in time matched samples of human wound fluid and plasma.

Plasma samples were obtained from whole blood samples while wound fluid specimens were collected on days 1, 3 and 5 from beneath semipermeable dressings. IGF-I was measured by RIA after HPLC separation of IGFs from IGFBPs as described in Materials and Methods.
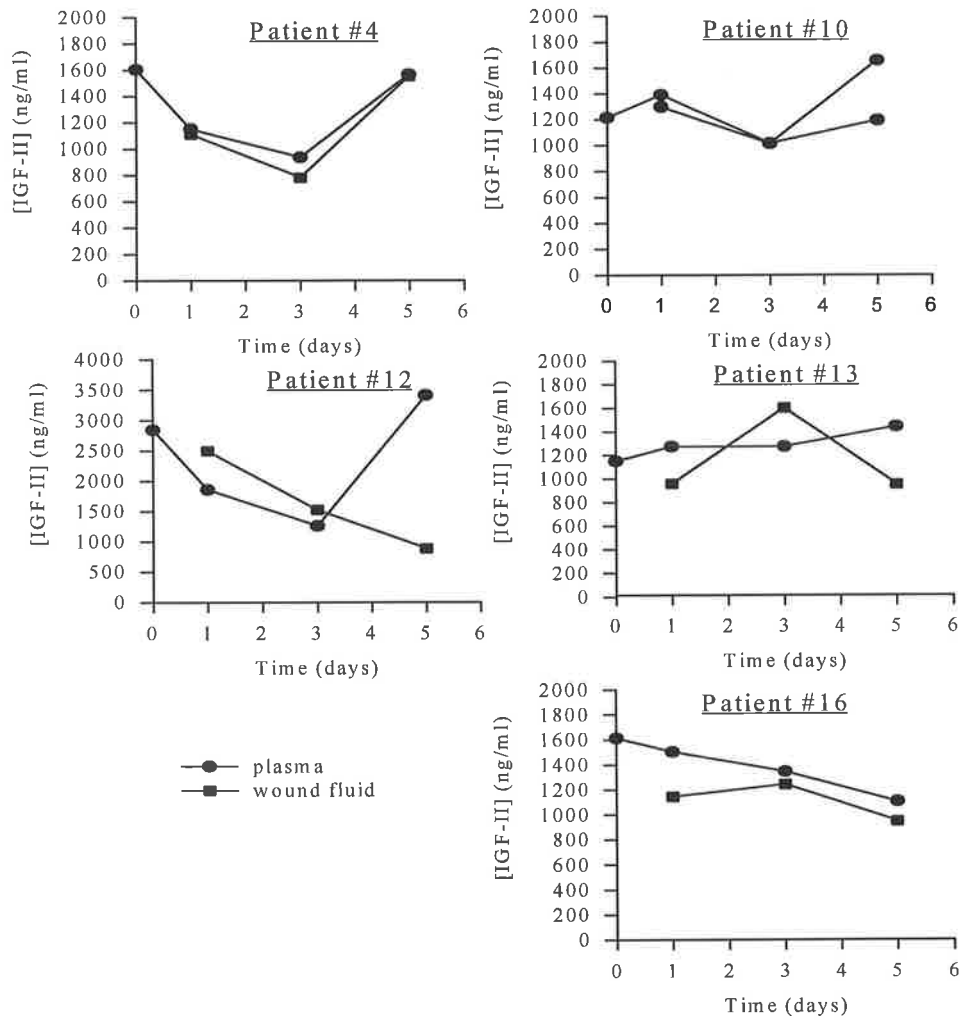
FIGURE 2.4. IGF-II concentrations in time matched samples of human wound fluid and plasma.

Plasma samples were obtained from whole blood samples while wound fluid specimens were collected on days 1, 3 and 5 from beneath semipermeable dressings. IGF-II levels were measured by RIA after HPLC separation of IGFs from IGFBPs as described in Materials and Methods.

2.4.2. Acid Ethanol Extraction

The values for IGF-I in plasma and wound fluid following AE extraction to separate IGFs from IGFBPs are illustrated in figures 2.5 and 2.6. These were about twice those observed following HPLC separation. The levels observed ranged from approximately 100 up to 800 ng/ml for both biological fluids. Plasma and wound fluid levels of IGF-I using this technique appeared to show greater similarity in both their variability and their actual levels compared to similar samples subjected to HPLC. However greater variability was observed for IGF-II levels during the same period. Interestingly the IGF-II values obtained in samples subjected to AE extraction proved to be 4 to 7 times lower than samples subjected AGPC. This contrasted with the results obtained for IGF-I where AE extracted values were higher than AGPC treated values.
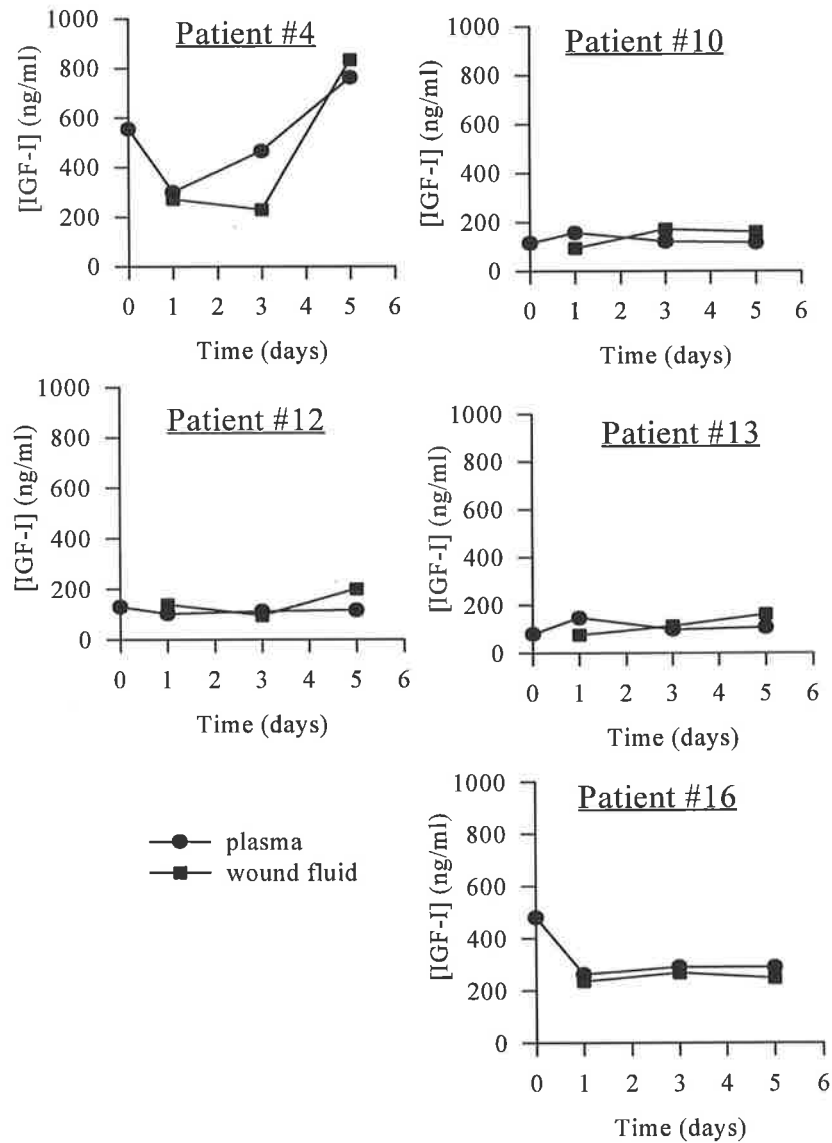
FIGURE 2.5. Comparison of IGF-I concentrations in time matched samples of wound fluid and plasma following.

Acid -ethanol extraction as described Daughaday et al was used to separate IGFs from their binding proteins. IGF-I was then measured by RIA as described in section 2.3.4.
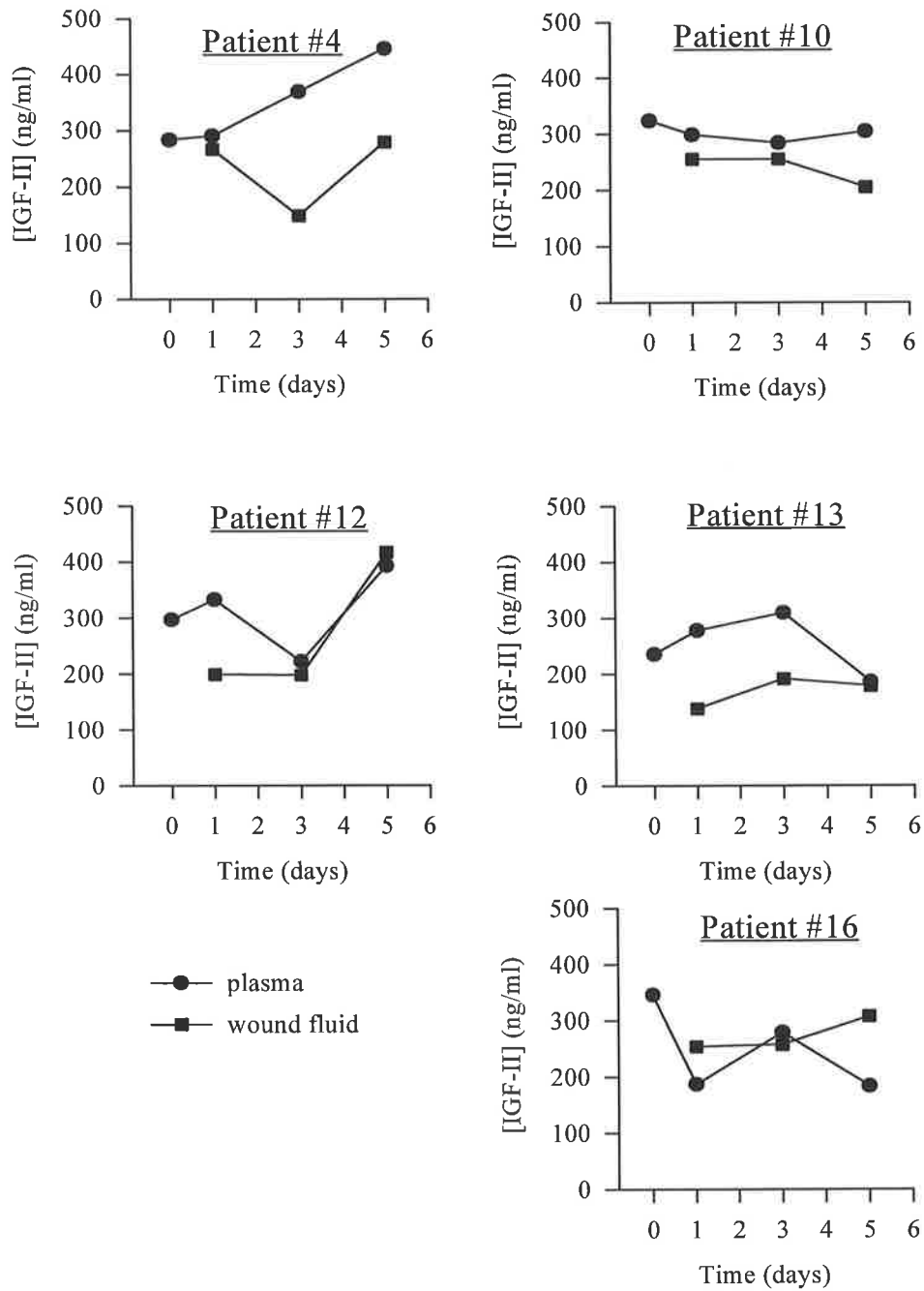
FIGURE 2.6. Concentration of IGF-II in time matched wound fluid and plasma.

Acid-ethanol extraction as described Daughaday et al was used to separate IGFs from their binding proteins. IGF-I was then measured by RIA as described in 2.3.4.

2.4.3. Comparison of AE Extraction and AGPC for Dissociation IGFBPs and IGFs.

The IGF values in plasma and wound fluid concentrations following AE extraction and AGPC were compared using linear regression (figures 2.7 and 2.8).

IGF-I values for the plasma samples demonstrated good correlation between concentrations obtained using the two different extraction methods ($p<0.001$; $r=0.84$). When a similar analysis was performed upon the results for IGF-II in human plasma no clear-cut relationship was observed ($p=0.2$; $r=0.54$; figure 2.7).

A similar pattern emerged for wound fluid, (figure 2.8) where IGF values following AE extraction correlated well with those after AGPC ($p<0.05$, $r=0.56$). However the values for IGF-II following the two extraction procedures correlated poorly ($p=0.783$; $r=-0.075$).

Electrophoresis and western ligand blots (WLB) were performed upon wound fluid and plasma samples that had been treated by acid-ethanol extraction to determine if IGFBPs remained within the extracts. Illustrated in figure 2.9 are the residual IGFBPs in AE extracts of both wound fluid and plasma samples. IGFBPs were detected in all samples except day 1 wound fluid from patient 13 (Q13WFd1) although band intensity varied dramatically between patients and time points. All bands detected were of 30kDa or less and therefore consistent with low molecular weight species of binding proteins. The specimens of Q12WFd3 and Q12WFd1 also showed bands that were clearly within the region of 14kD. Plasma samples retained higher quantities of low molecular weight IGFBPs than wound fluid.
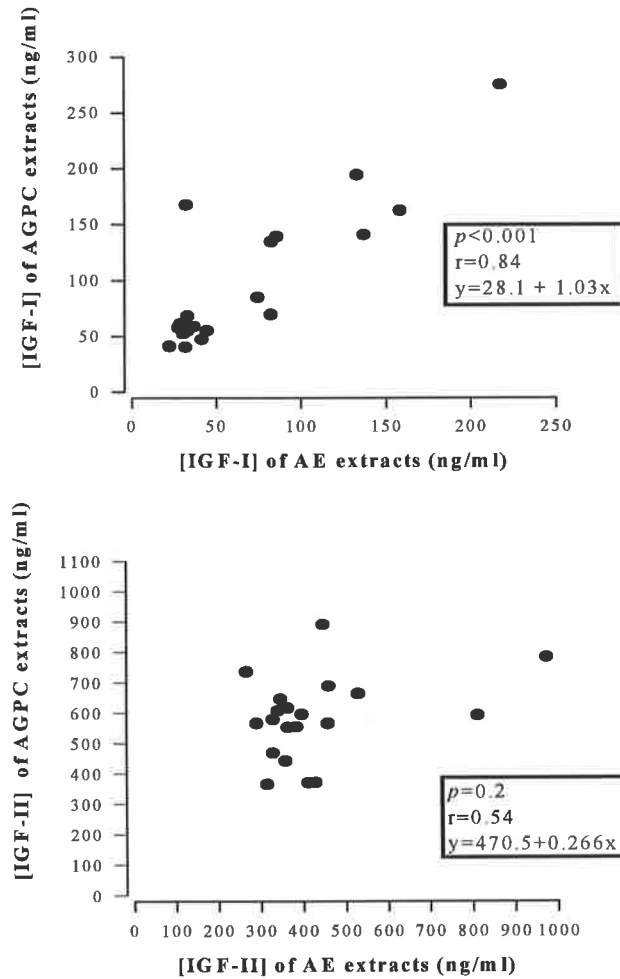
FIGURE 2.7. Relationship of the IGF-II and IGF-I concentrations in human plasma measured by RIA.

The data for 15 pairs of values for IGF-I and IGF-II following AGPC and AE extraction techniques were analysed by linear regression and the Spearman correlation coefficient calculated.
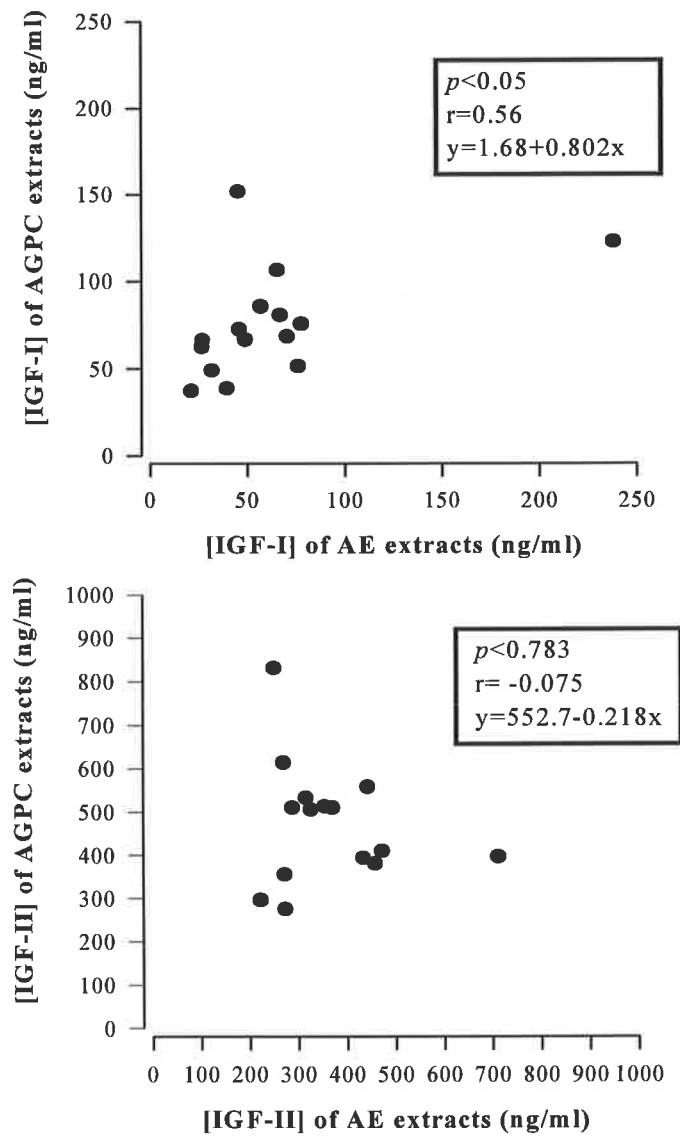
FIGURE 2.8. Comparison of IGF-I and IGF-II concentrations measured by RIA in wound fluid.

The data for 15 pairs of values for IGF-I and IGF-II following AGPC and AE extraction techniques were analysed by linear regression and the Spearman correlation coefficient calculated.

2.4.4. WLB and Immunoblot Analysis of Whole Human Wound Fluid and Plasma.

All raw samples were analysed for binding proteins using WLB analysis (figure 2.10). Immunoactivity suggestive of binding proteins was detected in all samples except Q13Pld0. Commonly a doublet was detected at ~46kDa consistent with IGFBP-3. Bands between 24 and 36 kDa confirmed the presence of other lower molecular weight IGF binding activity.

Western immunoblots performed to characterise the IGFBPs, revealed IGFBP-3 in all samples (figure 2.11). The doublets detected using WLB were identified by immunoblotting to be IGFBP-3. Interestingly, the intensity of the bands was greatest amongst wound fluid samples taken on the third and fifth days after wounding. In addition, wound fluid samples also contained lower molecular weight fragments of IGFBP-3 apparent at 30kDa and 21.5kDa. Such species may represent proteolytic fragments of IGFBP-3.

Further analysis of representative plasma was undertaken to determine the presence of IGFBP-1, -2, -4 and -5 (figure 2.12) in the sample. IGFBP-1 and -2 bands were identified. Also detected in the IGFBP-2 profile was the presence of some lower molecular weight bands samples. This may have represented cross reactivity however the existence of proteolytic degradation even in the plasma samples has not been excluded. IGFBP-4 was also found in both wound fluid and plasma while IGFBP-5 failed to be visualised with this system in either wound fluid or plasma. This suggested that it was not secreted into wound fluid and if it was then the amounts did not reach the level of sensitivity of this assay.

FIGURE 2.9 Western Ligand Blot of Acid Ethanol extracts of matched human wound fluid and serum from five patients undergoing skin grafting procedures.

Samples were incubated in acid-ethanol after Daughaday et al (190). The supernatant was removed and freeze-dried after separation. Freeze dried protein was reconstituted with 50 μl sample buffer and 40 μl of the solution was loaded into each well and electrophoresed on 12.5% SDS-PAGE gels. After electrotransfer of protein to nitrocellulose filters the filters were probed with $[^{125}I]$-IGF-II and the bands visualised autoradiographically after exposure at -70° C for 12 days.
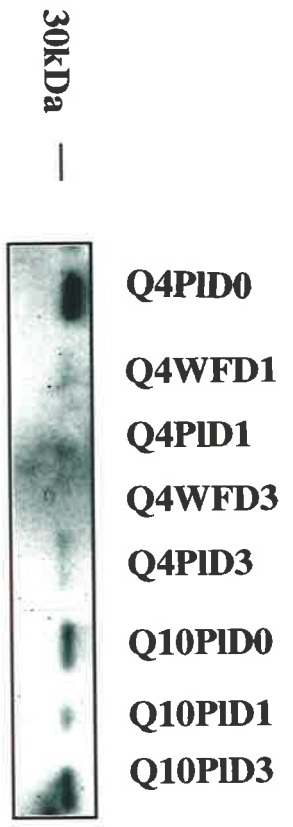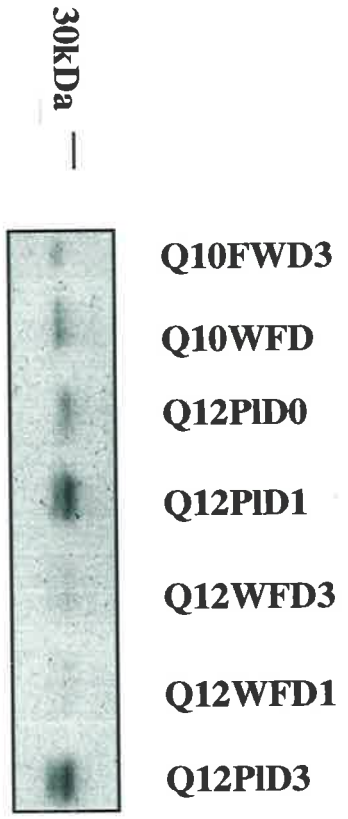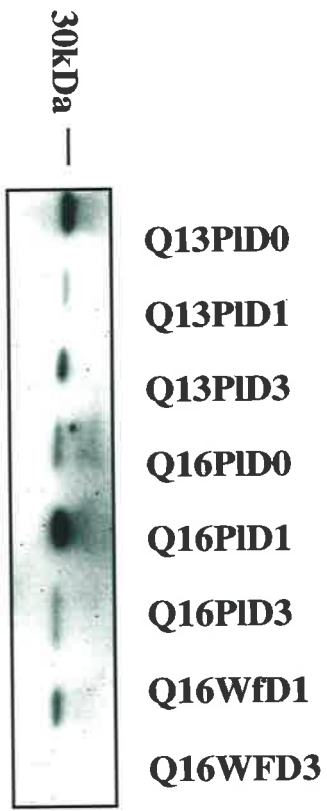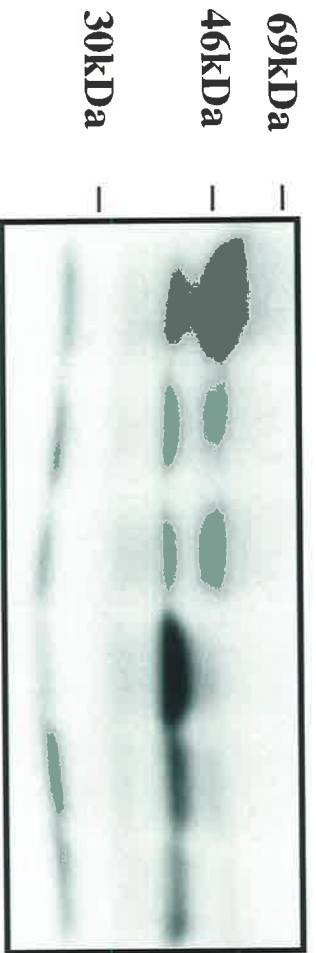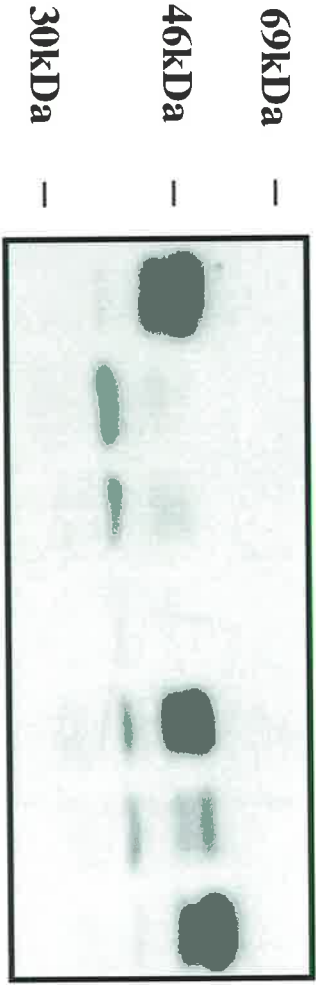
FIGURE 2.10. WLB of raw wound fluid and plasma.

Wound fluid and plasma were collected as described in Methods. One microlitre of plasma or 4 µl of wound fluid were mixed with non-reducing sample buffer and electrophoresed on 12.5% SDS-PAGE gels. Proteins were transferred to nitrocellulose filters which were then probed with [^{125}I]-IGF-II. The bands were visualised autoradiographically after exposure at -70°C for 7 days.

FIGURES 2.11. Characterisation of IGFBPs in human wound fluid and plasma.
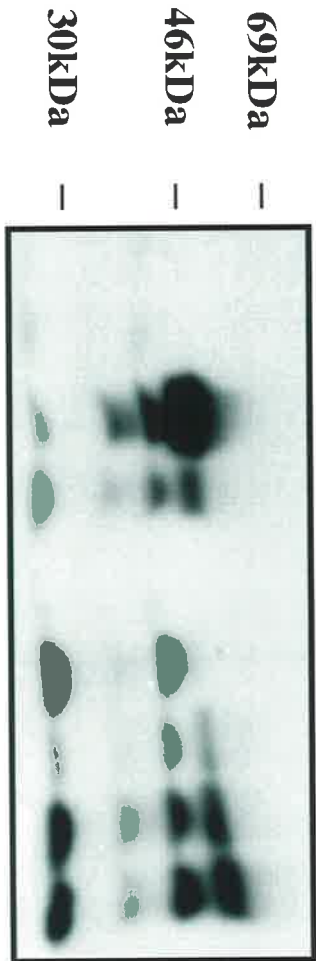
Wound fluid and plasma were collected as described in Methods. One microlitre of plasma or 4 µl of wound fluid was mixed with non-reducing sample buffer and electrophoresed on 12.5% SDS-PAGE gels before transfer of protein to nitrocellulose filters. After blocking overnight with 3% BSA/western saline, the filters were washed and incubated with the anti-IGFBP-3 antibody (1:5000) for 60 min at room temperature. The positions of SDS-PAGE molecular weight markers are shown.

IGFBP3

FIGURE 2.12. Characterisation of IGFBPs in human wound fluid and plasma.

