Insulin-like growth factors and insulin-like growth factor binding proteins in wounds

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Abstract

Insulin-like growth factor-I (IGF-I) appears to play an important role in wound repair. However, knowledge of how this role may be modulated by wound IGF binding proteins (IGFBPs) is limited. Therefore, the main aim of this Thesis was to determine general roles for IGFBPs in regulating IGF-I actions in the wound.

Preliminary experiments sought to characterise the alterations to IGF-I levels and IGFBP profiles that may occur during wound repair. Wound fluid IGF-I levels declined following wounding and were generally half to two-thirds those of plasma. IGFBP-3, the predominant IGFBP in plasma, was also present in wound fluid, however the presence of wound fluid proteases significantly diminished the ability of this IGFBP to bind IGFs. IGFBP-3 proteases may therefore play a role in the regulation of IGF bioactivity by increasing IGF interaction with IGF receptors.

The effects that interactions with IGFBPs may have on IGF actions in wounds were addressed by comparing the rate of elimination of IGF-I from the wound site with those of IGF analogues with differing affinities for IGFBPs. A bolus of each radiolabelled peptide was infused into the lumen of Hunt-Schilling chambers that had been implanted in rats. Clearance rates were increased for peptides that bound poorly to IGFBPs. These observations support a general role for wound IGFBPs in maintaining a pool of IGF at the wound site by limiting clearance.

The final experiments aimed to determine whether IGFBP-3 proteolysis observed in the initial work of this Thesis acted to increase IGF bioavailability. Comparisons of wound fluid and plasma from rats in competitive binding experiments revealed that IGF-I in wound fluid was

no more available to cell receptors than that in plasma. Indeed, IGF availability in both fluids was similar to that of pure IGF-I. These results contrasted with those obtained for human plasma in that IGF-I in normal human plasma was unable to compete for binding to cell receptors, whereas IGF-I in human pregnancy plasma competed to an similar extent to that of rat plasma and wound fluid. These observations may explain the increased responsiveness of rats to exogenous IGFs and furthermore, that those IGF analogues with reduced affinity for IGFBPs such as LR^3IGF-I may show potential as vulnerary agents in humans.

Statement of Originality

This work contains no material that has been accepted for the award of any 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. Furthermore, I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Signed:

James Gray Robertson

Date: 27/9/99.

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Robertson G, Belford D, Ballard J. IGF and insulin clearance from rat wounds. *The Wound Healing Society 8th Annual Meeting*, Salt Lake City, 1998.

Robertson G, Belford D, Ballard J. Half-life of IGF-I in a rat wound model. *36th National Scientific Conference of the Australian Society for Medical Research*, Adelaide, 1997.

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Abbreviations

This thesis uses standard abbreviations. Non-standard abbreviations are listed as follows:

°C	degrees Celsius
ALS	acid labile subunit
ANOVA	analysis of variance
BSA	bovine serum albumin
cpm	counts per minute
FBS	foetal bovine serum
FPLC™	fast-protein liquid chromatography
h	hour
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPLC	high performance liquid chromatography
ID	inner diameter
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IgG	immunoglobulin G
im	intramuscular
ip	intraperitoneal
iv	intravascular
kDa	kilodalton
LR^3IGF-I	long [Arg^3] insulin-like growth factor-I
M	molar
OD	outer diameter

P	probability
PAGE	polyacrylamide-gel electrophoresis
PBS	phosphate-buffered saline
RIA	radioimmunoassay
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane
WF	wound fluid

Chapter One

Literature Review

1 Literature Review

1.1 GENERAL INTRODUCTION

Injury invokes a series of biochemical and physiological events that aim to restore the anatomical and functional integrity of tissues. Regulation and coordination of these events is not completely understood. However, many cytokines, which act as messengers between cells, are produced and released into the wound environment. Polypeptide growth factors, a sub-class of cytokines, regulate the actions of many cells and appear to play important roles in the wound repair process.

The insulin-like growth factors (IGFs), structurally related to proinsulin, are potent regulators of cell proliferation and differentiation of many different tissues and cell types (Jones and Clemmons, 1995). Cell membrane-bound receptors for these growth factors are also widely distributed, although with varying degrees of abundance. Interaction of the IGFs with the receptors is strongly regulated by a family of IGF-binding proteins (IGFBPs) that generally inhibit the actions of the IGFs, yet in some instances may also enhance their effects.

IGFs have been identified in wounded and regenerating tissues suggesting a general role in the wound healing process. Indeed, recent studies have reported beneficial effects when wounds are treated with IGFs and IGFBPs conjointly. However, little information exists concerning the role of IGFBPs in the wound repair process and in the wider context how they may regulate IGF action in the extracellular environment. The aims of this thesis were i) to determine the types and forms of IGFBPs present in the wound during the acute stages of repair and ii) to explore the effects that IGFBPs may have on IGF actions in the wound.

This review provides a general background of the wound healing process, with a particular emphasis on growth factors and their role in dermal and epidermal repair. It also discusses the components of the IGF system, including analogues of IGF-I that bind IGFBPs poorly, and the postulated roles of IGFs in wound repair. The review covers the literature to the end of 1995, approximately the time that my research program started, whereas citations relevant to this thesis published since 1995 have been included in the appropriate chapters.

1.2 EPIDERMAL WOUND REPAIR: ASPECTS OF GROWTH FACTOR ACTIONS

In adult higher vertebrates, the wound repair process occurs as a fibro-proliferative response that leads to scar formation. Although some differences in the extent of the scarring may occur between tissues and species, the repair process exhibits common sequential elements. Convention breaks the process into three main phases: inflammation, proliferation and remodelling, which may overlap somewhat.

1.2.1 *Phase I*: Haemostasis and inflammation

Several events occur rapidly upon injury and disruption of blood vessel integrity such that blood components are exposed to the extravascular tissues. The purpose of these responses is to prevent blood loss by forming a clot and initiating the cellular and biochemical repair processes. This is principally achieved by initiating the intrinsic and extrinsic clotting pathways through activation of coagulation factors in plasma (Furie and Furie, 1988). These two pathways ultimately catalyse the conversion of prothrombin to thrombin, which in turn catalyses the degradation of fibrinogen to form fibrin. Fibrin, together with fibronectin, is an important biochemical constituent of the clotting process, forming strands that act to trap platelets to form a clot and prevent further bleeding.

Following binding to either fibrin formed during the clotting process or collagen types IV and V of the sub-endothelial membrane, platelets become activated and undergo morphological and functional changes that increase their stickiness and promote binding to other platelets. During this activation and aggregation, platelets release many pro-inflammatory chemical mediators, such as adenosine diphosphate and thromboxane-A_2, which serve to amplify the

initial inflammatory response. In addition, the contents of their α-granules are discharged releasing chemotactive and growth factors such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β) and IGF-I into the wound environment (Antoniades *et al.*, 1979; Kaplan *et al.*, 1979; Assoian *et al.*, 1984; Karey and Sirbasku, 1989; Sporn and Roberts, 1992). In the small amounts released by platelets, PDGF and TGF-β are strongly chemotactive for a variety of cells and provide the initial impetus for monocyte, fibroblast and endothelial cell migration into the wound. Thus, formation of the clot from fibrin and aggregated platelets is important in not only preventing further haemorrhage, but also in promoting the initial inflammatory response (Clark, 1995).

Early in the inflammatory phase, neutrophils and macrophages, drawn by the many chemotactive factors generated and released during the clotting process, begin migrating into the wound using the provisional extracellular matrix of the clot. Although the accumulation of neutrophils is rapid, as these cells are the most abundant in the circulation, their presence in non-septic wounds may not be essential for wound repair. Their main role is to phagocytose and destroy bacteria and infiltration ceases within a few days in the absence of substantial contamination of the wound (Simpson and Ross, 1971; Simpson and Ross, 1972). In contrast, macrophages attracted to the wound site from the circulation or the surrounding tissues (Leibovich and Wiseman, 1988) become particularly evident after the initial inflammatory events and remain at the wound site for days or weeks playing a central role in the wound repair process.

As demonstrated by Leibovich and Ross (1975) in their pivotal study, depletion of either circulating monocytes or wound macrophages by hydrocortisone or anti-macrophage serum results in a marked delay in fibroblast proliferation, reduced wound fibrosis and severe impairment of tissue debridement. Blood and tissue monocytes arrive at the wound in

response to chemoattractants generated during the inflammatory response by degradation of extracellular matrix or by release from cells already present at the wound site (Wahl *et al.*, 1987). TGF-β is a potent chemoattractant for monocytes and is released in the wound not only by platelets, but also by neutrophils, monocytes, lymphocytes and fibroblasts (Sporn and Roberts, 1986). PDGF is also a potent chemoattractant for monocytes (Pierce *et al.*, 1989), yet other chemoattractants such as thrombin, complement C5a, collagen, elastin and fibronectin and their degradation products may also draw monocytes to the wound (see Riches, 1995 for review). The principal effect of this initial inflammatory phase is to attract cells to the wound that will begin the repair and regenerative processes.

Upon arrival at the wound site, monocytes rapidly differentiate and become activated. The events that initiate activation are unclear, but may involve binding to fibronectin via integrin receptors, which are cell-surface adhesion receptors (Wright *et al.*, 1983; Hynes, 1992; Riches, 1995). Activation after binding to extracellular matrix proteins via integrin receptors is an important event for stimulating extracellular matrix phagocytosis as well as antibody- and complement-mediated phagocytosis (Brown and Goodwin, 1988). With the production of a variety collagenases (Wahl *et al.*, 1974; Hibbs *et al.*, 1987), elastases (Senior *et al.*, 1989) and other matrix degrading enzymes (see Clark, 1995), activated macrophages together with neutrophils (Janoff and Scherer, 1968; Lazarus *et al.*, 1968) are able to digest and remove contaminating organisms, tissue debris and dead or dying neutrophils (Newman *et al.*, 1982). Additionally, activated macrophages are a source of chemoattractive factors for other monocytes. These factors, including but not limited to PDGF, TGF-β, interleukin-8, monocyte chemoattractant protein-1, monocyte inflammatory protein-1α and interleukin-1, serve to attract additional inflammatory cells to the wound site and ensure that the initiated inflammatory response is amplified and sustained (Clark, 1995). Conversely, debridement, the

removal of contaminated or devitalised tissue by these migrating cells, is essential to the wound repair process as it also ensures that inflammatory stimuli are removed and that the tissue repair processes can proceed.

1.2.2 *Phase II*: Proliferation and new tissue formation

Initially it was thought that the sole function of the macrophage was that of wound debridement, however it is now clear that considerable functional heterogeneity occurs in macrophage populations. In addition to playing a pivotal role in the phagocytosis and removal of wound contamination and degraded or injured tissue, the activated macrophage has a role in the initiation of the repair and regenerative processes that lead to the formation of new tissue. Leibovich and Ross (1976) demonstrated that monocytes synthesised and secreted a macrophage-derived growth factor that was able to induce the proliferation of serum-deprived fibroblasts. Eventually it was recognised that the macrophage-derived growth factor was not a single growth factor, but rather a mixture of previously described growth factors. In particular, PDGF (Shimokado *et al.*, 1985), FGF-2 (Baird *et al.*, 1985), TGF-β (Assoian *et al.*, 1987), TGF-α and IGF-I (Madtes *et al.*, 1988) have been identified in media conditioned by macrophages and monocytes. Messenger RNA transcripts of these growth factors have also been found in macrophages isolated from wound chambers that had been implanted subcutaneously in mice (Rappolee *et al.*, 1988).

Fibroblasts, smooth muscle cells and endothelial cells, drawn to the wound site by similar chemoattractive factors to those acting on monocytes, are stimulated to grow and divide under the influence of the various macrophage-produced growth factors. These three key cell types provide the developing characteristics of the new tissue: fibroplasia and neovascularisation.

An additional process, re-epithelialisation, is present where those wounds involve the epidermis.

Fibroplasia

Migration of fibroblasts into the clot, beginning a few days after injury, is under the chemotactive influence of growth factors particularly TGF-β (Postlethwaite *et al.*, 1987) and PDGF (Seppa *et al.*, 1982) released by wound macrophages and injured endothelial cells (Sitaras *et al.*, 1987; Rappolee *et al.*, 1988). At this stage, the wound extracellular matrix is complex and lacks structure such that fibroblasts must migrate over a mixture of collagen, fibrin, fibronectin and vitronectin fibres. The ability of fibroblasts to migrate in such an environment is dependent on the increased expression of cell-surface integrin receptors in response to growth factors such as PDGF (Ahlen and Rubin, 1994) and TGF-β (Heino *et al.*, 1989). Once in the wound, a switch of phenotype occurs from one of migration to one of protein production resulting in *de novo* synthesis of new collagen and connective tissue matrix (Welch *et al.*, 1990). At this time, several growth factors act to enhance the fibrotic response. TGF-β and PDGF are potent inducers of collagen and fibronectin synthesis (Clemmons, 1984; Ignotz *et al.*, 1987; Blatti *et al.*, 1988) and in the case of PDGF, can act synergistically with IGF-I to promote fibroblast mitosis (Stiles *et al.*, 1979; Chen and Rabinovitch, 1989) and extracellular matrix deposition (Ross, 1987). Importantly, some of these growth factors are expressed by wound fibroblasts (Clemmons, 1984; Sporn and Roberts, 1986), suggesting that autocrine/paracrine mechanisms may be vital for maintaining the fibroplastic response.

Neovascularisation

Concurrent with the fibroplastic response is the migration and proliferation of endothelial cells to form new blood vessels. This process, known as neovascularisation or angiogenesis, is essential since the wound becomes highly cellular, develops a high demand for energy and essential nutrients, and produces metabolites and toxins that need to be removed. Two to three days after injury, endothelial cells of microvessels in tissue immediately adjacent to the wound margin become activated by a variety of soluble factors such as FGF-2, TGF-β, interleukin-1 and tumour necrosis factor (Madri *et al.*, 1991). Endothelial cell activation and migration is heavily dependent on the induction and expression of certain proteins by growth factors released from injured cells and the extracellular matrix (Forrest, 1983; Vlodavsky *et al.*, 1987; Tsuboi *et al.*, 1990; Madri *et al.*, 1991; Mignatti *et al.*, 1991). These proteins allow release of the cells from their basement membranes before the activated endothelial cells begin to migrate into the provisional matrix of the clot.

Two fibroblast growth factors, FGF-1 and FGF-2 (acidic and basic FGF, respectively), appear to be the predominant angiogenic growth factors. These growth factors are synthesised by many cells in the wound (Baird *et al.*, 1985; Blotnick *et al.*, 1994) and interact strongly with heparin-like molecules (Maciag *et al.*, 1984; Shing *et al.*, 1984). This may have biological significance as heparin and heparan sulfate proteoglycans may act to sequester and protect the factors from degradation in the wound. Thus, following extracellular matrix injury and degradation of these proteoglycans, the FGFs may be released to become available to bind cellular receptors and provide continual stimulation of the endothelial cells (Vlodavsky *et al.*, 1991a).

The continuation of new blood vessel formation is not only dependent on endothelial cell migration into the clot, but as with fibroblasts, a marked proliferation of endothelial cells occurs in response to local soluble autocrine and paracrine growth factors. Migrating endothelial cells form capillary sprouts (Gabbiani *et al.*, 1972; Ausprunk and Folkman, 1977), which eventually branch and join at the tips to form loops and a capillary plexus through which blood may begin to flow. In turn, more capillary sprouts may extend from these loops, the process continuing until the wound is completely revascularised. Although fibroblasts are the primary source of new extracellular matrix, endothelial cells in capillary sprouts may also synthesise and deposit fibronectin to form their own provisional matrix. In turn, the newly deposited provisional matrix may act to guide other endothelial cells as they continually migrate from the capillary sprouts into the clot and stabilise the developing vessels (Clark *et al.*, 1982).

Re-epithelialisation

Wounds to the epidermis create the need for an additional important wound repair process particularly as loss of skin integrity may result in significant losses of body fluids with associated increases in morbidity and mortality. Thus, rapid re-establishment of the epidermal barrier is paramount. Wound re-epithelialisation, beginning within hours of injury, necessitates the proliferation and migration of skin basal keratinocytes over the provisional wound matrix. The keratinocytes may originate from the wound margin, although in partial thickness injuries to the dermis, they may also originate from hair follicles. Specific morphological changes to basal epithelial cells occur upon the initiation of migration: the cells lose their basement membrane attachments, become flattened and disc-shaped, and lose their

apical/basal polarity. The signals for basal keratinocytes to undergo such changes are yet to be fully understood. However, growth factors have been shown to accelerate wound closure and promote keratinocyte migration *in vitro*, suggesting that they play an important role in this process.

The first evidence of epidermal responses to a growth factors were reported by Cohen and Elliott (1963) with a protein extract from mouse sub-maxillary glands later identified as epidermal growth factor. Following wounding and release from the α-granules of platelets (Oka and Orth, 1983; Pesonen *et al.*, 1989) and production by wound cells (Rappolee *et al.*, 1988), EGF may stimulate keratinocyte proliferation and migration (Barrandon and Green, 1987). TGF-α, an EGF-like growth factor, is also secreted by keratinocytes (Derynck, 1986) and along with EGF may bind to EGF receptors present on migrating epidermal keratinocytes (Wenczak *et al.*, 1992). Interestingly, both EGF and TGF-α are initially produced as transmembrane glycoproteins (Bell *et al.*, 1986; Massague, 1990) and it was once thought that proteolytic cleavage was required to release the biologically active forms. However, the transmembrane precursors may also bind EGF receptors on adjacent cells (Wong *et al.*, 1989; Anklesaria *et al.*, 1990; Massague, 1990) allowing juxtacrine actions to occur in addition to the autocrine and paracrine actions of the soluble forms. This is of particular interest as only TGF-α and not EGF is produced by keratinocytes (Coffey *et al.*, 1987), suggesting that TGF–α is a major autocrine growth factor for basal keratinocytes. However, as epidermal wounds in mice lacking the TGF-α gene heal normally (Luetteke *et al.*, 1993; Mann *et al.*, 1993), it is possible that other members of the EGF family may compensate for the TGF-α deficiency.

One such candidate, initially identified as a secretory product of macrophages that binds heparin (Higashiyama *et al.*, 1991), can be found in wound fluid within one day of injury at levels far greater than for PDGF or FGF-2 (Marikovsky *et al.*, 1993). Heparin-binding EGF may also be produced by keratinocytes (Marikovsky *et al.*, 1993), and although wound fluid levels are highest 2-3 days following injury, the exact role of this growth factor in the process of re-epithelialisation is unclear. Nonetheless, the *in vitro* mitotic activities of not only human keratinocytes (Hashimoto *et al.*, 1994), but also fibroblasts (Besner *et al.*, 1990), vascular smooth muscle cells (Higashiyama *et al.*, 1991; Higashiyama *et al.*, 1992) and bovine pericytes (Nomura *et al.*, 1995) are increased by heparin-binding EGF. Similarly, the motility of bovine smooth muscle cells is increased in response to treatment with heparin-binding EGF *in vitro*. Moreover, its heparin-binding characteristics may impart properties similar to those of FGF-1 and FGF-2 (Maciag *et al.*, 1984; Shing *et al.*, 1984), which are found in close association with extracellular matrix and whose bioactivity is increased by such association (Vlodavsky *et al.*, 1991b). Whilst a wider role in wound repair is suggested by its increased expression in some regenerating tissues such as the liver (Ito *et al.*, 1994) and kidneys (Homma *et al.*, 1995), at present much of the evidence linking this growth factor to re-epithelialisation is circumstantial and require further investigation. [It has been recently reported that HB-EGF is present in the wounds of paediatric patients that have partial-thickness burns (McCarthy *et al.*, 1996). Furthermore, Marikovsky (1996) have reported that HB-EGF acts synergistically with IGF-I to stimulate keratinocyte growth *in vitro*].