Wound fluid and plasma were collected as described in Methods. One microlitre of plasma or 4 μl of wound fluid was mixed with non-reducing sample buffer and electrophoresed on 12.5% SDS-PAGE gels before transfer of protein to nitrocellulose filters. After blocking overnight with 3% BSA/western saline, the filters were washed and incubated with the anti-IGFBP-1 or anti-IGFBP-2 antibody (1:5000) for 60 min at room temperature. The positions of SDS-PAGE molecular weight markers are shown. Detection of IGFBP-4 and IGFBP-5 were also attempted (results not shown).
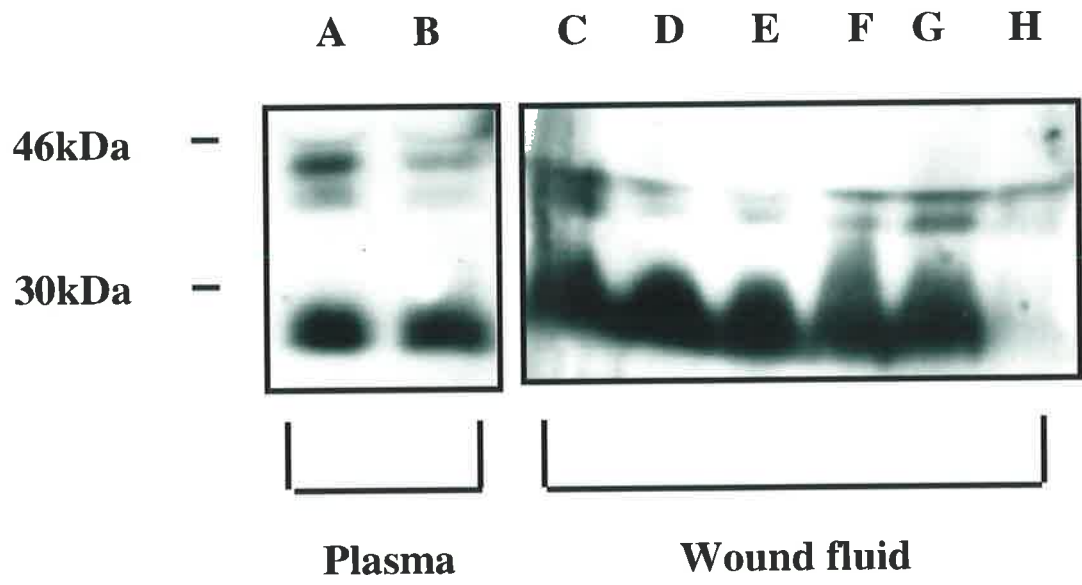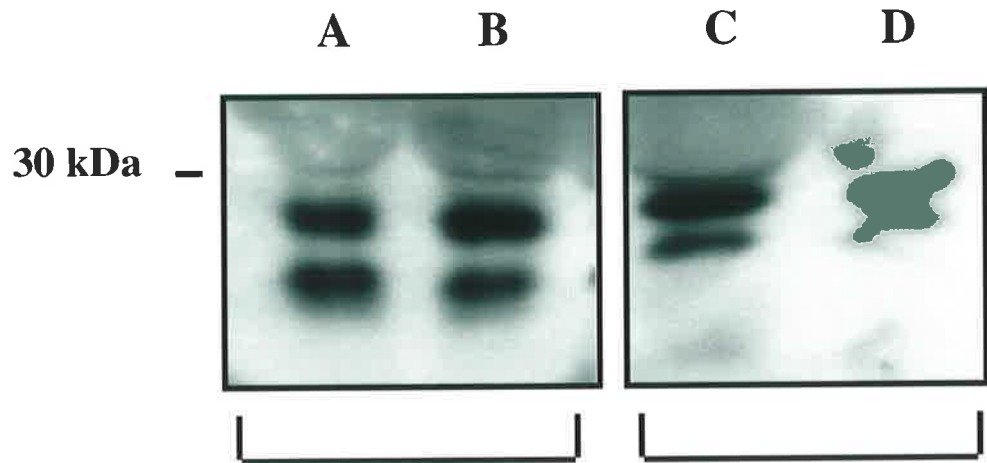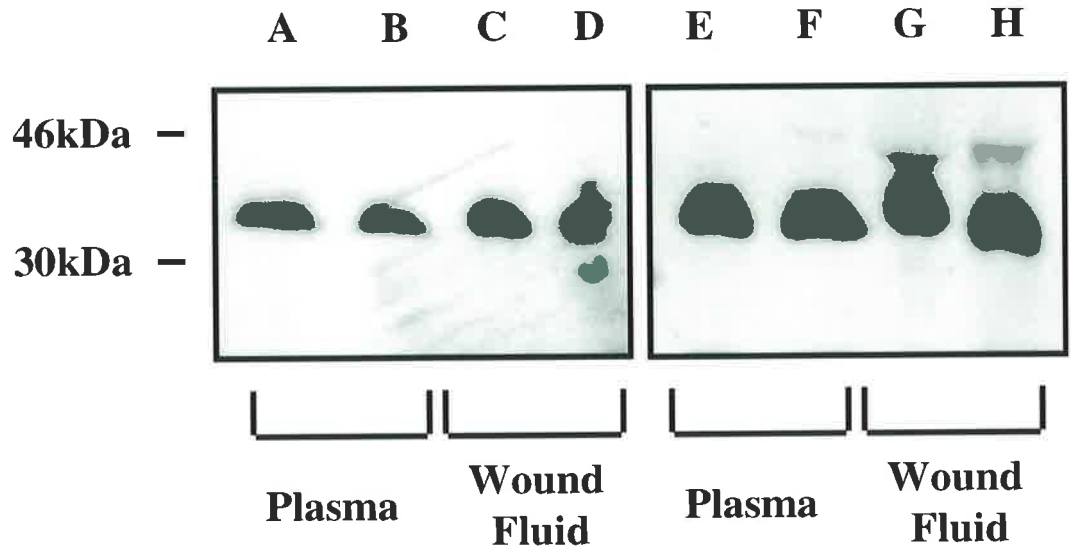
IGFBP-1

A   B   C   D

30 kDa —

Plasma   Wound Fluid

IGFBP-2

A   B   C   D   E   F   G   H

46kDa —

30kDa —

Plasma   Wound Fluid   Plasma   Wound Fluid

2.5. DISCUSSION

This series of experiments has sought to quantify changes in IGF-I and II levels in human wound fluid harvested from partial thickness dermal wounds and to compare these results with temporally matched peripheral blood samples. IGFBPs within wound fluid were identified along with changes in their profiles during the time course of sampling. These results demonstrated that IGFs are found in measurable concentrations in human wound fluid.

Minimising the impact of systemic factors was important in choosing our model to measure the concentrations of IGFs in wound fluid so that realistic conclusions could be drawn about the changes in homeostasis of the IGF system relating to the healing of a partial thickness wound. However, IGF-I reaches the wound from a variety of sources including platelet degranulation (15) and leakage through damaged capillaries. Fibroblasts and other cell lines also synthesise IGFs and their binding proteins. Exudates from mastectomy drains (112) and wound cylinders (96,126,191) have previously been analysed for IGF-I content. In those studies IGF-I levels increased transiently. Alternatively, as described by Xu et al (187), 'suction' blisters allow collection of interstitial fluid that can then be analysed for IGFs and their binding proteins. However this method tends to separate epidermis from basement membrane and the dermis may not be exposed. Therefore any aspirate may not accurately reflect dermal growth factor production.

The extraction method employed to separate IGFs from their binding proteins may influence significantly the final values obtained for IGFs measured by RIA. Many techniques have been devised to measure IGF concentrations. Bioassays were initially used,

however because of the complexity of IGF physiology, the results obtained with these techniques were unreliable and difficult to reproduce (196). Several studies and reviews have been published in recent years emphasising the short comings of the various techniques and provide answers to the problem of IGFBP interference in measuring IGF-I and II concentrations in various biological fluids (173,188,197-200). Despite a consensus that acid gel permeation chromatography, originally described by Horner et al in 1978, is the most effective method of IGFBP extraction, other methods continue to be used in the endocrine and surgical literature. Using our model, we concluded that IGF-I and IGF-II levels measured by RIA after either AE or AGPC extraction procedures generally did not alter dramatically during the healing of relatively minor wounds such as a partial thickness skin graft donor site wound. These data were consistent with Antoniades et al (189) who demonstrated little change in IGF-I receptor and IGF-I mRNA expression in acute wounds that heal primarily by re-epithelialisation.

Western ligand blots and immunoblots identified IGFBP-1, -2, -3 and -4 in raw wound fluid and plasma samples. In addition, low molecular weight fragments of IGFBP-3 and IGFBP-3 were found in wound fluid samples. Various proteases such as collagenase, gelatinase and other metalloproteinases are expressed in cutaneous wounds (201) and excreted into wound fluid (202,203). IGFBP-3 proteolytic activity has also been described (187,191). These proteases while not specifically assayed, may be responsible for the lower molecular weight immunoactivity that was observed.

Western ligand blots were also performed on acid-ethanol extracts examining for residual IGFBP. Residual IGFBPs were detected in the extracts thus invalidating this method as a technique for dissociating IGFs completely from IGFBPs in *human* wound fluid and plasma.

Linear regression analysis was used to compare these results with those values obtained using acid gel permeation HPLC. Correlation of AE extract levels of IGFs with AGPC extracted samples demonstrated the problems of different separating techniques and the influence upon IGF levels as measured by RIA.

Acidification of samples in a solution pH 2.5 is critical to adequate separation of the IGFs from IGFBPs as found by Owens et al (192). That particular paper detailed clearly the protocol for acid gel permeation HPLC and compared porcine IGF-I levels obtained with this technique and those following AE extraction. Interestingly a similar trend was found with AE extracted IGF-I concentrations by Owens et al and this study. This method of extraction produced IGF-I values lower than those following the HPLC technique. Adequate acidification was especially applicable to IGF-II as this will totally dissociate from the IGFBPs at a pH of less than 2.8. Consequently our laboratory conducts HPLC assays at a pH of 2.5 to maximise dissociation. Owens et al (192) and Mesiano et al (204) suggest that failure to maintain the samples at this pH may precipitate re-association of residual IGFBPs with IGFs when the solution was neutralised.

Neutralisation of the solution occurred with the addition of 0.855M Tris base before RIA when AE extraction was used. The HPLC method avoided this problem by fractionating the samples into pools that contain IGFBPs (pool 1) or IGFs (pool 3) so that the elutates were physically separated into pools containing IGFBPs or IGFs. Low molecular weight binding proteins or their degraded fragments may still fractionate into pool 3 and subsequently lead to inaccuracies. However most IGFBPs are detectable above 24kDa and would be excluded in this way from the IGF pool. The WLB of whole wound fluid and plasma suggested that BPs greater than 30kDa comprised the majority of IGFBPs in the samples tested and

therefore supported the above argument for the use of AGPC in preference to AE. Furthermore, results in this study showed that despite acid conditions and the precipitation of the pellet the resultant supernatant can still contain IGFBPs. This clearly demonstrates the inadequacy of the unmodified AE extraction technique. Therefore its use can lead to inaccuracies in the measurement of IGF levels, particularly IGF-II, in human biological fluids that contain low molecular weight binding proteins.

While correlation of AGPC derived IGF-I levels with those obtained following AE extraction suggested that these differences may not be marked, this is not true for IGF-II. This was most evident when measuring IGF-II in wound fluid. This disparity along with the reasonable correlation in IGF-I values could be related to the greater affinity IGF-II has for the IGFBPs overall and IGFBP-2 specifically. Clearly the separation profiles for IGF-I and IGF-II in wound fluid imply larger quantities of binding protein in the IGF-II assays than the immunoreactivity of IGF-I would suggest (figures 2.1 and 2.2).

Consideration of the complex set of equilibria that are established in performing an RIA may provide a clearer understanding of the lower values generally seen for both IGFs but particularly IGF-II. If a method is used that dissociates IGFBPs from IGFs less efficiently than another it will produce less free IGF to compete against $[^{125}I]$-IGF for binding sites on the primary antibody. Bound radioactivity is quantified and used to calculate the amount of IGF present in the pellet. Increasing radioactivity reflects lower levels of free unlabelled IGF in the extract. Three other factors can influence the amount of free unlabelled IGF:

1) the pH of the solution;

2) the overall concentration of the IGFBPs; and

3) whether they bind to free IGF-I when the solution is neutralised.

As IGF-II has a higher affinity for IGFBPs, IGF-II assays are likely to be more significantly affected. Two methods have been proposed to circumvent this effect. One method requires saturation of the extracted samples with IGF-I if IGF-II is being assayed and vice versa if IGF-I is being measured (204). The other method uses an IGF analogue with minimal binding affinity for IGFBPs, as the tracer (199).

In conclusion, this study has demonstrated the presence of levels of IGF-I and IGF-II in wound fluid taken from a partial thickness wound that were generally similar for time matched plasma samples. The measurement of IGFs in biological fluids may be confounded by incomplete separation of these peptides from their binding proteins as demonstrated by the comparison of IGF values obtained for the two different extraction techniques described in Materials and Methods. The presence of these IGFBPs has been confirmed using WLBs and they have been identified using immunoblots. WLBs of AE extracted samples confirmed the presence of residual low molecular weight binding proteins. Correlation of values obtained following AGPC or AE extraction supported the conclusion that IGF-II levels determined following AE extraction will prove to be inaccurate compared to results obtained following extraction using AGPC.

The presence of IGF-I in physiological concentrations prompted a series of studies aimed at investigating the role of IGFs in wound healing and to examine the influence of altering the affinity of IGF-I for its binding proteins.

CHAPTER 3

IGF-I AND ITS ANALOGUES:

IN VITRO RESPONSES OF FIBROBLASTS

3.1. INTRODUCTION

3.1.1. Wound Contraction

3.1.2. Growth Factors and Fibroblast Activity

3.1.3. Objectives

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3.3.2. Fibroblast Growth Assay

3.3.3. Protein Synthesis in Response to IGFs

3.3.4. The Fibroblast Populated Collagen Lattice

3.3.5. Incorporation of [^{3}H]-thymidine: A Model of DNA Synthesis

3.3.6. Statistics

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3.4.1. The Influence of GH and IGF-I upon Fibroblast Activity

3.4.2. Skin Fibroblast Responses to IGF-I Analogues

3.4.3. DNA Synthesis: Comparison of Growth factors

3.5. DISCUSSION

3.1. INTRODUCTION

The final quality of a healed wound is determined by the scar produced. Fibroblast activity within the wound is responsible for the deposition and maturation of the collagen matrix that ultimately forms this scar (205). Early in healing, fibroblasts also aid in the production of granulation tissue and wound edge contraction. Granulation tissue is composed of proliferating fibroblasts, ground substance proteins and proliferating endothelium and provides the foundation upon which re-epithelialisation will occur (18).

Migration of fibroblasts into a newly formed wound commences at about day three post-wounding. Chemoattractants that promote fibroblast migration include thrombin (206), tissue plasminogen activator (t-PA), ECM fragments (20), fibronectin (207), cytokines such as PDGF (208) and TGFβ (209), C5a from the complement cascade (210), histamine released from mast cells (211), interleukins as well as components of fibroblast and epithelial conditioned media (212). Proliferation of fibroblasts within the wound is also stimulated by a variety of influences including the relative hypoxia found in the centre of wounds (9) and growth factors such as IGF-I, PDGF (213-217) and TGFβ (65).

The proliferative phase features phenotypic changes to the fibroblast population (218) as well as deposition of a fibronectin and collagen matrix. The appearance and regression of myofibroblasts within wounded tissue are associated with the commencement and completion of wound contraction (219). As a presumed fibroblast derivative was first characterised by Gabbiani (50,51) in 1971, it is the cell believed responsible for wound

contraction. Myofibroblast activities are regulated by various factors including ECM components (56,220) and growth factors, for example TGFβ (221).

Bell demonstrated with an *in vitro* model of dermal contraction known as the fibroblast populated collagen lattice, that the contractile response of the fibroblast may be influenced by its proliferative potential as well as its phenotype (57). Normal dermal fibroblasts, being quiescent cells, do not appear to exhibit the same contractile activity as fibroblasts derived from a wound (63). This may also be related to collagen maturity as this too has been shown to modulate fibroblast mediated contraction as shown by Ehrlich's studies of gel contraction which utilised various forms of collagen (89,220). Ehrlich's results indicate that matrix composed of the more immature type III collagen will contract faster than one rich in type I collagen.

Finesmith et al (66) have provided further evidence that the age of wounds influences cell-mediated wound contraction. These authors examined the contraction of collagen gels seeded with fibroblasts derived from granulation tissue that had been sampled at various time points in a chronic healing wound. Their findings indicated that rates of gel contraction responses to TGF-β increased in a dose dependent fashion and these responses were even more pronounced with cells extracted from older granulation tissue. Growth factor responses in these studies were also influenced by the timing of peptide application as demonstrated by pretreatment of the gels with bFGF. Furthermore these authors showed that this tended to retard TGF-β mediated contraction in those gels seeded with cells derived from younger granulation tissue. A phenotypic change is therefore required for these cells to induce wound contraction. Such a change increases the complexity of this dynamic process

which requires the interaction of cells, ECM and growth factors to generate forces of contraction that can be attributed to fibroblast locomotion (52,56).

3.1.3. Growth Factors and Fibroblast Activity

Many growth factors have been linked with the activity of fibroblasts generally but also specifically within the wound (222). They may be chemotactic, mitogenic or induce synthesis of components of the extracellular matrix. Their influences may be effected by paracrine or autocrine mechanisms (11,223,224) and those growth factors produced by the fibroblasts themselves may act similarly upon other cell types, for example keratinocytes (225).

GH has been shown to promote growth in children with growth retardation (226) and it can reverse catabolic states (227) and it has mitogenic effects upon mesenchymal tissues. Well recognised for its ability to promote growth of cartilage and cell proliferation in osteoblast and chondrocyte cultures, it also has anabolic effects upon soft tissue in general (101,228-233). Until recently GH was the only growth factor to have an important clinical application in wound healing (98,104). As with most of its anabolic activities, GH probably acts indirectly in wound repair to influence healing as target tissues exhibit upregulation of IGF-I protein and mRNA production in response to GH (96).

The mitogenic activity of IGF-I (234), its role as the second messenger for GH in fibroblast cultures (124) and its presence in wound fluid (112) provide evidence that this growth factor has a significant role in wound repair. *Iin vitro* it has the capacity to regulate endothelial proliferation (11), collagen synthesis by fibroblasts (123) and to promote keratinocyte

migration and growth (146,223) in a paracrine fashion. IGF-I affects cellular activity in a paracrine manner independently of GH in other systems such as chondrocyte cultures (235).

Much of the literature surrounding IGF-I induced fibroblast activity *in vitro* indicates that some other factor is also required to maximise the response to IGF-I. The binding proteins, IGFBP-1 (236) and IGFBP-3 (237,238), PDGF (214), TNF-α and TGF-β1 (238) and even sodium chloride (239) influence the growth and contractile responses of fibroblasts to IGF-I. TGF-β1 is renowned for its capacity to promote collagen synthesis and to influence matrix reorganisation. It is associated with keloid scars (65,88,240,241), adult-type scar formation (242,243) and has been implicated as a modulator of IGF-I bioactivity by upregulating IGFBP mRNA expression (238). Like IGF-I, TGF-β may also play a key role in wound contraction either through its influence upon IGFBP physiology or by direct action on fibroblasts as suggested by Pena et al and Montesano and Orci (244,245). In animal studies PDGF has been shown to be important in activating an IGF-I related response and generally this is greater than that achieved with either IGF-I or PDGF alone (46,128,129,134,246). Consequently, IGF-I has earned the reputation as a "progression factor" rather than a "competence factor", a concept initially proffered by Van Wyk (214). As a progression factor it promotes cell growth and activity rather than simply rendering cells competent to enter the cell growth cycle.

The aspect of IGF physiology that presents an obstacle to assessing IGF-I's potency as a mitogen has been its family of carrier proteins, the IGFBPs. These peptides are produced by cultured cells as well as existing *in vivo*. Their influence upon *in vitro* models is unpredictable as they may either potentiate or inhibit IGF activity (247). The development of recombinant techniques for the production of peptides such as IGF-I has resulted in the

design of new growth factors or analogues of IGF-I and -II (141,248). The potencies of the analogues, long Arg^3 IGF-I (LR^3 IGF-I) and des(1-3)IGF-I have been assessed in cell cultures by examining [^{3}H]-leucine incorporation by L6 myoblasts, hepatoma cells and growth of murine fibroblasts (BALB c/3T3 fibroblasts) (140,249). These peptides exhibit greater activity *in vitro* than their parent peptides due largely to the reduced affinity they demonstrate for the IGFBPs. The effect of LR^3 IGF-I in cultures of human fibroblasts is not affected by the addition of IGFBP-3 nor is its effect influenced by other growth factors that increase IGFBP concentrations in tissue fluid as demonstrated by Yateman et al (238). This increased activity is not necessarily associated with greater affinity for the type 1 IGF receptor as LR^3 IGF-I actually has less affinity for this receptor than IGF-I (140). However, little information is available about their comparative efficacy in models incorporating human skin fibroblasts.

3.1.4. Objectives

As the second messenger for GH, it is reasonable to assume that IGF-I's activity *in vitro* would be equal or better than GH and consequently IGF analogues known to have reduced affinity for IGFBPs should exhibit greater potency than IGF-I. Therefore tissue culture assays were performed to examine:

- The activity of GH in models of fibroblast growth and fibroblast related gel contraction;

- The relative response of IGF-I compared to GH;

- The relative potency of IGF-I, IGF-II, GH and two IGF analogues, namely des(1-3) IGF-I, LR^3IGF-I.

3.2. MATERIALS

All IGF-I and -II peptides (recombinant human proteins and analogues) were obtained from GroPep Pty Ltd (Adelaide, SA, Australia) and were reconstituted in 0.01M HCl from lyophilised powders. Growth hormone was purchased from Bresatec (Adelaide, SA, Australia). All peptides were of receptor grade purity (95% by HPLC) for all *in vitro* studies. TGF-β1 and bFGF were purchased from Austral Biologicals (San Ramon, CA, USA), PDGF-AB from UBI (Upstate Biotechnology Incorporated, Lake Placid, NY, USA) and EGF was supplied by Chiron Corporation, (Emeryville, CA, USA). Bovine serum albumin (BSA; Sigma, Fraction V, RIA grade) was purchased from Sigma Chemicals (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) and foetal bovine serum (FBS; Cytosystems, Melbourne, VIC, Australia) were supplemented with benzylpenicillin (60mg/l, CSL, Australia.), streptomycin (100μg/ml; Jurox, Silverwater, NSW, Australia) and amphotericin-B (1mg/l; Bristol-Meyers-Squibb Pharmaceuticals, Noble Pk, VIC, Australia.). Radiolabelled isotopes (L-[2,3-^{3}H]-proline, [^{3}H]-inulin and methyl [^{3}H]-thymidine) were obtained from Amersham (Little Chalfort, Buckinghamshire, U.K.). Serum free medium (SFM) was prepared using DMEM and 0.1% wt/vol BSA. All reagents for phosphate buffered saline (PBS) were obtained from AnalaR BDH Chemicals.

Two human diploid dermal fibroblast cell lines were obtained from the Women's and Children's Hospital, North Adelaide, South Australia. These were derived from a one day old neonate who had died with congenital abnormalities (SF3169) and from a 7 month old

infant who had died from a respiratory illness (SF1967). All stocks were maintained in 75cm^2 flasks (Corning Inc, Corning, NY, USA).

3.3. METHODS

3.3.1. Harvesting Protocol for Fibroblasts

Fibroblast cultures were used at passages 11 to 14 and grown to confluence in 10% FBS/DMEM in Costar T_{75} tissue culture flasks in a humidified atmosphere of 5% CO_2, at 37°C.

The cells were harvested when confluent using 0.025% trypsin/0.01M EDTA. Ten per cent FBS/DMEM was added after two minutes to neutralise the trypsin. The suspension of cells was next transferred to 10ml eppendorf tubes and centrifuged at 4°C, 1500 rpm for five minutes. The pellet was washed twice more using SFM before determining cell numbers and viability using a haemocytometer with trypan blue exclusion. The suspension was again centrifuged and the pellet mixed with 1ml of SFM. An appropriate fraction was removed for use in the tissue culture experiments described.

All steps involving cell harvesting, preparation of solutions, plating of cells and formation of FPCLs, were performed under aseptic conditions in a laminar flow hood. All solutions apart from collagen were passed through a 22μm x 47mm filter (Millipore) prior to use in tissue culture experiments.

3.3.2. Fibroblast Growth Assay

Passage 12 SF 3169 human fibroblasts were subcultured at a density of 4 $\times 10^4$ cells in 100μl of 10% FBS/DMEM per well of 96-well tissue culture plates (Nunclon, Denmark). The

plates were incubated overnight to encourage cell attachment. On the second day the plates were washed twice with SFM and incubated for a further two hours in SFM. Treatment solutions were added to the fibroblast monolayer after aspiration of the wash medium.

Following a further 48 hour incubation, cell growth was assessed using the methylene blue dye binding assay described by Oliver et al (250). Briefly, methanol (100%) was used to fix the cells prior to staining with methylene blue in 0.01M borate buffer, pH 8.5. Following five washes with borate buffer, the methylene blue bound to the cell monolayers was eluted with acidified ethanol (1:1 v/v 0.1N HCl/ethanol). Photometric analysis was performed using an automated vertical light path photometer (Dynatec). These results provided an index of optical density at the filtered wavelength of 630nm (A_{630}) that reliably reflected changes in the mass of cells in the monolayers.

3.3.3. Protein Synthesis in Response to IGFs.

Confluent monolayers of passage 12 dermal fibroblasts (SF3169), cultured in 24-well tissue culture plates (Nunclon, Denmark), were washed with SFM and the cells incubated overnight in SFM prior to addition of treatments. L-[2,3-^{3}H]-proline was diluted in SFM and the equivalent of 0.5 μCi/ml of treatment was added with each solution to individual wells. The plates were incubated for 24 hours before harvesting the newly synthesised protein.

The cell monolayers were washed twice with Hanks' balanced salt solution (HBSS), once with 5% solution of trichloroacetic acid (TCA) to precipitate the cellular protein and finally with deionised water. Synthesised protein was solubilised using 0.1% Triton X-100/0.5 M

NaOH. Duplicate 100µl subsamples from each well were each diluted in 2ml of scintillation fluid (Ultima Gold™ LSC, Packard), before measuring the radioactivity on a β-counter.

3.3.4. The Fibroblast Populated Collagen Lattice

Type 1 collagen was acid-extracted from rat tail tendons using the technique described by Bell et al (57). This model has also been applied to other combinations of cell types such as endothelial cells and keratocytes (61,62) or other ECM proteins such as fibronectin (251). A modification of this model has been devised in our laboratory that incorporates [^{3}H]-inulin into the gel as a marker of the contractile response. It remains inert and it is not incorporated by the cells. It therefore tends to be expelled from the gel as it contracts. Residual radioactivity compared to that of control treated gels provides an index of the potency of a reagent to promote fibroblast-mediated contraction. This modification to the FPCL model measures residual radioactivity after a 48 hour incubation. This time point was chosen as the rate of gel contraction is maximal in this period and generally declines thereafter (57,64,220).

A standard gel mixture was prepared as follows:

The rat tail derived collagen was added to deionised (Milli-Q) water and 2 x DMEM in a volumetric ratio of 2:3:5 at 4°C. [^{3}H]-inulin (1 µCi/ml) was added according to the formula:

$$\text{Volume [}^3\text{H]-inulin (µl)} = \frac{6000 \times \text{number of gels required}}{\text{counts/µl inulin stock}} \times 20$$

1M NaOH ($1.25\mu l/ml$ gel mixture) was used to promote polymerisation of the collagen. The gel mixture ($500\mu l/well$) was seeded with $16\text{-}20 \times 10^{4}$ human dermal fibroblasts (SF3169) per ml of gel and poured into 48-well tissue culture plates (Costar, Cambridge, MA, USA).

The plates were incubated at $37°C$ in a humidified atmosphere of 5% CO_2 until the gels had polymerised. 'Rimming' of the gels with a 25FG hypodermic syringe needle released them from the well surface and allowed them to float freely in the treatment media. Doubling dilutions of the peptides were added in final volumes of 500 μl SFM to each well.

After incubating for another 48 hours the FPCLs were transferred to 2 ml of scintillant (Ultima Gold™ LSC, Packard), shaken and allowed to dissolve for another 48 hours to release the residual radioactivity. Each concentration of peptide was tested in triplicate and each experiment repeated to confirm initial results. Gel size was consistent in all experiments and their preparation was carried out under the same conditions.

3.3.5. Incorporation of [^{3}H]-Thymidine: A Model of DNA Synthesis

Fibroblasts were subcultured at a density of 2×10^4 cells/ well (16mm diameter) in serum supplemented medium and cultured for 72 hours at 37°C in an atmosphere of 5% CO_2. Once the monolayers were almost confluent they were washed with SFM and incubated for 24 hours in SFM. The growth factor dilutions were added at a final volume of 100 μl/well and the cultures pulsed 6 hours later with the [^{3}H]-thymidine (0.5 μCi/ml). After 18 hours the plates were washed twice with PBS, once with 10% TCA at 4°C and the cells lysed using 100 μl 0.5M NaOH. Samples of 85μl were taken from each well and placed in scintillant (2ml) prior to reading of the radioactivity on the β-counter.

3.3.6. Statistics

All results represented pooled data from duplicate experiments and are expressed as mean ± SEM. All analyses were conducted using Sigmastat v.2, (Jandel Scientific, San Ramon, CA, USA). One way analysis of variance was performed where multiple treatment solutions were compared. Levels of significance were $p < 0.05$ compared with serum free medium unless otherwise indicated.

3.4 RESULTS

3.4.1. Influence of IGF-I and GH upon fibroblast activity

<u>Cell growth</u>

Preliminary experiments were conducted comparing the potency of IGF-I as a fibroblast mitogen to that of GH using an automated dye binding assay (250). IGF-I and GH were also combined in equal concentrations to determine whether their effects were additive or even synergistic. Cell growth following these treatments is shown in figure 3.1. The most important observation in these experiments was that IGF-I was a significantly better mitogen than GH alone ($p < 0.001$). When treated with GH, only at the highest concentration tested (1000ng/ml) was fibroblast growth significantly increased ($121 \pm 7\%$ of SFM response, $p < 0.01$). This compares with the greater potency of IGF-I which produced a maximum growth response of $170 \pm 7.0\%$ of serum free controls at a concentration of 500ng/ml. It can be seen from figure 3.1 that the combination of IGF-I with GH proved no more effective than IGF-I alone at any tested concentration. It can be concluded that over the indicated dose range equal concentrations of IGF-I and GH were not synergistic in their effect upon cell growth.