Although several glycosaminoglycans are present in the wound, fibronectin derived from plasma and fibroblasts in the wound is the primary structural protein used for cell migration (Toda *et al.*, 1987). Indeed, keratinocytes may synthesise fibronectin in response to growth factors such as FGF-2 (O'Keefe *et al.*, 1988) and attach to the Arg-Gly-Asp (RGD) tripeptides

of fibronectin (Kim *et al.*, 1992). As TGF-β has been shown to stimulate expression of integrins by keratinocytes, a role for the macrophage and other TGF-β producing cells in stimulating epithelial cell migration has been proposed (Gailit *et al.*, 1994), although this observation seemingly contradicts the general inhibitory role of TGF-β on epithelial cell proliferation (Hebda, 1988).

Eventually, the migrating keratinocytes cover the provisional matrix and a new basement membrane is formed. The basement membrane is vital for the attachment of new keratinocytes to the dermis as well as the re-establishment of the epidermis as a protective organ. Of the many cytokines and growth factors that regulate deposition of the new basement membrane, TGF-β appears to be important in the up-regulation of the expression of basement membrane components (Uitto *et al.*, 1995). In addition to its function as a site of anchorage for the epidermis, the basement membrane is also an important store for growth factors. For instance, FGF-2 bound in the basement membrane by heparan sulphate (Folkman *et al.*, 1988; Bennett and Schultz, 1993a) may be released following secretion of proteinases and heparinases during keratinocyte migration (Vlodavsky *et al.*, 1991b). Other growth factors that act on keratinocytes and potentially may bind heparin or heparan sulfate include FGF-1, FGF-7 (Reich-Slotky *et al.*, 1994), PDGF (Ross *et al.*, 1986), vascular endothelial growth factor (Gitay-Goren *et al.*, 1992), TGF-β1 (McCaffrey *et al.*, 1992), hepatocyte growth factor (Naka *et al.*, 1993) and HB-EGF (Higashiyama *et al.*, 1991). Although heparin and heparan sulphate proteoglycans increases the affinity of FGF-2 for its receptor (Yayon *et al.*, 1991), it is not clear whether they act in a similar manner to facilitate the actions of other heparin-binding growth factors. Further investigations will ascertain whether this is the case or not.

1.2.3 *Phase III*: Remodelling

The final phase of wound repair begins at the wound margin and involves changes in the extracellular matrix composition and a reduction of cell numbers within the granulation tissue. Remodelling is an ongoing process that begins at the time of injury and may continue for weeks or years, well after the wound has visibly healed.

Successful remodelling of the scar is highly dependent on a balance between new matrix synthesis and degradation requiring the production of high levels of proteolytic activity. Proteases act to degrade damaged elements of the provisional matrix, such as fibronectin, elastin and collagen, to allow replacement by newly synthesised material. An important part of this process is the replacement of type III with type I collagen (Patt and Houck, 1983). TGF-β is an key growth factor for this process, concurrently stimulating production of type I collagen (Ignotz *et al.*, 1987) and collagenase expression in fibroblasts (Chua *et al.*, 1985; Overall *et al.*, 1989). On the other hand, TGF-β and PDGF may induce synthesis of inhibitors of metalloproteinases (Overall *et al.*, 1989) and other serine proteases, thereby providing a mechanism for moderating collagen degradation.

During remodelling there is a delicate balance between degradation and synthesis, however the factors that determine whether pro-inflammatory cytokines or growth factors are pro- or anti-fibrotic are unclear. Preliminary investigations of hypertrophic and chronic wounds have suggested that these wounds evolve as a result of inappropriate expression of growth factors such as TGF-β (Noble *et al.*, 1992; Sporn and Roberts, 1992). For example, enhanced expression of TGF-β1 has been observed in hypertrophic scars following burn injury (Ghahary *et al.*, 1993), whereas expression of this isoform is often absent in chronic non-healing ulcers (Schmid *et al.*, 1993). These observations are supported by data that showed that neutralising

antibodies to TGF-β1 and TGF-β2 decreased the accumulation of extracellular matrix and decreased scarring in rodents (Shah *et al.*, 1992; Shah *et al.*, 1994; Shah *et al.*, 1995). Thus, TGF-β appears to be an important growth factor in problem wounds. However, it should be noted that this is not true for all TGF-β isoforms as expression of TGF-β3 appears to be constitutive in intact skin and unaltered in acute or chronic wounds (Schmid *et al.*, 1993). Moreover, addition of TGF-β3 to experimental wounds produced a similar effect to that seen following the addition of neutralising antibodies to TGF-β1 and TGF-β2 (Shah *et al.*, 1995). The role of this isoform, in contrast to TGF-β1 and TGF-β2, appears to be related more to maintaining epithelial differentiation, making it difficult to ascribe a general role for TGF-β action in pathological wounds.

Wound contraction is one of the most visible changes that occur during remodelling. After migration into the wound, fibroblasts switch phenotype and begin to synthesis fibronectin and collagen (Gabbiani *et al.*, 1971; Gabbiani *et al.*, 1972). At this point, the collagen fibres are disorganised and the wound lacks strength. However, further changes to the phenotype of wound fibroblasts to become myofibroblasts with contractile properties (Gabbiani *et al.*, 1971) and remodelling of collagen fibres to form larger collagen bundles results in contraction and an increase in the strength of the wound. Although most myofibroblasts within granulation tissue are derived from fibroblasts, vascular pericytes and smooth muscle cells may also change phenotype to become myofibroblasts. Myofibroblasts acquire contractile properties through the expression of α-smooth muscle actin (Darby *et al.*, 1990) and contractile proteins such as myosin and vimentin (Desmouliere and Gabbiani, 1995). Cytokines and growth factors, as regulators of fibroblast proliferation and migration, also appear to regulate myofibroblast differentiation and expression of α-smooth muscle actin. Thus, PDGF, FGF-2, and tumour necrosis factor-α may decrease expression of α-smooth

muscle actin whereas the TGF-β1 isomer has been shown to increase expression (Desmouliere and Gabbiani, 1995).

The wound slowly gains tensile strength as remodelling continues and the scar matures, yet most normal wounds only attain approximately seventy percent of the full breaking strength of uninjured tissue (Levenson *et al.*, 1965). In the latter stages of repair, wound tensile strength and resiliency are primarily achieved through the formation of larger collagen bundles and alterations of cross-linking rather than through collagen synthesis (Bailey *et al.*, 1975). The maturing scar is also relatively acellular due to apoptosis of cells within the provisional matrix and the maturing scar. Apoptosis, or programmed cell death, is thought to be an important step in the resolution of the wound repair responses. Indeed, apoptosis of fibroblasts, myofibroblasts (Desmouliere *et al.*, 1995), endothelial cells (Bochaton-Piallat *et al.*, 1995) and granulocytes (Savill *et al.*, 1989) has been recently demonstrated.

1.3 THE INSULIN-LIKE GROWTH FACTORS

1.3.1 Historical aspects

Several unrelated observations were made of the effects of IGFs prior to the recognition that the same growth factors were responsible for a range of responses. Salmon and Daughaday (1957) observed that a defect in the incorporation of ^{35}S-sulphate into cartilage of hypophysectomised rats was not corrected by treatment with pituitary growth hormone *in vitro*, but was *in vivo*. Furthermore, the *in vitro* effect was reversed by the addition of serum from normal rats. They proposed that a "sulphation factor" present in serum mediated the actions of growth hormone on skeletal tissues. Several years later, it was discovered that neutralising antibodies to insulin were only partially successful in suppressing the effects of insulin (Froesch *et al.*, 1963). A significant amount of unaccounted insulin-like activity was present and was termed non-suppressible insulin-like activity or NSILA. NSILA activity was also observed in fractions that were partially purified from calf serum (Pierson and Temin, 1972) and media that was conditioned by rat hepatocytes (Dulak and Temin, 1973b). The fractions stimulated DNA synthesis in chicken-embryo fibroblasts and accordingly were termed multiplication-stimulating activity (Dulak and Temin, 1973a; Dulak and Temin, 1973b). Earlier, Daughaday and Reeder (1966) had observed that sulphation factor was able to stimulate uptake of ^{3}H-thymidine into DNA. When it was shown to also cause non-suppressible insulin-like activity in adipose tissue (Hall and Uthne, 1971), it was suggested that the term "sulphation factor" was inappropriate. The term "somatomedin", as the mediator of the effects of somatotrophin, was proposed as an alternative (Daughaday *et al.*, 1972).

Although all three activities were found in crude serum extracts, a concentrated organ source was not identified (Solomon *et al.*, 1967). More highly purified serum extracts formed the

basis of later studies in which NSILA and somatomedin stimulated glucose uptake into cartilage (Froesch *et al.*, 1976; Schlumpf *et al.*, 1976), suggesting that the structures of each were similar. Then in 1976, Rinderknecht and Humbel identified two polypeptides of NSILA, NSILA-I and NSILA-II, that had both insulin-like and cell-growth promoting activities. Further analysis revealed that the polypeptides contained amino acid sequences that were similar to proinsulin and the A- and B-chains of insulin (Rinderknecht and Humbel, 1976a; Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b). Similarly, Klapper *et al.* (1983) showed that somatomedin-C, which was purified from human serum (Van Wyk *et al.*, 1974), was identical to IGF-I/NSILA, while Spencer *et al.* (1983) reported that somatomedin-A (Hall, 1972), also purified from human serum, was a mixture of IGF-I and IGF-II. Multiplication-stimulating activity, eventually purified from serum-free media that was conditioned by rat hepatocytes, was characterised as rat IGF-II (Marquardt *et al.*, 1981). These various growth factor activities are now commonly known as IGF-I or IGF-II (Daughaday *et al.*, 1987).

1.3.2 IGF structure

The IGFs are members of the insulin super-family, which includes proinsulin, insulin, relaxin, bombyxin and molluscan insulin-like peptide (Blundell *et al.*, 1978; Chan *et al.*, 1990). IGF-I and IGF-II are single chain polypeptides having 70 and 67 amino acids, respectively, giving molecular weights of approximately 7.5 kDa (Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b). They share approximately 62% sequence homology (Rinderknecht and Humbel, 1978b) and in vertebrates, the sequences are highly conserved between species (Rotwein, 1991). For example, rat IGF-I only differs from human IGF-I by

three amino acids, whilst human, porcine and bovine IGF-I are identical (Rinderknecht and Humbel, 1978a; Francis *et al.*, 1988b; Tavakkol *et al.*, 1988). Similarly, rat IGF-II only differs from human IGF-II by four amino acids (Rotwein, 1991).

1.3.3 Sites of IGF synthesis

The original somatomedin hypothesis proposed that the effects of growth hormone were mediated by somatomedin, which was produced by the liver under the control of growth hormone and travelled via the bloodstream to the tissues (Daughaday *et al.*, 1972). Many lines of evidence support this model. For instance, treatment with growth hormone stimulates synthesis of IGF-I in the liver (D'Ercole *et al.*, 1984; Mathews *et al.*, 1986) and patients with liver cirrhosis have low plasma IGF-I levels (Schimpff *et al.*, 1977). However, whilst it is clear that IGF-I can behave in a classical endocrine manner, the identification of IGF-I mRNA in many tissues other than the liver (Han *et al.*, 1987; Adamo *et al.*, 1989) suggests that IGF-I can act as an autocrine or paracrine growth factor (Green *et al.*, 1985; Zezulak and Green, 1986). Moreover, mRNAs for IGF-I are produced by extra-hepatic tissues at all stages of life (Mathews *et al.*, 1986; Nilsson *et al.*, 1990). Although serum contains the highest levels, IGF–I can also be measured in lymph (Cohen and Nissley, 1975), cerebrospinal fluid (Yamaguchi *et al.*, 1990), urine (Hizuka *et al.*, 1987) and saliva (Hansbrough *et al.*, 1995). The liver, which contributes an estimated 90% or more of the serum pool, appears to be the predominant site of IGF-I synthesis in the body (Schwander *et al.*, 1983).

As plasma concentrations of IGF-II are high during foetal development in rats (Moses *et al.*, 1980), sheep (Handwerger *et al.*, 1983), guinea pigs (Daughaday *et al.*, 1986) and humans (Gray *et al.*, 1987), it has been suggested that IGF-II may be developmentally regulated.

Furthermore, levels of IGF-II protein and mRNA decline rapidly during the post-natal period of rats and mice such that expression in the adult is negligible in all tissues except the brain (Brown *et al.*, 1986; Donovan *et al.*, 1989). The notion of IGF-II as an important foetal growth factor is also supported by observations that some embryonic tumours, such as nephroblastomas (Scott *et al.*, 1985) and leiomyosarcomas (Hoppener *et al.*, 1988), may synthesise IGF-II. However, since IGF-II plasma levels do not change markedly during the post-natal period of either humans (Mignatti *et al.*, 1992), guinea pigs (Daughaday *et al.*, 1986) or pigs (Owens *et al.*, 1991) and infused IGF-II can exert somatic effects *in vivo* during the post-natal period, albeit less effectively than IGF-I (Conlon *et al.*, 1995), the distinction between the roles of IGF-I and IGF-II as foetal or adult growth factors is not so clear.

1.3.4 IGF receptors

There are two distinct IGF receptors known as the type 1 and type 2 receptors. While the insulin receptor may bind IGFs weakly, the IGF receptors show high affinity for IGFs. The type 1 receptor, comprising two alpha- and two beta-subunits linked by disulphide bridges in a manner homologous to the insulin receptor, has intrinsic tyrosine kinase activity. The type 2 receptor is identical to the cation-independent mannose-6-phosphate receptor (Kiess *et al.*, 1988).

Type 1 IGF receptor

IGF-I, IGF-II and insulin are all ligands of the type 1 IGF receptor. While this receptor may preferentially bind IGF-I over IGF-II, depending on the cell type and binding conditions

(Massague and Czech, 1982; Steele-Perkins *et al.*, 1988; Germain-Lee *et al.*, 1992), insulin is bound with an affinity 500- to 1000-times less than IGF-I (Massague and Czech, 1982). The structure of the receptor is such that the α- and β-subunits link to form a half-receptor: two half-receptors then link to form a hetero-tetrameric complex with a molecular weight of approximately 300-350 kDa (Czech, 1989). The complex has 50-60% sequence homology with the insulin receptor, which is also an α2β2 heterologous tetramer (Ullrich *et al.*, 1985). Ligand recognition and binding occurs on the α-subunits, which are positioned extracellularly, by virtue of a cysteine-rich region (Gustafson and Rutter, 1990; Schumacher *et al.*, 1991), whereas the β-subunits form a transcellular domain of which the intracellular portions possess the intrinsic tyrosine kinase activity (Czech, 1989). Signalling occurs after IGF binding to the extracellular domain of the α-subunit with subsequent autophosphorylation of the β-subunit and initiation of a series of cascades to effect the metabolic and mitogenic actions of the IGF ligand (reviewed by De Meyts *et al.*, 1994). Type 1 IGF receptors are found in almost all tissues and cells. Expression appears to be developmentally regulated with high level occurring in the foetus, especially in the central nervous system and skeletal musculature, while steady state mRNA levels appear to decrease during the post-natal period (Werner *et al.*, 1989).

Type 2 IGF receptor

Also known as the cation-independent mannose-6-phosphate receptor, the type 2 IGF receptor is a single polypeptide of approximately 250 kDa that spans the cell membrane (Morgan *et al.*, 1987). However, unlike the type 1 receptor, it lacks intrinsic tyrosine kinase activity (Corvera *et al.*, 1986; Kiess *et al.*, 1988) and preferentially binds IGF-II with high affinity

(Rechler *et al.*, 1980; Gelato *et al.*, 1989). Furthermore, binding of IGF-II occurs at a single site, which is different to the sites that bind proteins containing mannose-6-phosphate (Morgan *et al.*, 1987; Braulke *et al.*, 1988; Kornfeld, 1992). Following binding of IGF-II, the receptor is internalised and degraded (Oka *et al.*, 1985).

The physiological roles of the type 2 receptor as a component of the IGF system are unclear despite there being a reasonable understanding of the receptor's role in lysosomal enzyme transport (Braulke *et al.*, 1990; Kornfeld, 1992) as well as regulation of proteins that contain mannose-6-phosphate residues, such as thyroglobulin (Herzog *et al.*, 1987) and the latency-associated peptide of TGF-β (Harpel *et al.*, 1992). While some biological effects arising from IGF-II binding have been observed including an increased influx of calcium ions (Nishimoto *et al.*, 1987) and increased glycogen synthesis (Hari *et al.*, 1987), the specificity of these observations is questioned by studies suggesting that when the type 2 receptor is blocked with antibodies the biological responses to IGF-II are mediated by the type 1 IGF receptor (Kiess *et al.*, 1987b; Hartmann *et al.*, 1992). Similarly, there are conflicting results describing the involvement of G-proteins in type 2 IGF receptor signalling (Nishimoto *et al.*, 1989; Okamoto *et al.*, 1990; Ikezu *et al.*, 1995; Korner *et al.*, 1995), leaving the consequences of IGF-II binding to cell membrane type 2 IGF receptors unresolved.

A proteolytically cleaved 200-kDa form of the type 2 receptor can be found in culture media (MacDonald *et al.*, 1989; Clairmont and Czech, 1991) and in serum (Hey *et al.*, 1987; Kiess *et al.*, 1987a; Bobek *et al.*, 1992). Although the significance of this soluble form is unclear, it is found in higher concentrations in foetal serum than maternal or non-pregnant serum (Causin *et al.*, 1988; Gelato *et al.*, 1989) and together with the membrane-bound form may act as a specific binding protein for IGF-II (Lau *et al.*, 1994). These observations have generated the notion that the type 2 IGF receptor functions to remove IGF-II and possibly other growth

factors from the extracellular environment. This is given some credence by studies that show that deletion of the gene for this receptor in mice results in foetal overgrowth, increased serum levels of IGF-II and death during the perinatal period (Lau *et al.*, 1994; Wang *et al.*, 1994). Nevertheless, these and the aforementioned results describing possible roles for the type 2 IGF receptor are vexing and further investigation of this protein's role in the IGF system is warranted.

1.3.5 The IGF binding proteins

IGF levels in the mammalian circulation are approximately 750 ng/ml or almost 1000-times that of insulin (Zapf *et al.*, 1981; Daughaday and Rotwein, 1989) and although the hypoglycaemic potential of the IGFs is approximately 5% that of insulin and there is sufficient present to induce hypoglycaemia (Humbel, 1990), no such effect is seen. This paradox was explained following the discovery that IGFs circulate as high molecular weight complexes (Zapf *et al.*, 1975). The complexes, identified in plasma that was incubated with radiolabelled IGF-I and separated on the basis of molecular size, were observed to have molecular weights of 150 kDa and 50 kDa in adult and foetal plasma, respectively (Hintz and Liu, 1977; Kaufmann *et al.*, 1979). Acidification dissociated IGFs from the complexes, which then resolved to approximately 50 kDa and not 7.5 kDa. Furthermore, it was shown that the circulating half-lives of the IGFs are greatly decreased in hypophysectomised rats compared to normal (Zapf *et al.*, 1986), while the action of NSILA on fat cells is inhibited by the binding of a carrier protein (Zapf *et al.*, 1979). As no stores of IGFs were known, these initial observations suggested that the IGF binding proteins had a general role as inhibiters of IGF action and as carrier proteins

Characterisation of the IGFBPs was made when electrophoresis techniques were modified to allow visualisation of serum carrier proteins (Hardouin *et al.*, 1987; Hossenlopp *et al.*, 1987; Binoux and Hossenlopp, 1988). Several species of binding proteins with molecular weights between approximately 24 and 42 kDa were identified by Western-ligand blotting with radiolabelled IGFs. A variety of IGFBPs was initially identified in many biological sources, a feature reflected in the early names for IGFBPs. A system of common terminology was applied by Ballard *et al.* (1989) and at the time of the commencement of this thesis, IGFBPs –1 to –6 had been described (Baxter and Martin, 1989a; Rechler and Brown, 1992).