<u>FPCL contraction</u>

Having identified that GH was relatively ineffective as a fibroblast mitogen but that IGF-I promoted growth, these same peptides were tested alone and in combination at equal concentrations using our model of fibroblast contraction. The results are summarised in

figure 3.3. When applied at increasing concentrations, the residual radioactivity in the gel after 48 hours decreased across all IGF-I concentrations indicating significant contraction of the gel in response to IGF-I. GH failed to significantly influence the contractile response when used alone. Its maximal effect was noted at a concentration of 250 ng/ml (contraction to 90% of residual radioactivity of SFM treated gels). In comparison, IGF-I produced signicant gel contraction with treated gels having a minimum residual radioactivity that was 72% of control gels after 48 hours. Furthermore, GH and IGF-I mixed in equal concentrations were no more effective than IGF-I alone indicating no synergistic relationship between the two peptides in this model.

Similar experiments testing the concentration range of 7.8 ng/ml to 250 ng/ml once again demonstrated the weak activity of GH in the FPCL model with maximal gel contraction to 88%± 2% at 125 ng/ml. In contrast IGF-I was able to induce contraction of the gels to 60% of the size of time matched controls within 48 hours. The relative inactivity of GH in this range confirmed GH to be a weak fibroblast mitogen at physiological concentrations.
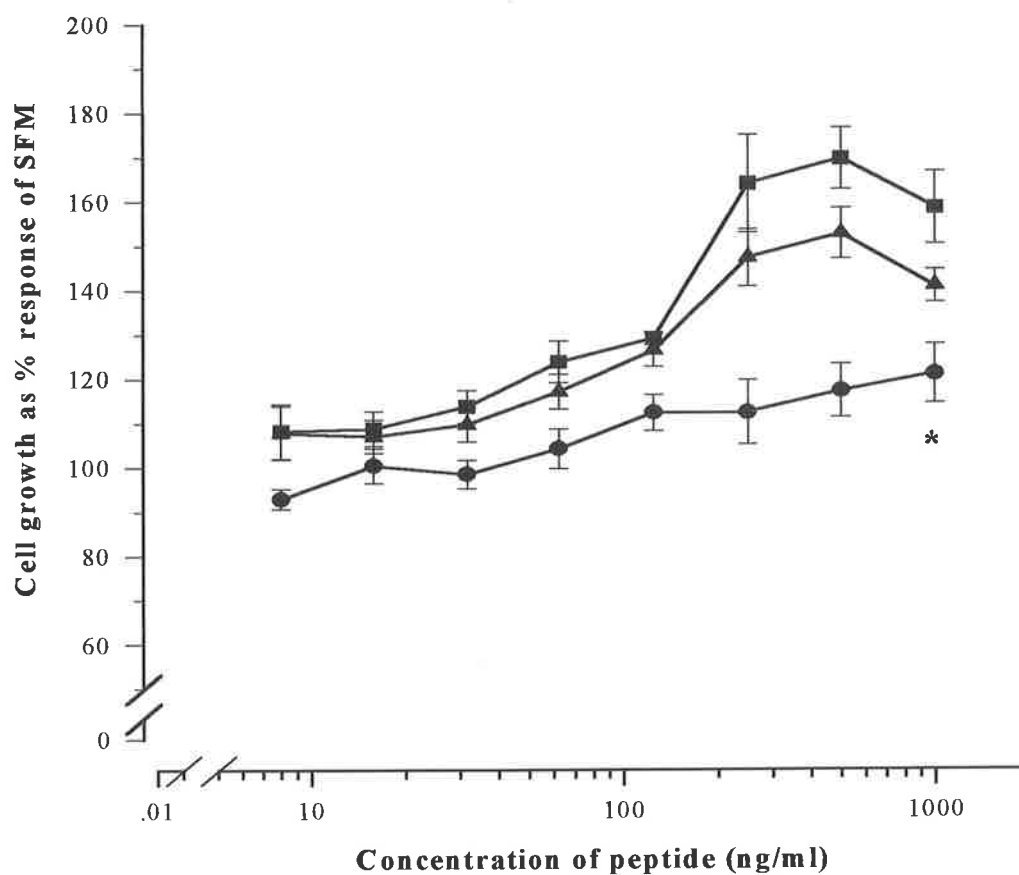
FIGURE 3.1. Effect of IGF-I, GH, IGF-I combined with GH on fibroblast growth.

Monolayer cultures of human skin fibroblasts were treated with increasing concentrations of IGF-I (■) met hGH (●) or met hGH combined with IGF-I (▲) at equivalent concentrations for 48 hours. Cell growth is expressed as a mean percentage ± SEM (n=6) of that observed under serum-free conditions (* $p < 0.0001$ vs IGF-I and IGF-I plus GH).

FIGURE 3.2: Colour plate of FPCL in wells after incubation for 48 hours in peptide supplemented SFM.
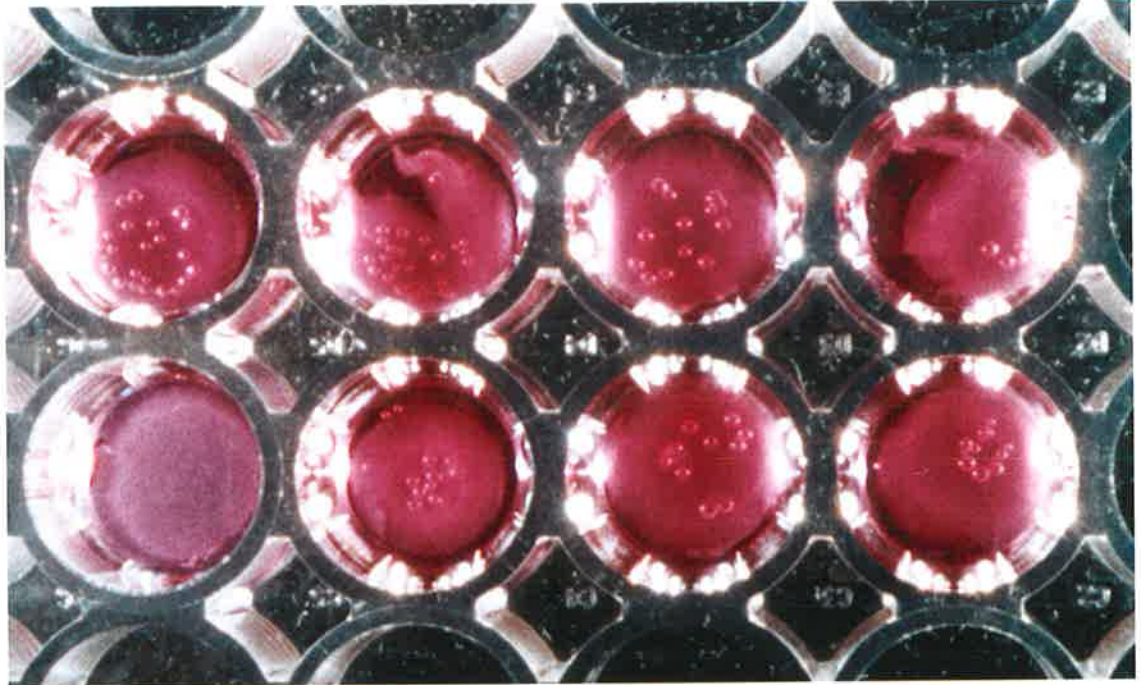
IGF-I
10 μg/ml

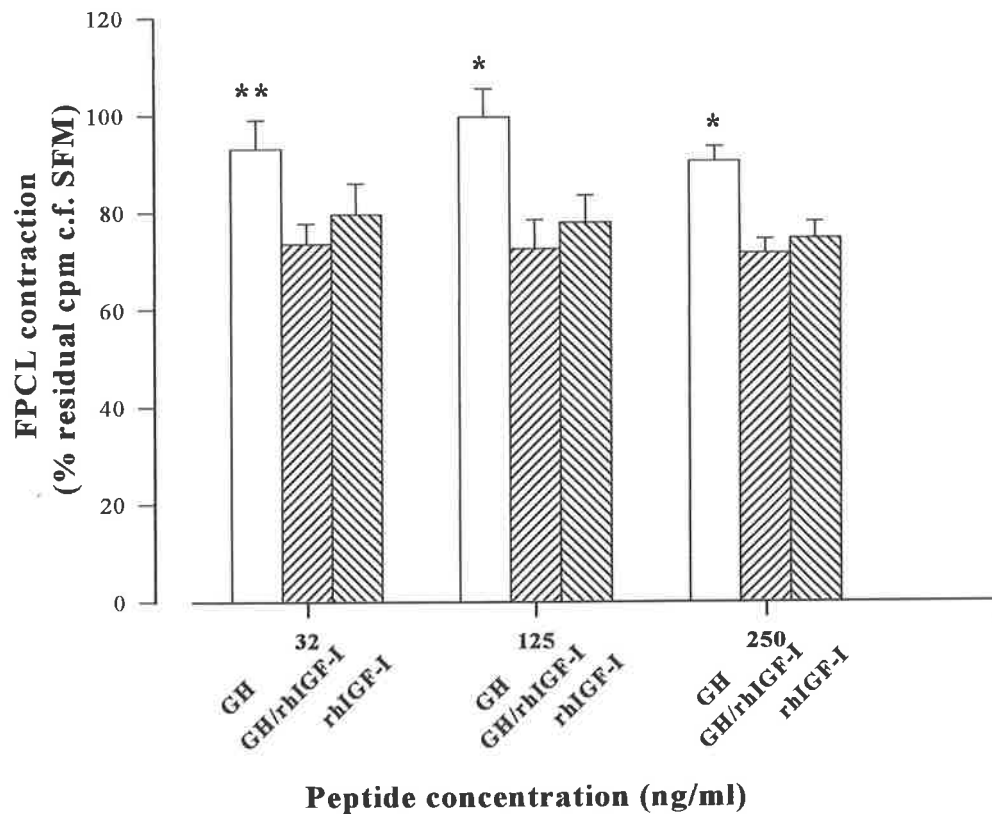**IGF-I**
100 μg/ml

SMF

FCS

FIGURE 3.3. Contraction of collagen gel lattices seeded with fibroblasts (FPCL) determined by the percentage residual radioactivity compared to SFM treated controls.

Gel lattices populated with 10^5 cells per 500 µl gel were incubated for 48 hours in SFM supplemented with IGF-I, met-hGH, or IGF-I and GH mixed in equal concentrations. Residual radioactivity was assessed 48 hours after removal of the gels from their media. The bars represent the mean ± SEM of 6 wells. *$p < 0.001$ vs GH and rhIGF, rhIGF-I; **p < 0.01 vs GH and rhIGF-I, rhIGF-I.

3.4.2. Skin Fibroblast responses to IGF Analogues

Experiments in 3.4.1 clearly demonstrated that fibroblasts will both grow and induce contraction of a collagen matrix in response to IGF-I but the response to GH is weak. As their activity was not synergistic, consideration was given to examining other aspects of IGF-I physiology that could enhance its activity. Experiments were designed to examine the influence of reduced binding protein affinity upon activity of IGF-I in contraction of a collagen gel using the IGF-I analogues, des(1-3)IGF-I and LR^3 IGF-I. Having established that IGF-I does promote cell growth, two other assays that examine specific aspects of growth, namely DNA synthesis ($[^3H]$-thymidine incorporation) and protein production ($[^3H]$-proline incorporation) were used to assess the relative potencies of the IGFs.

Fibroblast contraction

When the gels were suspended in media containing the analogues, a difference in potency of up to a factor of 10 was demonstrated when compared to rhIGF-I (figure 3.4). Half maximal effective concentrations (ED_{50}) were 40 ng/ml, 6.2 ng/ml, 4 ng/ml for native IGF-I, des(1-3)IGF-I and LR^3 IGF-I respectively. Data obtained from contraction studies performed using a range of doses from 0.78 ng/ml to 50 ng/ml revealed that these analogues were active in concentrations as low as 1ng/ml in this model (data not shown).

Fibroblast-populated lattice contraction was also examined comparing des(1-3)IGF-I with PDGF-AB, TGF-β1, bFGF, EGF and 10% FBS. Maximal gel contraction using TGF-β1 occurred with a concentration of 20 ng/ml (88% ± 4.7% of the serum free controls) producing a result which was not statistically significant. In contrast PDGF and des(1-3)IGF-I significantly reduced gel size compared to controls ($p < 0.0001$). This result

demonstrated that the contraction induced by these two growth factors was as great as that exhibited by gels exposed to 10% FBS (figure 3.5).

The poor efficacy demonstrated by TGF-β1 prompted examination of the contraction response of two separate fibroblast lines (SF1967 and SF3169) to treatment with TGFβ1. Similar studies were conducted to provide a comparison with IGF-I. The results obtained revealed that the contraction of gels demonstrated previously by both IGF-I and TGFβ1 were not specific to the fibroblast line (SF3169) that was used for the majority of the experiments (data not shown).
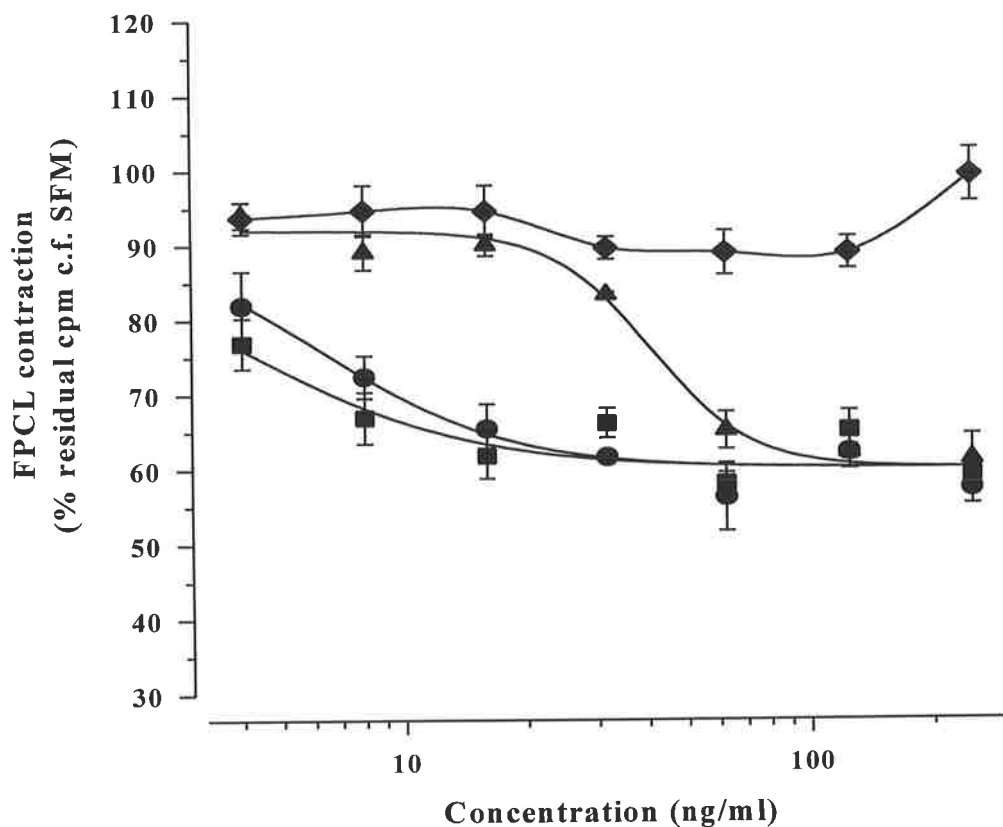
FIGURE 3.4. Collagen lattice contraction stimulated by IGF-I (▲) and its analogues (des(1-3)IGF-I (●), LR^{3}IGF-I (■)) and compared to GH (◆).

Symbols represent the mean ± SEM of six values. The gels were incubated for 48 hours in media containing the indicated concentrations for each growth factor. The results are presented as the percentage of contractile response observed under serum-free conditions.

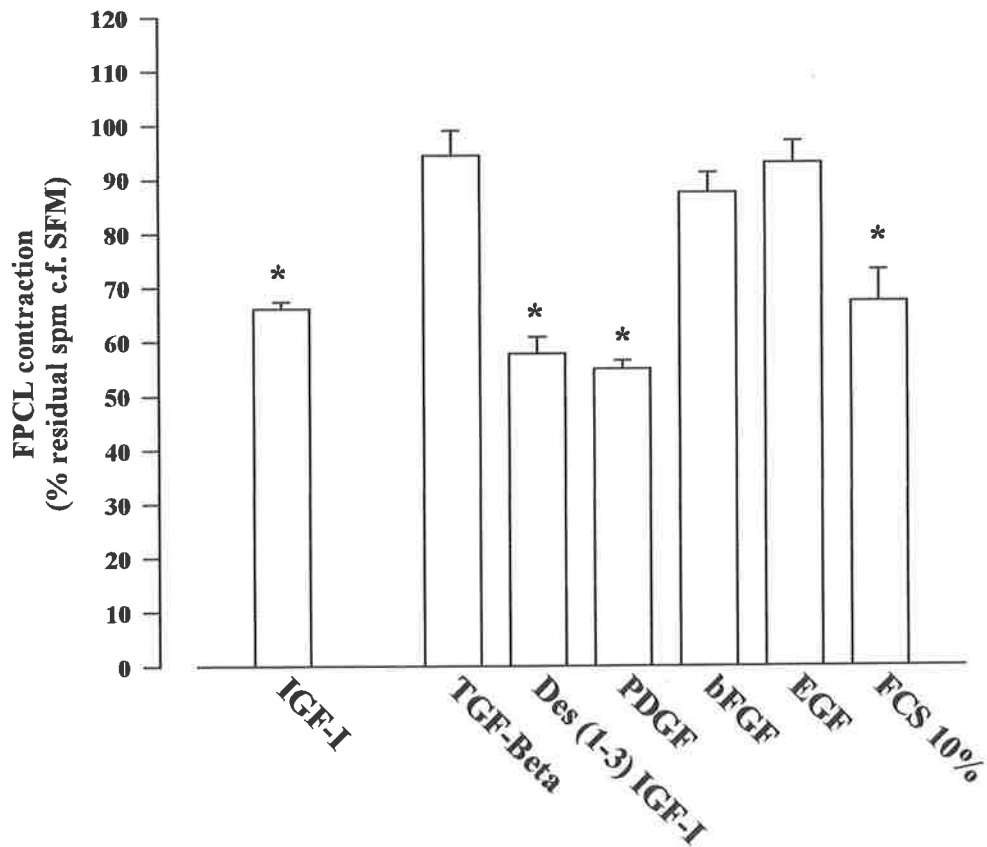**FIGURE 3.5.** Contraction of FPCLs using other growth factors.

FPCL contraction for the indicated peptides was tested over a full dose response range Maximal responses are illustrated and were observed at des(1-3)IGF-I, 62.5 ng/ml; TGFβ1, 40 ng/ml; PDGF-AB, 20 ng/ml; bFGF, 20 ng/ml; EGF, 25 ng/ml and rhIGF-I at 62.5 ng/ml. The error bars represent mean ± SEM, n=3 for each growth factor. *$p < 0.01$.

3.4.3. Cell Growth in response to the IGF Analogues

Having performed experiments comparing the potency of IGF-I and GH as fibroblast mitogens more specific assays were employed to examine fibroblast protein and DNA synthesis in response to IGF-I and its analogues.

Protein Synthesis - Incorporation of Tritiated Proline

Protein synthesis by fibroblast in response to stimulation by IGF-I and its analogues was examined by assessing the incorporation of $[^3H]$-proline into cellular protein. This activity was further compared with that observed for IGF-II (figure 3.6).

IGF-I stimulated protein synthesis as measured by proline incorporation approximately 240% more than the baseline levels observed for controls. The IGF-I analogues, particularly LR^3 IGF-I, proved even more potent as anabolic agents. The flattening of the dose response curve and an associated shift to the left of the dose response curve was an important feature of the observed results. This suggests that there may be a more direct relationship between dose and availability of free peptide for receptor binding of the IGFs to their target cells. The order of potency observed was LR^3 IGF-I > des (1-3) IGF-I > IGF-I > IGF-II.

DNA Synthesis - fibroblast incorporation of tritiated thymidine

Des(1-3) IGF-I, rhIGF-I and rhIGF-II were applied to monolayer cultures of fibroblasts exposed to tritiated thymidine tracer. These results are represented in figure 3.7. The maximum response for all three peptides was approximately 220% of the level observed for controls. The greater potency of the IGF-I analogues was once again demonstrated by a shift

to the left of the dose response curve for des(1-3)IGF-I as well as flattening of the curve when compared with the recombinant peptide.
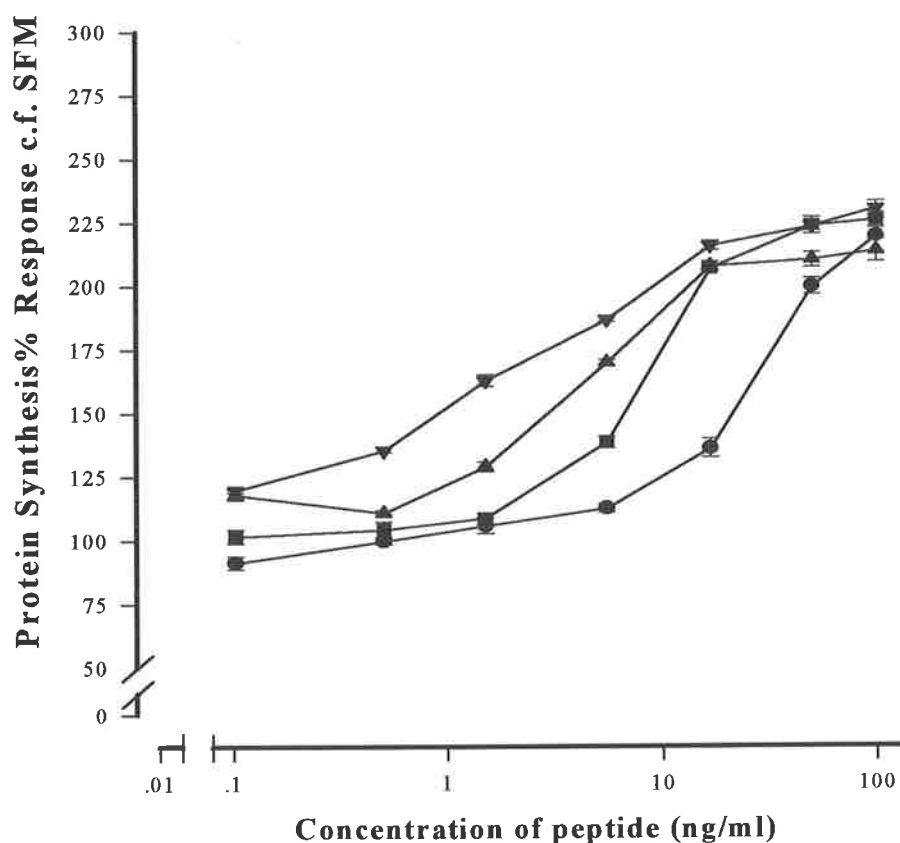
FIGURE 3.6. Stimulation of protein synthesis by human skin fibroblasts in response to IGF-I, its analogues and IGF-II.

Confluent monolayers of human skin fibroblasts were incubated in media containing tritiated proline (0.5 μCi/ml) and increasing concentrations of IGF-I (■), IGF-II (●), des(1-3)IGF-I (▲), LR³IGF-I (▼) for 24 hours. Radioactivity was measured in precipitated protein obtained following cell lysis. The symbols represent the mean ± SEM of 3 determinations.

FIGURE 3.7. DNA synthesis by human dermal fibroblasts following exposure to IGFs.

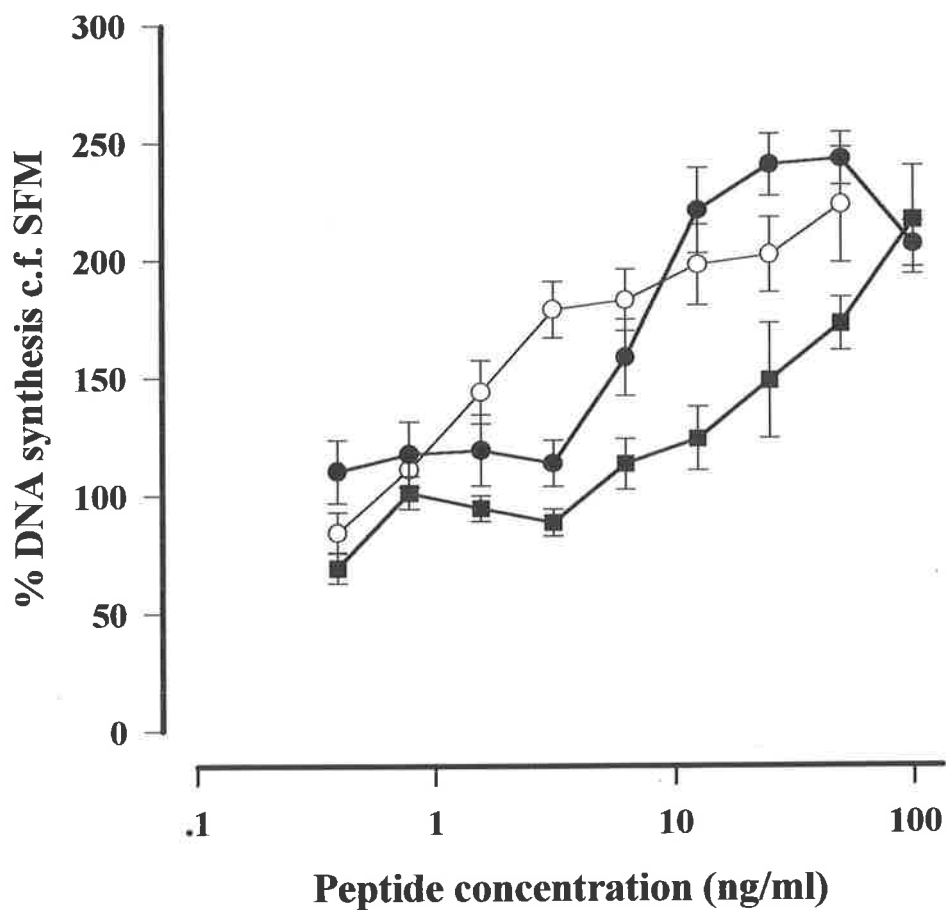Monolayers of fibroblasts were incubated for 6hrs in the growth factor solutions prior to pulsing with [^{3}H]-thymidine for a further 18 hrs before measuring reactivity in percipitated DNA. Dose response curves were obtained for rhIGF-I (●), rhIGF-II (■), and des(1-3)IGF-I (O). The symbols represent the mean ± SEM (n=9).

The anabolic activity of IGF-I is these particular assays can be compared with the less specific assay of cell growth that relies on optical density of monolayer cultures (see 3.4.1). This assay provided a maximal IGF-I response of 170% which contrasts with that of 240% for protein synthesis and about 250% for DNA synthesis. This highlights the methylene blue absorbance assay as a good screening assay for cellular growth but does not allow accurate interpretation as to whether the observed response is related to an increase in cell numbers or if it is also due to increased cell size.

3.4.4. DNA synthesis: Comparison of Growth Factors

The activity of IGF-I was compared with several growth factors in fibroblast monolayer cultures. All of these peptides are recognised mitogens in fibroblast cultures. The group of growth factors tested included PDGF-AB, bFGF, EGF and TGFβ1. They were compared with IGF-I and des(1-3)IGF-I. Maximal responses for each growth factor are illustrated in figure 3.8 and were attained at the following concentrations: PDGF-AB 20 ng/ml; TGFβ_1 1.25 ng/ml and bFGF 0.625 ng/ml; IGF-I 50 ng/ml; des(1-3)IGF-I 50 ng/ml and EGF 12.5 ng/ml. PDGF demonstrated greatest activity at the highest concentration of 20 ng/ml while des(1-3)IGF-I and IGF-I both demonstrated greater potency than the remaining growth factors in this assay. TGFβ_1 elevated DNA synthesis only 50% above that of SFM treated controls while IGF-I was able to produce a 250% increase compared to controls.
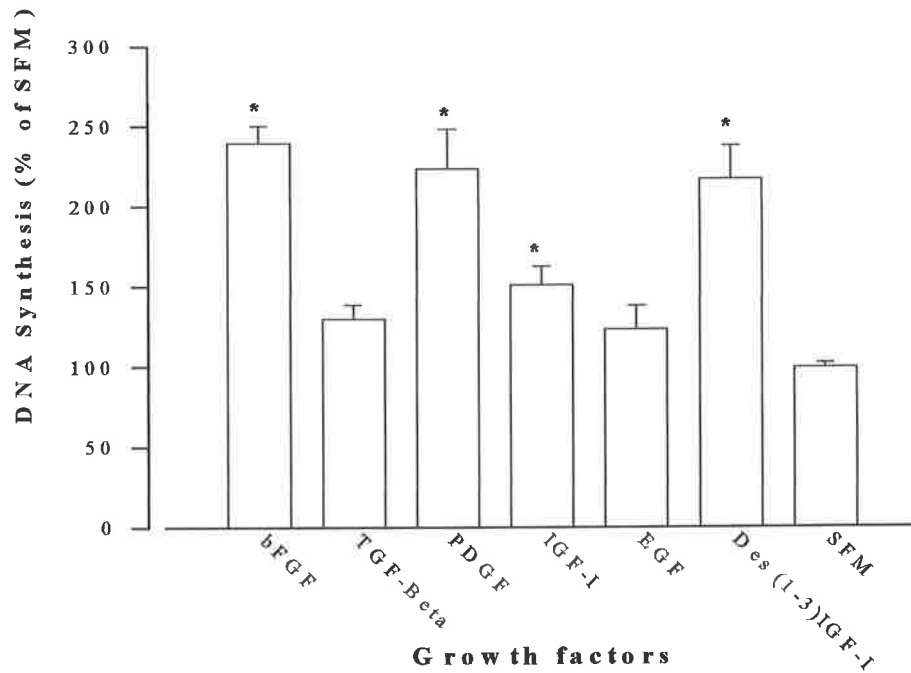
FIGURE 3.8. DNA Synthesis by human dermal fibroblasts.

Monolayers of fibroblasts were incubated for 6 hrs in the growth factor solutions prior to pulsing with [^{3}H]-thymidine for a further 18 hrs. Radioactivity was measured in percipitated DNA. The maximum responses illustrated were attained at the following concentrations: PDGF-AB 20ng/ml, TGF-β1 1.25ng/ml, bFGF 0.625ng/ml, IGF-I 50ng/ml, des(1-3)IGF-I 50ng/ml and EGF 12.5ng/ml. Results are expressed as mean $\pm$ SEM (n=9); *p < 0.0001 vs SFM by ANOVA.

The relative inactivity of TGFβ_1 was confirmed in assays using another fibroblast line (SF1967). These data complemented that obtained for FPCL contraction and suggested that TGFβ_1 may only be a weak promoter of fibroblast activity in these assays (data not shown).

3.5. DISCUSSION

IGF-I can modulate the behaviour of a variety of cells important to wound healing in a paracrine (223,224), endocrine or autocrine (11,225) manner at the wound site. Following injury, platelet and tissue macrophages release IGF-I into the wound (15), supplementing the IGF-I derived from the systemic circulation where it is largely bound to IGFBP-3. Physiologically, IGF-I activity, expression and regulation is closely linked to GH (105).

Uncertainty exists as to whether GH directly regulates mitogenesis or whether its effects are dependent upon the presence and/or local production of IGF-I. Epiphyseal plate growth in particular responds to IGF-I stimulation (230,232,252) and systemic GH regulates local tissue expression of the IGF-I gene (253). This theory is supported by the expression of GH receptors by chondrocytes juxtaposed to chondrocytes from the more mature proliferative zones of epiphyseal cartilage.

Proliferation of human lung fibroblasts appears to be modulated in a paracrine fashion by IGF-I produced by epithelial cells (120) as well as by autocrine pathways. In skin IGF-I is produced by melanocytes and fibroblasts but not by keratinocytes, however IGF-I is regarded as cell survival factor for epidermal cells (90,223). IGF-I has been shown to induce cell proliferation and protein synthesis by foreskin fibroblasts where GH has not been effective (254). Eming et al (12) demonstrated this principle of paracrine IGF-I activity in the skin using genetic modification of human diploid keratinocytes. In this study only those keratinocytes that had been genetically modified secreted IGF-I while endogenous IGF-I

was not detected in unmodified keratinocytes. Ewing study demonstrated that IGF-I was required for keratinocyte growth. Given that IGF-I is not produced by normal keratinocytes, this effect must be a paracrine phenomenon presumably associated with dermal derived IGF-I.

The synthesis of DNA by fibroblasts is a physiological event that is at best only minimally stimulated by GH supplemented SFM (124,254). Using the methylene blue absorbance assay, met-hGH was shown in figure 3.1 to only weakly stimulate growth at supraphysiological concentrations (maximum concentration of 1000ng/ml). Cook et al (124) showed a similar effect where fibroblast DNA synthesis was increased only marginally above baseline with SFM supplemented with up to 100ng/ml hGH. The authors reported that when applied in the presence of 0.5% HPS (hypopituitary serum), GH at 1000 ng/ml increased thymidine incorporation five fold. It can be concluded that stimulating growth of dermal fibroblasts by GH requires some other factor to produce significant biological effect *in vitro*. The cell growth and FPCL data for GH in 3.4.1 support this finding.

A similar minor effect was reported by Cook et al for IGF-I. However this was only at a concentration of 10ng/ml. IGF-I in SFM remains capable of inducing cell growth (170% of SFM at a concentration of 500ng/ml) but growth is only weakly enhanced by GH in these same serum depleted conditions. Similarly these cells exhibit significant responses under serum free conditions for thymidine incorporation into cellular DNA (figure 3.7) and synthesis of cellular protein (figure 3.6). In both of these assays IGF-I had an ED_{50} of 10mg/ml.

Results for cell growth and gel contraction indicated that IGF-I potentiates fibroblast activities in the absence of hGH while hGH is ineffective when used alone. GH was not

synergistic with IGF-I under the conditions of the models used. These results are consistent with Salmon and Daughaday's hypothesis (105) and complements the work by Cook et al (124) who demonstrated that GH activity in fibroblast cultures is primarily mediated by the IGF-I. Other cell lines, in particular chondrocytes and osteoblasts, exhibit similar growth related activity with IGF-I (233). However Clemmons and Van Wyk (214) reported that pre-incubation or co-administration of other growth factors augmented IGF-I activity. They introduced the concept of a conditioned medium containing competence factors one of which was considered to be PDGF. Several animal studies involving dermal and periodontal wounds have demonstrated that this synergism is not isolated to tissue culture (46,129,131,134). The influence of competence factors such as PDGF has not been fully addressed in this study although this is an important issue in understanding the cellular events associated with IGF-I. The contraction exhibited by fibroblasts populated gels in response to IGF-I may be partly related to priming of the fibroblasts by factors present in 10% FBS/DMEM. The sequence of harvesting of fibroblasts and lattice construction requires that the cells be primed by FBS however the assays were conducted in solutions of SFM containing IGFs or GH as the only additives.

The incorporation of [^{3}H]-proline into precipitable protein and [^{3}H]-thymidine into DNA were used to measure other fibroblast-related events in response to IGF-I and its analogues. In these experiments the influence of FBS and priming of cells were minimised by incubating the subcultures for 24 hours in SFM before addition of treatment peptide solutions. IGF-I was able to increase protein production up to 250% more than that for serum free conditions as shown in figure 3.6. The analogues, LR^3 IGF-I and des(1-3)IGF-I, were more potent than IGF-I as demonstrated by the shift to the left of the dose response curve. This result was consistent with other data relating to the activity of these peptides in

L6 rat myoblasts cultures (140). This response is neither species nor cell line specific with several studies supporting the potential use of these analogues *in vivo* (143,145,146,255) as anabolic agents with greater potency than IGF-I. Protein synthesis and FPCL contraction were also assessed using the IGF-II analogues, R^6 IGF-II and des (1-6) IGF-II (data not included). IGF-II and its analogues were effective in both models but all were less effective than IGF-I. However the analogues were no more potent than the native IGF-II. This demonstrated that the structural changes to the N-terminus of the IGF-II molecule weakly, if at all, affected biological activity of IGF-II even though IGFBP affinity is reduced to 1% of IGF-II by such alterations. These results were consistent with those observed for protein synthesis in L6 rat myoblast (249) and contrast dramatically with the markedly different potencies that similar structural changes can have for IGF-I. The reduced activity of IGF-II compared to IGF-I and its analogues may be explained by the lower binding affinity that IGF-II has to the type I IGF receptor (256) compared to IGF-I and the effect of the IGF-II receptor mopping-up free IGF-II (personal communication G. Francis).

The functional activity of fibroblasts in response to various mitogens was extensively investigated using the FPCL model. The collagen gel lattice is a versatile *in vitro* model of wound contraction that can be used to explore cell-cell and cell-matrix interactions as well as to study growth factors alone or in various combinations. The change in gel size has traditionally been recorded as residual area (57). Assouline et al (61) chose to use optical transmission coupled with residual gel area to quantitate a change in the collagen density and size, presumably to correlate this with gel contraction. Our modification incorporates a small amount of radioactivity ($[^3H]$-inulin) into the gel and allows residual gel volume to be measured and expressed as a function of residual radioactivity. Thus it also allows assessment of contraction of floating gels in three dimensions. Other factors that have been

shown to influence the results in this model include number of cells seeded into the gel, the type of collagen used in its composition and whether or not they are allowed to float freely in their medium (56,57,220).

Several important findings were apparent from this study of FPCL contraction.

- *In vitro* IGF-I did not require FBS even in small concentrations to activate human dermal fibroblasts to contract collagen lattices although priming of the cells prior to harvesting cannot be entirely disregarded.

- Gel contraction was not markedly improved when met-hGH was added with rhIGF-I nor did met-hGH have any great influence upon contraction by itself. These observations are important in light of the work of Gillery et al (123) where IGF-I was added in the presence of varying amounts of FBS and its effect upon collagen lattice contraction was only noticeable when low concentrations of FBS were used. The significance of even a small amount of serum lies in the provision of other cytokines to the medium which may influence the IGF-I response.

- The analogues demonstrated even greater ability to enhance contraction than IGF-I. This was illustrated by a shift to the left of the dose response curve and straightening of the curve for the more potent peptides. This suggests that as the ratio of free IGF-I like peptide to IGFBP bound peptide increased the level of IGF receptor activation probably also increased. Therefore a smaller concentration of peptide is required to produce a comparable response to rhIGF-I.

The exposure of this model to IGF analogues is unique to this study. IGFBP influences can therefore be examined indirectly. In addition, these peptides have been shown to promote

DNA synthesis and cell proliferation in other cell types, including H35 hepatoma cells and L6 rat myoblasts (140). In neonatal fibroblasts cultures they are up to 20 fold more potent than IGF-I (238). The type 1 IGF receptor is the primary receptor mediating IGF activity including IGF-II related events. Similarity of the maximal responses of the IGF-I species implies that they are all exerting their effects through the same receptor type rather than through for example the type II receptor or even the insulin receptor.

The mechanisms by which IGFBPs modulate IGF-I effects are not clearly defined. They may augment IGF-I activity *in vivo* (257) but they impede smooth muscle cell migration and inhibit embryonic fibroblast proliferation in tissue culture preparations (237,258). Fibroblast cultures are also known to produce endogenous IGFBPs (259) and their production may be modulated to some extent by other growth factors (238). Therefore it is useful to examine the IGF related effects in such a way that their influence is minimised. This has been achieved using IGFs with low affinity for the binding proteins. The results described using analogues with known affinity of less than 5% for the IGFBPs (142) suggest that binding proteins produced endogenously may restrict contraction stimulated by IGF-I. This suggestion concurs with the work of Yateman et al (238) who demonstrated that the proliferative activity of neonatal fibroblasts was more responsive to LR^3 IGF-I than IGF-I.

The differences observed between natural IGF-I and its analogues are possibly partly due to the greater affinity of IGF-I for IGFBPs and endogenous production of these proteins., It has been postulated that the N-terminus and particularly Glu^3 are important for IGFBP binding as modifications in this region provide the structural differences between the analogues and IGF-I (140,142,260).

TGFβ1 has long been associated with scar contraction and has been shown by other investigators to be active in gel contraction models (66,245,261,262). However in my studies it produced only weak gel contraction and was only weakly mitogenic also. The range of concentrations used in these experiments was 0.7 ng/ml to 20 ng/ml which are similar to those doses used by Finesmith et al (66) and MacNeil et al (262). In these two studies TGFβ produced significant gel contraction. The marked differences in response may be related to several factors including the time allowed for contraction to occur and the preparation of the gel lattice, particularly the cell numbers seeded into the gels. In MacNeil's study, contraction was measured over a 24 hour period and 5×10^5 cells were seeded into 1 ml gels while 1×10^5 cells/0.5ml gel lattice were used in my experiments. In the experiments by Pena et al, 0.5ml gels were used however they were inocculated with 250,000 rabbit dermal fibroblasts per gel. Piscatelli et al seeded 100,000 fetal wound fibroblasts into a 2ml lattice that also incorporated the growth factor additive unlike my experiments where the gels were allowed to float in their treatment solutions.

Timing and methods of assessing contraction may be influenced by observer error when determining diameter of the contracted gel and if gels are allowed to contract over a prolonged period of time the influence of endogenous factors and possibly proliferation of cells within the lattice may come to bear. The relatively short period allowed for contraction in our study minimises the influence of endogenous produced peptides while permitting observation of a significant response. In addition 48 hours appears to be the time point at which thet majority of contraction has occurred (245,261).

Finesmith et al (66) used large gels in petri dishes prepared the day before the experiments and quantitated gel contraction in relation to the uncontracted area determined from

photographs. In addition their cell inocculated gels were preincubated for up to 24 hours in 10% FCS prior to addition of treatment solutions. My impression is that this would prime the fibroblasts and therefore influence the final response to the growth factors being tested.

Cellular DNA synthesis (thymidine incorporation) is accentuated by TGFβ in certain mesenchymal cells (241) including fibroblasts and smooth muscle cells, but was not as pronounced as that observed for IGF-I in this study. TGFβ1 is known to have inhibitory influences upon proliferation and differentiation primarily in epithelial cell cultures (248). Therefore the biological responses prompted by TGFβ1 may be cell line dependent. A possible explanation for my results is that TGF-β1 is not a potent mitogen in either of the human skin fibroblasts used for my experiments. Therefore the function of TGFβ1 in the wound may relate more to fibroblast recruitment to the wound and regulation of ECM protein secretion and reorganisation (263). Application of TGFβ1 neutralising antibodies influence these parameters lending support to such a supposition (72,243,264). In this study, TGF-β1 was not as mitogenic as IGF-I in two different human skin fibroblast lines suggesting that IGF-I may play a greater role than TGF-β1 in fibroblast proliferation and wound contraction in the wound milieu. Another possible explanation is provided by the work of Ghahary et al (265) who have demonstrated that IGF-I is actually esponsible in part for the induction of TGF-β expression and activity in dermal fibroblasts.

As collagen is one of the fibroblast's principle synthetic products, the magnitude of the protein production in response to IGF-I suggests this peptide may be more important in collagen production and remodelling than TGF-β1. Comparison with other isoforms of TGF β may have been useful to examine difference in the TGF family response in the FPCLs but this was beyond the scope of the study.

In conclusion, in these models of *in vitro* fibroblast behaviour it has been shown that GH is largely impotent while IGF-I stimulates dramatic fibroblast growth and protein synthesis and may do so in the absence of other so-called competence factors such as PDGF. Secondly, fibroblast associated gel contraction and protein synthesis responses indicate that the IGF-I analogues are more potent than IGF-I while once again GH was unable to influence gel contraction to any significant degree. There was also an absence of any additive effect of GH and IGF-I. Finally, stimulation of both gel contraction and DNA synthesis by IGF-I and the truncated analogue des(1-3)IGF-I were only surpassed by PDGF when the IGFs were compared with a range of growth factors known to be active in the wound milieu. This implies that IGF-I is a potent fibroblast mitogen and that the IGFBPs may tend to blunt the magnitude of this effect.
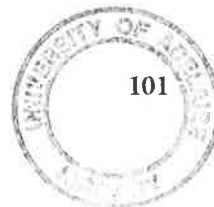
CHAPTER 4

APPLICATION OF IGFs TO INCISIONAL MODELS OF WOUND REPAIR

4.1. INTRODUCTION

4.1.1. The IGF-I Axis and *In Vivo* Studies

4.1.2. Diabetes

4.1.3. Aims

4.2. MATERIALS

4.3. METHODS

4.3.1. Surgery and Care of Animals

4.3.2. Peptide Preparation

4.3.2 i) GH and IGFs

4.3.2 ii) IGFBP-2

4.3.3. Confirmation of IGF-I Affinity for IGFBP-2

4.5. DISCUSSION

4.6. SUMMARY

4.1. INTRODUCTION

Experiments in chapter 3 demonstrated that cultured fibroblasts exhibit greater growth, synthetic and contractile activity following exposure to the IGF analogues compared to rhIGF-I or GH. These fibroblast responses may be important *in vivo*. This chapter explores the effects of IGF-I and its analogue LR^{3}IGF-I in animal models of healing.

Models of incised wounds allow examination of fibroblast activity within the healing wound, particularly extracellular matrix deposition following topical or systemic administration of treatment preparations (93,94,125,127,132,266-269). The data collected are generally presented in either histologic or tensiometric formats. Various tensiometry parameters have been described. The basic parameter is the 'breaking strength' that is the elongating force required to disrupt a given sample. This can be converted to the 'tensile strength' by incorporating into the analysis the cross-sectional area of the specimen at the point of disruption (87). Furthermore during the elongation of a strip of tissue the force absorbed up to breaking point can be used to calculate the amount of energy absorbed prior to wound dehiscence known as 'the yield energy'. Levenson (87) reported that uncertainties of wound thickness measurements made the exact tensile strength curve less precise than the breaking strength curve. However using groups of animals with paired control and treatment wounds, Jyung et al suggested that the skin thickness becomes less important for comparison of wound strength data within treatment groups (132). Therefore breaking strength remains as valid a parameter as tensile strength. That particular paper compared

IGF-I alone and in combination with IGFBP-1 as topical wound healing agents and utilised paired wounds on individual animals.

Examination of histological sections from wounds allows non-parametric data to be obtained relating to fibroblast responses within the wound. Relative amounts of newly produced to old collagen can be depicted using image analysis technology. These features can be correlated with tensiometric data (119,267). Immunohistochemical staining, in situ hybridisation and protein extraction techniques can also generate data that can be similarly compared (270).

4.1.1. The IGF Axis and *In Vivo* Studies

Few papers have been published that examine the effects of IGF-I in animal wound healing models (46,119,127,247,271-274). Greenhalgh et al (46) used a murine model of excisional wound repair to examine PDGF combined with IGF-I or IGF-II. The authors reported accelerated healing using IGF-II alone, but that PDGF potentiated both IGF-I and IGF-II activity. Mueller et al (119) demonstrated that infused IGF-I administered using osmotic pumps inserted subcutaneously, increased wound macrophage populations and collagen synthesis in hypophysectomised animals. Suh et al (127) reported a similar result for collagen synthesis using steroid affected rats.

The efficacy of IGF-I as a topical wound healing agent appears to rely on the presence of IGFBPs (132,247,271,275) or another growth factor, such as PDGF (46,134,274). It has been reported to be effective in models of compromised healing when administered alone

(119,127,273). Other reports have demonstrated that the IGFBPs, particularly BP-1 and BP-3 can potentiate the activity of IGF-I *in vivo* (132,247,271,273,276). Little is known about the influence of IGFBP-2 in the wound environment except that it exists in wound fluid (see figure 2.11) and, it appears to inhibit the *in vitro* activity of IGF-I (277).

Altering the affinity of IGF-I for its binding proteins also influences the *in vitro* activity of IGF-I as was shown in chapter 3 using the analogues, des(1-3)IGF-I and LR^{3}IGF-I. IGF analogues have not previously been tested in wound repair models but they have exhibited greater potency than rhIGF-I in promoting gastro-intestinal growth (149) and general somatic growth (255,278). Their increased bioavailability may also provide some advantage over the native peptide where levels of specific IGFBPs are increased (180).

4.1.2. Diabetes

Diabetes is the most common endocrine disorders afflicting humans. Wound healing complications in diabetics account for substantial numbers of hospital admissions worldwide despite improved methods for controlling hyperglycaemia (152,156,279). Ferguson et al (280) reported on the pathology and healing patterns of diabetic ulcers. They noted features of retarded epidermal migration, acute on chronic inflammation and changes in pericapillary fibrin deposition. However the authors concluded that local ischaemia from 'small vessel' occlusion was not an important feature.

Several models exist that allow investigation of the influence of diabetes upon wound healing parameters. These include toxin-induced and genetically selected models.

Streptozotocin (STX), the broad spectrum antibiotic produced by *Streptomyces acromogenes* (281), has been used as an agent to induce experimental diabetes for many years. It is toxic to the insulin producing β cells of the islets of Langerhans found in the pancreas, causing degranulation and necrosis (282) and thereby causing a clinical state similar to type 1 insulin dependent diabetes mellitus. Limitations of this model include regeneration of the β cells, which reverses the hyperglycaemia or the development of pancreatic adenomata (162) which secrete insulin, causing hypoglycaemia. Phillips et al (283) and Masiello et al (284) have reported that animal weight and age also affect the induction of diabetes by STX. Extrapancreatic effects of STX have been reported to reduce bile salt-independent bile flow (285) and induce nephrotoxicity and ketoacidosis that can progress to acute renal failure.

4.1.3. Aims

The aims of the *in vivo* experiments were to:

1) Develop a reliable rodent model of type I insulin dependent diabetes mellitus that could be used to examine the effects of the IGFs on impaired healing using incisional and excisional wound models;

2) Examine for differences in wound strength after topical applications of GH and rhIGF-I applied in several different concentrations;

3) Test for any advantage that may be conferred by IGF-I analogues;

4) Determine the effect on wound strength of coapplication of IGFs with IGFBP-2.

4.2. MATERIALS

Recombinant human IGF-I, (rhIGF-I, animal grade peptide >75% pure by HPLC), rhIGF-II and LR^3IGF-I (receptor grade peptide; >95% pure by HPLC) were obtained from GroPep Pty Ltd, Adelaide, SA and formulated as described in Chapter 3. Recombinant met-human GH (GH) was purchased from Bresatec (Thebarton, SA) and stored at 4°C after being solubilised in sterile deionised water. Type I collagen was acid-extracted from rat-tails tendons as described by Bell (57) for use as the peptide delivery vehicle. Streptozotocin (STX), 500 mg (Sigma Chemicals, St. Louis, MO) was dissolved in 10 ml of 0.9% NaCl in initial experiments and later in citrate buffer (pH 4.5). All other reagents were obtained from AnalaR Chemicals or Sigma Chemicals, St. Louis, MO. Male Sprague-Dawley rats, aged 7 to 8 weeks (250 to 300 g) were purchased from the Central Animal House, the University of Adelaide, Adelaide, SA. For the diabetic studies, body weights of 300-350 g at the time of arrival were selected. Glucose reagent strips (Ames Glucostix®, Bayer Diagnostics, Mulgrave, Vic.) were used to measure blood glucose levels. Gaseous anaesthesic agents used were halothane (Fluothane®, Zeneca, Macclesfield, Cheshire, U.K.) 2.5%, mixed with a 2:1 ratio of oxygen (O_2; Linde, Fairfield, NSW) and nitrous oxide (N_2O; Linde, Fairfield, NSW). Dynek Australia Pty Ltd (Adelaide, SA, Aust.) supplied suture material (4/0 silk; Dysilk®). Wound peak loads (breaking strengths) were measured using a tensiometer (M 1000 E, Mecmesin, U.K. with calibration package model MFG 25).

4.3. METHODS

Experiments were conducted using an incised wound model. The primary objective was to examine the influence of IGF-I upon the dermal healing response as reflected by wound breaking strength. General aspects of the studies will first be described before detailing specific experiments.

4.3.1. Surgery and Care of the Animals

The animals were delivered one week prior to surgery to permit acclimatisation to individual cages. They were fed water and rat chow *ad libitum* while housed in an environment with a 14 hour/10 hour light/dark cycle and an atmospheric temperature of 22°C. Approval for all animal studies was granted by the Animal Ethics Committees of The Queen Elizabeth Hospital (Woodville, SA) and The University of Adelaide (Adelaide, SA, Australia).

The rats were weighed individually on the day of surgery (day 0), then anaesthetised in a clear perspex box and the dorsal fur clipped before placing the snout into a hood connected in series with the induction box to maintain anaesthesia (figure 4.1). Two 7cm full thickness parasagittal incisions (including panniculus) equidistant from the midline were made on the back of each animal after cleaning the operating field with a 1:1 solution of Betadine™ and 70% ethanol (see figure 4.2).

The animals were randomly allocated to treatment groups (n=8 unless otherwise stated) after ranking the animals according to weight on the day before surgery. Treatment preparations were applied using a pipette set at a volume of 100 µl to one wound and 100 µl of the vehicle (collagen) was applied to the contralateral wound. The surgeon was blinded to which treatment preparation he applied. The wounds were closed with simple interrupted 4/0 silk sutures. Recovery from anaesthesia was observed by the operating team prior to the animal's return to its cage. Post-operatively all rats were weighed at day 1 and every third day thereafter.

Seven days after surgery the animals were sacrificed using CO_2 inhalation and cervical spine dislocation. The dorsal pelt was removed from the carcass and each wound was divided into four parallel strips 4 mm to 5mm wide at right angles to the wound (see figure 4.2.) These strips were cut out and individually placed into the clamps of the tensiometer. Each wound strip was distracted at a rate of 10 mm/min until disruption to determine the wound breaking strength which was recorded in kilograms (kg).

Samples at the cranial end of the wound were taken for histological studies and fixed in methacarn or 10% formalin for future analysis. In the diabetic studies, samples were fixed in Bouin's fixative for immunohistochemical studies.

FIGURE 4.1. Apparatus for providing general anaesthesia for all experiments

Fume hood
Anaesthetic machine
Isoflurane cannister
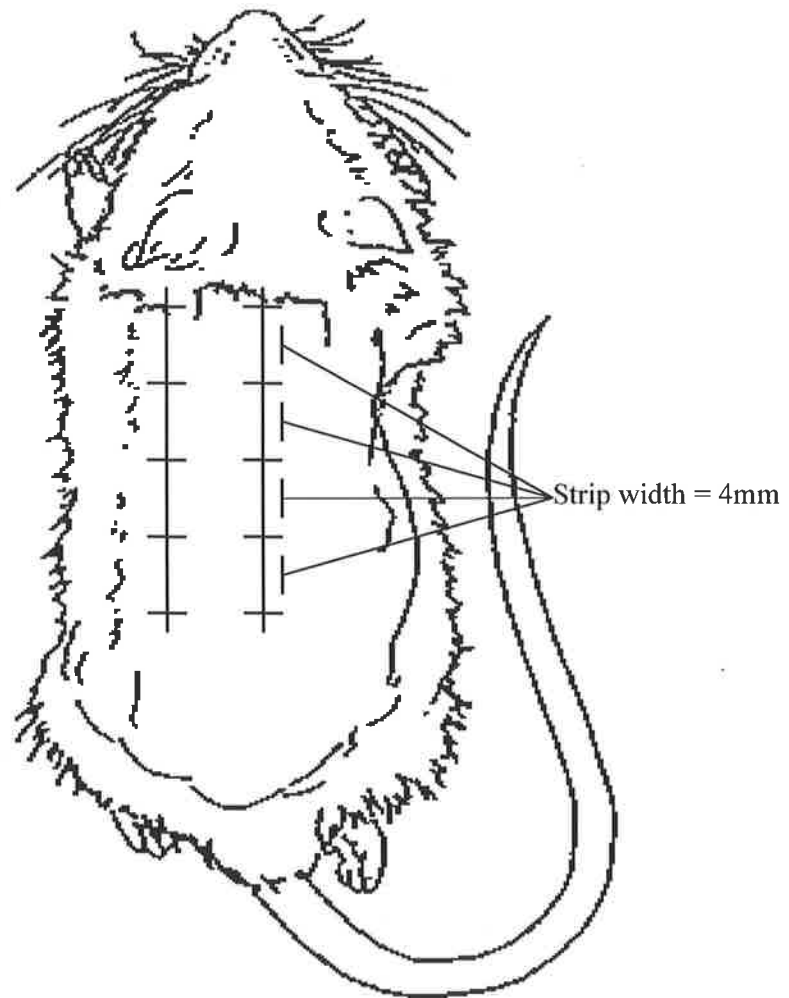Perspex induction box
O_2
N_2O

Strip width = 4mm
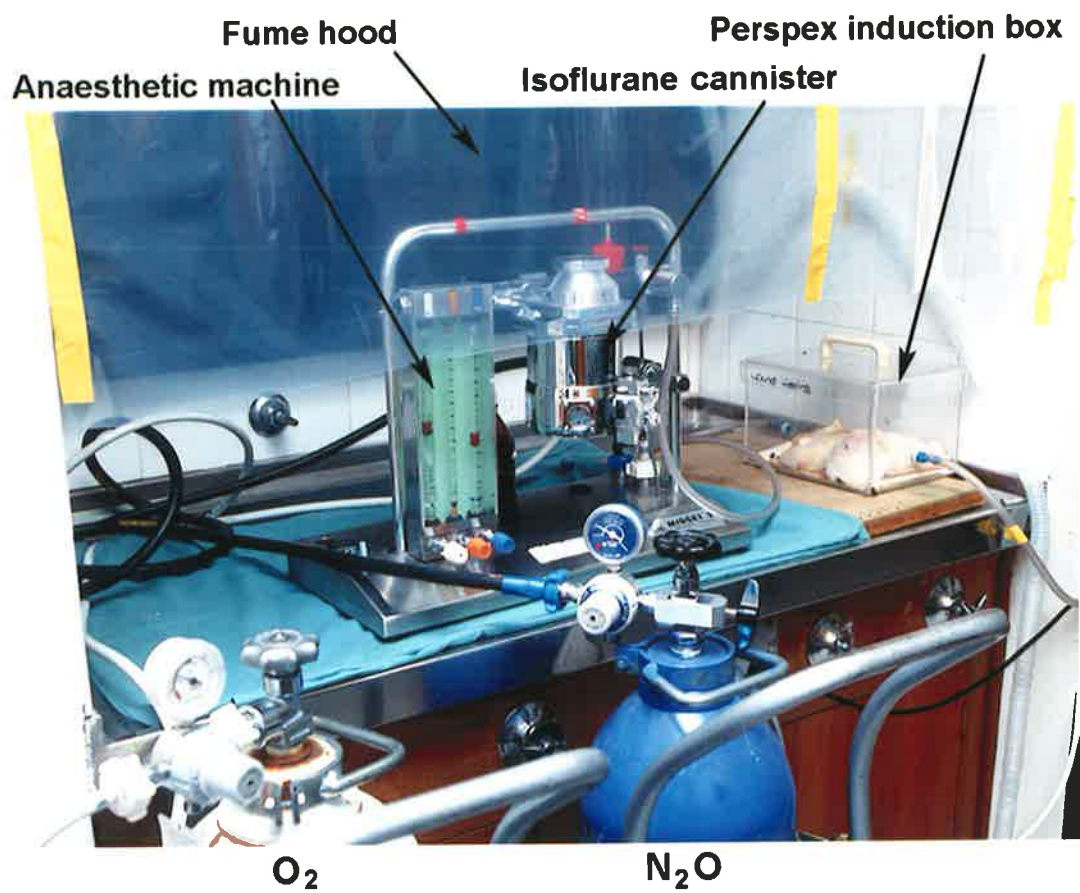
FIGURE 4.2. Schematic representation of the rodent incisional wound model.