1.3.6 IGFBP structure

All the IGFBPs so far identified share sequence homology both within and between species, yet variations in the levels of production between cell types and tissues at different developmental stages suggests differing physiological roles. The N- and C-termini of the human IGFBPs contain 16 to 18 cysteine residues (Rechler, 1993). Sequence homology in these regions is high also, possibly reflecting their importance to IGF binding. In contrast, the mid-regions of the IGFBPs generally lack cysteine residues, IGFBP-4 being the exception, and display considerable heterogeneity between IGFBPs within a species.

The biological activity of many IGFBPs may be determined by several structural features, some of which are dependent on post-translational modification. IGFBP-1 and IGFBP-2 contain a RGD sequence near the C-terminus, conferring the ability to bind cell membrane integrin receptors (Bourner *et al.*, 1992; Jones *et al.*, 1993c), a process that may help localisation of IGFs to the cell surface. A similar role has been postulated for other IGFBPs that possess heparin-binding consensus sequences. For example, all human IGFBPs, except

IGFBP-4, contain putative glycosaminoglycan-binding domains (Hodgkinson *et al.*, 1994) and may potentially associate with either cell surfaces or extracellular matrix (Shimasaki *et al.*, 1991; Rechler, 1993). Although it is unclear how such association may regulate IGF interactions with the cell-bound IGF receptors, proteolytic degradation of IGFBP-5 by fibroblast-conditioned medium has been reported to be inhibited by glycosaminoglycans such as heparin, heparan sulphate and dermatan sulphate (Arai *et al.*, 1994a; Nam *et al.*, 1994). The affinity of IGFBP-5 for IGF-I is decreased by proteolysis. Thus, the presence of glycosaminoglycan-rich extracellular matrix rich may inhibit proteolysis of this IGFBP, resulting in sequestration of IGF-I by IGFBP-5 and decreased access to IGF receptors. Alternatively, binding of other IGFBPs to glycosaminoglycans may facilitate presentation of IGFs to the receptors as has been described for FGF-2 (Yayon *et al.*, 1991).

The native structure of the IGFBPs may be altered by post-translational processes such as glycosylation, phosphorylation, or limited proteolysis. These, in turn, may alter the affinity of the IGFBP for IGFs and consequently the effect of the IGFs. For instance, it has been reported that phosphorylated IGFBP-1 has an affinity for IGF-I that is several-fold higher than the unphosphorylated form (Jones *et al.*, 1991). Moreover, Jyung *et al.* (1994) showed that concomitant treatment of wounds with IGF-I and de-phosphorylated IGFBP-1 resulted in increased wound breaking strength. Thus, the state of phosphorylation may regulate IGF bioactivity. A similar effect may also explain the different states of glycosylation seen for IGFBP-3, although Conover (1992) did not report it as being a determining factor of IGF-I action *in vitro*.

1.3.7 Synthesis of IGFBPs *in vivo*

Expression of the IGFBPs occurs throughout the body, although the patterns of expression may vary between tissues. Furthermore, mRNA for IGFBPs has been detected in mice as early as day 11 of gestation (Schuller *et al.*, 1994). Expression of each IGFBP appears to be tissue-specific (Cerro *et al.*, 1993), such that variations in the proportions of each IGFBP can be found in different body fluids. For example, mRNA for IGFBP-3, the predominant IGFBP found in serum, is concentrated in the portal venous and hepatic endothelium (Harrington *et al.*, 1994), whereas IGFBP-1 protein is found predominantly in amniotic fluid.

The identities of the factors that regulate synthesis of IGFBPs *in vivo* are not entirely clear. Certainly, the IGFs are potent regulators of IGFBP expression (Clemmons, 1992). For example, IGF-I has been shown to increase IGFBP-5 expression by human neonatal fibroblasts (Martin and Baxter, 1990). However, some indication of the tissue specificity of IGFBP expression is revealed by the knowledge that IGF-I decreases IGFBP-1 expression in human hepatoma cells (Lee *et al.*, 1993). Similarly, IGFBP-3 expression by the liver is strongly dependent on growth hormone status (Baxter and Martin, 1986; Walton and Etherton, 1989; Zapf *et al.*, 1989), while IGFBP-1 expression by the liver appears to be regulated by nutritional status (Murphy *et al.*, 1990; Lewitt and Baxter, 1991). Other factors, such as the inflammatory cytokines and glucocorticoids may also play an important role in regulating IGFBP expression (Okazaki *et al.*, 1994; Scharla *et al.*, 1994). Nevertheless, how the many factors thus far shown to alter expression of IGFBPs regulate IGF action is unclear and deserving of further study.

1.3.8 Functions of IGFBPs

Regulation of IGF actions: inhibition or potentiation?

The classical view of IGFBPs is that they have an inhibitory role on the effects of IGFs. As their affinity for IGFs is typically higher than the IGF receptors, they sequester IGFs and may block interaction with cellular receptors, thereby preventing the IGFs from effecting a biological response. (IGF-I and IGF-II are very tightly bound by IGFBPs with association constants generally between 0.1 and 1.0 nM, which is equal to or higher than the affinity of the IGF receptors for the IGFs; Rechler, 1993). This model of IGFBP action was developed following observations of the effects of preparations of IGFBPs on IGF action *in vitro* (Zapf *et al.*, 1975; Meuli *et al.*, 1978; Ross *et al.*, 1989). Thus, an IGFBP purified from bovine kidney (MDBK) cells strongly inhibited the ability of IGF-I and IGF-II to stimulate DNA synthesis and protein accumulation *in vitro* (Ross *et al.*, 1989), while the addition of NSILA carrier protein ablated the biological activity of NSILA on rat heart (Meuli *et al.*, 1978). In many instances, co-treatment of *in vitro* cultures of cells with IGFs and IGFBPs results in the inhibition of growth (Rechler, 1993).

The observation that approximately 75% of IGF-I in serum is present in a complex comprising IGF-I, IGFBP-3 and an acid-labile subunit (Baxter and Martin, 1989b; Gargosky *et al.*, 1991; Baxter, 1993) supports the concept of IGFBPs as carrier proteins and inhibitors of IGF action. IGF-I present in the ternary complex has a half-life of 12 to 15 hours in humans (Guler *et al.*, 1989). It is thought that the complex, at approximately 150 kDa, is too large to cross the endothelial barrier and is retained in plasma (Binoux and Hossenlopp, 1988; Martin and Baxter, 1992). In contrast, free IGF-I has a half-life of approximately 10 min in plasma (Davis *et al.*, 1989; Hodgkinson *et al.*, 1989a) and 30-90 min when bound to IGFBPs (Young

et al., 1992; Lewitt *et al.*, 1993b). The decreased plasma half-lives of IGFs in hypophysectomised rats, in which levels of circulating IGFBP-3 are greatly reduced, also underscores the role of IGFBPs as carrier proteins (Moses *et al.*, 1979; Zapf *et al.*, 1986).

Sequestration of IGFs by plasma IGFBPs appears to be important in the context of glucose homeostasis. Lewitt *et al.* (1991) infused a bolus dose of human recombinant IGF-I into rats and measured an approximately 72% fall in plasma glucose levels after 15 min, whereas an equimolar amount of IGFBP-1 co-infused with IGF-I abolished the effect. Similarly, infusion of radiolabelled IGF-I was shown to associate with the low molecular weight IGFBPs in humans, suggesting that these binding proteins are unsaturated in the circulation and that the IGFBPs have a protective effect against the potential insulin-like effects of IGF-I (Guler *et al.*, 1989). Further support for this hypothesis is derived from the observation that little or no IGF is unbound in plasma (Zapf *et al.*, 1986). However, a further study by Lewitt *et al.* (1993a) clouded this view by suggesting that there is a readily available pool of IGF-I available to complex with infused IGFBP-3 because complex formation was very rapid following injection of IGFBP-3. Furthermore, the acid-labile subunit is 2- to 3-times in excess of the complex (Baxter, 1990a). The source of readily available IGF-I is unknown especially in light of the fact that IGF-I is not stored within cells.

Although the actions of IGFs may be inhibited due to association with IGFBPs, under certain conditions the IGFBPs may potentiate IGF effects. Such functional duality has been observed, in particular, for IGFBP-1, -3, and -5. Initially, Elgin *et al.* (1987) showed that an IGFBP preparation obtained from human amniotic fluid, presumably IGFBP-1 (Busby *et al.*, 1988b), was able to potentiate DNA synthesis by fibroblasts and porcine aortic smooth muscle cells in response to IGF-I. Moreover, the response was synergistic: treatment with IGF-I or IGFBP alone resulted in increases of 8% and 17%, respectively of that achieved when in

combination. Subsequent work revealed that the binding protein contained two forms of IGFBP-1, each of which had differing abilities to bind to cell surfaces (Busby *et al.*, 1988b; Jones *et al.*, 1991). Further work revealed that a de-phosphorylated form showed the best cell-surface association and enhanced IGF-I action the greatest (Jones *et al.*, 1993a).

De Mellow and Baxter (1988) showed that IGFBP-3 was also able to potentiate IGF-I effects on DNA synthesis in human fibroblasts. However, as a clue as to the mechanism by which IGFBP-3 was able to achieve this, pre-incubation of the cells with IGFBP-3 prior to the addition of IGF-I was necessary to demonstrate such an effect. Simultaneous addition of the binding protein and IGF-I resulted in inhibition of IGF-I action, findings that were also reported by Conover *et al.* (1990). Furthermore, De Mellow and Baxter (1988) were able to demonstrate that pre-incubation resulted in association of IGFBP-3 with the cell surfaces, whilst Conover extended this finding by showing that potentiation of the effects of IGF-I by this IGFBP was dependent on proteolytic processing of the membrane-bound IGFBP (Conover, 1992).

The common theme of the enhancement of IGF-I action by these IGFBPs is cell-surface association, albeit by slightly different means. IGFBP-1 has been shown to bind the $\alpha 5\beta 1$ integrin, which is abundant on fibroblasts isolated from Chinese hamster ovaries (Jones *et al.*, 1993c). On the other hand, furtherance of IGF-I actions by IGFBP-3 may be dependent on attachment to the cell surface via a putative glycosaminoglycan-binding consensus sequence (Hodgkinson *et al.*, 1994). Nevertheless, the events that occur after IGFBP binding to the cell surface are unclear. In the case of IGFBP-1, binding to integrins may generate specific cell signals (Hynes, 1992), while cell-bound IGFBP-3 appears to be degraded by proteases into smaller fragments that have lowered affinity for IGF-I (Conover, 1991; Conover, 1992). This

latter process may then allow transfer of IGF-I from the IGFBP fragments to the IGF-I receptor.

Tissue and cell targeting

Whilst a large proportion of serum IGF is found in the 150 kDa complex (Jones *et al.*, 1991), the roles of the smaller molecular weight complexes of IGFBPs and IGFs are unclear. However, some lines of evidence suggest a possible role in the transfer of IGFs from the circulation to peripheral tissues (Sara and Hall, 1990). Specifically, IGFBPs present in the smaller complexes, namely IGFBP-1, -2 and -4, have decreased circulating half-lives in serum compared to the 150-kDa complex (Young *et al.*, 1992); compared to serum, a greater proportion of the lower molecular weight IGFBPs was observed on Western-ligand blots of lymph (Binoux and Hossenlopp, 1988); almost all of the IGF-I present in lymph eluted from a size-exclusion chromatography column in the lower molecular weight region; and finally, these complexes are of a small enough size to cross the endothelial barrier. Indeed, functional studies supporting these observations were made in a series of studies by Bar and colleagues. Using preparations of isolated and perfused beating rat hearts, they demonstrated that IGFBP–1 and -2 crossed intact endothelium and capillaries when complexed with radiolabelled IGF-I (Bar *et al.*, 1990a; Bar *et al.*, 1990c). Additionally, an endothelial cell-derived IGFBP later identified as IGFBP-4 (Boes *et al.*, 1992), also crossed the endothelial barrier (Bar *et al.*, 1990b). Interestingly, human growth hormone did not affect the transcapillary movement of these IGFBPs, whilst insulin increased the movement of IGFBP-1 and decreased transport of IGFBP-4, but had no effect on IGFBP-2 (Bar *et al.*, 1990a). Thus

these IGFBPs, under the control of other hormones other than IGF-I, may selectively shuttle IGFs from the vasculature to peripheral tissues.

An interesting aspect of the IGFBP translocation studies in isolated and perfused beating rat hearts was the observation that the IGFBPs localised to different tissues within the heart. The proportion of IGFBP-4 found in the connective tissue of the heart was greatest when infused alone (Bar *et al.*, 1990c; Boes *et al.*, 1992). However, when cross-linked to IGF-I, the IGF:IGFBP complex was found in greater proportions within the heart muscle (Bar *et al.*, 1990b). Thus, the presence or absence of IGF-I dictated the destination of the IGFBP.

The observations by Bar and co-workers raised the possibility that IGFBPs may selectively target different tissues. Indeed, earlier studies had suggested that IGFBPs might specifically bind target cells thereby facilitating delivery of IGFs to membrane-bound receptors (Clemmons *et al.*, 1986; Madtes *et al.*, 1988). As IGFBP-1 and IGFBP-2 have RGD consensus sequences and specifically bind the $\alpha 5\beta 1$ integrin receptor (Jones *et al.*, 1993c), these IGFBPs may be directed to those cells which express the appropriate integrin receptor (Ruoslahti and Pierschbacher, 1987). Similarly, putative glycosaminoglycan consensus sequences, which have been identified in IGFBP-5 (Hodgkinson *et al.*, 1994), may allow this IGFBP to tightly adhere to extracellular matrix (Jones *et al.*, 1993c). (The predicted heparin-binding consensus sequences are XBBXBX and XBBBXXBX where B is any basic and X is any hydropathic amino acid; Cardin and Weintraub, 1989). The specificity of this interaction was demonstrated by the inhibition of binding by synthetic peptides containing the putative heparin-binding domain of IGFBP-5 (Arai *et al.*, 1994b). It has been also noted after iv infusion of radiolabelled IGFBPs *in vivo* that there were unequal amounts of radioactivity found between the tissues (Arany *et al.*, 1993), supporting the notion that IGFBPs may selectively target tissues for IGF delivery.

Putative glycosaminoglycan-binding sequences are present in human and rat IGFBP-3 (Hodgkinson *et al.*, 1994), but this binding protein appears to associate with cell-surfaces rather than extracellular matrix (Smith *et al.*, 1994). This may be an important difference as cell-associated IGFBP-3 has a ten-fold reduced affinity for IGF-I (Conover, 1991), far less than the affinity of IGF-I for the type 1 receptor. With one study suggesting that nearly all the cell-surface associated IGF-I is bound to cell-associated IGFBP-3 (McCusker *et al.*, 1990), targeting of tissues may substantially increase the IGF-I levels of the cell micro-environment. Although the traditional view of IGFBPs is as modulators of IGF action and as carrier proteins, further studies of cell-associated IGFBP-3 have revealed another important aspect of IGFBP functions. Thus, De Mellow and Baxter (1988) showed that co-incubation of IGF-I with IGFBP-3 resulted in inhibition of IGF-I-stimulated tritiated-thymidine incorporation by human skin fibroblasts. Conversely, pre-incubation of the cells with IGFBP-3 potentiated the actions of IGF-I, increasing the maximal rate of DNA synthesis. These findings were extended in a similar manner by Conover (1992). The significance of this phenomenon is not known, but may represent a means by which a pool of IGF is maintained in close proximity to the target cells and of which could be mobilised during times of metabolic need.

IGF-independent actions

Whilst the cell-associated IGFBPs may potentiate IGF action, there is some evidence to suggest that bound IGFBP may act in a manner independent of IGF involvement. In a study of the effects of growth factors upon DNA synthesis, mouse IGFBP-3, then known as inhibitory diffusible factor of 45 kDa (Blat *et al.*, 1989), inhibited both serum- and fibroblast growth factor-induced DNA synthesis (Villaudy *et al.*, 1991; Liu *et al.*, 1992a). The inhibitory action

was also active in the presence of IGF-depleted serum, suggesting that IGFBP-3 was able to act independently of the IGFs (Liu *et al.*, 1992b). Then Oh *et al.* (1993a) demonstrated dose-dependent and IGF-independent inhibition of growth of human Hs578T breast cancer cells, a cell-line which lacks oestrogen receptors and fails to grow in response to insulin or IGF treatment (De Leon *et al.*, 1992). Native IGFs were able to inhibit binding of IGFBP-3 to the cell surface, yet analogues of IGFs that don't bind IGFBPs well yet retain affinity for the IGF receptors, were unable to stimulate growth in these cells. Likewise, IGFBP-3 added to the culture medium was able to inhibit cell growth, the effect of which was partially blocked by co-incubation with native IGF-II. This indicated that endogenously secreted IGFBP-3 was not responsible for the lack of IGF-stimulated growth. A further study using affinity cross-linking strongly suggested the presence of specific IGFBP-3-binding surface proteins (Oh *et al.*, 1993b).

Proteolysis of IGFBPs

An additional level of regulation of IGF action was surmised from observations that limited proteolysis of IGFBPs, such that their affinity for IGF is reduced, may occur both *in vitro* and *in vivo*. Varieties of proteases have been described for IGFBPs -2 to -5 (Jones and Clemmons, 1995), yet most are yet to be fully characterised. The initial observations of this phenomenon were made when serum from pregnant humans and rodents was analysed by Western-ligand blotting (Davenport *et al.*, 1990; Fielder *et al.*, 1990; Giudice *et al.*, 1990; Hossenlopp *et al.*, 1990). In these studies, the characteristic 43-/45-kDa doublet of IGFBP-3 was undetectable in serum collected from soon after conception to the early post-partum period. However, when IGFBP-3 content was measured by radioimmunoassay, the levels were normal (Baxter and

Martin, 1986; Gargosky *et al.*, 1992). Although further studies revealed that the predominant form of IGFBP-3 in these sera was a proteolysed fragment of approximately 28-30 kDa that binds iodinated IGF-I with reduced affinity (Suikkari and Baxter, 1991), the modified IGFBP–3 can still associate with IGF-I and the acid-labile subunit to form 150-kDa complexes (Suikkari and Baxter, 1992; Bang *et al.*, 1994b).

After the reports of proteolysis in pregnancy plasma, it was recognised that IGFBP-3 may be proteolysed in the sera of patients following major or elective surgery (Cwyfan-Hughes *et al.*, 1992; Davenport *et al.*, 1992a), severe illness (Davies *et al.*, 1991), development of malignancy (Muller *et al.*, 1994), and renal failure (Lee *et al.*, 1994a). Additionally, IGFBP-3 proteolysis was observed in the serum of patients with non-insulin-dependent diabetes mellitus or prostate cancer (Cohen *et al.*, 1992; Bang *et al.*, 1994a). In these circumstances, the proteolytic activity was largely due to cation-dependent serine proteases. However, matrix metalloproteases have also been observed to have IGFBP-3 proteolytic activity *in vivo* (Fowlkes *et al.*, 1994b) and *in vitro* (Fowlkes *et al.*, 1994a).