Parallel parasagittal dorsal incisions were made with a scalpel and closed with interrupted sutures after treatment application. Four x 4 mm strips were cut perpendicular to the wound between sutures. Subsequently these strips were stretched to breaking point.

4.3.2. Peptide Preparation

<u>4.3.2. i) GH and IGFs</u>

All treatments were prepared within a laminar flow environment and stored at 4°C until required. Collagen, 500 µl for each ml of treatment solution, was used as the delivery vehicle for the growth factors. Sufficient peptide was added to produce concentrations of 20 µg/ml (met-hGH or rhIGF-I), 100 µg/ml (met-hGH, LR³IGF-I, rhIGF-I), or 1 mg/ml (met-hGH, LR³IGF-I, rhIGF-I, or rhIGF-II). Sterile deionised water and 10 x PBS (100 µl/1ml of solution) were added to bring the volume of solution to 1 ml along with 7.5 µl of 1 M NaOH/ml of collagen solution to adjust the pH to between 7.0 and 7.4. The vehicle control solutions consisted of 1500 µl of collagen, 300 µl of 10 x PBS, 22.5 µl of 1M NaOH, 1177.5 µl deionised water.

All wounds received a volume of 100 µl whether this was the control preparation alone or one of the treatment solutions. For the diabetic study involving the application of IGFBP-2 all preparations were applied in a volume of 120 µl.

<u>4.3.2. ii) IGFBP-2</u>

IGFBP-2 peptide was produced by Dr. John Wallace (Dept. of Biochemistry, University of Adelaide) by transient transfection of COS cells with an expression vector containing the coding sequence of bovine IGFBP-2. Conditioned medium was collected and IGFBP-2 purified on an IGF-I affinity column followed by cation exchange and reverse phase HPLC. Final purity was greater than 95%.

IGFBP-2 was dissolved in 10 mM HCl before being mixed with rhIGF-I or LR^{3}IGF-I in a 1:1 molar ratio. Treated wounds received 120µl of solution containing rhIGF-I 10 µg: IGFBP-2 40 µg per wound, rhIGF-I 10 µg only, IGFBP-2 40 µg only, LR^{3}IGF-I 10 µg only or LR^{3}IGF-I 10 µg : IGFBP-2 40 µg.

4.3.3. Confirmation of IGFBP-2 binding of IGF-I

WLB analysis was performed using 12.5% SDS-PAGE under reducing conditions as described in chapter 2 (see 2.3.4). After electrophoresis the nitrocellulose membranes were probed with [^{125}I]-IGF-I and [^{125}I]-LR^{3}IGF-I for one hour before being visualised autoradiographically.

4.3.4. Development of the Model of STX-induced Diabetes

4.3.4. i) Induction of Diabetes

STX was administered intramuscularly to the thigh muscle of the right hind leg. It was dissolved in its vial in 0.9% NaCl to a concentration of 50 mg/ml. The solvent was changed to citrate buffer (pH 4.5) in later experiments as the stability of STX is better maintained in this medium than in physiological saline. The required doses were calculated using the formula;

$$\text{Volume of STX (ml)} = \text{Weight x [dose]/50 mg/ml}$$

4.3.4. ii) Blood glucose testing

Blood samples for testing glucose concentration were obtained by sectioning the terminal 1-2 mm of the tail tip. Sufficient blood was then expressed and applied to a Glucostix reagent strip for analysis in an Ames Glucometer® M blood glucose meter. These strips rely upon a glucose oxidase reaction to produce changes in colour of two pads. This system allowed the blood sugar levels (BSL) to be measured within a range of 2-22 mmol/l.

4.3.4 iii) Preliminary Experiment

Animal weights and BSL were recorded prior to this procedure. The body weights were recorded every second or third day. Detection of glycosuria was attempted once the animals developed polyuria (around day 3). BSLs were determined on the seventh day and subsequently twice weekly to confirm the development of hyperglycaemia. Diabetes was confirmed by recording three consecutive high BSLs (>11 mmol/dl) over a period of 2 weeks with associated polyuria and weight loss.

On the fourteenth day single incisions were created as described in 4.3.1. Body weights and BSLs were recorded on the day of surgery and on the day of sacrifice (see 4.3.1). Four transverse wound strips from the pelts of each animal were subjected to wound strength analysis. The pancreas from one representative animal in each group was also removed and processed for immunohistochemical staining of insulin and glucagon content within the pancreatic islets using a commercially available kit (Histostain-DS™ Kit, Zymed Laboratories, South San Francisco, CA, USA).

4.3.4. iv) Animal Care

Animals were acclimatised to individual wire-bottomed cages for one week during which time they were weighed twice and fed standard laboratory rat chow. The sixteen animals were divided into four groups. Each group received a different dose of STX (0 mg/kg (controls or vehicle only, 45 mg/kg (group I), 55 mg/kg (group II), and 65 mg/kg (group III)). These values were determined following communication with Dr Frank Tomas (Cooperative Research Centre for Tissue Growth and Repair) and from a review of relevant literature (150,161,162,164,180,283,284,286,287).

4.3.5. rhIGF-I and LR³IGF-I in Diabetic Wounds

Diabetes was induced using STX 55 mg/kg as an intramuscular injection. BSLs were conducted only weekly while weights were recorded each second or third day. The animals were allocated to either the diabetic or normal group depending upon their weight with the heaviest animals placed in the diabetic group. The controls received a weight calculated volume of buffer/vehicle (see 4.3.4.).

Three weeks after being rendered diabetic all animals were again randomised on the basis of weight to two treatment groups. Recombinant human IGF-I and LR³IGF-I were applied at a concentration of 1 mg/ml. The surgery and recovery were undertaken in the same manner described in 4.3.1.

4.3.6. The Influence of rhIGF-I and IGFBP-2 upon Wound Strength in Diabetic Wounds

Animals were allocated to groups of eight and surgery was performed over two days. Anaesthesia and wounds were produced as previously described and wound closure completed with 4/0 silk. One group received only rhIGF-I and were wounded on the first day and the other two groups 2 were wounded on the second day. These received solutions containing IGFBP-2. After seven days the wounds were harvested and analysed in the standard manner (4.3.1).

4.3.7. Statistics

The values of the individual strips were pooled for each treatment to give a mean value ± 1 SEM in kilograms (kg). Only dehisced strips (~5% of all specimens) were excluded from analysis. Analysis of results was performed using paired student t-tests comparing treated and paired contralateral control wounds. Statistical analysis was performed using Sigmastat v2.01 software package (Jandel Corporation, San Ramon CA, USA).

4.4. RESULTS

4.4.1. Met-hGH and rhIGF-I as Topical Agents in Wound Repair.

Rats with normal wound healing properties were used to assess the effect of met-hGH and rhIGF-I as topical wound healing agents where healing was not compromised. Preliminary experiments using a concentration of 20 μg of peptide/ml failed to influence wound breaking strength for any treatment compared to their controls (data not shown). The effect of met-hGH, rhIGF-I and LR^{3}IGF-I were subsequently tested at doses of 100 μg/ml and 1 mg/ml. The results for those experiments that tested a dose of 100 μg/ml (10 μg/per wound) are illustrated in figure 4.3.

Two experiments were used to test LR^{3}IGF-I (n=8), met-hGH (n=8), rhIGF-I (n=8) and a combination of these last two (n=7). Met-hGH and rhIGF-I were repeated but results have not been pooled as vehicle treated wounds were significantly different between the two experiments. Data for met-hGH and rhIGF-I have been presented from the first experiment and are representative of the results (figure 4.3).

Wounds that received 10 μg/wound met-hGH (79.8 g ± 6), rhIGF-I (77.4 g ± 6) and LR^{3}IGF-I (74.8 g ± 7) were no stronger than their paired control wounds. The combination of met-hGH/rhIGF-I (76 g ± 6) also did not improve wound strength after 7 days than their paired vehicle treated wounds. This was confirmed statistically using a paired t-test.

A separate set of experiments was used to test met-hGH, rhIGF-I, LR^{3}IGF-I and rhIGF-II at concentrations of 1 mg/ml. The first two experiments tested met-hGH, rhIGF-I and LR^{3}IGF-I (n = 15 animals/treatment) (figure 4.4). The results for these two experiments were combined as there was no significant difference in weights or vehicle treated wounds between the two studies. The potency of rhIGF-II (1 mg/ml) was also tested and compared with rhIGF-I and met-hGH (n = 8 per group) (figure 4.5).

Topical met-hGH failed to improve wound breaking strength significantly compared to paired vehicle treated wounds. This response remained consistent between experiments regardless of met-hGH concentration. In contrast rhIGF-I (79.4 g ± 8) at a concentration of 1 mg/ml applied as a volume of 100 µl to incisional wounds produced significantly stronger wounds compared to the matched controls (56 g ± 5; $p < 0.01$). This represented a 41% improvement in wound breaking strength. LR^{3}IGF-I did not influence wound breaking strength (70.9 g ± 7). Collagen treated wounds did not reveal a statistically significant difference between groups (one way ANOVA).

When rhIGF-II was applied in the same concentration (1 mg/ml) it did not demonstrate any influence upon wound breaking strength (see figure 4.4).

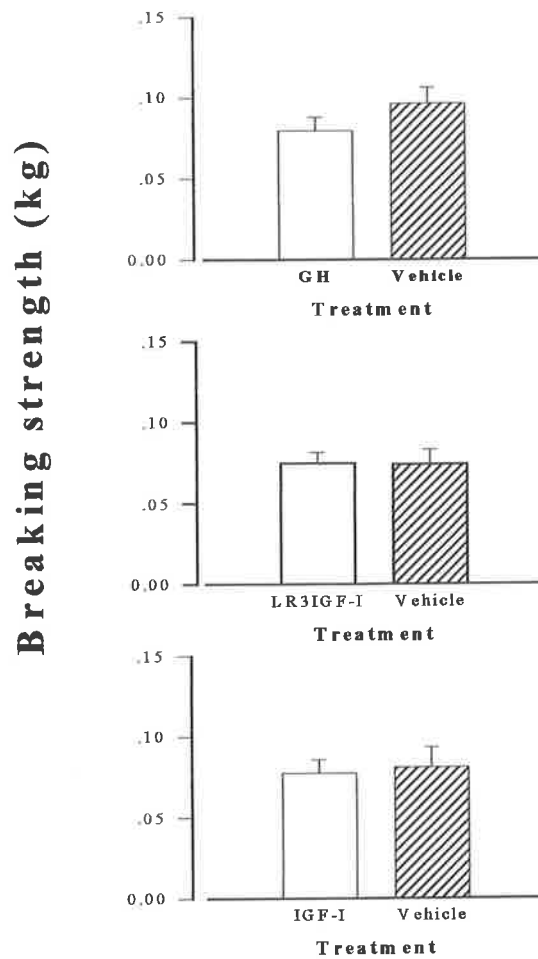**FIGURE 4.3.** Effect of met-hGH, rhIGF-I, LR^{3}IGF-I upon incisional wound breaking strength in rats.

Treated wounds received 100 µl of peptide (100 µg/ml per wound, n = 8 rats per group) and their wound strengths were compared statistically with those that received a collagen-based vehicle solution using paired Student's t-test (*p* = NS). The results represented the mean ± SEM (n=32 strips).
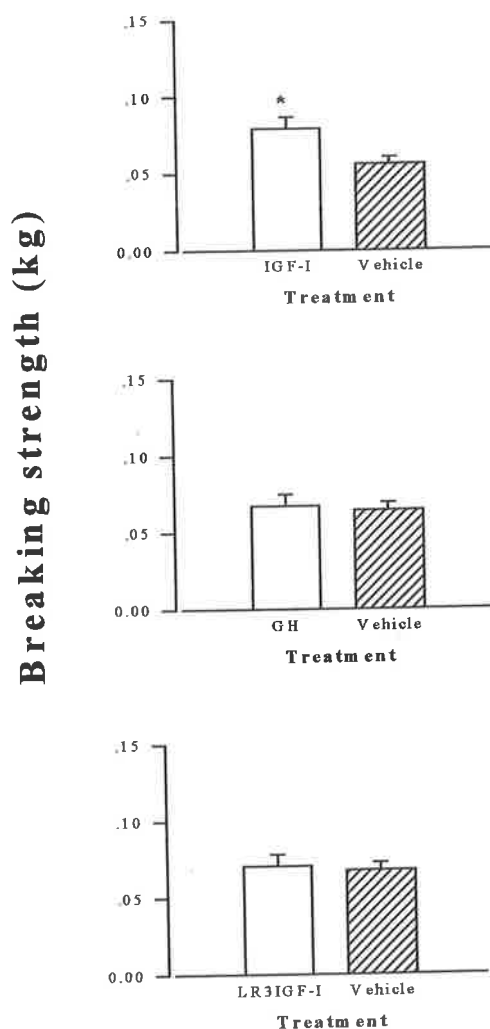
FIGURE 4.4. Wound breaking strength of rodent incisional wounds treated with increased peptide concentrations.

The indicated peptides were applied at a concentration of 1 mg/ml in a volume of 100 μl. The breaking strength of wound strips was determined seven days after wounding. Paired student's t-tests were performed on the mean values (n=15 animals, 60 strips). Error bars represent one SEM. *$p < 0.01$ vs control (paired Student's t-test).

FIGURE 4.5. Wound breaking strength of rodent incisional wounds treated with rhIGF-II (1mg/ml).

A volume of 100 μl of vehicle or treatment peptide was applied. Wound breaking strengths were measured after 7 days. Paired Student's t-test was used to compared mean values (n=8). Error bars represent one SEM.

4.4.2. The Influence of IGFBP-2 upon wound strength
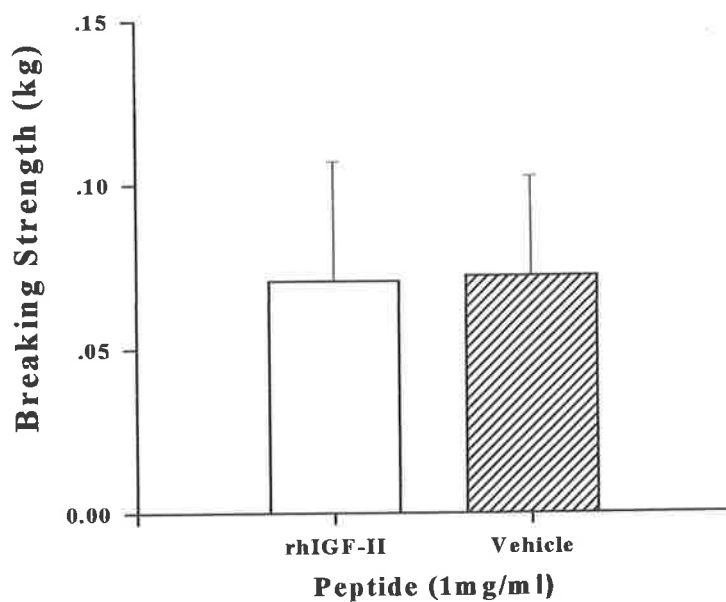
As IGFBP-2 is thought to inhibit IGF-I related responses, experiments were performed comparing the influence of IGFBP-2 upon IGF-I effects where binding affinity of IGFBP-2 to IGF-I is normal and also where binding affinity is reduced such as with LR^3IGF-I.

IGF-I binding to IGFBP-2 was confirmed using WLB (figure 4.6). Comparison of the blots incubated with $[^{125}I]LR^3IGF$-I or $[^{125}I]IGF$-I clearly demonstrated the presence of a band in the region of 30kDa on the membrane exposed to $[^{125}I]IGF$-I. This band is consistent with the molecular weight for IGFBP-2 and its absence in the gel incubated with LR^3IGF-I indicated little if any binding of the analogue to IGFBP-2. Good affinity of rhIGF-I for the binding protein was confirmed by these results.

Forty rats divided randomly into five groups (mean weight at time of surgery was 316.5 g; range 298.3 g - 367 g), were used for this study. Each group received collagen to one wound and rhIGF-I, LR^3IGF-I, IGFBP-2, IGF-I/IGFBP-2 or LR^3IGF-I /IGFBP-2 to the other (see 4.3.2. for peptide concentrations) to determine whether the addition of IGFBP-2 to an incised wound significantly altered the effect of IGFs on wound healing variables. The results are summarised in figure 4.7.

While rhIGF-I 10 µg alone marginally increased wound strength compared to paired controls, this was not significant when analysed using Student's paired t-test. Breaking strengths in the other groups showed no statistically significant difference between peptide treated wounds and paired vehicle treated wounds. IGFBP-2, 40 µg/wound, did not significantly influence rhIGF-I activity in this model. However a small trend towards

inhibition of incisional wound healing was observed when IGFBP-2 was applied with rhIGF-I but when used alone it had no effect.

FIGURE 4.6. Confirmation of IGFBP-2 binding of IGF-I.

WLB using 12.5% SDS-PAGE were performed and proteins were electrotransferred to nitrocellulose sheets before visualising the IGFBP-2 bands by probing overnight with $[^{125}I]LR^3IGF-I$ or $[^{125}I]IGF-I$. Lanes 1, 5, 6 and 10 represent molecular weight markers, lanes 2, 3, 4 were loaded with IGFBP-2 5 μg in 100 μl of sterile water and probed with $[^{125}I]IGF-I$ while lanes 7, 8, 9 were loaded with IGFBP-2 and probed with $[^{125}I]LR^3IGF-I$. IGFBP-2/IGF-I complexes were visualised while the absence of binding protein/ LR^3IGF-I confirms the reduced affinity of IGFBP-2 for the analogue.

$[I^{125}]$-IGF-I

M_r   1   2   3

30kDa —

$[I^{125}]$ -LR^{3}IGF-I

M_r 5   6   7   M_r

30kDa —
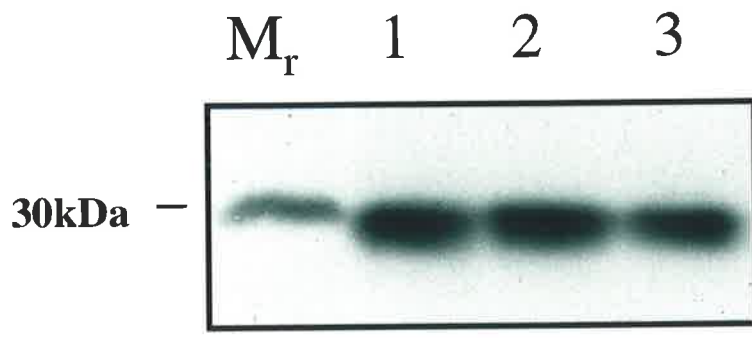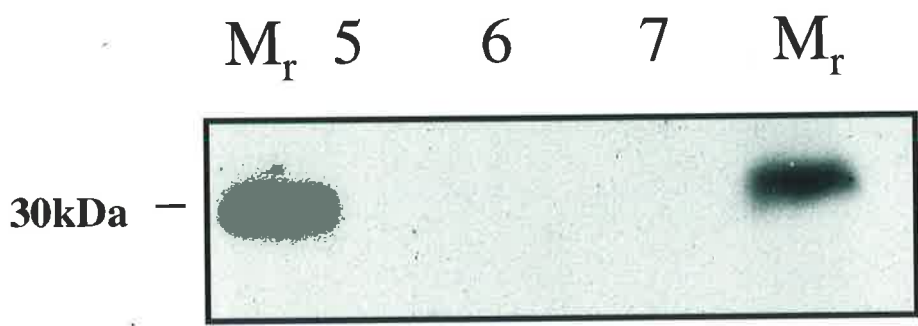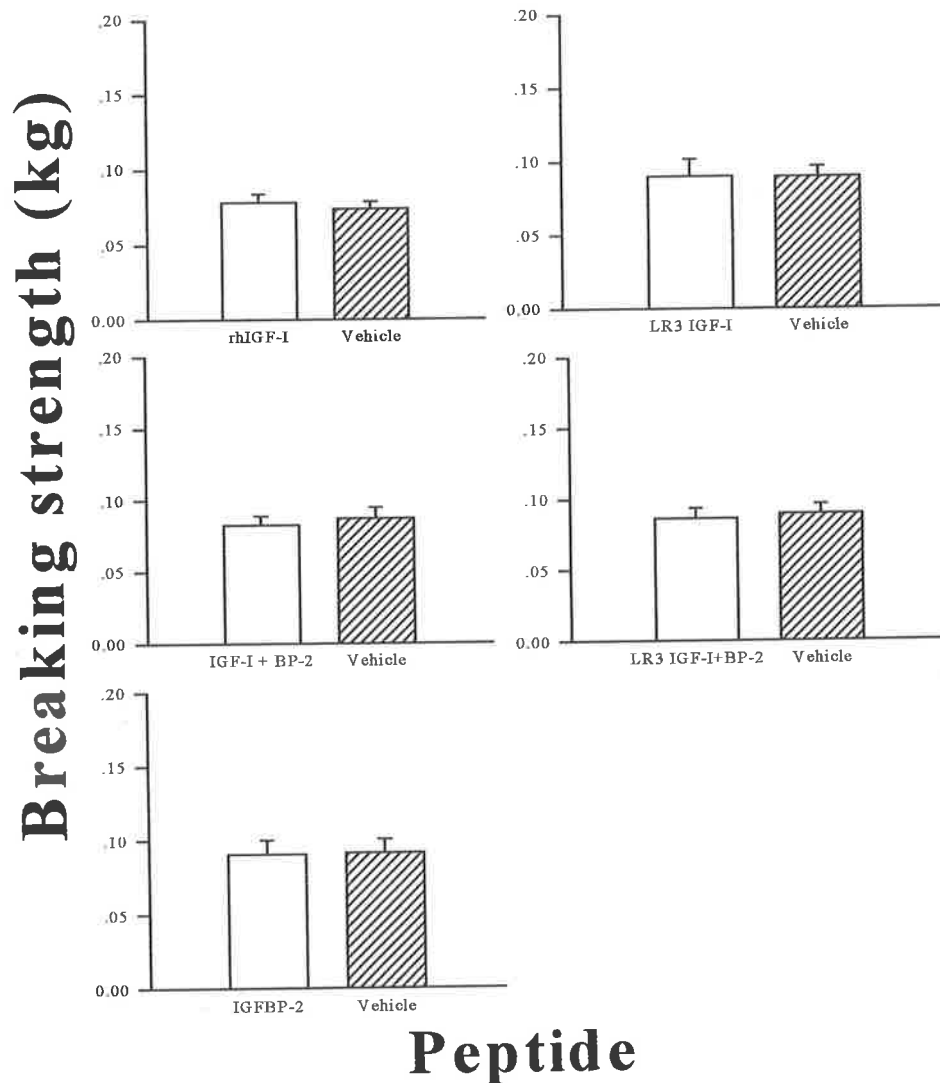
FIGURE 4.7. Strength of 7 days old incised wounds following application of IGF-I (10 µg/wound), LR³IGF-I (10 µg /wound), with and without IGFBP-2 (40 µg/wound). Error bars represent mean ± SEM (n=32 strips from 8 animals per group). No significant difference was demonstrated between controls and peptide treated wounds (paired Student's t-test).

4.4.3. Development of a Rodent Model of Diabetes Impaired Wound Healing

4.4.3. i) Weight loss and Hyperglycaemia:

Sixteen rats divided into four equal groups were used for this experiment. Diabetes is defined clinically in humans as the combination of hyperglycaemia and glycosuria where random blood glucose levels are greater than 11 mmol/l or fasting glucose concentrations are greater than 8 mmol/l. The normal range of blood glucose concentrations for rats is 189.4 mg/dl $\pm$ 41.1 (10.4 mmol/l $\pm$ 2.3) (288). Accumulated results using the Ames® Glucometer indicate that the normal range of blood sugar levels (BSL) in male rats of 8 to 12 weeks of age between is 4.5 and 8 mmol/l. The criteria adopted for confirmation of diabetic state in this rodent model were:

a) failure to thrive characterised by weight loss or reduced gain in weight compared to age-matched controls;

b) BSL greater than 13 mmol/l;

c) polydipsia, polyuria and hyperphagia. Metabolic cages were not used therefore urine volumes and the quantity of food consumed by the animals were not accurately measured, however the diabetic animals were obviously polyuric judged by the accumulated moisture of their bedding.

The BSLs recorded for those animals (n=4 per group) injected with STX at day 21 were 21.5 mmol/dl $\pm$ 1.1 for group I (STX 45 mg/kg), 20.9 mmol/dl $\pm$ 1.3 for group II (STX 55

mg/kg) and 21.9 mmol/dl $\pm$ 1.1 for group III (STX 65 mg/kg) (figure 4.8). This compared with the BSLs of the control group taken just prior to being sedated on the day of surgery (7.35 $\pm$ 0.8mmol/l). The technique used for measuring the glucose levels in these rats produced a range of measurements between 2.0 and 22.1 mmol/l. BSLs that registered as a "high" reading (>22.1) were recorded as 22.1 tended to skew the results towards a lower average. No "low" results were obtained in any of the four groups. All animals that were injected with STX developed hyperglycaemia and no deaths were recorded in this trial.

Change in animal body weights are summarised in figure 4.9. Over the first three weeks, all the animals given STX lost weight whereas age-matched controls continued to gain weight. Weight loss was most dramatic in the first week and slowed as the trial proceeded. The body weights of the group II plateaued early after the first week. The magnitude of total weight change correlated well with the dose of STX administered (n = 16; r = 0.969).

The effect of STX on β cells of the pancreatic islets was confirmed using immunofluorescent and immunohistochemical staining for glucagon and insulin in a representative pancreas of each group. Standard H & E stained sections revealed reduced cellularity of the islets along with evidence of pyknosis (plates A and B, figure 4.10). When stained with anti-insulin and anti-glucagon antibodies, the sections demonstrated that there was a diminution of insulin storage and production (plates C and D). The colour plates also demonstrate the lack of fluorescence obtained in a STX affected pancreas (plates E and F). This can be interpreted as indicating either 'drop-out' of insulin producing cells or near absence of insulin production by the pancreatic islets, resulting in a hyperglycaemic state similar to Type I diabetes mellitus.
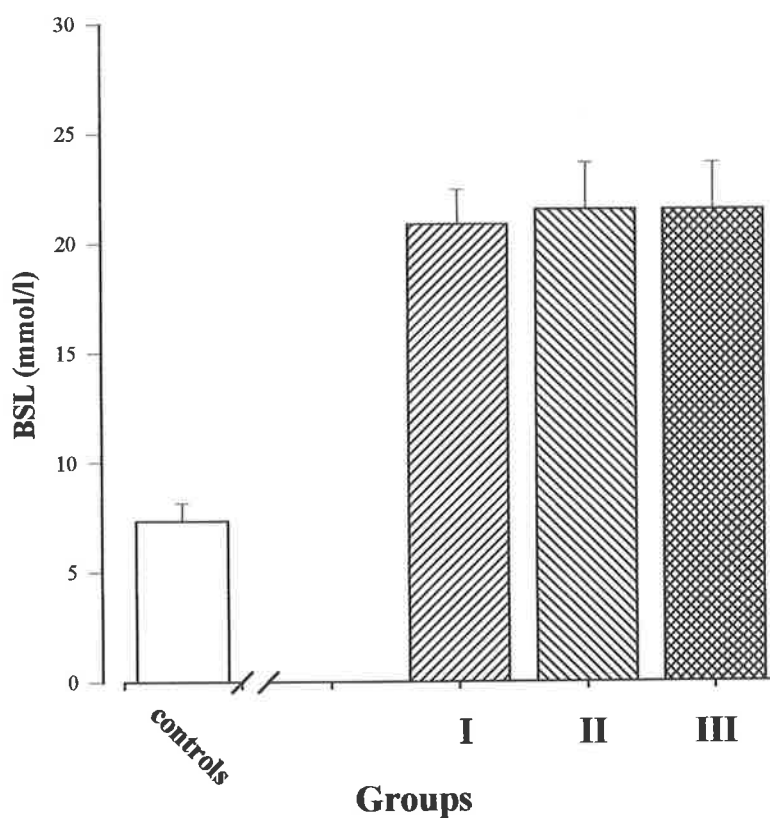
FIGURE 4.8. Blood glucose levels in rats 21 days after being rendered diabetic with the indicated doses of STX.