The function that proteolysis of IGFBPs may serve is unclear. It has been proposed that by reducing the affinity of the IGFBPs for IGFs, which reduces sequestration by IGFBPs, the IGFs could become more bioavailable (Lamson *et al.*, 1993). For example, prostate-specific antigen added to prostate epithelial cells proteolyses IGFBP-3 and abolishes the normal inhibitory effect of IGFBP-3 on the proliferation of these cells *in vitro* (Cohen *et al.*, 1994b). Similarly, Blat *et al.* (1994) reported that DNA synthesis in chicken embryo fibroblasts was stimulated to a greater extent by sera from women in their last trimester of pregnancy than by serum from non-pregnant adults. This proposal is also supported by a report that free IGF-I in plasma is increased during the last trimester of pregnancy (Hasegawa *et al.*, 1995). Furthermore, Lassarre and Binoux (1994) reported that during pregnancy the modified

IGFBP-3 has a decreased affinity for the IGFs and accelerated kinetics of dissociation. They also reported more IGF in a free form in plasma as well as a redistribution of IGF from the 150-kDa complex to the 50-kDa complex. These data agrees with earlier reports that IGFBPs -1, -2 and -4 can cross the endothelial barrier (Bar *et al.*, 1990b; Bar *et al.*, 1990c; Boes *et al.*, 1992), that radiolabelled IGF-I present in binary complexes with IGFBPs has a shorter circulating half-life than that present in the 150-kDa complex (Zapf *et al.*, 1986), and that the half-life of serum IGF-I is decreased in pregnant rats (Davenport *et al.*, 1990). Thus, proteolytic modification of IGFBP-3, the main plasma carrier protein for IGF-I, appears to increase dissociation of the IGF/IGFBP complexes enabling the IGFs to more readily interact with cellular receptors. However, definitive evidence of such a mechanism operating *in vivo* has yet to be demonstrated.

Proteolysis of IGFBPs other than IGFBP-3 may also occur during pregnancy and appears to occur normally in the extravascular tissues. Incubation of iodinated IGFBP-5 with pregnancy serum or amniotic fluid results in its proteolysis and the generation of two fragments of approximately 22 kDa and 15 kDa (Claussen *et al.*, 1994). Similar observations have been made using media conditioned by human osteosarcoma cells (Conover and Kiefer, 1993), fibroblasts (Conover and Kiefer, 1993; Nam *et al.*, 1994), or osteoblasts (Kanzaki *et al.*, 1994). IGFBP-5 proteolytic activity, partially purified from fibroblasts, was inhibited by heparin as well as a synthetic peptide containing the heparin-binding consensus site (Nam *et al.*, 1994), suggesting that association with the extracellular matrix may protect IGFBP-5 from degradation. Indeed, IGFBP-5 incubated with extracellular matrix may potentiate the actions of IGF-I, yet when added to the medium is cleared and has no effect (Jones *et al.*, 1993b). Further, the addition of IGF-I or -II was shown to protect IGFBP-5 from proteolysis in the

conditioned media of human osteosarcoma cells, whereas analogues of IGF-I that don't bind IGFBPs were ineffective (Conover and Kiefer, 1993).

Other examples of IGFBP proteolysis also exist. IGFBP-4 generally acts to inhibit the actions of IGFs, however, in contrast to IGFBP-5, proteolysis of IGFBP-4 consistently diminishes the inhibitory effect of this binding protein (Conover *et al.*, 1995). Additionally the IGFs, particularly IGF-II, promote the proteolysis of IGFBP-4 (Conover *et al.*, 1993). Thus, it has been proposed that IGF-II, which is the predominant IGF found in human bone (Mohan *et al.*, 1994), may act to increase available concentrations of IGF-I (Mohan *et al.*, 1994). The essence of this model is that there is a fine balance between those factors that promote bone lysis and those that promote bone cell proliferation and new bone formation. Factors that may act to alter protease or IGFBP expression, such as the IGFs and other growth factors or hormones including TGF-β and progesterone, may therefore alter the balance and regulation of bone formation. However, it remains to be determined whether such mechanisms may occur in other tissues that also produce these IGFBPs.

1.3.9 The acid-labile subunit

The acid-labile subunit of the serum ternary complex was originally purified from human plasma (Baxter *et al.*, 1989). The core protein has a molecular weight of approximately 70 kDa, but glycosylation yields two forms in plasma with molecular weights of approximately 84 and 86 kDa (Baxter *et al.*, 1989). The acid-labile subunit is expressed in the liver (Dai and Baxter, 1992) and expression appears to under the control of growth hormone and not IGF-I (Walton and Etherton, 1989; Zapf *et al.*, 1989; Baxter and Dai, 1994). The possibility that the acid-labile subunit was produced locally in peripheral tissues was raised by the initial

demonstration of mRNA in a wide range of tissues (Dai and Baxter, 1992). Indeed, acid-labile subunit protein was measured in amniotic and cerebrospinal fluids, albeit at amounts less than 0.5% of plasma (Baxter, 1990a). However, revision of the study of mRNA distribution with shorter and more specific probes revealed that expression was confined exclusively to the liver (Baxter *et al.*, 1994). Thus, it seems that expression of the acid-labile subunit is restricted to the liver and its presence in extravascular fluids is a result of transfer from the circulation.

In serum, the acid-labile subunit, present in amounts excess to IGFBP-3, binds IGFBP-3 and IGF-I to form a stable ternary complex with a molecular weight of approximately 150 kDa (Baxter and Martin, 1989b). Binding of the acid-labile subunit to IGFBP-3 does not alter binding of IGF-I to IGFBP-3, though binding to IGFBP-3 doesn't appear to occur in the absence of IGF-I (Baxter *et al.*, 1989). Furthermore, binding appears to be specific for IGFBP-3 as attempts to form the ternary complex using IGFBP-1 or -6 were unsuccessful (Baxter and Martin, 1989b; Martin *et al.*, 1990). [Although not reported by the time of this review, Twigg and Baxter (1998) have recently described the ability of IGFBP-5 to form ternary complexes with IGFs and the acid-labile subunit]. Notwithstanding those results, heparin, heparan sulphate, and chrondroitin sulphate have been reported to inhibit the formation of the ternary complex (Baxter, 1990b). This has possible implications for the stability of the ternary complex when in close association with capillary endothelium: cell-surface glycosaminoglycans may bind IGFBP-3, disrupt the complex and allow IGF-I to be released and transferred to adjacent tissues (Baxter, 1990b; Booth *et al.*, 1995). The binary complexes may also be subject to the same phenomenon (Arai *et al.*, 1994b).

1.3.10 IGF-I analogues with reduced affinities for IGFBPs

The development of recombinant DNA technology enabling modification of the primary structure of proteins and production of large quantities of pure peptide has contributed greatly to the understanding of how such modifications may alter protein-protein interactions. The first insights into IGF modifications that may affect interactions with IGFBPs and IGF actions were gained when a natural variant of IGF-I was isolated from *in vivo* sources. Thus, a truncated version of IGF-I missing the first three N-terminal amino acids, des(1-3)IGF-I, has been isolated from bovine colostrum (Francis *et al.*, 1986; Francis *et al.*, 1988b), brain tissue (Carlsson-Skwirut *et al.*, 1986; Sara *et al.*, 1986), and human platelet lysates (Karey *et al.*, 1989; Karey and Sirbasku, 1989). This form of IGF-I has a potency five- to ten-fold greater than that of native IGF-I in stimulating DNA and protein synthesis and inhibiting protein breakdown *in vitro* (Ross *et al.*, 1989). At first the functional significance of this truncated IGF-I variant was not fully understood and seemingly contradicted early models of IGF tertiary structure that supposed that the N-terminus of the IGF molecule was highly flexible and not involved in receptor binding (Blundell *et al.*, 1978). However, it is now known that the principal effect of the amino acid deletions is to reduce the affinity of this IGF for IGFBPs.

Further understanding of the effect of the deletion of the Gly-Pro-Glu tripeptide from the N-terminus of IGF-I was made using chemically synthesised analogues that were truncated by 1 to 5 amino acids (Bagley *et al.*, 1989). Compared to native IGF-I, deletion of the first two amino acids did not alter the concentration of peptide required to achieve half-maximal stimulation of protein synthesis by rat L6 myoblasts, whereas deletion of the first 3 amino acids resulted in an approximately ten-fold decrease in the concentration of peptide required. Des(1-4)IGF-I was less potent than des(1-3)IGF-I, while des(1-5)IGF-I was approximately

1200-times less potent than native IGF-I. When the ability of the respective peptides to bind IGF receptors in the same cells was examined, it was found that des(1-3)IGF-I was bound less well than IGF-I. Thus, the increased potency of des(1-3)IGF-I could certainly not be explained by increased binding to the IGF receptors. However, the paradox in this data was clarified by binding studies using media conditioned by L6 myoblasts and IGFBP purified from bovine kidney (MDBK) cell-conditioned media. The data showed that the increased potency of the shorter IGFs was due to much lower binding to IGFBPs, so enabling increased interaction of the growth factor with the IGF receptors (Szabo *et al.*, 1988; Bagley *et al.*, 1989).

Other analogues of IGF-I have properties that identify glutamic acid at position three as the critical amino acid for IGFBP binding (Francis *et al.*, 1992; King *et al.*, 1992). IGFs with substitutions of this amino acid with either glycine or arginine, but without any further changes, also bind IGFBPs less well and are more potent *in vitro* than native IGF-I (King *et al.*, 1992). Similar results were observed with the analogue [Gln^3,Ala^4,Tyr^{15},Leu^{16}]IGF-I (Bayne *et al.*, 1988), although substitutions elsewhere in the IGF-I molecule do not permit the observed reduction in affinity for IGFBPs to be attributed solely to substitution at the third amino acid position. This point was illustrated by Baxter and co-workers who showed that [Gln^3,Ala^4]IGF-I and [Tyr^{15},Leu^{16}]IGF-I bound purified IGFBP-3 with similar affinity, but less than that observed for native IGF-I (Baxter *et al.*, 1992).

Notwithstanding these results, assessment of the decline in affinity of IGF-I analogues is also dependent on the IGFBP used. Clemmons *et al.* (1990) reported that substitution of residues 3, 4, 15, and 16 were required to detect dramatic losses in affinity for IGFBP-3, yet only substitutions at residues 3 and 4 were needed to observe greater than 30-fold losses in affinity for IGFBP-1. Similar results were obtained with an IGF-I analogue with mutations at

positions 49, 50, and 51 in the A domain (Clemmons *et al.*, 1992): the affinity for IGFBPs -1, -2 and -4 was reduced by almost 20-fold whilst the affinity for IGFBP-3 was minimally affected. These data highlighted not only the increased potency of IGF-I that can arise through reduced interactions with the IGFBPs, but also those residues that are important for the binding of IGF-I to IGFBPs.

Similar observations have been made of analogues of IGF-II with N-terminal truncations or amino acid substitutions a position 6, the residue equivalent to position 3 in IGF-I (Francis *et al.*, 1993). However, the increase in potency *in vitro* is not as large as that observed with IGF-I analogues as the responses to the modified IGF peptides are attenuated by the presence of the type 2 receptor. The IGF-II analogues have an affinity 100-fold higher for the type 2 IGF receptor than either IGF-I or IGF-I analogues (Francis *et al.*, 1993). Thus, in cells lacking the type 2 receptor, such as chicken embryo fibroblasts (Kallincos *et al.*, 1990), the ability of the IGF-II analogues with reduced IGFBP affinity to inhibit protein breakdown is similar to the IGF-I analogues (Francis *et al.*, 1994).

The increased *in vitro* anabolic potency of these analogues is attributed to the reduced affinity for IGFBPs, a situation that provides indirect evidence of the inhibitory role of the IGFBPs (Forbes *et al.*, 1988; Szabo *et al.*, 1988). A similar increased potency of the IGF-I analogues is also observed in some *in vivo* models. Long [Arg^3]IGF-I, with a thirteen amino acid N-terminal extension and a substitution of arginine for glutamine at position 3, was 3- to 5-times more potent than IGF-I in stimulating growth and nitrogen accumulation in catabolic rats (Tomas *et al.*, 1991a; Tomas *et al.*, 1991b; Tomas *et al.*, 1992). This form of IGF-I also stimulated gut growth in rats after intestinal resection (Lemmey *et al.*, 1991). However, clearance of the infused peptide from the circulation of normal rats was increased and a greater proportion was in a degraded form (Francis *et al.*, 1988a; Ballard *et al.*, 1993). The

results of these two latter studies support the postulated role of the IGFBPs not only as inhibitors of IGF action, but also as carrier proteins that prolong IGF half-lives and protect against degradation.

1.4 THE ROLES OF IGFS AND IGFBPS IN DERMAL AND EPIDERMAL WOUND HEALING

Although the somatomedin hypothesis states that the IGFs are produced by the liver in response to pituitary-derived GH, sites of IGF-I synthesis are readily identified in a range of extra-hepatic tissues (D'Ercole *et al.*, 1984; Lund *et al.*, 1986; Murphy *et al.*, 1987). Moreover, basal production of IGF-I mRNA at those sites suggested some, but not exclusive, dependence on GH action (D'Ercole *et al.*, 1984; Mathews *et al.*, 1986). However, it is important to note that expression of IGF-I within a given tissue or organ is not uniform, being restricted to particular cell types within that organ (Han *et al.*, 1987).

IGF-I synthesis in tissues other than the liver, especially in response to trophic factors other than GH (Clemmons, 1984), suggests that IGF-I may have roles throughout the body distinct from being a regulator of somatic growth. Some of the first indications of this were evident when immunoreactive IGF-I was identified in regenerating nerves and endothelial cells (Hansson *et al.*, 1986; Hansson *et al.*, 1987). A wider role for IGF-I during tissue repair and injury was proposed when mRNA for IGF-I was identified in wound macrophages (Rappolee *et al.*, 1988) and later in granulation tissue (Gartner *et al.*, 1992; Steenfos and Jansson, 1992b). IGF-I protein is also present in the α-granules of platelets (Karey and Sirbasku, 1989) and acute wound fluid (Spencer *et al.*, 1988; Steenfos and Jansson, 1992a), whilst wound fibroblasts synthesise IGF-I *in vitro* (Sumi *et al.*, 1984). Although at the time of this review IGFBP-3 had been identified in platelet releasate (Spencer *et al.*, 1993), little was known of the expression and production of IGFBPs during the wound repair response.

The discoveries that IGF-I is expressed and produced at the wound site and that depletion of certain wound elements that produce IGF-I results in delayed wound healing (Leibovich and

Ross, 1975; Rappolee *et al.*, 1988; Noble *et al.*, 1993), strongly suggest that IGF-I may act as a vulnerary agent. This notion is supported by the observation that acute human wound fluid stimulates fibroblast and endothelial cell growth *in vitro* (Katz *et al.*, 1991) and early observations of the *in vivo* effects on wound healing of exogenous EGF (Buckley *et al.*, 1985; Brown *et al.*, 1988), PDGF (Grotendorst *et al.*, 1985), FGF-2 (McGee *et al.*, 1988; Slavin *et al.*, 1992) and TGF-β (Mustoe *et al.*, 1987; Sprugel *et al.*, 1988; Pierce *et al.*, 1989). However, the effects of exogenous IGF-I on cutaneous wound repair are equivocal. Whilst positive responses to IGF-I in several *in vitro* models of wound healing have been documented (Taylor and Alexander, 1993), the effects of exogenous IGF-I on cutaneous repair *in vivo* are more obscure. Thus, a single dose of up to 1500 ng of IGF-I added to partial thickness porcine skin wounds at the time of wounding had no effect upon the mean thickness of the newly formed dermis seven days after wounding. A similar lack of effect was noted of the hydroxyproline content of wound tissue (Lynch *et al.*, 1989). In contrast, Suh *et al.* (1992) treated rats with methylprednisolone and infused IGF-I into subcutaneous implanted wire mesh chambers for seven or fourteen days to find that the steroid-induced reductions in hydroxyproline, DNA and total protein content were reversed. This study followed an earlier observation that showed IGF-I had similar effects upon indices of wound repair in hypophysectomised rats (Steenfos *et al.*, 1989).

Some insight into the mechanism of these responses has been revealed by Mueller *et al.* (1994), who showed that infusion of IGF-I locally into the wound chambers implanted in hypophysectomised rats increased wound macrophage numbers. Although the central role of macrophages in wound healing had been demonstrated much earlier (Leibovich and Ross, 1975), and it while appears logical that restoration of wound macrophage levels to near normal may reverse the deleterious effects of steroid-administration, the evidence linking

IGF-I- and macrophage-dependant events is still circumstantial and requires further investigation. This is especially so in light of the failure of other laboratories to demonstrate favourable effects of IGF-I added alone in other *in vivo* models of wound healing.

The ambiguous results of IGF-I are puzzling. It was recognised several years ago that in addition to its anabolic effects, growth hormone (GH) was also a potent vulnerary agent. GH treatment increased hydroxyproline deposition in wounds (Kowalewski and Yong, 1968) and wound bursting strength in normal rats (Zaizen *et al.*, 1990). GH has also been shown to improve wound strength in rats treated with glucocorticosteriods (Takano *et al.*, 1994). Such results have lead to the use of GH to increase the rate of healing of donor sites of thermal injury patients that undergo skin grafting (Herndon *et al.*, 1990; Gilpin *et al.*, 1994; Herndon *et al.*, 1995). Promising therapeutic results have also been made with growth hormone releasing factor, though the response appears inferior to that of GH (Garrel *et al.*, 1991). However, the diabetogenic properties of GH favour IGF-I as a replacement for GH therapy in a range of conditions including that following injury.

In light of the wound healing responses to GH, the less than favourable results achieved with IGF-I treatment require further exploration. Some possible explanations may be sought from studies in which IGF-I was applied in combination with other growth factors. Thus, Lynch *et al.* (1994) reported a synergistic effect of PDGF when applied to porcine partial thickness skin wounds conjointly with IGF-I. Seven days after wounding, they observed an increase in epidermal thickness and a 2.5-fold increase in the width of newly formed connective tissue compared to controls, effects that were greater than those seen in wounds treated with either vehicle, PDGF alone, or IGF-I alone. The observations were also confirmed in a later study by these authors (Lynch *et al.*, 1989).

The mechanism by which this synergy occurs is not immediately clear, however PDGF is a potent mitogenic and chemotactic growth factor for fibroblasts (Seppa *et al.*, 1982) and arterial smooth muscle cells *in vitro* (Grotendorst *et al.*, 1981) and has also been shown to stimulate collagen formation *in vivo* (Grotendorst, 1984; Sprugel *et al.*, 1988; Pierce *et al.*, 1991). The synergy observed between IGF-I and other growth factors may be partially explained by the contrasting effects that each growth factor has on the cell cycle of quiescent cells. PDGF may be classified as a "competence factor" in that it is capable bringing cells to the first growth arrest point. IGF-I has been described as a progression factor, allowing cells to progress from a state of rest to one of division and proliferation (Pledger *et al.*, 1978; McGrath, 1990). In the wound environment, initial release of PDGF from platelet α-granules and later production by fibroblasts and epithelial cells from one day post wounding (Antoniades *et al.*, 1991) may "prime" wound fibroblasts and sensitise them to the mitogenic and anabolic actions of IGF-I. Stimulated to produce IGF-I within the wound, fibroblasts may also act in an autocrine or paracrine manner.

A scenario of regulation of wound repair by local factors does not preclude an endocrine role for liver-produced IGF-I. However, evidence that expression of IGF-I mRNA is up-regulated in regenerating skeletal muscle of normal and hypophysectomised rats suggests a central role for locally produced factors in wound repair (Edwall *et al.*, 1989). Similarly, demonstration of mRNA for receptors to GH in skin fibroblasts, but not in keratinocytes (Tavakkol *et al.*, 1992), further reinforces the view that IGF-I may act in a manner other than that of a classical endocrine hormone.