Symbols represent the mean ± SEM (n=4 per group). Hyperglycaemia consistent with diabetes mellitus was defined as a random BSL greater than 11mmol/l which was sustained over three separate determinations. Animals in group I received 45mg/kg, group II 55 mg/kg and group III 65 mg/kg.

FIGURE 4.9. Change in body weight subsequent to STX administration.

A) Results are represented as mean weight ± SEM (n=4). Doses of 45mg/kg (▲), 55mg/kg (●), 65mg/kg (■), or saline only (O) were administered intramuscularly 3 weeks prior to surgery.

B) Percentage change in body weight of animals by dose group 21 days (ie the day of surgery) after administration of STX. *p=0.0001 vs 45 mg/kg, 55 mg/kg, 65 mg/kg ANOVA with post-hoc Bonferroni test.

A)
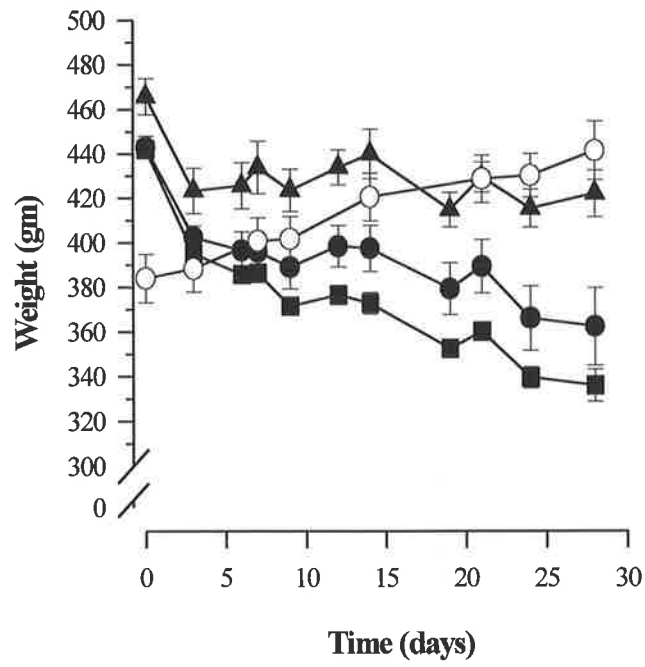
B)
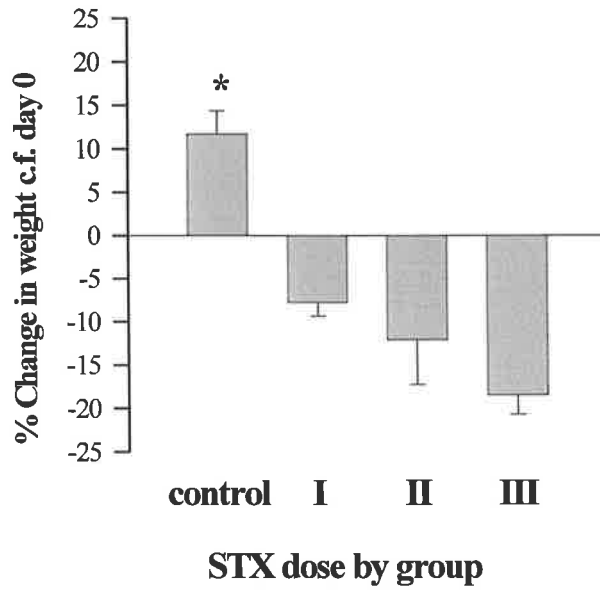
FIGURE 4.10. Colour plates of pancreas tissue from STX treated rats compared with normal groups.

H & E stained sections (Plates A and B); immunohistochemical staining (plates C and D) and immunofluorescent staining (plates E an F) of paraffin embedded sections of pancreas from control rats and animals treated with 55mg/kg STX

Sections were double stained using the Histostain-DS kit with α-insulin (red) and α-glucagon (blue/purple). Normal pancreatic tissue is shown in plates A, C and E: diabetic tissue is shown in plates B, D, and F. *Islet* = islet of Langerhans, β cells = insulin producing cells: α cells = glucagon producing cells; *pyk* = pyknotic cells

Normal   **STX - D.M.**

A

B

PYK

MØ

C

α Glucagon
stained cells

α Insulin
stained cells

D

E

F
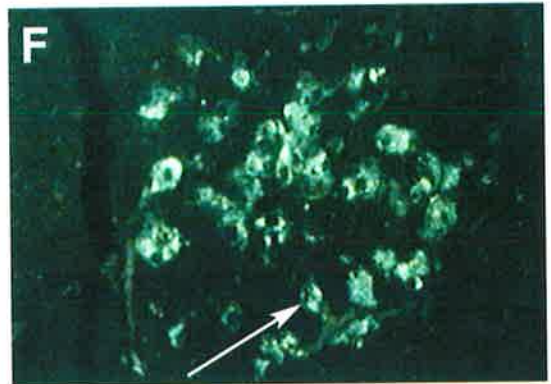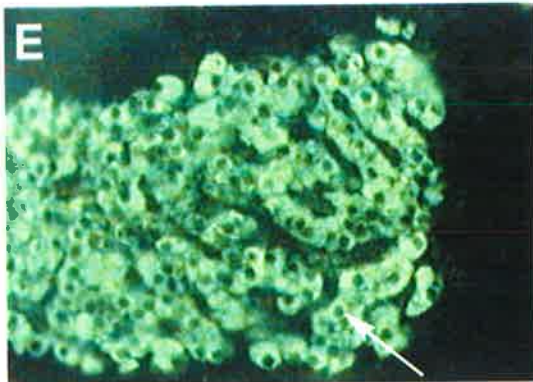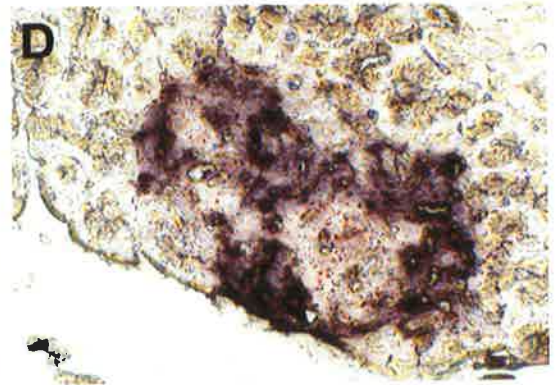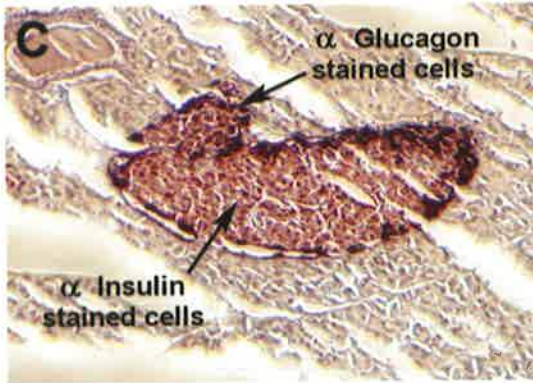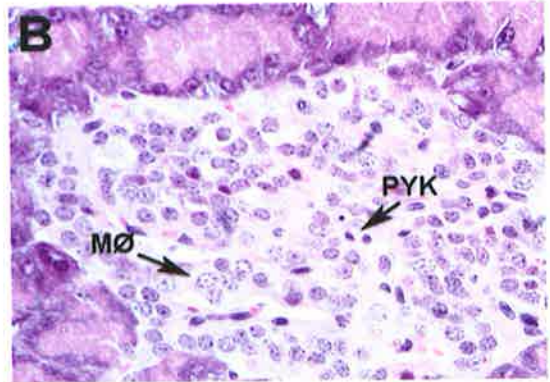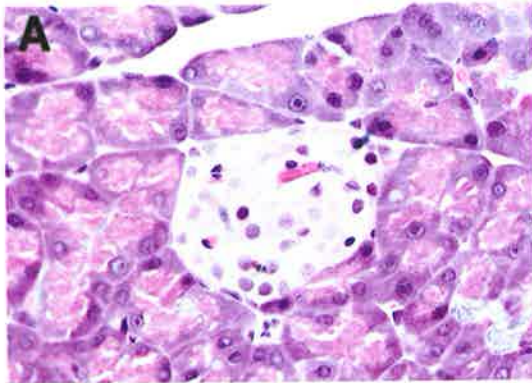
Insulin producing cells

4.4.3. ii) Wound Strength

When the pelts were harvested from these animals several observations were made. The skin was noticeably thinner than that of the normal animals, and the subfascial fat pads usually found in the region of the hind limbs had atrophied. The same features were noted of the body wall musculature. The graph in figure 4.11 shows how this catabolic state impacted upon wound strength. All groups treated with STX had significantly weaker wounds (n=16 strips per group) compared to the controls (ANOVA with Dunnett's post-hoc test $p < 0.001$ control vs group I-III)

A dose of 55 mg/kg of STX was determined as the most appropriate doses for the ensuing experiments using the diabetic model. This decision was based upon the reliability of the induction of diabetes, the weight loss data and that it was a dose that produced a sustained diabetic state. This compares to the lowest dose where animals began to gain weight towards the end of the time course and the highest dose which was associated with one death.
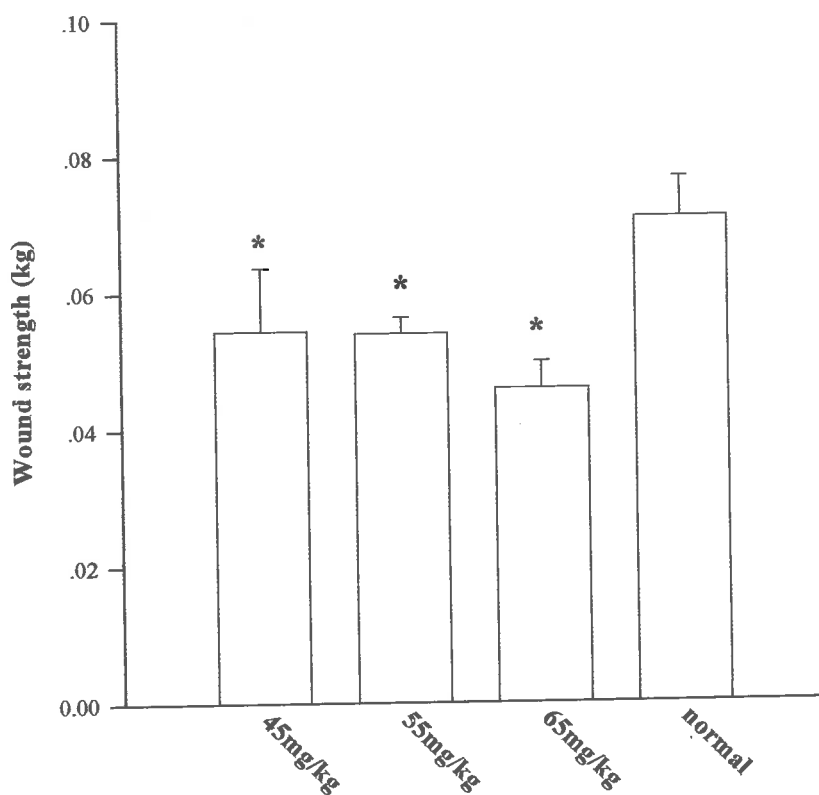
FIGURE 4.11. Wound strength related to dose of STX.

STX was administered as an intramuscular injection dissolved in 0.9% NaCl solution at a concentration of 50mg/ml. 'Normal' animals received an equivalent volume of 0.9% NaCl solution. Bars represent mean $\pm$ SEM (n=16). $*p=0.004$ (one way ANOVA, with Dunnett's post-hoc test).

4.4.4. rhIGF-I and LR³IGF-I in diabetic wounds

The effects of topical applications of rhIGF-I and LR³IGF-I were assessed in two experiments involving diabetic rats utilising the incised wound. In the first experiment 14 out of 16 were successfully rendered diabetic while there was one recorded death. The second trial included 20 animals that were successfully rendered diabetic. In each experiment six were kept euglycaemic to compare the vehicle treated wounds between diabetic and normal animals.

The animals used in both experiments did not differ statistically in their mean weights either at the commencement of the study or at the time of surgery on day 21. Data are summarised in figure 4.12 and represent the combined results from both experiments.

When applied to the incisional wounds of diabetic rats both peptides increased wound breaking strength compared to their paired controls but the change did not reach statistical significance for those wounds treated with rhIGF-I in contrast with the response to LR³IGF-I (100 µg/wound). In these wounds, wound strength was increased 30% compared to the paired control wounds (69 g ± 5 and 53 g ± 4) ($p = 0.01$; n=14).

The breaking strength of the vehicle treated wounds showed no overall difference between the two groups of animals and they were similar to the mean result obtained in the preliminary experiment 4.4.4 (wound strength for rats given 55 mg/kg was 54 g ± 2.4).
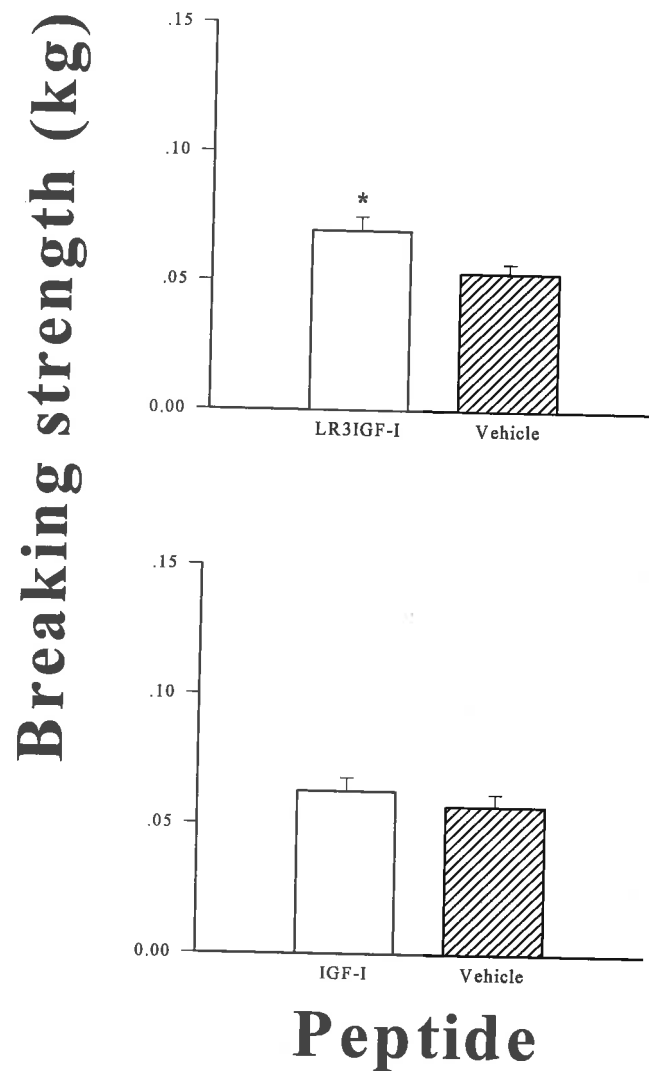
FIGURE 4.12. Comparison of wound breaking strengths in diabetic rats treated with rhIGF-I 100 µg/wound, LR^3IGF-I 100 µg/wound or collagen (vehicle).

Peptides were added at a concentration of 1mg/ml and wound strengths determined 7 days after surgery. Four strips were harvested from each wound and stretched to breaking point. The bars represent mean values ± SEM (rhIGF-I, n=14; LR^3IGF-1, n=15 animals) determined from combined results of two experiments. $*p = 0.01$ (paired t-test).

4.4.5. The influence of IGF-I and IGFBP-2 upon Wound Strength in Diabetic Rats

The influence of IGFBP-2 upon IGF-I activity in wounds was examined in this experiment which incorporated incised wounds in diabetic animals. Wounds were treated with rhIGF-I alone, IGFBP-2 alone or a combination of rhIGF-I and IGFBP-2 (see figure 4.13). None of the treatments significantly altered wound strength with the dosage used. While IGF-I appeared to increase wound breaking strength by 14% (80.6g $\pm$ 9 vs 70.2 g $\pm$ 8 for controls) this was not statistically significant. The combination of rhIGF-I with IGFBP-2 (56.7 g $\pm$ 6) appeared to reduce wound strength by 27% compared to those wounds which received collagen (78 g $\pm$ 9). Statistical significance was not reached for these data. IGFBP-2 alone (97.3 g $\pm$ 10) had no statistically significant effect upon wound breaking strengths compared to the vehicle (90 g $\pm$ 10).
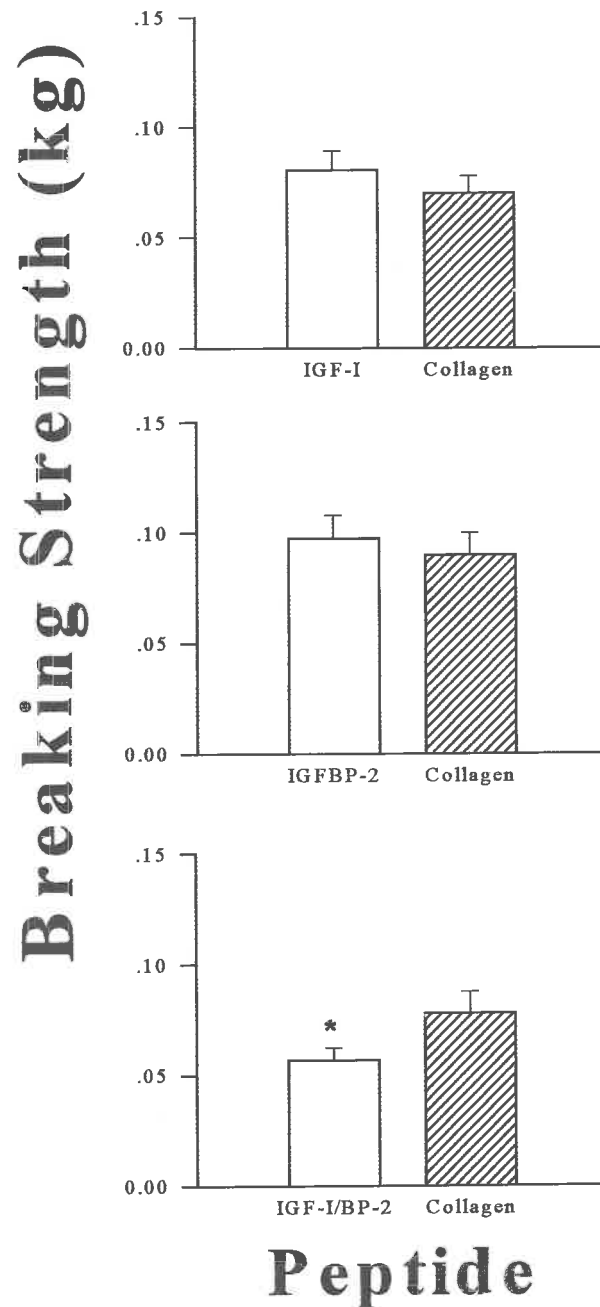
FIGURE 4.13. Effect of IGF-I/IGFBP-2 combination upon wound breaking strength. Incisional wounds in diabetic rats received IGF-I, 10 µg/wound; IGFBP-2, 40 µg/wound and IGF-I with IGFBP-2 (10 µg and 40 µg per wound respectively). Contralateral control wounds received an equivalent volume of collagen vehicle. The results represent the mean $\pm$ SEM of 32 wound strips (n=8 animals/group). $*p$ = NS vs vehicle (paired t-test).

4.5. DISCUSSION

Insulin-like growth factors have been applied in various concentrations to a rodent model of incisional wound healing to determine their efficacy as promoters of wound healing. The study also aimed to demonstrate the effect of IGFs on the delayed healing associated with diabetes and to determine the influence that IGFBP may have on IGF related changes in normal and diabetic wounded tissue. This study concentrated on the parameter of wound strength to provide a standardised method for analysing the end result of collagen synthesis and remodelling *in vivo* following topical application of growth factors. Several other studies in the literature have examined the effect of IGFs in wound healing (132,247,273,276). However they have used other parameters such as fibroplasia, neovascularisation or the counting of inflammatory cells which rely upon subjective interpretations of non-parametric data.

Human and animal studies support the theory that the physiological effects of GH are linked to IGF-I (89,92-95,268). Systemic exogenous GH can influence several acute metabolic stress parameters associated with injury. Gatzen et al (97) demonstrated an improvement in body cell mass and dampened changes in the relationship between extracellular to total body water in critically ill surgical patients. Herndon et al (98) used systemic GH to demonstrate its benefit in paediatric burns. Rates of donor site re-epithelialisation and total hospital stay were decreased in those patients prescribed GH. In an era of escalating costs associated with hospital inpatient care, studies such as this have established GH as the only systemically administered growth factor with a defined clinical application in the wound healing arena.

Normal subjects exhibit gains of net skeletal muscle protein when given GH (100,101). If administered via subcutaneous or intravenous routes, exogenous GH produced stronger wounds in laboratory rats (268) as well as increased collagen content of both intact skin (93) and granulation tissue (92,93). It has also been reported to increase the strength of incisional wounds in hypophysectomised animals (289) and animals affected by catabolic states including diabetes (290) and malnutrition (94). Zaizen et al used the bursting strength of laparotomy wounds in rats as their model and reported a dose dependent relationship between subcutaneous GH and wound strength which peaked at 500ng of GH/day with the greatest advantage seen in the first week.

Immunoreactivity for GH receptor is found in all layers of the skin, however it appears to be most pronounced in the epidermis and particularly the epidermal appendages as described by Lobie et al (91). Tavakkol et al (90) used polymerase chain reaction (PCR) techniques to better localise GH receptor transcript to melanocytes and dermal fibroblasts and specifically noted very little expression by keratinocytes. It can be hypothesised that GH directly influences hair growth and melanocyte differentiation or proliferation but its effect upon the keratinocytes is at best indirect. A proposed pathway would involve activation of dermal fibroblast proliferation induced by IGF-I secreted in response to systemic GH. IGF-I may activate keratinocytes in a paracrine fashion to promote epidermal cellular proliferation and differentiation.

Results from this study demonstrated that a single dose of topical GH did not influence the strength of incisional wounds when assessed seven days after application (see 4.4.1 and 4.4.2). This contrasts with the findings of Zaizen et al (94) and may reflect inadequate activation by a single topical dose of the cellular mechanisms that mediate GH responses.

Alternatively GH principally promotes IGF-I mRNA expression and particularly hepatic IGF-I protein production. However GH acts in selected tissues such as growing bone (291) and granulation tissue (95,96) to influence IGF-I secretion by target cells. Granulation tissue produced within Hunt-Schilling chambers expressed increased IGF-I mRNA in response to GH stimulation. However such expression has not been examined using incisional wound models. In addition it is unclear whether the changes seen in healing dermis are truly reflected by formation of granulation tissue within Hunt-Schilling chambers where the newly formed tissue is bathed in 'wound fluid'.

The minimal mitogenic response to GH documented in this study and also reported by Tavakkol et al (90) and Cook et al (124) provided evidence that GH is only a weak fibroblast mitogen. In addition IGF-I is more potent when used alone or where its secretion within a target tissue is promoted by systemic GH. The wound strength data clearly demonstrated the inactivity of GH *in vivo* when used topically (see 4.4.1)

Breaking strengths of wounds treated with IGF-I (100 μg/ml (100 μl/wound) were similar to those of the controls. Topical application of IGF-I improved wound strength at the higher dose of 1 mg/ml, 100 μl/wound. This represented a breaking strength about 40% greater than controls. Enhancement of breaking strength using IGF-I as a single large topical dose has not previously been demonstrated to be effective in normal animals (132,247,273,276). In states of impaired healing, such as diabetes or steroid affected animals, other parameters including hydroxyproline content and DNA synthesis are improved with IGF-I alone (46,127). However both studies used different models. Suh et al (127) utilised the Hunt-Schilling chamber and Greenhalgh et al (46) primarily studied responses in excisional wounds. In this study IGF-I has been used at concentrations that are higher than in previous

reports. Therefore variations in techniques of treatment administration and dosage may contribute to contradictory evidence between studies.

Replacing IGF-I with one of its analogues, LR^3IGF-I, did not replicate the effect obtained with the native peptide in normal animals. This contrasts with data obtained *in vitro* (see Chapter 3) and other animal studies that have shown systemic analogues of IGF-I's to have a greater general anabolic effect than IGF-I, particularly in metabolically impaired animals (143,145,146,150,255,292). Scheiwiller et al (286) demonstrated that infusions of IGF-I restored growth in insulin deficient diabetic rats as reflected by weight gain and tibial bone growth. Other studies have reported that N-terminus modified analogues of IGF-I, when administered systemically and continuously are able to restore growth in diabetic rats and therefore show greater potency than the native molecule (146). Similar effects are noted with the catabolic state induced by high dose systemic steroids (145) where weight loss can be reversed. Read et al (149) described the analogues as being several fold more potent than IGF-I in their observations of intestinal growth in rats pretreated with high dose dexamethasone. Tomas et al (150) suggested the effects of IGF-I and their analogues in diabetes are complimentary to those of insulin and may in fact facilitate to insulin activity by increasing tissue sensitivity to insulin. Elevated IGFBP concentrations, particularly serum IGFBP-3, provided an explanation for the difference between analogue and native peptide. They reduce the amount of free IGF-I but the analogues with their diminished affinity for IGFBP's, will not be affected in this manner.

The nature of IGFBPs in the wound and their roles may be important in finding an explanation for the efficacy of analogues in diabetic but not normal wounds. While IGFBPs are present in rat wound fluid their function at the wound site remains unclear (191). If they

protect IGF-I or help bind IGFs to the ECM (293) or if one of their functions is to present the peptide to cell surface receptors, then reduction of the binding affinity for these proteins may have several effects upon IGF activity *in vivo*. Firstly, IGF half life may be reduced due to the reduced protection from proteolytic enzymes within the wound. Secondly they may be cleared more rapidly because IGFBPs associated with ECM proteins will be unable to retain the peptide within the wound environs. In addition, IGFBPs -1, -2 and -3 contain within their sequence the arginine-glycine-D-asparagine (RGD) sequence which is the recognition sequence for integrins (257,294). These are a group of cell surface receptors which generally bind extracellular matrix molecules such as laminin, fibronectin and collagen and are believed to be associated with cell locomotion (70).

The attachment of an IGF/IGFBP complex to an integrin may enable presentation of the IGF peptide to its own cell surface receptor. This may potentiate the IGF response but may be an IGFBP dependent activity. Alternatively, as demonstrated by Jones et al (294), they may stimulate cellular events such as cell migration by themselves by using an RGD-mediated mechanism. While integrin binding is related to the RGD recognition sequence for IGFBP-1 and -3, IGFBP-2 is not known to bind integrin family members (295). Galiano et al (295) examined the significance of the RGD sequence in the IGFBPs and reported that IGFBP-2 did not influence healing in a rabbit ear dermal ulcer model either when applied alone or in combination with IGF-I. Further evidence for IGFBP-2 being an inhibitor of IGF-I activity has been produced by *in vitro* studies where it has been shown to inhibit IGF-I migration of smooth muscle cells (258) possibly by preventing IGF-I binding the type I IGF receptor. These reports correlate well with the present study's results for incisional wounds where neither diabetic nor normal wounds were influenced by the combination of IGFBP-2 and rhIGF-I.

Gockerman et al (258) has demonstrated that IGF-I analogues exhibited greater activity *in vitro* than native peptide in circumstances where there was an excess of IGFBP-2. In this study this situation was replicated in two ways by either adding exogenous IGFBP-2 or inducing an increase in endogenous IGFBP-2 which is a feature of diabetes (180,296-298). Lower molecular weight binding proteins (LMW-BPs) can be detected at increased concentrations in diabetic rat wound fluid (180) while diabetic wound fluid has characteristics which inhibit fibroblast growth (178). In both normal and diabetic experiments, the combination of IGF-I/IGFBP-2 had no effect upon wound healing and neither did IGF-I when used alone in the diabetic model. LR^3IGF-I improved wound strength in a model that was known to be associated with excess IGFBP-2 production.

The experiments described in this chapter confirmed impairment of the healing response in STX-diabetic rats. This was reflected by the reduced wound breaking strength of those animals rendered diabetic in the first experiment. Bitar and Labbad (161) similarly found that wound strength and collagen content were STX dose dependent. All doses of STX caused a significant reduction in wound strength compared with the normal animals. BSL and body weight data confirmed the diabetogenic effect of the STX. This was further supported by immunofluorescent staining of sections of pancreas taken from representative animals in each group. These sections confirmed the loss of β cells from the islets of Langerhans. The effect of insulin supplementation in these animals was not established as this would possibly confound the results obtained with the IGFs in the ensuing studies. The severity of diabetes ie weight loss that the animals could tolerate without insulin had to be determined. This preliminary study confirmed that while weight loss can exceed 20% of original weight over five weeks, this was tolerated by the animals. The catabolic state induced by STX and the consequent biomechanical changes might be construed as being

secondary to STX, however previous investigators have shown that these effects are prevented by insulin (299).

Experimental and clinical studies have documented factors which contribute to impaired healing in diabetics. They include clinically large vessel arterial disease, neuropathies (156) and a reduced collagen content (180,299). This reflects lower collagen synthesis and glycosaminoglycan (GAG) content as well as increased proteolytic activity within the skin (164). There may also be deficiencies of growth factors particularly IGFs and TGFβ (300).

Application of IGFs and other growth factors to experimental diabetic wounds is not unprecedented. Greenhalgh et al (46) examined the effect of IGF-I, IGF-II alone and in combination with PDGF-BB in a murine model of inherited diabetes. Their major finding was the synergism observed between IGF-II and PDGF-BB in healing and that all three factors improved the rate of healing. This same model has also been used to demonstrate efficacy for the application of acidic FGF (301).

Comparison of the recombinant protein with its analogue suggested that both were capable of improving wound strength and that LR^{3}IGF-I was more potent in reversing the detrimental effects of diabetes than IGF-I. The wounds treated with the analogue exhibited significantly increased wound strength compared to the paired control wounds. This suggested that the reduced binding protein affinity of LR^{3}IGF-I was beneficial at enhancing IGF-I efficacy in diabetic states. This finding contrasted with that observed in uncompromised wounds where IGF-I was more potent.

The influence of one of the IGFBPs (IGFBP-2) was tested in the diabetic model. The results suggested that this particular binding protein neither impairs nor augments the activity of

IGF-I efficacy in diabetic states. This finding contrasted with that observed in uncompromised wounds where IGF-I was more potent.

The influence of one of the IGFBPs (IGFBP-2) was tested in the diabetic model. The results suggested that this particular binding protein neither impairs nor augments the activity of IGF-I in these wounds. However given the effect of LR^3IGF-I it may be postulated that the accepted increase in IGFBP-2 concentrations in uncontrolled diabetes may inhibit IGF-I activity and this may account for the lack of response to rhIGF-I or rhIGF-I combined with IGFBP-2. In contrast other IGFBPs, BP-1 and -3, will augment the effects of IGF-I in compromised (276) and uncompromised wound environments (247). These effects appear to be molar ratio and model dependent (247,258).

4.6. SUMMARY

In summary GH alone was inactive in this model of wound repair where a single dose was applied at the time of wound closure. In contrast IGF-I was able to increase breaking strength. The lack of GH effect may be explained by the observation that in other studies multi-dosing of GH may be required to stimulate expression of IGF-I which then exerts autocrine and paracrine effects upon dermal and epidermal elements. Therefore GH may exert its effects in a fundamentally different manner in peripheral tissues compared to its influence in the liver where it promotes the synthesis and release of IGF-I into the systemic circulation which in turn acts in an endocrine fashion. The presence of GH receptors and binding protein on dermal fibroblasts supports this hypothesis. However this cell line does not exhibit proliferative or synthetic responses when isolated and cultured in serum free media containing only GH (see chapter 3).

Manipulation of the binding affinity of IGF-I to its binding proteins has been explored in normal and compromised rodent models of incisional wound repair. Only in normal animals and at a high dose (100 µg/wound, 1 mg/ml) can exogenous IGF-I improve the breaking strength of incisional wounds. In compromised metabolic states where IGFBPs are probably elevated compared to normal animals the analogue demonstrated its potential as a more effective topical agent. This suggested that endogenous IGFBPs impact upon the potency of exogenous IGFs perhaps by 'soaking up' the IGF-I thus preventing them from having an immediate effect at the cellular level. The functions of IGFBP-2 may not include presentation of IGF-I to cells therefore it may not promote receptor binding and activation

by the IGFs. For the normal wounds large exogenous doses of IGF-I may have allowed sufficient free IGF-I to reach the cell surface. The lack of effect of LR^3IGF-I in the normal model remains difficult to explain. It may partly relate to the role some IGFBPs have in binding the IGF/IGFBP complex to cell surface integrins and then presenting the IGF molecule to the IGF receptor.

Diabetes does affect incsional wound repair in rodents and this effect is partly reversed by LR^3IGF-I but not IGF-I. This effect can probably be explained through a knowledge of the balance of IGFBPs reported in this model. As previously discussed IGFBP-2 is inhibitory to IGF function and its concentration is elevated in diabetes. Therefore exogenous rhIGF-I with its greater affinity for IGFBPs may be less effective than the analogue, since the response to IGF-I was further depressed when administered with exogenous IGFBP-2 although not significantly.

While some implications of this series of studies at a clinical level remain unclear, LR^3IGF-I may have a place as a topical agent to augment healing of incised wounds in diabetics.

CHAPTER 5

EXCISIONAL WOUNDS: RESPONSE OF NORMAL AND DIABETIC WOUNDS TO TOPICAL IGF-I AND TOPICAL LONG R^3 IGF-I

5.1 INTRODUCTION

5.1.1. Objectives

5.2 MATERIALS

5.3 METHODS

5.3.1. Preparation of Treatment Solutions

5.3.2. Induction of Diabetes

5.3.3. Surgical Technique and Post-operative Care

5.3.4. Data Collection and Monitoring of animals

5.3.5. Statistics

5.5. RESULTS

5.4.1. Blood Sugar Levels and Weight Change

5.4.2. Wound Healing Data

5.5. DISCUSSION

5.1. INTRODUCTION

Excisional wound models have been developed to examine granulation tissue formation, wound contraction and wound epithelialisation. Previous chapters have discussed IGF physiology and examined the effect of IGFs on compromised incised wounds in both normal and compromised healing states. Also the effects of IGF-I and its analogues have been explored using cultured fibroblasts in models of lattice contraction, cell proliferation and protein synthesis. In particular the FPCL contraction studies demonstrated that IGF-I and especially the analogues can promote fibroblast contractile activity. These results encouraged further investigation of the effects of IGF-I and LR^{3}IGF-I on healing by secondary intention in both normal and diabetic rats.

While treatment of diabetic excisional wounds with growth factors including IGF-I (46,135), acidic FGF (301) and PDGF (46) has been described in the literature, obese genetically diabetic mice were used rather than rats that have a chemically induced diabetic state. One of the principal findings with these studies is that the use of topical agents, in particular aFGF alone and IGF-I or IGF-II combined with PDGF, can accelerate wound closure in a diabetic animal however the rate of healing is not returned to that of normal animals.

States of impaired healing due to steroids, diabetes, malnutrition or vascular insufficiency cause significant morbidity and consume health care resources (302). Aberrations in IGF physiology due to diabetes include altered IGFBP profiles, particularly increases in IGFBP-

2 (180) and reduced levels of 46 kDa species (consistent with IGFBP-3) in conjunction with reduced systemic IGF-I concentrations (161). Collagen metabolism is also impaired resulting in reduced collagen content in the skin of diabetic animals (299).

Given the tissue culture results presented in chapter 3 demonstrating the anabolic activity of IGFs, particularly the analogues and the effect of diabetes upon the bioactivity of IGF-I exogenous analogues of IGF-I may potentially reverse impaired healing responses associated with diabetes.

5.1.1. Objectives

The aim of this study was to examine the effect of topical IGF-I and its analogue LR^{3}IGF-I on excisional wound healing in both normal and diabetic animals.

A)
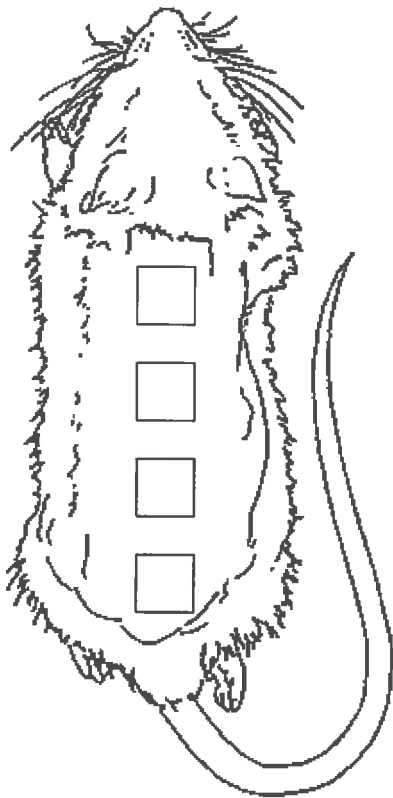
B)
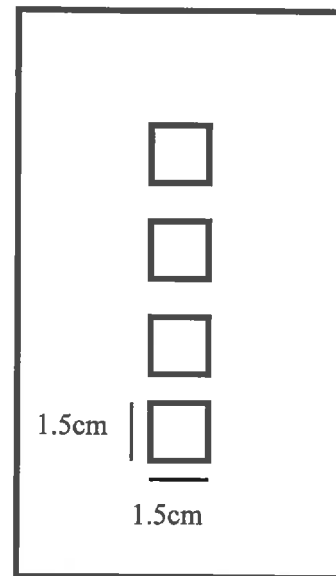
FIGURE 5.1. Schematic representation of the rodent wound contraction model (A).

Wounds were created under general anaesthesia using a template of four squares each measuring 15 mm x 15 mm along the inside margins (B). Wound closure was documented by tracing both the dermal and epithelial margins on the day of surgery and thereafter until day 28. Histological samples were obtained from the two cranial wounds with tensiometry performed only on those wounds not previously biopsied.

5.2. MATERIALS

Sprague-Dawley rats were obtained from the Central Animal House, University of Adelaide, and divided into two groups with weight ranges of 200-250 g and 250-300 g on arrival. The animals in the heavier group were given STX.

Collagen, STX, IGF-I and LR^3IGF-I were obtained from the same sources as chapter 4. Isoflurane (Forthane, Abbott Australasia, Sydney, Australia) mixed with N_2O: O_2 was used to provide general anaesthesia for the days of shaving, wounding and tracing of wounds. Povidone-iodine (Betadine, Mundipharma, Switzerland) and 70% ethanol were used to clean the dorsal skin. A semipermeable polyurethane dressing, Opsite®, was supplied by Smith & Nephew, Hertfordshire, U.K. Wound strips were stretched using a Mecmesin tensiometer (Model PCM 2500 EL, Mecmesin, U.K.).

5.3. METHODS

5.3.1. Preparation of Solutions

LR^{3}IGF-I solutions were prepared in concentrations of 100μg/ml and 1mg/ml mixed with collagen while rhIGF-I was prepared at a concentration of 1mg/ml. The control solution (collagen only) and the peptides were applied in volumes of 100μl to each wound on alternate days for 7 days.

5.3.2. Induction of Diabetic State

One week before surgery the animals were divided into two groups and the heaviest 32 animals were allocated to the diabetic group. The rats in this group were injected intramuscularly with STX, 55mg/kg (50mg/ml in citrate buffer, pH 4.5), while the control group received an injection of citrate buffer vehicle. Weights and BSLs were recorded on the day before surgery after which these groups were further randomised into four treatment subgroups as detailed in table 5.1.

Table 5.1. Treatment Groups

Diabetic (n=32)		Normoglycaemic (n=32)	
(dose/wound/treatment)	No. per gp	(dose/wound/treatment)	No. per gp
100 μg IGF-I	8	100 μg IGF-1	8
10 μg LR^{3}IGF-I	8	10 μg LR^{3}IGF-I	8
100 μg LR^3 IGF-I	8	100 μg LR^{3}IGF-I	8
Vehicle only	8	Vehicle only	8

5.3.3. Surgical Technique and Post-operative Care

Animals were shaved under gaseous anaesthesia (Isoflurane 2.5% with 2:3 N_2O and O_2) the day before wounding.

The rats were anaesthetised and the skin prepared with povidone-iodine and 70% ethanol. Wounds were created on the dorsum of each animal using a template of four 15 mm x 15 mm squares (figure 5.1). After tracing the fresh wounds onto transparent acetate sheets the first of five treatment applications were applied. Each animal's wounds were dressed with Opsite® after each treatment application. This procedure aimed to maximised peptide exposure to the wound before the solution dried or was licked off by the rats. Within a few hours each rat had removed its dressing.

All animals were housed in individual wire bottomed cages (50 cm x 30 cm x 25 cm) that contained a terracotta pot in which the animal could shelter. They were kept in standard animal laboratory conditions of 22°C with a 14 hour light/ 10 hour dark cycle with water

and food provided *ad libitum*. Adequate water supply was ensured with a 600 ml water bottle one per cage.

5.3.4. Data Collection and Monitoring of Animals

All tracings were made under general anaesthesia and treatments were applied on days 0, 1, 3, 5, 7 from time of surgery. Body weights were recorded on each day of surgery and the wounds were traced prior to application of treatment solutions. Tracing of wounds was conducted on days 0, 1, 3, 5, 7, 9, 12, 14, 16, 19, 21, 23, 26, 28 and 29. These tracings included both the wound and the advancing epithelial edges once it was apparent. Wound strengths were determined on day 28 or 29.

The collected wound tracings were scanned using a Color One Scanner (Apple Computer Inc, Cupertino, CA, USA) and Ofoto v2.0 software (Light Source Computer Images Inc, Marin County, CA, USA). These images were colour coded and digitised using the Prism Image Analysis software system (Dapple systems Inc, Sunnyvale, CA, USA) to allow computation of wound image areas.

Curves were plotted using Sigmaplot Scientific Graphing Software v 2.01 (Jandel Scientific Inc, San Ramon, CA, USA).

Sampling for histology was performed using four animals per group on days 3, 7, 14, 21 and 28 or 29. A single wound on each of these animals was used only on days 3, 7 and 14 to assemble a chronological representation of the wounds. Once wounds were biopsied they were excluded from tensiometric analysis and further wound area analysis. Tissue specimens were fixed in 4% wt/vol paraformaldehyde/PBS solution.

BSLs were recorded weekly for the diabetic groups and at the time of surgery and on the day of sacrifice in normoglycaemic animals with samples being obtained as described in 4.3.1.

On kill days all animals were weighed and blood obtained for BSLs prior to death as described in 4.3.1. The dorsal pelts were harvested, four wounds/animal excised for tensiometric studies except where biopsies had been taken. These biopsy wounds were excluded from statistical analysis and tensiometry.

5.3.5. Statistics

Data were expressed as mean values $\pm$ 1 SEM and were analysed using Sigmastat v.2.0 (Jandel Scientific, San Ramon, CA, USA). Body weight and BSL data for the diabetic animals and the normoglycaemic rats were compared using unpaired Students t-test and the effects of the treatment preparations by two-way ANOVA partitioned according to diabetes and treatment with Student-Newman-Keuls post-hoc multiple comparison analysis.

Wound epithelialisation

The rate of wound re-epithelialisation was calculated using the same formula as that used for contraction. The results for wound epitheliasation and contraction were similar for the first 9 days therefore it can be concluded that epithelialisation did not contribute significantly to wound closure until day 9 post-operatively. This component of wound healing appeared to account for the final 20% of total wound closure although it commenced earlier when closure was 65% to 70% complete.

In contrast to contraction, epithelialisation was not retarded by diabetes in this model (p = NS, two way ANOVA) nor was it influenced by topical IGF-I or LR^{3}IGF-I. Those diabetic animals that received LR^{3}IGF-I 10μg/wound/application re-epithelialised their wounds faster than the diabetic animals which received collagen only ($p < 0.05$, Students t-test). However it needs to be emphasised that there were only 6 animals in this group as one died and another failed to become diabetic. Therefore confirmation of the apparent acceleration in re-epithelialisation noted for the group of *diabetes x LR^{3}IGF-I 10μg/wound/application* would be required to exclude a falsely positive result.

5.4. RESULTS

5.4.1. Blood Sugar Levels and Weight Change

Conversion to a diabetic state was confirmed using the same criteria used in the incisional wound studies (4.4.2). BSLs for the time course of the study are shown in figure 5.2. The BSLs for the STX-treated rats rose dramatically by the end of the first week after they were injected with STX. The BSLs remained elevated for the duration of the study confirming, established diabetes. One rat succumbed within the first week and two others failed to develop hyperglycaemia.

Body weight data are shown in figure 5.3 and these confirmed the effect of diabetes upon growth rates. Growth of STX-treated rats exhibited a plateau which was maintained for the time course of the study. Another important observation was the effect that multiple anaesthetics had upon the normal animals. These animals exhibited a slowing of their growth with each anaesthetic. Therefore protein malnutrition may be another feature of this particular model.

5.4.2. Excisional Wound Closure:

This is divided principally into three sections:

- wound contraction;

- wound re-epithelialisation;

- wound strength.

Wound contraction and epithelialisation data are presented as a ratio of the residual wound area divided by the original wound area recorded on the day of surgery and expressed as a percentage (% $Area_{t0}$). A similar technique has been utilised by Matuszewska et al (301).

potential sources of error arise in the actual tracing of wounds both the remaining ulcerated area and the rim of neoepithelium. By having the same person perform this task this error of interpretation would be consistent. Its accuracy was enhanced by having the animals anaethetised unlike Cross et al (303) and Teo (304). Digitalising the tracings provides an other important source of observer error but again the same operator was used and therefore the error was consistent.

Means with one SEM at each time point for each group of animals were plotted (figure 5.4).

Wound contraction

Wound contraction was retarded in those animals rendered diabetic. This was most apparent between day 3 and day 12. Statistical comparison using two way analysis of variance revealed significant retardation in all diabetic animals regardless of the treatment peptide at days 5, 7 and 9 ($p < 0.001$ days 7 and 9 diabetic vs normal). This significant retardation of wound contraction represented a delay of approximately 2 days in the healing of diabetic wounds when compared with those wounds on the backs of normal animals. Importantly, wound contraction appeared to plateau between day 11 and day 13 of healing leaving a residual percentage of original wound area (x) that closed principally by re-epithelialisation. In all treated and control groups this value was approximately 20% of $Area_{t0}$.

Topical administration of rhIGF-I or LR³IGF-I at either dose did not influence the rate of wound contraction in either diabetic or normal animals.
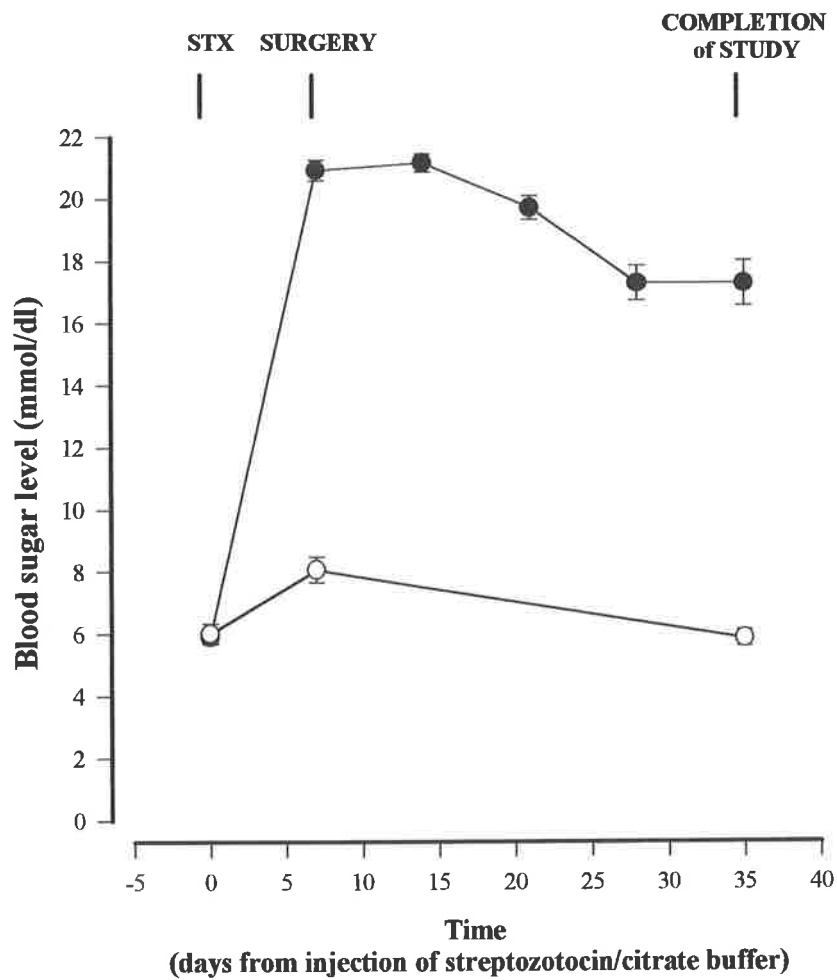
FIGURE 5.2. Blood sugar levels of diabetic (●) and normal (○) rats recorded during the experiment.

All animals administered I.M. STX (55mg/kg) or citrate buffer. Each point represents the mean ± SEM of 32 animals (normal) and 30 animals (diabetic).
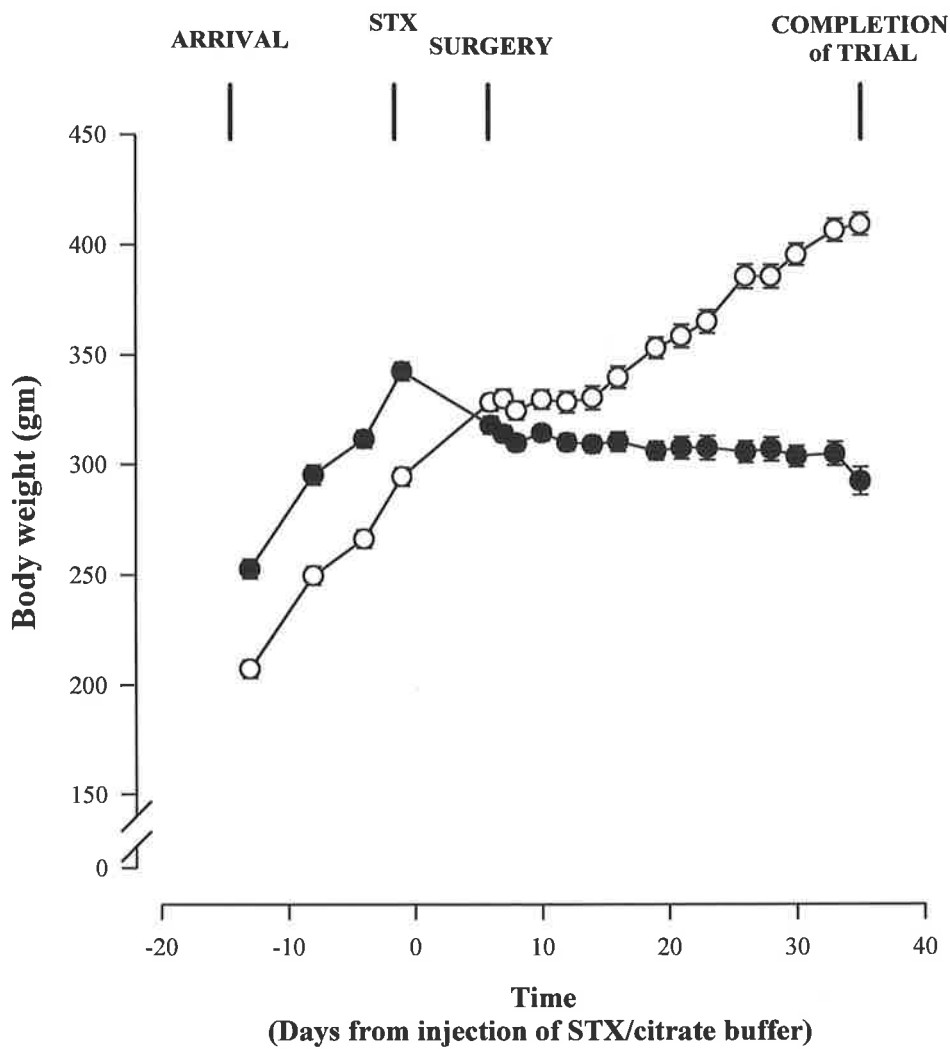
FIGURE 5.3. Body weights over the time course of the study.

Rats were administered vehicle (O) or STX (55mg/kg) (●) on day 0. Surgery (ie creation of wounds) was performed on day 7 post-injection. Each point represents the mean ± SEM represent (n=30 for diabetic rats and n=32 for normal animals).

FIGURE 5.4. Excisional wound contraction produced by IGFs.

The graphs compare: A) LR^{3}IGF-I 10 μg/wound/treatment (diabetic, ●; normal, ○); B) LR^{3}IGF-I 100 μg/wound/treatment (diabetic, ●; normal; ○); C) IGF-I 100 μg/wound/treatment (diabetic, ●); normal, ○); with vehicle only (diabetic, ■; normal, □). The symbols represent the mean ± SEM, n = 6-9 animals (no. of wound ≥ 18 wounds/group). *p<0.05; **p<0.01; ***p<0.001 (two-way ANOVA with Student-Newman-Keuls post-hoc test). ('x' represents the residual area of wound once the contribution to closure associated with wound contraction has plateaued.)

A)

B)

C)
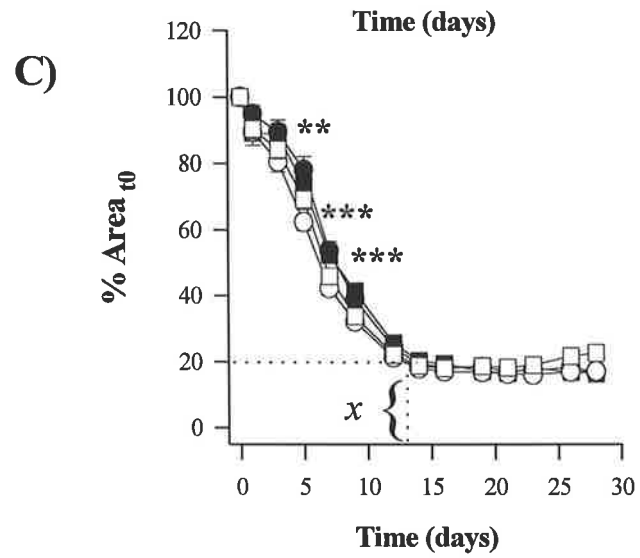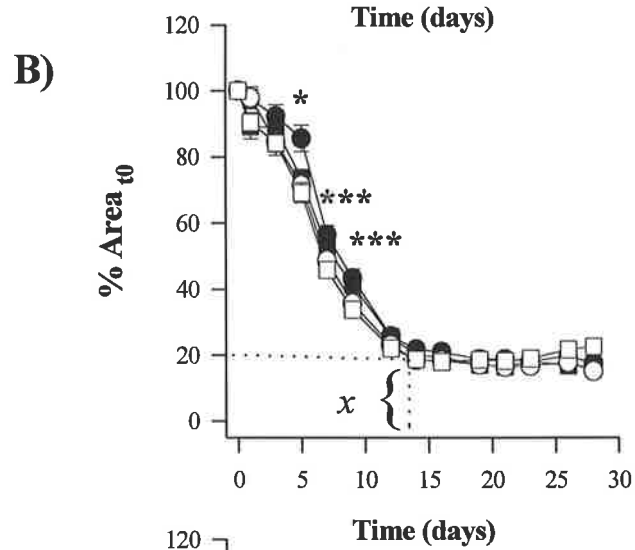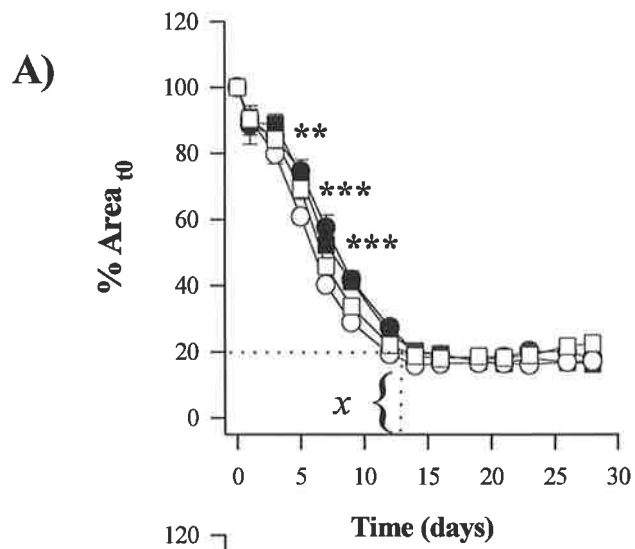
FIGURE 5.5. Wound epithelialisation following 5 treatment applications over 7 days.

The graphs represent the mean values ± SEM (n = 6 - 9) comparing A) LR^{3}IGF-I 10 µg/wound/treatment (diabetic, ●; normal; ○); B) LR^{3}IGF-I 100 µg/wound/treatment (diabetic, ●; normal, ○); C) IGF-I 100 µg/wound/treatment (diabetic, ●; normal, ○); with vehicle only (diabetic, ■; normal, □). Data were statistically compared using two-way ANOVA with Student-Newman-Keuls post-hoc test.