A further clue as to the possible reasons for the failure of IGF-I to promote surface wound repair comes from the knowledge that IGFBPs modulate IGF-I action. More specifically, IGFBP-3 can be found in platelet lysates (Spencer *et al.*, 1993) and is presumably released

into the wound environment upon injury. Although synthesis of IGFBP-3 by the liver appears to be predominantly regulated by GH (Blum and Ranke, 1990), it is not known whether GH also acts to modulate extra-hepatic expression of this IGFBP. Similarly, the patterns of expression of IGFBPs during the wound repair response have yet to be described.

The presence of IGFBPs in the wound milieu may serve several functions. They may prolong IGF-I action through protection from premature degradation and diffusion from the wound site. Limiting access of IGF-I to cellular receptors may also be important in preventing IGF receptor down-regulation in response to high levels of free exogenous IGF-I (Conover and Powell, 1991). These observations may explain the absence of effect of application of exogenous IGF-I to wounds, often at concentrations 100- to 1000-fold that needed to stimulate DNA synthesis or cell migration *in vitro*, as well as the enhanced anabolic response to a combination of GH and IGF-I (Kupfer *et al.*, 1993; Hussain *et al.*, 1994). Nonetheless, mRNA for GH receptors has been demonstrated in skin fibroblasts (Tavakkol *et al.*, 1992), indicating that GH may act locally in dermal wounds to facilitate the actions of IGF-I. How such an arrangement may work *in vivo* has yet to be determined.

Although data that gives clear indications of the interactions IGFs and IGFBPs may have in the wound are limited, several recent studies have highlighted the possible importance of IGFBPs in wound repair. Both IGFBP-1 (Jyung *et al.*, 1994; Tsuboi *et al.*, 1995; Zhao *et al.*, 1995) and IGFBP-3 (Sommer *et al.*, 1991; Mueller *et al.*, 1994) have been shown to improve wound repair indices under normal and pathological conditions. Application to a wound of an IGFBP conjointly with IGF-I may localise IGF-I to the cell surface and make it more readily accessible to IGF receptors: IGFBP-1 acting via its $\alpha 5\beta 1$ integrin binding sequence, or as in the case of IGFBP-3, via heparin-binding domains (Hodgkinson *et al.*, 1994). However, the effects appear to require precise regulation. For instance, only the dephosphorylated form of

IGFBP-1 was effective in enhancing the vulnerary effect of IGF-I. Moreover, equimolar amounts of IGFBP-1 and IGF-I were ineffective, whereas a ratio of 11:1 or 5:1 were effective. This contrasts with the result obtained with IGFBP-3, with which equimolar amounts were effective. Although IGFBP-1 may mediate IGF-I signalling by binding to $\alpha 5\beta 1$ integrin receptors (Jones *et al.*, 1993c), the intracellular events that follow binding are obscure and may be dependent on the target cells. Notwithstanding their IGF-independent effects, it is possible that the IGFBPs may act as co-stimulatory molecules with IGF-I, the integrin receptors being the co-receptors (Slootweg *et al.*, 1990; Hynes, 1992).

1.5 REGULATION OF THE EXTRAVASCULAR ACTIVITY OF IGF-I BY IGFBPS

Approximately three-quarters of the plasma IGFs circulate in a high molecular weight complex with IGFBP-3 and the acid-labile subunit (Zapf *et al.*, 1975; Baxter and Martin, 1989b; Gargosky *et al.*, 1991; Baxter, 1993), whilst almost all of the remaining IGF is present in binary complexes with lower molecular weight IGFBPs. Only a small amount of the total IGF contained within plasma is unassociated with binding proteins making the IGFBPs significant determinants of IGF action. Direct evidence supporting this notion has also been gained from many *in vivo* studies: plasma clearance of IGFs is greater in hypophysectomised rats, which have significantly reduced levels of IGFBP-3 (Moses *et al.*, 1979; Zapf *et al.*, 1986); the IGFs do not exert a hypoglycaemic effect despite being in amounts greater than 750 ng/ml and having a hypoglycaemic potential approximately 5% that of insulin (Zapf *et al.*, 1981; Daughaday and Rotwein, 1989; Humbel, 1990); IGF analogues with reduced affinity for IGFBPs are cleared from plasma more rapidly, are degraded to a greater extent and are transferred to the tissues more extensively (Francis *et al.*, 1988a; Ballard *et al.*, 1991; Ballard *et al.*, 1993); and clearance of IGFs is greater when proteolytic modification of IGFBP-3 occurs such as in pregnant rats (Bastian *et al.*, 1993). However, it is not clear from this data whether the same regulatory mechanisms that determine the intravascular dynamics of the IGFs also occur in wounds, or more generally in extravascular spaces.

IGFs and IGFBPs can be found in many extravascular fluids including lymph (Cohen and Nissley, 1975) and cerebrospinal (Yamaguchi *et al.*, 1990), synovial (Schalkwijk *et al.*, 1989), follicular (Owen *et al.*, 1991), and amniotic fluids (Adashi *et al.*, 1985). Although the compositions of these fluids are reflections of factors synthesised locally or derived systemically from the circulation, it is often not clear which source makes the greater

contribution. An initial study by Binoux and Hossenlopp (1988) showed that more than 90% of the IGFs in human lymph were associated with the low molecular weight IGFBPs, suggesting that the 150-kDa complex in plasma is too large to cross the endothelium. Moreover, the authors proposed that the low molecular weight IGFBPs acted to transport plasma IGFs to the tissues, a view supported by the longer half-life of IGF-I in the ternary complex compared to that within the binary complexes (Young *et al.*, 1992; Lewitt *et al.*, 1993b). However, this may not be the case in all instances as chromatographic studies of lymph from other mammals has revealed binding of IGF radioactivity in high molecular weight fractions (Hodgkinson *et al.*, 1989b; Gargosky *et al.*, 1990; Lord *et al.*, 1991). Notwithstanding that the lymph samples were collected from different species, other reasons for the apparent discrepancies may be due to differences in the structure of the capillaries and endothelia supplying the tissues and differential production of IGFBPs by those tissues. Thus, cerebrospinal fluid from lambs that was incubated with radiolabelled IGF-II and fractioned under neutral conditions yielded no radioactivity in the 150-kDa region. In contrast, lymph analysed in the same manner did, albeit in varying amounts according to the region that the lymph nodes drained (Lord *et al.*, 1991).

Although *in vitro* studies of extravascular fluids may yield data on the steady state kinetics, transport to and expression of IGFBPs by extravascular tissues may vary considerably depending on the nutritional and hormonal status (Bar *et al.*, 1990a; Rotwein, 1991). In this context, it may be reasonable to assume that the extravascular regulatory mechanisms for the IGFs may also be altered following injury. Whilst altered IGF and IGF receptor gene expression following injury has been documented (Gartner *et al.*, 1992; Steenfos and Jansson, 1992a; Antoniades *et al.*, 1993), the effect that injury has on the types and forms of IGFBPs present in a wound produced has yet to be described. It is assumed that secretion of IGFBPs

during wounding reflects that seen when the cells are "injured" *in vitro*. This is of particular interest as the IGFBPs may be judged as the primary regulators of IGF action so that alterations to their extravascular composition or function may have dramatic effects upon IGF bioactivity. For instance, if cells decrease secretion of IGFBPs in response to injury, then clearance of IGFs from the wound tissue may be increased, while increased secretion may lead to sequestration. This is especially so given the perceived importance of locally produced IGF acting in an autocrine or paracrine manner (Gluckman and Butler, 1994). Moreover, an understanding of the extravascular kinetics and clearance following treatment with IGFs may allow the design of appropriate treatment regimes that, for example, maximise the half-life of added growth factor. Although some attempts to measure clearance of growth factors from extravascular sites or wounds under normal conditions have been made previously (Goodson *et al.*, 1980; Buckley *et al.*, 1985; Sprugel *et al.*, 1988), studies that specifically examine IGF clearance and the modulating effects IGFBP interactions may have on IGF bioavailability are yet to be performed.

1.6 STATEMENT OF THE PROBLEM

There is some evidence that IGF-I plays a role in the repair of injured tissue. Thus, IGF-I is expressed by many cells in the wound environment (Gartner *et al.*, 1992; Steenfos and Jansson, 1992a) and may be found in the normal wound milieu in amounts similar to those found in plasma, whilst there are decreased amounts in compromised wounds (Suh *et al.*, 1992). This last observation in particular suggests that application of IGF-I to wounds may aid the wound repair process. However, whilst a general role for IGF-I in wound repair is inferred by these data, results obtained following application of IGF-I to wounds have been equivocal. My review of the literature shows that whilst IGF-I may increase DNA and hydroxyproline content in wound chambers following infusion locally into the wound site, topical application to dermal/epidermal surface wounds has been less productive. The most favourable results have come from some recent studies where IGF was conjointly administered with an IGFBP, suggesting a role of IGFBPs in protecting IGF-I from premature clearance or degradation.

At the time of commencing my research program, information concerning the types and forms of IGFBPs at the acute wound site was limited. Similarly, how they may regulate the actions of IGF-I at the wound site was unclear. It has been established that in the circulation, IGFBPs serve to increase the half-life of IGF-I and protect it from degradation and clearance. Thus, analogues of IGF-I that bind poorly to IGFBPs have increased rates of clearance and diminished half-lives. Although similar mechanisms are presumed to exist at the extra-vascular wound site, no investigations of this possibility had been reported. Synthetic IGF-I analogues that have reduced affinities for the known IGFBPs present as useful tools for examining the effects that IGFBPs have upon the clearance of the IGFs and the role IGFBPs may play in protecting IGFs from degradation in the wound. I hypothesised that the IGFBPs

could play an important role at the wound site in limiting degradation and clearance of IGFs from the wound.

Accordingly, the specific aims of this thesis were:

- to measure and characterise the types and forms of IGF and IGFBP proteins present in the wound milieu during normal acute wound repair *in vivo*;
- to develop a model of wound repair that would allow estimation of the rate of clearance of IGFs from a wound *in vivo*, with particular emphasis on dermal/epidermal wounds; and
- to compare the rate of clearance of IGF-I with those of analogues of IGF-I that bind to IGFBPs poorly as well as with IGF-II, which has a higher affinity than IGF-I for several IGFBPs.

Chapter Two

Measurement of IGFs and characterisation of IGFBPs in rat wound fluid

2 Measurement of IGF-I and Characterisation of IGFBPs in Rat Wound Fluid

2.1 INTRODUCTION

The insulin-like growth factors (IGF-I and IGF-II) exert a range of mitogenic, differentiating and anabolic effects in a variety of tissues (Daughaday and Rotwein, 1989) and *in vivo* are bound to at least six specific IGFBPs (Ballard *et al.*, 1989; Shimasaki and Ling, 1991). Most IGF is present in the circulation as part of a 150-kDa ternary complex, comprising IGFBP-3 and an acid-labile subunit (Baxter and Martin, 1989b). The size of the ternary complex is thought to restrict IGF to the intravascular space thereby regulating access of IGF to tissues (Binoux and Hossenlopp, 1988). Indeed, the half-lives of IGF-I or IGF-II are greatly reduced when present in the lower molecular weight binary complexes (Zapf *et al.*, 1986; Hodgkinson *et al.*, 1987; Hodgkinson *et al.*, 1989a; Lewitt *et al.*, 1993a). The functions of circulating IGFBPs other than IGFBP-3 are not clear, but some evidence suggests that they may be involved in the transfer of IGF-I from the circulation to the tissues. In this context, studies of isolated rat heart preparations have demonstrated that IGFBP-1, -2, and -4 can cross intact endothelial barriers (Bar *et al.*, 1990a; Bar *et al.*, 1990c; Boes *et al.*, 1992). Further, these IGFBPs are present in sera in the highest concentrations after IGFBP-3 (Rechler, 1993), and their half-lives, when complexed with IGF-I, are less than that of the ternary complex (Young *et al.*, 1992).

IGFs have been measured in many extra-vascular fluids (Daughaday and Rotwein, 1989). Similarly, IGFBPs have been detected in extravascular fluids such as lymph, amniotic fluid, and cerebrospinal fluid (Rechler and Nissley, 1990). At the commencement of this study, evidence of the presence of the 150-kDa complex in lymph was conflicting and the acid-labile

subunit had yet to be measured in any extravascular fluids (Jones and Clemmons, 1995). Binoux and Hossenlopp (1988) detected IGFBP-3 in human lymph, but were unable to demonstrate binding of radiolabelled IGF-II to IGFBPs in the 150-kDa range of fractions separated by size-exclusion chromatography. However, Gargosky *et al.* (1990) found that IGFBP-3 was present in the high molecular weight fractions of adult rat abdominal lymph analysed by size-exclusion chromatography, suggesting that species differences exist or that the acid-labile subunit and the ternary complex can access the extravascular space.

The IGFs are thought to play an important role in regulation of the tissue repair and regeneration process. Thus, IGFs are specifically expressed in granulation tissue (Gartner *et al.*, 1992), while the infusion of IGF-I into the local wound environment can correct the healing deficit induced by administration of glucocorticoids (Suh *et al.*, 1992). Additionally they act in synergy with other growth factors to promote healing (Lynch *et al.*, 1989). On the other hand, the role of IGFBPs in tissue repair is less clear. Some studies have shown that the co-administration of IGFBP-1 with IGF-I can enhance the stimulatory actions of IGF-I on healing wounds (Jyung *et al.*, 1994; Tsuboi *et al.*, 1995). Similarly, IGF complexed with IGFBP-3 has a greater effect on wound tissue formation than IGF-I alone (Sommer *et al.*, 1991), suggesting a role for the IGFBPs in the regulation of IGF-I action at a cellular level.

When this study began, there were few reports on the IGF content of wound fluid and whether IGF levels varied throughout the wound repair response. Likewise, no attempts to measure the amounts of IGFBPs in wounds or wound fluids had been made. To address this deficiency, I adopted a model of wound repair that would allow sampling of the wound milieu. Several wound repair models that allow wound fluid sampling exist such as poly-vinyl alcohol sponges and wound drains (Viljanto, 1969; Viljanto, 1976), polytetrafluoroethylene tubes (Goretex™; Goodson and Hunt, 1982), and the Hunt-Schilling chamber. The last, a stainless

steel wire mesh chamber, was first described by Schilling *et al.* (1959) and was later employed by Hunt and colleagues to investigate the wound environment (Hunt *et al.*, 1967). An in-growth of granulation occurs around the chamber and through the wire mesh into the lumen of the chamber, which is implanted in the subcutaneous tissues. Fluid and granulation tissue accumulate within the lumen of the chamber that can be sampled for analysis. Eventually, the lumen of the chamber becomes totally filled with tissue. This model of wound repair was chosen over the others for the following reasons: the chambers are simple and reproducible to construct; they are inexpensive; the implantation procedure is uncomplicated; abundant amounts of wound fluid can be easily recovered and analysed with little risk of contamination with plasma or blood; and the inflammatory response is less than that which may occur with the other implants such as poly-vinyl alcohol sponges (Alaish *et al.*, 1995).

The aim of the research described in this chapter was to examine the IGF content and the IGFBP profile of wound fluid. In order to achieve this I implanted Hunt-Schilling chambers in rats and collected wound fluid at selected intervals over the following 21 days.

2.2 MATERIALS AND METHODS

2.2.1 Peptides

Recombinant human IGF-I and IGF-II were obtained from GroPep Pty Ltd (Adelaide, South Australia, Australia). Peptides were iodinated with carrier free $Na^{125}I$ using the chloramine-T method (Van Obberghen-Schilling and Pouyssegur, 1983) to a specific activity of 70-80 μCi/μg (Gargosky *et al.*, 1990; Ballard *et al.*, 1991). The radiolabelled IGFs were separated from free $Na^{125}I$ on a Sephadex G-50 column (30 cm x 1 cm) in a sodium phosphate buffer (pH 6.5; 50 mM NaH_2PO_4, 150 mM NaCl) containing 0.25% BSA. The peptide containing fractions identified by precipitation in 10% (w/v) TCA. The biological activity of IGF peptides iodinated in this manner has been previously described (Ballard *et al.*, 1986).

2.2.2 Animals

Male Sprague-Dawley rats, weighing 250-340 grams, were obtained from the Central Animal House, University of Adelaide. The rats were housed in separate cages under constant temperature and humidity with 12-h light/12-h dark cycles. Rats were the preferred species for this study as they are inexpensive and easy to handle. Additionally, they are of sufficient size to receive the chambers that I had designed to be large enough to provide useful amounts of wound fluid. The rats were weighed at regularly at 2 to 3 day intervals throughout the experiment and were allowed *ad libitum* access to water and standard rat feed. Prior approval for the experimental protocol was gained from the Animal Ethics Committee of the Adelaide Women's and Children's Hospital.

2.2.3 Wound chamber implantation

Hunt-Schilling wound chambers (Schilling *et al.*, 1959; Hunt *et al.*, 1967), 3 cm long and 1 cm in diameter, were constructed from #40 stainless steel mesh (Crestware Industries, Adelaide, Australia) and the ends sealed with silicon polymer (Figure 2.1). After curing, the chambers were washed in 100% ethanol and rinsed several times in distilled water before autoclaving.

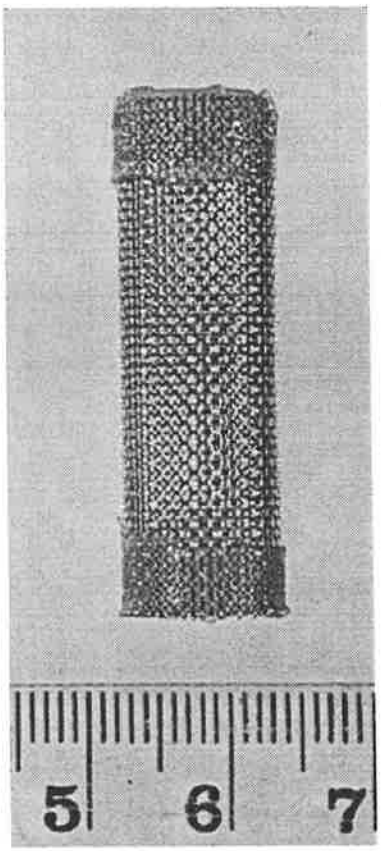

Figure 2.1 Hunt-Schilling chamber

At the time of chamber implantation, each rat was anaesthetised with isoflurane (Forthane; Abbott Australasia Pty Ltd, Sydney, Australia) and an oxygen/nitrous oxide mix administered by a nose cone. The rats were placed on a heating pad to help maintain body temperature. The hair of the dorsum was clipped and skin aseptically prepared with povidone-iodine (Betadine; Mundipharma A.G., Switzerland) and 70% ethanol with any excess fluid removed to prevent loss of body heat. Using a #14 scalpel blade, two abaxial incisions were made cranial to the sacrum so that one chamber could be introduced to the subcutaneous space cranial to each incision. Each chamber finally rested in a separate space in a dorso-lateral position above each scapula (Figure 2.2). The skin incisions were sutured with a non-absorbable material (Vetafil; Clements Stansen Medical, Sydney, Australia) after which the rats were allowed to recover on the heating pad before returning to their cages.