A)
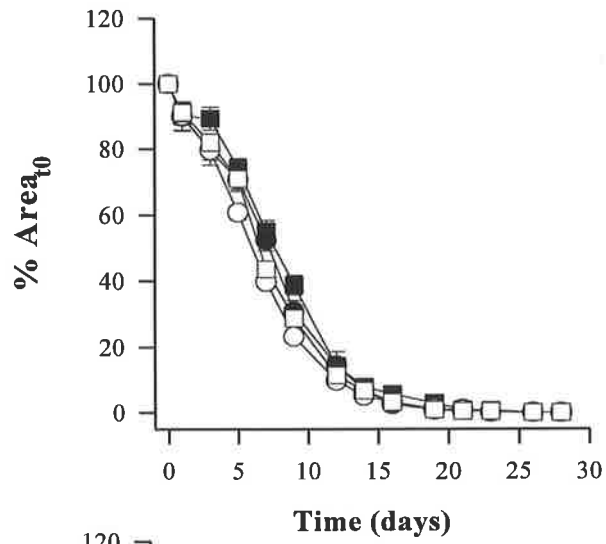
B)
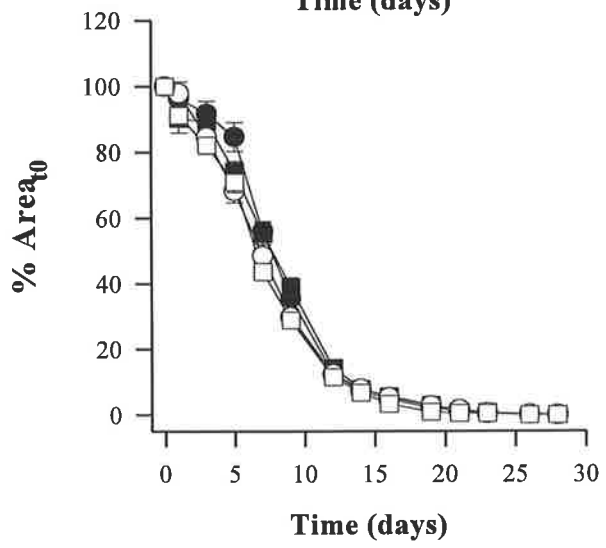
C)
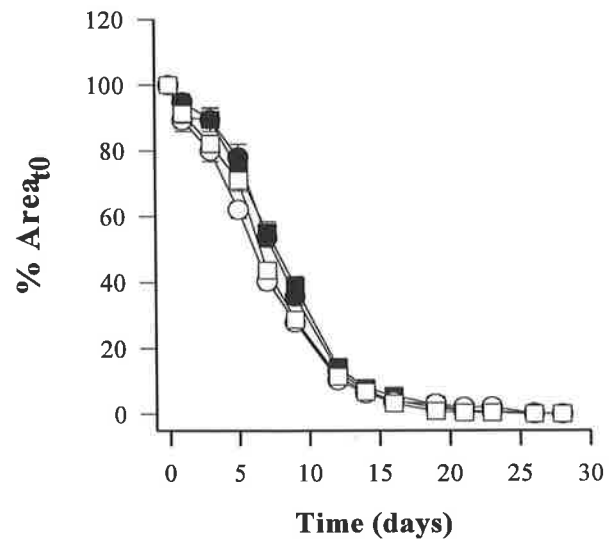
5.4.3. Wound Breaking Strength

The healing response in excisional wounds can be assessed also by a comparison of wound strengths and associated parameters such as yield energy and wound breaking strength or peak load. Strips 5mm in width were cut from the contracted wounds and stretched to breaking point thus giving the wound breaking strength. The yield energy (or relative failure energy) was calculated from the area beneath the force vs time displacement curve and represents the energy absorbed prior to sample disruption. Wound breaking strengths were significantly decreased by diabetes in all groups ($*p < 0.001$). Furthermore application of IGF-I or its analogue LR^3IGF-I did not enhance final wound strength using a regimen of five doses of over the first 7 days from wounding (figure 5.6).

Statistical analysis of the data confirmed that only diabetes contributed to any significant alteration in the yield energy of the healed contracted wounds ($*p < 0.001$, two way ANOVA).

A)

B)
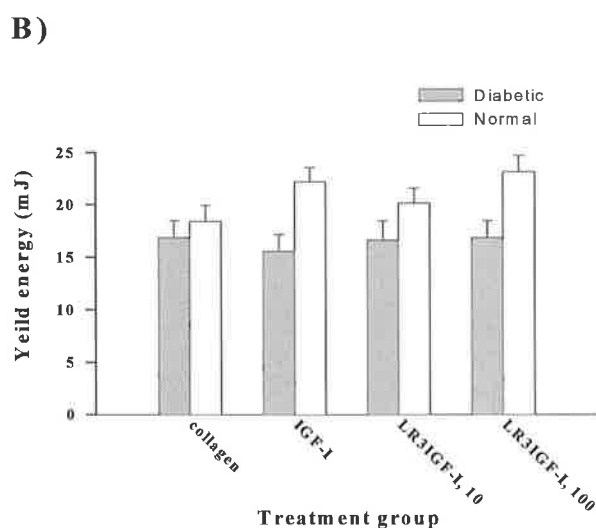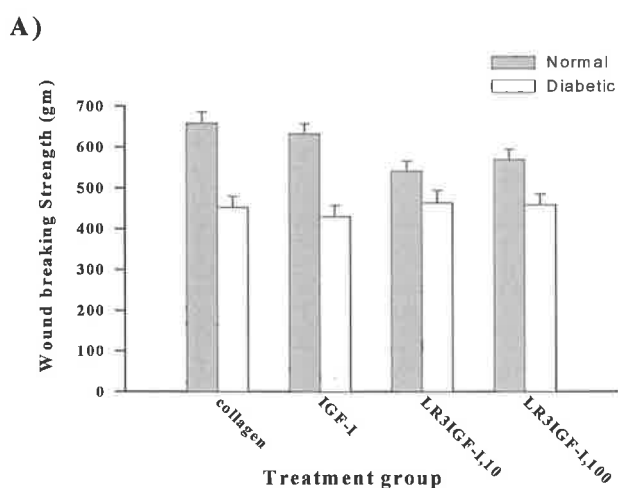
FIGURE 5.6. Tensiometry data for wound strips harvested from the contracted wounds at day 28.

Wound breaking strength (A), and Yield energy (B) for the indicated treatment groups were determined stretching wound strips 5mm in width, to breaking point. The bars represent mean values ± one SEM (n=18 to 24 wounds/group). Diabetes significantly reduced wound breaking strength and yield energy ($p < 0.0001$, two way ANOVA)

5.5. DISCUSSION

Andreassen et al (305) and Greenwald et al (306) have previously shown that streptozotocin induced diabetes is associated with weakened incisional wounds. This has been confirmed by the studies reported in chapter 4. Therefore diabetes was induced in a model of healing by secondary intention.

As in chapter 4, STX induction of diabetes produced sustained hyperglycaemia and weight loss for the 5 week period of the study. Therefore it can be concluded that this model is reliable even up to 5 or 6 weeks after administration of STX. This model of diabetes has not been described in the wound healing literature for excisional wound studies. Previous studies involving STX-induced diabetes have used this model to examine healing within three to four weeks from the time of STX administration and these have predominantly been incisional wound studies (305-307). Where animal models have been employed to examine the effect of growth factors upon wound contraction and re-epithelialisation a genetic murine model of diabetes has been utilised (45,46,135,301). While reliable and convenient the mouse model is limited by the size and the number of wounds that can be created on each animal. The same criticism can be levelled at the rat model in comparison to larger animal models. However this model is more convenient, allows multiple wounds and can be manipulated easily if systemic agents such as insulin are to be administered. This study has clearly demonstrated that untreated diabetes impairs wound healing by not only reducing the final strength of wounds but also retarding wound contraction. Therefore this model can be used successfully as one of impaired healing by secondary intention.

Wound closure was completed in both diabetic and normal rats within 15 to 18 days from surgery. All groups demonstrated maximum contraction by day 14 with complete epithelialisation by day 18. While this did not represent a delay in total wound closure associated with diabetes, contraction from the first to the ninth day for diabetic wounds was retarded by 2 days compared to normal wounds. This effect was related statistically to the state of diabetes and not to any of the applied treatments. The contraction of normal rat wounds is most pronounced during the first ten days after wounding (205,308) as previously observed by Cross et al (303) in the validation of their model.

Wound contraction takes place during the proliferative phase of wound repair and is characterised by increasing fibroblast/myofibroblast population size (309). Factors at a biochemical and cellular level that may contribute to the delay in contraction of the diabetic wounds include altered leucocyte function during the inflammatory and proliferative phases (5) and reduced collagen synthesis in this model (299,310). Other factors in this model that may influence contraction include reduced migration of fibroblasts and inflammatory cells into and around the wound (45) as well as absolute reductions in fibroblast numbers. An important distinction to make between this rat model and the genetic model used by Greenhalgh et al (45,46) is that the mouse model is obese and as stated by Greenhalgh wound contraction accounts for only 30 - 40% of closure compared to the 80% documented in this study. Therefore the contribution to wound closure that can be attributed to epithelialisation becomes difficult to assess accurately as it is relatively small. While fibroblast and inflammatory cell numbers have not been compared in this current experiment it remains the subject of ongoing research and analysis of histological sections in our laboratory.

Wound strength is determined principally by two factors;

(i) the density of newly synthesised collagen and

(ii) the reorganisation and remodelling of that collagen from type III collagen to type I (205).

Therefore wound strength is influenced by the intensity of fibroblastic activity which tends to markedly increase towards the end of the first week of healing while collagen remodelling continues for months after complete closure of the wound as demonstrated by Levenson et al (87). Wound strength is therefore a function of the maturity of collagen remodelling and the amount of newly formed collagen. Collagen cross-linking may have been retarded significantly by diabetes as wound strengths in the diabetic animals were generally lower than their normal counterparts. Collagen deposition may also be reduced by any retardation of fibroblast and inflammatory cell migration into the wound (45). Increased collagenase activity, increased glycosylation of collagen and reduced hydroxyproline concentrations have also been implicated (311). Certainly *in vivo* studies exist to demonstrate reduced collagen content in intact skin (299) and reduced collagen synthesis in incised wounds (306,307). In addition, diabetic serum can inhibit collagen production by fibroblasts (178).

In excisional wounds treated five times over seven days neither IGF-I nor LR^{3}IGF-I affected wound healing parameters. Failure of the growth factor treatments to influence wound closure may be related to several factors. The adherent coagulum that exists in the first three to five days on excisional wounds could provide an impervious barrier to peptide absorption. While occlusive dressings were used to maximise treatment exposure to the wound and attempt the simulation of a moist wound healing environment the dressings could not be maintained more for than 6

hours on the backs of the animals. This subsequently allowed the formation of the coagulum. It is also possible that a significant amount of the treatment preparation may have adhered to the dressing even though the collagen was allowed to form a gel before the dressings were applied.

While technical factors may compromise treatment absorption other more fundamental physiological factors may also explain the lack of response to IGFs. The expression of cell surface receptors for IGF-I and the secretion of IGFBPs will also affect the response at a cellular level. Antonaides et al (189) showed that in acutely wounded explants of porcine skin IGF-I mRNA expression and IGF type I receptor were present before and after injury, but were not up-regulated in either the dermis or the epidermis following wounding. Even though IGFs improve excisional wound closure in diabetic mice, (46), the lack of IGF receptor up-regulation related to PDGF and reduced endogenous IGF-I expression, may have contributed to our failure to demonstrate a positive IGF-I effect. Therefore a competence factor such as PDGF may be required to maximise any IGF response in our model including the response to the analogues.

The influence of IGF-I may not be directed primarily to the dermis. Several studies have shown that IGF-I mRNA is expressed by fibroblasts originating from the dermis (125) and from granulation tissue (126). In addition epithelial cells, namely keratinocytes, express IGF-I receptors during healing (312,313). IGF-I produced by fibroblasts has been demonstrated by Barreca et al (223) to be partly responsible for keratinocyte growth when cultured fibroblasts are employed as a 'feeder' layer. These studies indicate a paracrine activity of IGF-I. Therefore it is possible that IGF-I exerts its major influence upon re-epithelialisation rather than wound contraction. As up to

80 % of wound closure in this model is attributable to contraction, any delay in re-epithelialisation may be masked by the large contribution that contraction has to the healing response. A tight skin mouse model exists to examine re-epithelialisation of full thickness wounds (205). Therefore the ability of IGFs to promote epithelialisation may be better examined in such a model.

The complex modulation of IGF activity in the wound is increased by the known existence of IGFBPs in rat wound fluid (191) and alteration of their profiles with diabetes (180). IGFBPs, along with proteases found in wound fluid (180,191,202,203), influence the balance of free IGF-I within the wound milieu by protecting it from degradation and providing a slow release pool of active peptide. They may simultaneously prevent IGFs binding to cell surface receptors which contributes to inactivation of the peptide. The issue surrounding IGFBPs is especially relevant to diabetic models where IGFBP-2 is known to be proportionally increased in experimental insulin dependent diabetes mellitus (257,314) and is recognised as an inhibitor of IGF-I activity (277).

5.6. SUMMARY

In summary this chapter presents a combination of two rodent models, the excisional wound and the STX-induced diabetic rat. The data show that diabetes will persist in these animals for nearly six weeks and therefore this excisional wound model of diabetes can be used to investigate effects of diabetes on open wound healing. This has relevance to human diabetic ulcers or acute wounds that have been surgically

debrided. That the rat is a valid and useful diabetic model is reflected in the wound contraction delay of 2 days when compared to normal animals.

Tissue culture studies (chapter 3) showed some promise for IGFs as agents to promote excisional wound healing. However other factors including mechanisms of vehicle delivery, the presence of proteolytic enzymes, receptor expression and levels of various IGFBPs may play a role in determining the net effect attributable to IGF-I. A greater understanding of anabolic events within the wound would be gained by examination of processes involved in the induction of wound derived IGF peptides and receptor mRNA transcripts by exogenous peptides. This is especially important in metabolically compromised states such as diabetes.

CHAPTER 6

DISCUSSION AND SUMMARY

6.1. IGF PHYSIOLOGY

6.2. IGFS AND IGFBPS IN WOUND FLUID

6.3. *IN VIVO* WOUND HEALING AND THE IGFS

6.4 CONCLUDING REMARKS

This study has examined the ability of IGF-I to facilitate dermal healing as a topical agent with a focus on diabetic wounds.

6.1. IGF PHYSIOLOGY

The anabolic characteristics of IGF-I (289), its activity in those cell lines associated with wound repair (71,90,133) and its expression by fibroblasts at the wound site (313) all suggest that IGF-I could play an important role in wound repair. These characteristics are similar to those of insulin (109) in that under normal circumstances both insulin and the IGFs have anabolic effects but insulin has a much greater influence upon glucose homeostasis.

The bioavailability and production of IGF-I is regulated by a wide variety of influences including GH (253), nutrition (315), metabolic status and the IGFBPs. It exhibits endocrine, paracrine and autocrine activity. IGF-I is produced by the liver and complexed with IGFBPs in the systemic circulation. IGFBPs have several important roles including prolonging the half lives of IGF-I and II as well as providing a means for distributing IGFs from the intravascular to the interstitial spaces (257). Of the six known binding proteins, IGFBP-3 carries tne majority of IGF peptide systemically (257). The plasma levels of IGF-I correlate with GH secretion

and activity. Thus IGF-I has earned a reputation as the second messenger for GH and therefore accounts for the majority of physiological responses associated with GH.

The somatomedin hypothesis proposed in 1972 by Daughaday et al was an attempt to define somatomedins (IGFs) and their relationship with GH (106). It states that "a GH-dependent plasma factor stimulates in cartilage not only the incorporation of sulfate into chondroitin sulfate but also the incorporation of thymidine into DNA, proline into hydoroxyproline of collagen and uridine into RNA" (106). GH is the major stimulus of IGF-I production particularly by the liver. However other factors influence IGF production by target tissues including nutritional state (316) and conditions specific to the model used to investigate expression of IGF such as monolayers of endothelial cells (11) or granulation tissue obtained from implanted wound chambers as described by Steenfos et al (96,126).

6.2. IGFS AND IGFBPS IN WOUND FLUID

IGFs have been measured in most body fluids, including wound fluid exudate (112). Therefore the identification of IGFs in reasonable concentrations in the wound fluid of skin graft donor sites was not unexpected. It was interesting to discover that no discernible pattern of IGF concentration existed in any of the five patients over the time course of this current study. Greater numbers and segregation by age group may provide a better indication of any trend.

By time matching wound fluid and plasma samples it was possible to show that the IGF levels in both were similar. It is difficult to draw any conclusion from this study as to whether the main source of IGFs in wound fluid was local production or plasma transudation. It does provide an avenue for further research exploring the transport of IGFs from blood to the wound, expression of IGF mRNA as well as protein production in this model. Questions also arise from this curent study concerning clearance of IGFs and the effects of tissue metalloproteinases, such as gelatinase and collagenase, upon the equilibrium of IGFs at a wound site. As discussed in chapter 2, the technical exercise of measuring IGF levels will also impact upon the recorded concentration of IGFs. Correlation of the two techniques described clearly indicates that complete removal of IGFBPs from the samples is important if one technique is to be used reliably in place of another to measure IGFs. This applies particularly to IGF-II.

WLB and immunoblots confirmed the presence of low molecular weight IGFBPs in raw samples including proteolytic fragments of IGFBP-3. Low molecular weight binding proteins such as these moieties tend to be retained by many IGFBP extraction procedures thus interfering with measurement of IGF-I concentration (317). Furthermore, WLBs of the acid-ethanol extracts revealed residual IGFBPs in almost all samples.

As with the IGF levels, inter-patient variation made it difficult to determine if any specific relationships existed between the IGFBPs detected, IGF levels and time of healing. IGFBP-1, -2, and -3 were the main species found in wound fluid, however IGFBP-4 was also detected but less reliably.

6.3. *IN VIVO* WOUND HEALING AND THE IGFS

Experimental wounds have provided a means of exploring the effects, expression and fate of IGF-I at various sites. Models of wound contraction, granulation tissue growth, wound fluid production and wound epithelialisation have been utilised to identify IGFs in the wound as well as mRNA expression and receptor localisation (96,125,126,133,189). A hybrid model of toxin induced diabetes and excisional wound healing has been introduced in this study to examine the influence of IGFs in a compromised healing state.

In vitro effects attributable to IGF-I include fibroblast mitogenesis, collagen production as well as keratinocyte proliferation and migration. For these activities IGF-I appears to act via both autocrine (fibroblast-fibroblast) and paracrine (fibroblast-keratinocyte) mechanisms. The results in Chapter 3 corroborate with these previous findings. In addition IGF-I was shown to induce fibroblast-mediated contraction of collagen lattices. Cell growth and FPCL contraction suggest that IGF-I is the growth factor responsible for the majority of GH's physiological effects and that GH alone is relatively ineffectual as a mitogen for fibroblasts. Other studies have demonstrated that the influence of GH in fibroblast culture is largely due to endogenous IGF-I production (124) although these experiments were conducted in the presence of 0.5% hypopituitary serum. This contrasts with those results observed in Chapter 3 where the cell growth and contraction assays were performed under serum-

free conditions. The failure of GH to augment IGF-I's effect would suggest that the observed responses of IGF-I combined with GH were due to IGF-I alone. This lack of GH response was also observed in the incisional wound repair studies.

Wound strength was improved above paired controls where a sufficiently high IGF-I concentration was administered. Whereas no significant response was observed for an equal concentration of GH. Such a finding contrasts with those of several other studies where GH has been shown to increase granulation tissue production, deposition of collagen as well as to strengthen wounds (89,95). Porcine data from our laboratory support the improvement in wound healing parameters produced by systemic GH use (318). However a major difference exists between these studies and those in Chapter 3. GH was administered subcutaneously or given as an infusion or, in the case of Garrel's study (89), growth hormone releasing hormone (GHRH) was used. Whereas in the current study, a single dose of GH was administered directly into the wound as a topical preparation (refer Chapter 3).

Several factors may contribute to the observed absence of GH effect. Firstly, a single dose may be insufficient to activate cell surface receptors for GH in the skin and thereby induce IGF-I activity. Secondly GH 'related effects' must be mediated by hepatic-derived IGF-I and skin lacks GH receptors. Thirdly GH is denatured within a fresh wound before it can exert any effect. The first proposal would presuppose a time lag between GH stimulation, IGF-I production and IGF-I secretion by the local tissue. Certainly this is supported by evidence that IGF-I mRNA expression and peptide concentrations within adipose and granulation tissue increase following GH administration (126,319) while serum and extracellular levels of IGF-I can also improved (126,192). The weakness of the second proposal is that rat dermal

fibroblasts do express GH receptors. The third proposal may have merit given the intense proteolytic activity that occurs within a new wound.

If tissue proteases are responsible for the lack of GH effect, a similar influence upon IGF-I should have been observed. However the homeostatic mechanisms protecting IGF-I within the ECM may also protect it from degradation. This is especially applicable to IGFBP-1 and IGFBP-2 which contain the Arg-Gly-Asp *(RGD)* sequence within their structures allowing them to bind to cell membrane integrin receptors and to the ECM (320). Thus IGFBPs can increase the half life of IGFs within both the systemic circulation and target tissues.

Further support is provided by the relative lack of response in wound strength noted with the analogue LR^{3}IGF-I. *In vitro* experiments presented in the current study suggest that this peptide has greater anabolic potency than rhIGF-I. However it appears that reduction of binding affinity to IGFBPs may offset this greater potency in wound healing models because reduced binding to IGFBPs leaves a greater proportion of the analogue exposed to be denatured by tissue. Alternatively LR^{3}IGF-I may simply be cleared from the tissues intact by transport mechanisms or degraded by tissue proteases at the wound site. The ability of the binding proteins to provide a means of slow release of active growth factor may be also important in ensuring a biological response. However, *in vitro* studies do not truely replicate the *in vivo* response, as IGFBPs differ in their ability to inhibit or facilitate IGF activity in vitro and their influence may be dictated by the environment in which they are expressed.

The increased potency of IGF-I analogues upon cultured fibroblasts was expected as previous studies have demonstrated these peptides' greater ability to induce cell

proliferation and protein synthesis in a variety of other cells. In addition, studies of somatic growth indicate that this potency is not confined to cell culture (145,146,255,292). However in experiments using IGF-II analogues, these peptides were no more effective than rhIGF-II in the models described (results not shown). This suggests that while the IGF-I analogues provide more free IGF-I-like peptide to bind cell surface receptors, the same characteristic for IGF-II analogues may allow more 'free' IGF-II to bind to the type II receptor for which the physiological response is not known.

The influence of binding protein affinity in incisional wound repair was investigated by combining IGF-I and LR^{3}IGF-I with IGFBP-2 and applying these combinations to wounds in normal rats. The strength of wounds treated with IGF-I combined with IGFBP-2 was not significantly different to that of their controls. The results obtained suggest that the IGFBP-2 could possibly inhibit exogenous IGF-I within the wound.

Other investigators have used IGF-I in compromised states of wound repair producing improved wound strength, new collagen deposition and rates of excisional wound closure (46,127,273,276). However the common finding is that IGF-I influences wound repair greatest when administered in combination with either an IGFBP (132,247,275) or another growth factor. PDGF has been found to be the most effective of these in promoting the ability of IGF-I and IGF-II to close full-thickness skin wounds in mice (46), periodontal wounds in primates and beagles (134,246) as well as partial thickness porcine wounds (274). Previous studies differ from those described in Chapter 3 as the models are different and parameters other than wound strength were measured. It is possible that IGF-I is capable of improving wound

strength by itself as described in chapter 3 but may require other factors to promote more rapid wound closure.

As models of impaired healing the diabetic models provide two significant obstacles in the assessment of topical IGF-I treatment. The proteolytic enzyme content of the wounds (161) and circulating levels of IGFBP-2 (161,180,296) are increased in acute untreated diabetes (181). The influence of exogenous insulin in models of controlled diabetes must be considered as it can activate the type 1 receptor and consequently mimic any IGF-related effect. For this reason insulin was not given to the diabetic animals used in this study. Again it was postulated that in a diabetic wound the reduced binding protein affinity of LR³IGF-I would improve wound strength more than the recombinant peptide. The results imply that in a diabetic model the analogue may confer some benefit over the native peptide and that exogenous IGFBP-2 added to IGF-I will tend to reduce its effect or even inhibit the restoration of wound strength.

These results support IGF-I's potential as an agent to promote wound repair, however the analogues may be better utilised in compromised states where increased concentrations of binding proteins tend to down-regulate IGF activity. The net effect of this can be to increase wound strength as shown in Chapter 4. This is further supported by the *in vitro* findings of greater protein production and fibroblast contraction observed in the cell culture studies. Exogenous IGFBPs may also be useful as agents to modulate IGF-I activity produced within the wound whether this is related to exogenous or endogenous IGF-I.

Further studies of collagen physiology, epithelialisation, inflammatory cell infiltration, and fibroblast modulation within a wound by IGFBP-2, IGF-I or their

combination are required. Their binding to glysoaminoglycan molecules and heparin may be important to understanding why their combination was ineffective (321). Indeed recent evidence produced by Hoflich et al would suggest that IGFBP-2 does sequester IGFs thereby minimising their effects. These observations lend support to the impressions formed by this thesis that IGFBP-2 inhibits IGF-I activity in would healing as demonstrated by the lack of rhIGF-I in diabetic animals where IGFBP-2 levels are elevated and in those experiments where IGF-I was used in combination with IGFBP-2 in normal animals In addition, other objective indices of IGF-I activity and collagen deposition are required for complete interpretation of the responses observed. These would provide a better insight into IGF-I's role in dermal wound repair. This information would be important in answering the question as to whether IGF-I facilitates collagen *maturation* rather than promoting collagen production and deposition. Some evidence for this has been observed in studies examining the response of fibrobalsts in ligaments where IGF-I can increase the ratio of Type I to type III collagen (323). If the latter was true then this growth factor may contribute to hypertrophic scarring as postulated by Ghahary et al (324).

6.4 CONCLUDING REMARKS

Despite clearly demonstrated increases in activity of the analogues using tissue culture models, this activity could not be translated to the enhancement of dermal wound repair. The failure of *in vitro* success to translate to animal models raises

clinically relevant points. It underpins the well accepted edict that any product being investigated as a topical wound healing agent should be tested *in vivo* as well as *in vitro*. Secondly, these studies suggest that many single agents may be ineffective when used alone although success has been observed with the use of rh-PDGF-BB for lower extremity diabetic ulcers (104). The timing of treatment application and a myriad of other factors within the wound, may be important variables in determining the role of IGF-I in wound repair. In wound fluid, the presence of low molecular weight binding activity for IGF-I in the form of IGFBP fragments suggests that proteolysis within the wound maybe an important influence upon growth factor responses generally.

Topical wound healing agents that are effective in reversing the aberrations encountered in chronic wounds, can only be developed with a better understanding of the signalling mechanisms that regulate wound repair including growth factor physiology. While the success of rh-PDGF-BB has not evolved from absolute understanding of the pathways it is certainly recognised for its activity in cell cultures of fibroblasts and its activity in animal models both alone and in conjunction with IGF-I (40,45,262,325-329). IGF-I is one of many such agents that has shown potential that is yet to be realised because of its complex physiology.

<u>Demographic Data Sheet For IGF-I Concentration Protocol</u>

*Name:*_____

*Unit Record Number:*_____ *Date of birth:*_____ *Sex: M___ F___*

*Weight:*___   *Consultant:*_____

*Reason For Current Admission:*_____

Condition of site to be grafted at the time of admission:

       Unwounded ___

       Wounded ___   1) Clean ___

                       2) Contaminated ___

       Compromised ___

Past Medical History:

                                              Y   N

      1. Peripheral Vascular Disease   ___ ___

      2. Diabetes mellitus   ___ ___

      3. Deep Venous Thrombosis   ___ ___

4. Varicose Veins   —   —

5. Chronic Airways disease   —   —

6. Rheumatoid Arthritis   —   —

7. Hypertension   —   —

8. Congestive Cardiac Failure   —   —

9. Malignancy   —   —

10. Neuropathy   —   —

11. Cerebro-vascular disease   —   —

12. Sickle Cell Disease/ Trait   —   —

Details:_____

Medications:

1. Steroids   —   —

2. Other Immunosuppressive Agents   —   —

3. Chemotherapeutic Agents   —   —

4. Antibiotics   —   —

5. Insulin   —   —

6. Oral Hypoglycaemics   —   —

7. Antiplatelet drugs/ anticoagulants   —   —

Smoking history: —   —

            Reformed _   _

Alcohol:

Amount per week _____

*Date of Operation:*_____

*Site of Donor Site:*_____

Size of Donor site: **Day 0** _____   **Day 1** _____   **Day 3** _____   **Day 7** _____

Microbiology: _____   _____   _____   _____

Volume Aspirated (mls):   _____   _____   _____   _____

Total Vol. Aspirated: _____

Need for Change of Dressing: Y__   N__   **No. of Changes:** ____

No. of blood Samples Obtained: ___

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