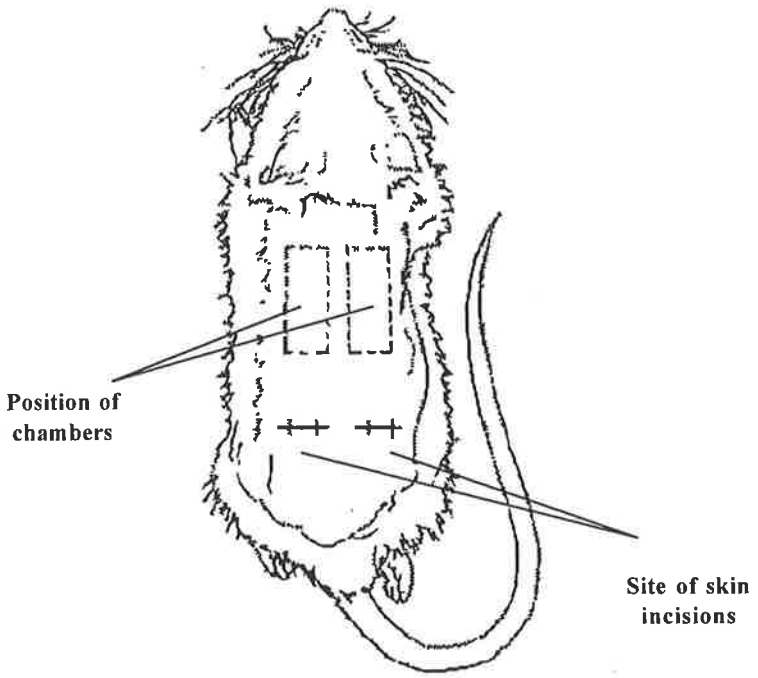

Figure 2.2 Schematic of rat showing sites of skin incisions and final position of Hunt-Schilling chambers

2.2.4 Sample collection

One day after surgery, four rats were randomly chosen for plasma and wound fluid collection and anaesthetised as described above. Whole blood was collected by cardiac puncture, heparinised to a final concentration of 12.5 IU/ml (Heparin Injection BP; Commonwealth Serum Laboratories, Melbourne, Australia), and stored on ice. Each rat was then killed and the skin overlying the chambers shaved and disinfected with 70% ethanol. A 19-gauge needle was carefully inserted through the silicon plug of the chamber to allow aspiration of the chamber contents. Care was taken to avoid traumatising the ingrowing granulation tissue on the luminal surface of the chamber. The collected fluid was also heparinised (10 IU/ml) and stored on ice with samples from each chamber kept separate. This collection procedure was repeated 3, 5, 7, 10, 14, and 21 days after implantation.

2.2.5 Biochemical and haematological comparisons of wound fluid and blood

Complete blood cell counts and differential counts of leucocytes in day 10 whole blood or wound fluid were conducted using an automated flow cytometer (Technicon H*1: Technicon Instruments Corporation, Tarrytown, NY). The pH of whole blood or wound fluid was measured using an automated blood/gas analyser (Radiometer ABL30, Copenhagen, Denmark). The remaining whole blood and wound fluid was centrifuged at 4°C to allow collection of plasma and cell free wound fluid supernatants. Subsamples of the supernatants from day 10 wound fluid were measured for total protein and albumin using an automated biochemical analyser (Technicon RA-XT: Technicon Instruments Corporation, Tarrytown, NY). In addition, subsamples of day 10 wound fluid were diluted in Brain Heart Infusion solution (DIFCO Laboratories, Detroit, Michigan, USA) for plating on blood agar nutrient

plates. The plates were incubated in air at 37°C for 48 h to estimate the degree of possible bacterial contamination. The remaining plasma and wound fluid were either stored on ice for immediate analysis within 4 h or frozen at -20°C.

2.2.6 Measurement of IGFs in plasma and wound fluid

Chromatography

IGFs were measured in plasma or wound fluid samples by radioimmunoassay (RIA) after acid gel-permeation HPLC chromatography to separate IGFs from the IGFBPs as previously described by Owens *et al.* (1990), except that the samples were diluted at pH 2.5. Briefly, 40 µl of plasma or wound fluid was added to 260 µl of water and 100 µl of 4x running buffer (pH 2.5: 200 mM acetic acid and 50 mM trimethylamine), defatted using an equal volume of Freon (1,1,2,trichloro-1,2,2-trifluoroethane) and centrifuged at 10 000 x g and 4°C for 10 min. The supernatants (200 µl) were chromatographed using a Protein-Pak 125 gel-permeation column (Waters, Lane Cove, Sydney, Australia) mounted on a HPLC. Fractions (0.25 ml) were collected and assayed to determine the presence of IGFs and IGFBPs.

Radioimmunoassay

The HPLC fractions were assayed for IGF content according to the method of Owens *et al.* (1990). Triplicate subsamples (50 µl) of each fraction were neutralised by the addition of 30 µl of Tris base (0.4 M), and diluted by the addition of 200 µl of assay buffer [30 mM NaH_2PO_4, 0.2 g/l protamine sulphate, 10 mM EDTA, 3.8 mM NaN_3, 0.05% (v/v) Tween-20, pH 7.5].

Serially-diluted standards of recombinant human IGF-I or IGF-II, reference tubes, and blank tubes containing mobile phase alone (all in triplicate) were firstly acidified by the addition of 50 µl of 4x running buffer (see above) before neutralisation with 0.4 M Tris. Thereafter, approximately 20 000 cpm of [^{125}I]IGF-I or [^{125}I]IGF-II in 50 µl was added to all tubes except the blanks. Finally, 50 µl of anti-human IGF-I (GroPep Pty Ltd) or anti-rat IGF-II (Amano Pharmaceutical Co, Ltd, Nagoya, Japan), diluted to the manufacturer's recommendations (final concentration 1:80 000), was added to all tubes except the references. The tubes were incubated for 16-18 h at 4°C after which 10 µl of sheep anti-rabbit immunoglobulin (Silenus Laboratories, Hawthorn, Australia; final concentration 1:200) and 50 µl of rabbit immunoglobulin-G (Dako Australia Pty Ltd, Botany, Australia; final concentration 1:5200) were added. After 30 min incubation at 4°C, 1 ml of 5 % (w/v) polyethylene glycol 6000 in 0.9% NaCl was added to the tubes, which were incubated for a further 10 min on ice. The supernatant was aspirated after centrifugation at 4000 x *g* for 20 min at 4°C and the radioactivity of each pellet measured by a gamma counter. Radioactivity present in the reference tubes (no anti-IGF antibody) was subtracted from the unknown values before comparison to the standards. The anti-human IGF-I antibody used in this assay shows approximately 25% cross-reactivity with rat IGF-I (Upton *et al.*, 1996).

2.2.7 Neutral gel-permeation chromatography

Pools of day 14 plasma or wound fluid (200 µl) were added within 4 h of collection to 80 000 cpm of [^{125}I]IGF-I and incubated at 4°C for 18 h. The pools were defatted by adding an equal volume of Freon, rapidly vortexing and centrifuging at 10 000 x *g* for 10 min. The supernatants were applied to a Superose 12™ column (HR10/30; Pharmacia, Sweden)

connected to a FPLC™ that had been previously equilibrated in PBS (pH 7.2; 50 mM NaH_2PO_4, 150 mM NaCl, 3.1 mM NaN_3, 10 IU/ml heparin), and eluted at 0.5 ml/min. The column was previously calibrated with molecular weight standards (human immunoglobulin-G, 150 kDa; BSA, 69 kDa; carbonic anhydrase, 30 kDa; and cytochrome C, 12.5 kDa; all from Sigma Chemical Co., Missouri, USA), and [^{125}I]labelled IGF-I (7.5 kDa). Fractions (0.5 ml) were collected and the radioactivity in each measured using a gamma counter.

In a further experiment, samples of day 14 wound fluid and plasma were collected from 6 rats, chromatographed on a Superose 12™ column, and individual fractions (0.5 ml) subjected to Western-ligand blot analysis.

2.2.8 Western-ligand blot analysis

The technique used to examine IGFBPs profiles by Western-ligand blotting has been previously described (Hossenlopp *et al.*, 1988) with modifications (Gargosky *et al.*, 1990). Briefly, plasma, wound fluid or molecular weight marker proteins (Amersham Rainbow Markers; Amersham International plc, Buckinghamshire, UK) were diluted in non-reducing sample buffer (Laemmli, 1970) and incubated at 65°C for 15 min. The samples and markers, and a pooled rat plasma standard from animals of a similar age and sex, were electrophoresed on a SDS polyacrylamide gel consisting of a 4% (w/v) stacking gel and a 12.5% (w/v) separation gel. The separated proteins were transferred to nitrocellulose membranes (0.45 μm; Schleicher and Scheull, Dassel, Germany) using a semi-dry blotter (Multiphor II NovaBlot; Pharmacia, Sweden) and then washed in Tris buffer (pH 7.4; 10 mM Tris; 150 mM NaCl) for 30 min and 1% (w/v) BSA in Tris buffer for 2 h. Thereafter 0.1% (w/v) Tween-20 was added followed 10 min later by 500 000 cpm of [^{125}I]IGF-II. The membranes were incubated with

the tracer overnight before rinsing in fresh Tris Buffer and 0.1% Tween-20 for 2 h, changing every 15 min, and drying.

The IGFBPs were visualised by exposure to X-ray film (RX Medical film; Fuji Photo Film Co. Ltd, Japan) in the presence of intensifying screens (Cronex Hi-Plus; Du Pont, Wilmington, MA) or phosphor images (Molecular Dynamics, Sunnyvale, CA) which were later quantified by laser scanning densitometry. The area under the curve of each band was calculated by integration of the optical density, and standardised against pooled plasma standards incorporated onto each gel to allow for differences between gels. To compare the intensity of equivalent bands in wound fluid and plasma, wound fluid samples were placed adjacent to plasma samples from the same rat and the intensity of IGFBP bands in wound fluid expressed as proportion of the corresponding molecular weight band in plasma.

2.2.9 Detection of IGFBP proteolysis in wound fluid

To measure the concentration of proteolytic activity directed at IGFBP-3 in wound fluid, equal volumes of pools of plasma and wound fluid were mixed and incubated at 37°C. All samples were collected and held on ice until the incubation began within 4 h of collection. In addition, the plasma/wound fluid mixes were incubated in the presence of four protease inhibitors - phenylmethylsulfonyl fluoride (PMSF), antipain, aprotonin (all Sigma), and EDTA (BDH Chemicals, Australia) at final concentrations of 5 mM, 25 mM, 2 mg/ml, and 10 mM respectively. Subsamples of the incubations were taken at 0 and 6 h, diluted in SDS sample buffer, and electrophoresed as described in section 2.2.8. The loading volume of the plasma/wound fluid mixes was twice that of plasma alone to allow for the dilution of the plasma by wound fluid and a pooled rat plasma standard was incorporated on each gel. In an

additional experiment, the intensity of the plasma IGFBP bands after 6 h of incubation was measured by laser scanning densitometry of phosphor images as described above, and expressed as a ratio of the intensity at time zero.

2.2.10 Statistical analysis

All data were analysed by one-way analysis of variance. Post hoc comparisons of temporal variations of IGF-I and IGFBP levels were made using Dunnett's procedure with the day 1 data serving as the control group (SuperANOVA, Abacus Concepts, Inc., Berkeley, CA, 1989). Comparisons of IGFBP levels at each time point between plasma and wound fluid were made using Students t-tests. Values are expressed as the means ± SEM except the biochemical and haematological data (means ± SD). Differences that gave $P < 0.05$ were judged to be statistically significant.

2.3 RESULTS

2.3.1 Biochemical and haematological comparisons of wound fluid and blood

The volumes of wound fluid aspirated from the chambers are shown Table 2.1. The greatest volumes were collected at days 10 and 14. Subsamples of day 10 wound fluid were plated onto blood agar nutrient plates and incubated at 37°C for 48 h. Bacterial growth was not detected under these culture conditions.

Table 2.1 Volumes of wound fluid collected

Day	Volume (µl)
1	543 ± 57
3	529 ± 31
5	700 ± 70
7	1250 ± 93
10	1517 ± 131
14	1512 ± 80
21	1067 ± 183

Data represent the means ± SEM of 2 chambers and 4 rats per time point.

Plasma and wound fluid samples were analysed for selected biochemical parameters, the results of which are shown in Table 2.2. The pH of wound fluid collected on day 10 was the same as plasma. However, total protein and albumin were approximately two thirds the amounts found in plasma ($P < 0.001$). Less potassium and calcium ($P < 0.01$ and $P < 0.001$) and more sodium and chloride ($P < 0.001$) were found in wound fluid than in plasma.

Table 2.2 Biochemical analysis of day 10 plasma and wound fluid

	Plasma	Wound fluid
pH	7.46 ± 0.02	7.42 ± 0.06
Sodium (mM)	139.3 ± 1.0	145.4 ± 1.1 *
Potassium (mM)	5.3 ± 0.2	4.8 ± 0.2 †
Chloride (mM)	101.8 ± 0.5	104.6 ± 0.5 *
Calcium (mM)	2.6 ± 0.1	2.2 ± 0.1 *
Total Protein (g/l)	56.8 ± 2.6	34.8 ± 2.3 *
Albumin (g/l)	34.8 ± 1.3	23.5 ± 1.7 *

Samples of wound fluid collected 10 days after implantation of Hunt-Schilling wound chambers were analysed and compared to plasma from the same animals. Plasma was collected from 4 rats and wound fluid from 8 chambers (total of 4 rats). Values are means ± SD. *, $P < 0.001$; and †, $P < 0.01$ *vs.* plasma

Compared to whole blood, wound fluid was largely acellular. Thus, red blood cell and platelet counts were approximately 1% of whole blood, and total leukocyte counts were 12% (Table 2.3). The count showed a composition in wound fluid that was similar to whole blood with lymphocytes being the predominant leukocyte type. Monocyte and lymphocyte numbers were approximately 11% of whole blood, and neutrophil numbers approximately 5%. However, due to the presence of tissue cells and cellular debris in the wound fluid some 35% of cells could not be classified.

Table 2.3 Analysis of cell content of plasma and wound fluid at day 10

	Plasma	Wound fluid
Red Blood Cell Count ($x10^6/\mu l$)	7.37 ± 0.13	0.11 ± 0.05 *
Platelet ($x10^3/\mu l$)	732 ± 88	8 ± 5 *
White Blood Cell Count ($x10^3/\mu l$)	12.07 ± 1.52	1.42 ± 0.51 *
Neutrophil ($x10^3/\mu l$)	4.67 ± 0.27	0.25 ± 0.11 *
Lymphocyte ($x10^3/\mu l$)	5.04 ± 0.93	0.52 ± 0.33 *
Monocyte ($x10^3/\mu l$)	1.42 ± 0.84	0.15 ± 0.08 *
Unclassified ($x10^3/\mu l$)	0.76 ± 1.27	0.50 ± 0.11

Samples of wound fluid collected 10 days after implantation of Hunt-Schilling wound chambers were analysed and compared to plasma from the same animals. Plasma was collected from 4 rats and wound fluid from 8 chambers (4 rats). Values are means ± SD. *, $P < 0.001$ versus plasma.

2.3.2 Plasma and wound fluid IGF-I concentrations

Initially all fractions were assayed individually for IGF-I and IGF-II (Figure 2.3). After demonstrating baseline separation of the fractions containing the IGFBPs and IGF-I (Figure 2.3A), the fractions for each sample were pooled on the following basis: Pool 1 (fractions 1-8) containing IGFBPs; Pool 2 (fractions 9-10) intermediate region; Pool 3 (fractions 11-18) containing IGF-I; and Pool 4 (fractions 19-20) tail region. As IGF-II was not detected at any time in either plasma or wound fluid, other than through interference by IGFBPs (Figure 2.3B), no further assays for IGF-II were attempted.

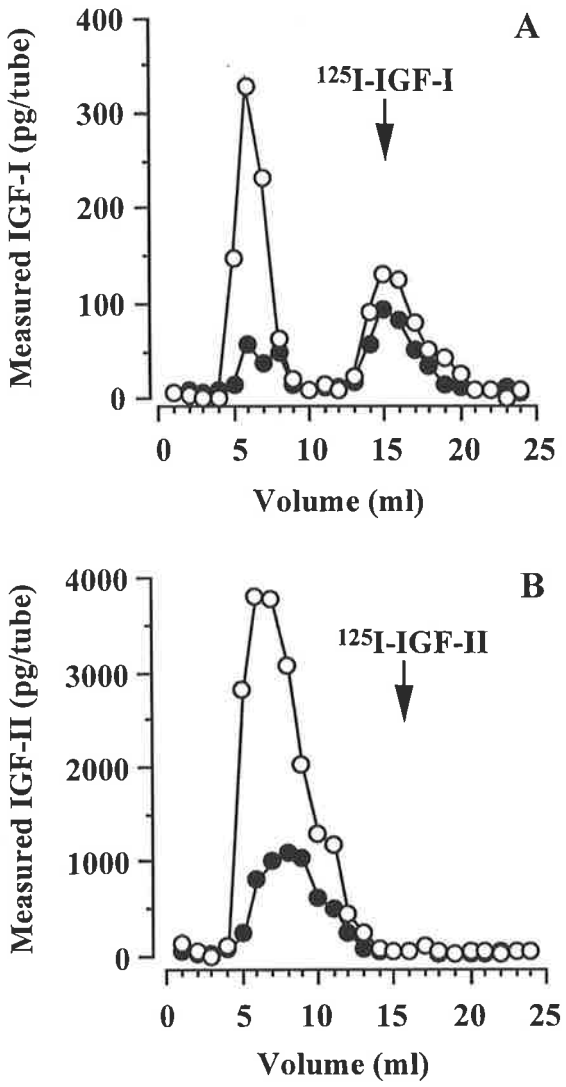

Figure 2.3 Assay of IGFs in HPLC fractions of plasma or wound fluid

Samples of day 10 plasma (○) or wound fluid (●) were subject to gel-permeation HPLC at pH 2.5, and each fraction assayed by IGF-I (A) or IGF-II (B) RIA. The volumes at which [^{125}I]IGF-I and [^{125}I]IGF-II were eluted are indicated.

After gel-permeation chromatography at pH 2.5 to separate IGF-I from IGFBPs in samples of plasma and wound fluid, fractions were pooled as described above and the amount of IGF-I measured by RIA (Figure 2.4). Plasma contained 147.5 ± 13.9 ng/ml of IGF-I one day after implantation of the chambers. The plasma IGF-I level did not significantly differ from this level for the remainder of the trial. In contrast, wound fluid IGF-I levels varied considerably (Figure 2.4) and fell significantly from 152.1 ± 14.7 ng/ml at day 1 (104% of plasma) to

79.5 ± 5.4 ng/ml (47% of plasma) by day 7 ($P < 0.01$), before increasing to 117.7 ± 8.8 ng/ml (78% plasma) at day 21.

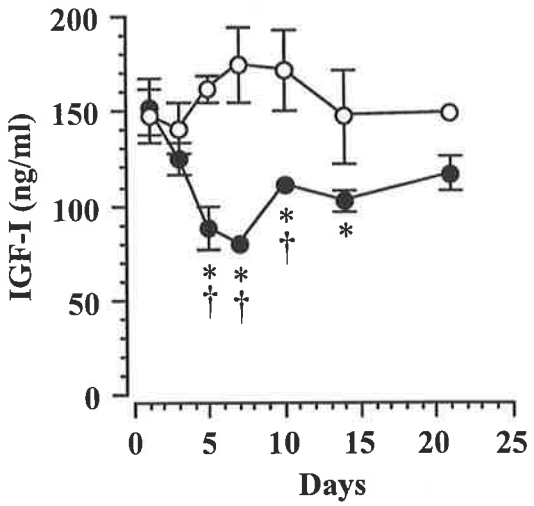

Figure 2.4 IGF-I in plasma and wound fluid

Samples of plasma (○) or wound fluid (●) from each collection day were subject to gel-permeation HPLC at pH 2.5, and assayed by IGF-I RIA as described in section 2.2.6 Data points represent means ± SEM (n = 4 rats for plasma and n = 6-8 separate chambers for wound fluid). *, P < 0.01 versus day 1. †, P < 0.01 versus plasma at the same time point.

2.3.3 Wound fluid and plasma IGFBPs

The IGFBP profiles of wound fluid from each day of collection were visualised after electrophoresis under non-reducing conditions and Western-ligand blotting with [^{125}I]IGF-II. Representative autoradiograph profiles of day 10 wound fluid and plasma are shown in Figure 2.5A. Electrophoresis of equivalent volumes of plasma or wound fluid (1µl; Figure 2.5A) revealed a qualitatively similar banding pattern, although the intensities of the 46-kDa IGFBP-3 and 28-/30-kDa doublet bands in wound fluid were significantly diminished compared to plasma. In contrast the 24-kDa band was of similar intensity. Three bands of approximately 44, 30, and 24 kDa were identified when 4 µl of wound fluid was loaded onto the gel (Figure 2.5B). A fourth band was faintly seen at approximately 28 kDa.

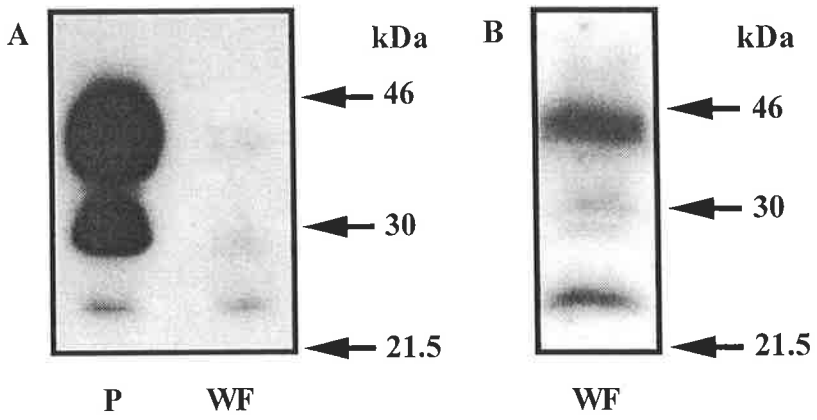

Figure 2.5 Autoradiographic profile of day 10 plasma or wound fluid

A, One microlitre of day 10 samples of wound fluid (pooled from 8 chambers) or plasma (pooled from 4 rats), and B, a 4 µl sample of day 10 wound fluid (pooled from 8 chambers), were added to sample buffer and electrophoresed on 12.5% SDS-PAGE gels under non-reducing conditions. The proteins were transferred onto nitrocellulose filters, incubated with [^{125}I]IGF-II, and exposed to X-ray film for 7 (A) or 14 (B) days. The positions of SDS-PAGE molecular weight markers (kDa) are shown.

Quantitative analysis of individual day 10 samples by laser showed that the intensity of 46-kDa IGFBP-3 and the 28-/30-kDa doublet (which were not measured separately due to their low intensities and close proximity) averaged around 6% of plasma levels (Figure 2.6A). The difference in intensity of the 24-kDa band between wound fluid and plasma was not significant. The intensities of each band in wound fluid over the 21 day trial, expressed as a ratio of the equivalent molecular weight band in plasma of the same rat, are shown in Figure 2.6B. Over the time course of the trial the intensity of the IGFBP-3 band in wound fluid ranged between 5% and 15% of the equivalent band in plasma, although the differences were not statistically significant over time. In contrast, the intensity of the 24-kDa band was 82% of plasma levels at day 1, rose significantly during the first 7 days of the trial to 129% of plasma levels ($P < 0.05$), then fell for the remainder of the trial so that by day 21 wound fluid levels were 37% of plasma.

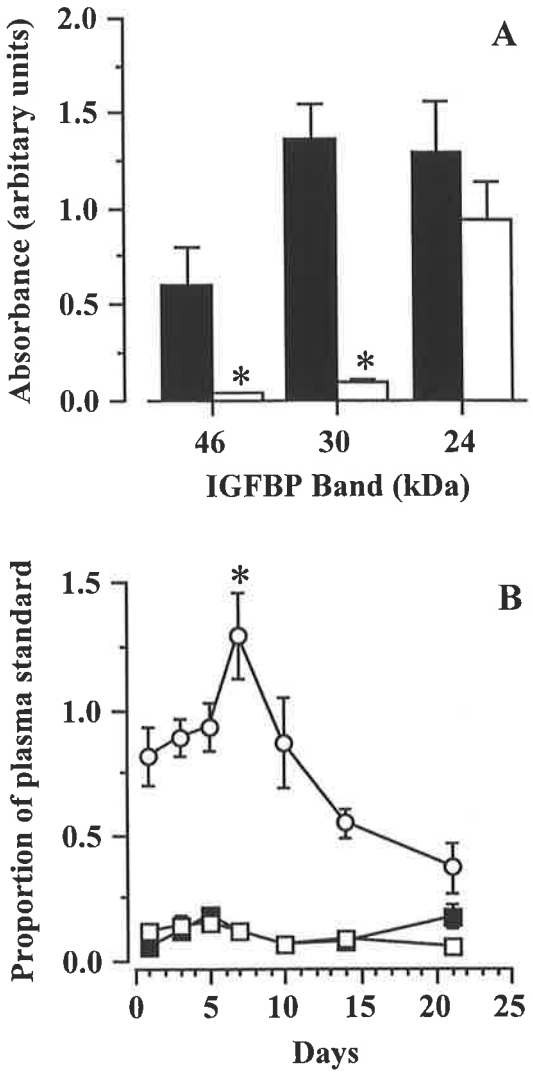

Figure 2.6 Measurement of wound fluid IGFBP bands

One-microlitre samples of plasma or wound fluid from the same rat were subjected to non-reducing SDS-PAGE, transferred onto nitrocellulose sheets, and incubated with [^{125}I]IGF-II. After washing and drying, the filters were exposed to phosphor screens for 5 days. The intensities of the IGFBP bands were measured by laser scanning densitometry and the area under the curves calculated by integration. A, The intensities of the major IGFBP bands in day 10 plasma (■; n = 4 rats) or wound fluid (□; n = 7-8 separate chambers) are expressed as a ratio of a pooled age- and sex-matched plasma standard incorporated on each gel. *, $P < 0.05$ versus plasma. B, The intensity of the 46- (■), 28-/30- (□), and 24-kDa (○) IGFBP bands in wound fluid were expressed as a proportion of the corresponding band in plasma from the same rat, and plotted for each day of sample collection (means ± SEM; n = 5-8 separate chambers at each time point). *, $P < 0.05$ versus day 1.

2.3.4 Neutral gel-permeation chromatography

Radiolabelled IGF-I was added to pools of plasma and wound fluid within 4 h of collection on day 14 and incubated at 4°C for 18 h before chromatography on a size-exclusion column at neutral pH. Three distinct peaks of radioactivity were recovered from both plasma and wound fluid corresponding to molecular weights of 150, 40-50, and 7.5 kDa (Figure 2.7A and Figure 2.8A). Compared to plasma, less radioactivity in wound fluid eluted in the 150-kDa region, and recovery of the [^{125}I]IGF-I tracer was increased in the 40-50 kDa region. Radioactivity also eluted at a position corresponding to the unbound ligand.

In a separate experiment, pools of day 14 plasma and wound fluid were chromatographed on a Superose 12™ size-exclusion column at neutral pH and subsamples of the fractions subjected to electrophoresis and Western-ligand blot analysis. Plasma IGFBP-3 was recovered in the fractions eluting from the column at a volume corresponding to 150 kDa (Figure 2.7B; lanes 3-5). A smaller 30-kDa band was also present in these fractions as was a fainter band at 24 kDa (Figure 2.7B; lanes 8-9). Figure 2.8B shows that, as visualised by Western-ligand blotting, IGFBP-3 is also recovered in the 150-kDa region in wound fluid (lanes 3-5). Again, several faint bands were observed in the lower molecular weight region. No IGFBPs were detected in the free IGF (7.5 kDa) regions of either plasma or wound fluid.

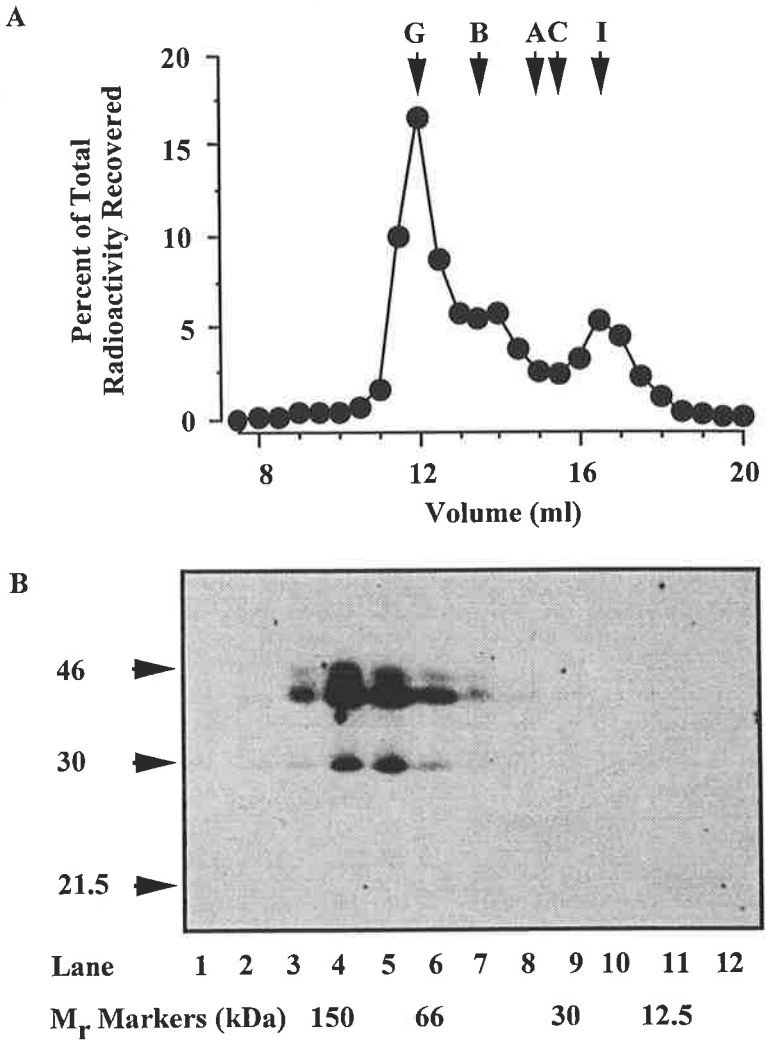

Figure 2.7 Characterisation of the IGFBPs in day 14 plasma by neutral gel-permeation chromatography followed by Western-ligand blotting

A, Samples of plasma (200µl) were pooled and mixed with [^{125}I]IGF-I (80,000 cpm) within 4 h of collection, incubated overnight at 4°C, and applied to a Superose 12™ column equilibrated in PBS (pH 7.2). The samples were eluted at 0.5 ml/min with the same buffer. The column was standardised with γ-globulin (G), BSA (B), carbonic anhydrase (A), cytochrome-C (C), and [^{125}I]IGF-I (I; 150, 66, 30, 12, and 7.5 kDa, respectively). Fractions (0.5 ml) were collected and the radioactivity measured on a gamma counter. B, Western-ligand blot analysis of column effluent fractions. Plasma fractions (50 µl) were analysed by Western-ligand blotting and autoradiographs obtained after exposure for 14 days at –70°C. Gel electrophoresis molecular weight markers are indicated on the *left*, and lane numbers and the elution position of molecular weight markers are shown at the *bottom*.

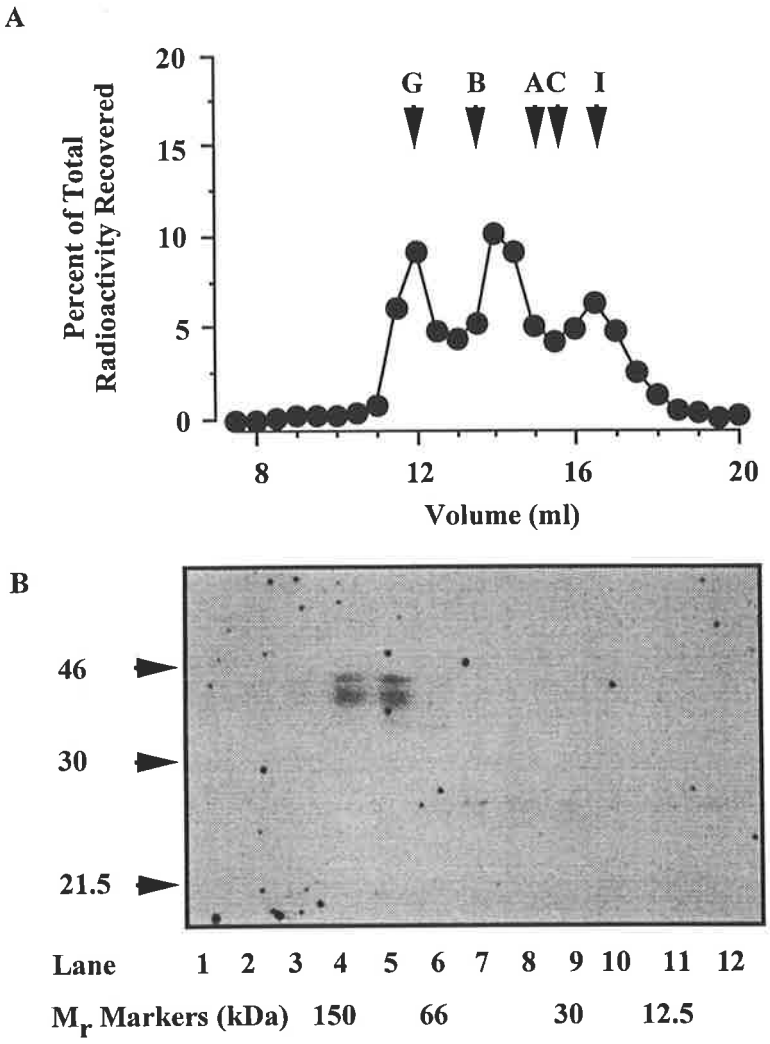

Figure 2.8 Characterisation of the IGFBPs in day 14 wound fluid by neutral gel-permeation chromatography followed by Western-ligand blotting

A, Samples of wound fluid (200µl) were chromatographed as in Figure 2.7. B, Western-ligand blot analysis of column effluent fractions. Subsamples of wound fluid fractions (175 µl) were analysed by Western-ligand blotting and autoradiographs obtained after exposure for 14 days at -70°C. Gel electrophoresis molecular weight markers are indicated on the *left*, and lane numbers and the elution position of molecular weight markers are shown at the *bottom*.

2.3.5 Degradation of IGFBP-3 by wound fluid

The possibility that the observed low level of IGFBP-3 in wound fluid was due to accelerated degradation in the wound environment was investigated by a series of co-incubation experiments. Pools of day 14 plasma were incubated in the presence of an equal volume of wound fluid at 37°C for 6 h (Figure 2.9). Subsamples were taken at 0, 1, and 6 h and subjected to Western-ligand blot analysis. A second set of incubation tubes contained plasma or wound fluid only. Exposure times of X-ray film were such that the IGFBP-3 band in wound fluid alone was barely visible. At time zero the plasma/wound fluid mix IGFBP profile was similar to plasma. However, a decline in the intensity of the IGFBP-3 band in both the plasma/wound fluid mix and plasma alone was observed, although degradation of IGFBP-3 was accelerated by the presence of wound fluid.

To quantify the decline in the IGFBP bands, a further experiment was conducted using samples of plasma and wound fluid collected and pooled from a separate group of 4 rats and the ligand blots exposed to phosphor screens. Mean values were calculated from scans of three separate lanes for each pooled sample. After incubation for 1 h, the intensity of the IGFBP-3 band in the plasma/wound fluid mix had fallen to 59% of the starting intensity compared with 77% for plasma alone. By 6 h the intensity of the IGFBP-3 band in the plasma/wound fluid mix had decreased to 25% of the starting intensity, compared to 69% in plasma alone. The 28-/30-kDa doublet also showed a decline falling to 28% of the starting intensity after 6 h of co-incubation however, this did not differ from the equivalent molecular weight bands in plasma alone (33%). In contrast, the intensity of the 24-kDa band changed little either in the plasma/wound fluid mix or in plasma alone (100% and 91% respectively). This experiment

was repeated with pooled samples obtained from a separate group of animals with similar results.

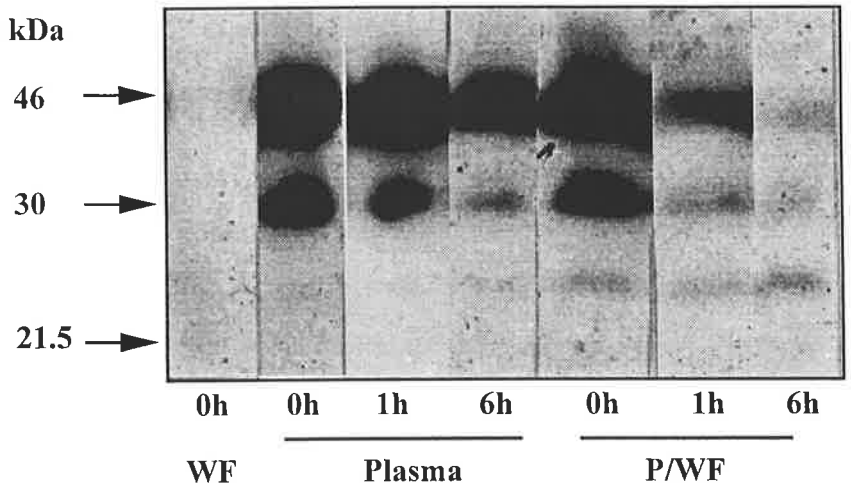

Figure 2.9 Loss of intensity of plasma IGFBP-3 after co-incubation in wound fluid

Pooled samples of day 14 plasma (P) from 4 rats, and wound fluid (WF) from 8 chambers, collected within the previous 4 h, were mixed (P/WF) and incubated at 37°C for 6 h. Samples were drawn at 0, 1 and 6 h and diluted with SDS-PAGE sample buffer, electrophoresed, transferred to nitrocellulose, and probed with [^{125}I]IGF-II. Autoradiographs were produced by exposing to X-ray film for 3 days. The positions of SDS-PAGE molecular weight markers are shown on the *left*.

2.3.6 Inhibition of IGFBP-3 degradation activity in wound fluid

Selected protease inhibitors were added to plasma/WF mixes to determine whether the degradation of plasma IGFBP-3 by wound fluid was due to proteolysis. The addition of the protease inhibitors was effective in decreasing the loss of intensity of plasma IGFBP-3 in the presence of day 14 wound fluid after 6 h of incubation at 37°C. Thus, Figure 2.10 shows that the addition of EDTA (10 mM), antipain (25 mM), or aprotonin (2 mg/ml) reduced the proteolysis of IGFBP-3, whereas PMSF (5 mM) did not. In a separate experiment, using pools of fluids that were collected on day 10, the degree of inhibition of IGFBP-3 proteolytic activity was measured by laser scanning densitometry. EDTA, antipain, and aprotonin reduced

IGFBP-3 degradation by 53%, 59%, and 74% respectively. However, the inhibitory activity of PMSF (5 mM) was variable and in this experiment had a smaller effect in only being able to reduce IGFBP-3 degradation by 26%. (Values represent means of scans of 3 separate lanes for each protease inhibitor.)

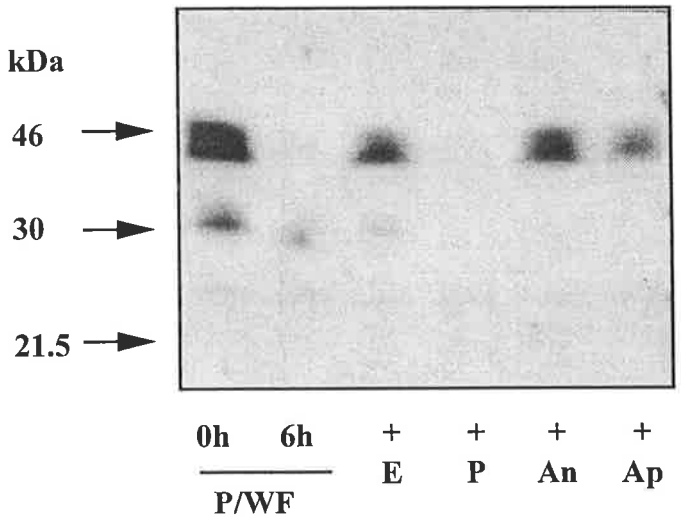

Figure 2.10 Inhibition of IGFBP-3 proteolysis by protease inhibitors

Equal volumes of pools of day 10 plasma from 4 rats, and wound fluid from 8 chambers were mixed (P/WF) and incubated for 6 h at 37°C with or without 10 mM EDTA (E), 5 mM PMSF (P), 25 mM antipain (An), or 2 mg/ml aprotonin (Ap). Subsamples of the mixture were withdrawn at 0 and 6 h, added to SDS-PAGE sample buffer and the IGFBPs visualised by Western-ligand blotting.

2.4 DISCUSSION

The Hunt-Schilling chamber is a useful model of wound repair with all phases of the repair process present, except for contraction and re-epithelialisation are present. The tissue response and new growth associated with this model have been well described (Schilling *et al.*, 1959; Hunt *et al.*, 1967). The model readily allows collection of the wound milieu. Some red blood cells were present in wound fluid, perhaps indicative of contamination with blood. However, new blood vessels, such as those found in granulation tissue, are "leaky" and red blood cells may extravasate (Nobuto *et al.*, 1988). Additionally, any cells introduced to the chamber during the implantation procedure may still be present after 21 days. The presence of leukocytes in wound fluid is not surprising given their role in inflammation and immune surveillance (Chapel *et al.*, 1984). Importantly, culture of wound fluid failed to yield a positive result, confirming the implantation procedure did not introduce bacteria into the wound that could potentially degrade and consume IGFs or other growth factors.

Other models of wound repair were considered, but judged technically difficult or flawed in some respect. For instance, the polyvinyl alcohol sponge model, whilst easy to implement, has been reported to provoke a strong foreign body response (Schilling *et al.*, 1959; Alaish *et al.*, 1995). Furthermore, recovery of adequate amounts of wound fluid for analysis is difficult. Micro-dialysis was ruled out on the bases that only very small amounts of fluid could be obtained; there was a significant risk of contamination of samples with blood and plasma following probe insertion, and commercial probes that did not bind IGFs and IGFBPs were not available.

Significant amounts of immunoreactive IGF-I were measured in wound fluid obtained from the Hunt-Schilling chambers at all times of collection. The concentrations in wound fluid,

relative to plasma, are in agreement with previous reports (Steenfos and Jansson, 1992b; Suh *et al.*, 1992), although they are higher than those reported for human lymph (10-30% of plasma levels as reported by Binoux and Hossenlopp, 1988). Despite the absence of significant changes in IGF-I plasma concentrations during the study, IGF-I levels in wound fluid declined significantly over the first 7 days after chamber implantation, presumably reflecting uptake of IGF-I by ingrowing granulation tissue, decreased local production, or decreased transport into the chamber from the circulation. It is plausible that wound fluid IGF-I levels at day 1 are largely the result of plasma exudation associated with chamber placement, although a component derived from platelet degranulation may contribute (Karey and Sirbasku, 1989). With the ingrowth of granulation tissue into the chamber, production and release of IGF-I into wound fluid by invading fibroblasts and macrophages (Rappolee *et al.*, 1988; Gartner *et al.*, 1992) or endothelial cells (Taylor and Alexander, 1993) may become significant.

In the rat, IGF-II is synthesised in many tissues (Daughaday and Rotwein, 1989), but levels of expression of IGF-II, which are highest during foetal development, decline postnatally in all tissues except the brain (Rotwein *et al.*, 1988). However, in two rat models of wound repair, Gartner *et al.* (1992) demonstrated that expression of IGF-II increased over time to reach a peak around 10-15 days post-wounding. The authors postulated that expression of IGF-II appeared to be regulated in the wound and that a role in wound healing appears likely. Accordingly, I attempted to measure IGF-II in rat wound fluid, but in contrast to IGF-I did not detect significant amounts of IGF-II. The monoclonal antibody used in the RIA was raised against rat IGF-II (Conlon *et al.*, 1995) and shows minimal cross-reactivity with IGF-I (S. E. Knowles, personal communication). Thus, given the known expression pattern of IGF-II, the most likely explanations are that production of IGF-II is subject to post-transcriptional

regulation (Rotwein *et al.*, 1988) and IGF-II is secreted into the surrounding environment at levels below the minimum detectable level of the assay.

While plasma is the most likely source of wound fluid IGFBPs, contributions by components of granulation tissue (Conover, 1990; Boes *et al.*, 1992; Camacho-Hubner *et al.*, 1992; Taylor *et al.*, 1993) as well as platelets (Spencer *et al.*, 1993) may also occur. Comparison of wound fluid and plasma showed that major IGFBP bands visualised in plasma by Western-ligand blotting were also present in wound fluid. However, the intensity of 46-kDa IGFBP-3 in wound fluid was significantly reduced. Laser densitometry of phosphor images revealed the intensity of this band was only 5-15% of that in plasma over the 21-day time course of the study. The reduced intensity is seen as soon as 1 day after chamber implantation, at a time when IGF-I levels are equivalent to plasma. A similar decrease in intensity of the 28-/30-kDa doublet, which in rat plasma and lymph is known to include IGFBP-1 and -2 (Donovan *et al.*, 1989), was observed. Similar reductions in the intensity of the IGFBP-3 band on Western-ligand blots have been observed in human lymph (Binoux and Hossenlopp, 1988), lymph derived from the sheep popliteal lymph glands (Lord *et al.*, 1991), and rat amniotic fluid and rat foetal serum (Gargosky *et al.*, 1990). In contrast, the 24-kDa band corresponding to the molecular weight of non-glycosylated IGFBP-4, was relatively abundant in wound fluid throughout the trial. The significance of this last observation is unclear, however it is possible that this IGFBP may play a role in the transfer of IGF-I from plasma to the wound.

Neutral gel filtration after overnight incubation with [^{125}I]IGF-I was performed with samples that had not been previously frozen but rather held on ice before incubation. In agreement with previous studies, the bulk of the [^{125}I]IGF-I in plasma was recovered at column volumes corresponding to a molecular weight of 150 kDa (Gargosky *et al.*, 1990; Lord *et al.*, 1994c). Similarly, ligand blot analysis of column fractions revealed that IGFBP-3 was predominantly

found in this region. Significantly, the IGFBP-3 identified in wound fluid was also recovered from the 150-kDa region. Although current evidence indicates that the acid-labile subunit is derived from the liver and largely confined to the intra-vascular compartment (Baxter *et al.*, 1994; Chin *et al.*, 1994; Jones and Clemmons, 1995), this finding suggests the presence of the acid-labile subunit in the extra-vascular space.

The reduced intensity of the 46-kDa IGFBP-3 in wound fluid compared to plasma could be attributed to lower concentrations of this IGFBP in wound fluid or degradation in the wound environment. I investigated the possibilities that first, degradation of this IGFBP was occurring in wound fluid, and second, that it was due to proteolysis. Pure IGFBP-3 for degradation studies was not readily available at the time of this study, so plasma, as a source of IGFBP-3, was incubated with an equal volume of wound fluid. The intensity of the plasma-derived IGFBP-3 band declined with time and the decline could be inhibited with EDTA, antipain, and aprotonin suggesting proteases were acting on this IGFBP. In this context, an IGFBP-3 protease activity has been reported in the pregnancy plasma of rats, humans, and mice (Davenport *et al.*, 1990; Fielder *et al.*, 1990; Giudice *et al.*, 1990), severely ill patients (Davies *et al.*, 1991), after major surgery (Cwyfan-Hughes *et al.*, 1992), in the reproductive tissues of 12-15 day pregnant rats (Davenport *et al.*, 1992b), and more recently in a variety of other disease states (Cohen *et al.*, 1994a; Lee *et al.*, 1994a), biological fluids (Giudice *et al.*, 1994; Lee *et al.*, 1994b), as well as in the normal state (Lalou and Binoux, 1993). Subsequent studies with human pregnancy plasma have revealed that IGFBP-3 is proteolytically modified such that its affinity with IGF-I is reduced (Baxter and Skriver, 1993; Baxter *et al.*, 1993; Blat *et al.*, 1994; Lassarre and Binoux, 1994), allowing redistribution of IGF-I from IGFBP-3 to other IGFBPs that may then transport IGF-I from the circulation to the tissues. Alternately, the amount of "free" or unbound IGF-I, which is normally very low, could increase (Hasegawa *et*

al., 1995; Juul *et al.*, 1996). This would therefore provide a means by which the bioavailability of IGF-I, or ability to interact with IGF receptors, could be increased. One could predict such a requirement in the local environment of a healing wound and it is possible that wound fluid IGFBP-3 exhibits a similar binding deficit for IGF-I to that described in pregnancy plasma. Indeed, my observations of the degradation of IGFBP-3 in wound fluid have been independently confirmed (Fournier *et al.*, 1996).

In summary, I have shown that the IGF:IGFBP profile in the wound environment varies during the tissue repair response and these changes are not reflected in the circulation. The presence of IGFBP-3 proteases may add a further level to the regulation of IGF action in the healing wound. The effect that this phenomenon may have on the bioavailability and clearance of IGF-I from extravascular wound sites will be addressed in the next chapters.

My research on the IGFBPs present in wound fluid and the presence of IGFBP-3 proteases described in this chapter has been published and appears as "Insulin-like growth factor I (IGF-I) and IGF-binding proteins in rat wound fluid" by J.G. Robertson, K.J. Pickering and D.A. Belford in *Endocrinology* **137**:2774-2781, 1996.

Chapter Three

Clearance of Insulin-like Growth Factors from Wounds: Development of Methodologies

3 Clearance of Insulin-like Growth Factors from Wounds: Development of Methodologies

3.1 INTRODUCTION

In the previous chapter, changes in the IGF/IGFBP profile of rat wound fluid collected over a 21-day period after subcutaneous implantation of Hunt-Schilling wound chambers were examined. The IGFBP profile, as shown by Western-ligand blotting, was similar to that of plasma except that the intensity of the IGFBP-3 band in wound fluid was greatly reduced. Subsequent investigation revealed the presence of factors within wound fluid responsible for the proteolysis of IGFBP-3, leading to reduced affinity for IGF-I. Similar findings with human, rat, and mouse pregnancy plasma have been reported (Davenport *et al.*, 1990; Fielder *et al.*, 1990; Giudice *et al.*, 1990) as well as in many other pathological disturbances (Davies *et al.*, 1991; Cwyfan-Hughes *et al.*, 1992; Lee *et al.*, 1994a).

It has been estimated that 75% of circulating IGF-I is present in the 150-kDa complex that supposedly restricts access of IGF-I to the tissues (Guler *et al.*, 1989). It has also been proposed that partial proteolysis of IGFBP-3, leading to decreased affinity for IGF-I, may occur during times of increased metabolic demand allowing redistribution of IGF-I to the low molecular weight binding proteins IGFBPs -1, -2, and -4 (Davenport *et al.*, 1990; Lamson *et al.*, 1993; Lassarre and Binoux, 1994). These IGF:IGFBP complexes may then cross the endothelial barrier and gain access to the tissues. In support of this hypothesis, Blat *et al.* (1994) demonstrated that human plasma obtained during the last trimester of pregnancy was able to stimulate DNA synthesis to a greater extent than plasma from non-pregnant

individuals. Thus, the bioavailability of the IGF-I may be increased by the actions of the IGFBP-3 protease.

Bioavailability may be also defined as the ability of a growth factor to interact with cellular receptors and effect a cellular response. To improve healing, an exogenous growth factor may need to be protected from degradation and premature clearance from the wound site so that it can access the appropriate cellular receptors at the required concentrations (Bennett and Schultz, 1993b). Therefore, decreasing the clearance or degradation at the site of action should increase the bioavailability of the growth factor. However, few models exist that allow examination of the *in vivo* regulation of growth factor movement between extravascular spaces, and consequentially, detailed knowledge of the fate of growth factors after application to wounds and their interactions with components of the wound is limited.

Several earlier studies have attempted to measure the transfer of IGFs from plasma to tissues *in vivo* (Hodgkinson *et al.*, 1991; Lord *et al.*, 1994a). Most of the data concerning the regulation of the movement of IGFs between extracellular compartments have been derived from plasma pharmacokinetic experiments which identify target organs or tissues, but do not provide information on the extravascular kinetics of the IGFs. Knowledge of the extravascular kinetics of IGFs would reveal important information about factors that regulate IGF bioavailability. In the previous chapter it was shown that more IGF-I tracer could be recovered in the low molecular weight IGFBP region in wound fluid, presumably due to partial proteolysis of IGFBP-3 (see 2.3.5). As Lewitt *et al.* (1993a) reported that IGF-I present in the 150-kDa complex was cleared less quickly from the circulation than that present in the lower molecular weight binary complexes, one may expect that IGF-I associated with the low molecular IGFBPs may be cleared from wound fluid more quickly.

Analogues of IGF-I that exhibit poor binding characteristics to IGFBPs are more potent *in vitro* compared to native IGF-I (Francis *et al.*, 1992), a potency that is attributed to reduced interactions with IGFBPs secreted into the media by cells in culture. Yet, these analogues are cleared from the circulation more quickly and are degraded to a greater extent than IGF-I (Bastian *et al.*, 1993), further supporting the notion that IGFBPs may regulate the bioavailability of IGFs. I propose that by measuring the rate of clearance of IGF analogues with reduced affinities for IGFBPs from a wound site, the effect that IGFBPs may have on the bioavailability of IGFs can be determined. Thus, the purpose of the research described in this chapter was to develop and describe an *in vivo* model of wound repair that would allow the measurement of the rate of clearance of IGFs from the wound site.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

Thiobutabarbital (Inactin™) was purchased from Research Biochemicals International (Natick, MA). Sodium iodide ($Na^{125}I$) was obtained from Amersham Australia (Castle Hill, Australia). Heparin, Evans Blue dye, recombinant human insulin, and the size-exclusion molecular mass markers were obtained from Sigma Chemical Co. (St Louis, MO). Recombinant human IGF-I was obtained from GroPep (Adelaide, Australia). The peptides were ^{125}I-labelled using the chloramine-T method to a specific activity of 75-85 μCi/μg, as described in section 2.2.1, and stored at -20°C.

3.2.2 Animal surgery and preparation for sample collection

All animal procedures were approved by the Animal Ethics Committee of the Adelaide Women's and Children's Hospital, following the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Male Sprague-Dawley rats (175-200g) were housed under constant temperature and humidity with 12-h light/12-h dark cycles. Each rat was individually caged and *ad libitum* access to food and water was allowed. Hunt-Schilling chambers were constructed from stainless steel mesh as described in section 2.2.3. One chamber was implanted subcutaneously per rat and left *in situ* for 14 days. This study was performed at 14 days after implantation of the chambers for several reasons. Firstly, from the time of implantation there is an accumulation of fluid within the lumen of the chamber and an ingrowth of granulation tissue. Secondly,

there was potential for volumetric disturbances caused by injection of the tracer or sampling of wound fluid to occur. By conducting the experiment 14 days after implantation when the wound fluid volume was greatest and by limiting the injection or sampling volumes to 10% or less of the wound fluid volume, I aimed to minimise the risk of such problems. Finally, the presence of a large and well-perfused vascular structure at this time facilitated the measurement of clearance rates.

Fourteen days after implantation each rat was weighed and anaesthetised by a single ip injection of Inactin™ (60 mg/kg). Body temperature was maintained with a heating pad set so that core body temperature was kept at 37°C. The rats were placed in dorsal recumbency and using aseptic techniques an incision was made though the skin parallel to and overlying the right jugular vein. The vein was exposed by blunt dissection of the overlying subcutaneous tissues and a catheter (OD 0.97 mm; ID 0.58 mm) introduced into the lumen of the vein. The catheter was immediately flushed with 100 µl of 0.15 M PBS containing 10 IU/ml heparin and affixed by means of a silk ligature. To prevent respiratory problems, a tracheostomy tube was inserted to allow aspiration of accumulated secretions. The medial edge of the existing skin incision was retracted to allow blunt dissection and exposure of the trachea. A small incision was made between two cartilage rings and a 3-4 cm bevelled cannula (OD 2.5 mm; ID 1.5 mm) was inserted approximately 5 mm into the airway. A ligature was placed around the trachea to hold the cannula in place. The cannula was then passed through the skin at a point approximately 1 cm cranial to the site of the tracheal incision and the skin incision sutured with silk.

A catheter was also placed in the bladder to allow collection of urine. Hair over the caudal abdomen was clipped and a 1 cm mid-line skin incision extending cranially from the pubis was made. The abdominal musculature was incised along the *linea alba* to allow

exteriorisation of the bladder. After the abdominal cavity was packed off with gauze, a small 2-3 mm incision was made at the pole of the bladder, taking care to avoid any visible blood vessels. A catheter (OD 2.1 mm; ID 1.0 mm) with a cuff (3.1 mm) was inserted into the bladder lumen and affixed with a purse-string suture after which the other end of the catheter was passed cranially within the abdominal cavity and exited through the abdominal wall at the umbilicus. Finally, a loose ligature was tied around the penis and urethra to prevent passage of urine via the urethra. The abdominal and skin incisions were closed routinely with silk sutures. The rat was then placed in ventral recumbency and supplemented with 100% oxygen.

The hair overlying the chamber was clipped, and the skin disinfected with 70% ethanol. A sterile 23-gauge needle was inserted percutaneously so that the tip of the needle was approximately in the centre of the lumen of the chamber and a micro-syringe was then attached to the needle, using a short flexible catheter to allow injection of the tracers and subsampling of the wound fluid within the chamber. After the surgical procedures were completed, the rats were allowed to stabilise for 20-30 min.

3.2.3 Tracer infusion and sample collection

In this experiment, an estimate of the rate at which radiolabelled IGF-I or $Na^{125}I$ was eliminated from wound fluid was made. Preliminary results had indicated that a sampling duration of 6 h was required to obtain a decline of approximately 50% in the amount of labelled IGF-I remaining in the chamber. Samples of blood (90 µl) and wound fluid (19 µl) were first withdrawn, heparinised to a final concentration of 10 IU/ml, and stored on ice, before 0.35 ml of 10 mg/ml Evans Blue dye in 0.15 M NaCl was injected into the jugular catheter. The catheter was washed by several flushings of jugular blood plus a final flush with

100 µl of heparinised saline. Ten minutes after injection of the Evans Blue dye, a second subsample of blood (170 µl) was withdrawn. Additional samples were withdrawn according to the schedule in Table 3.1.

All samples were heparinised (10 IU/ml final concentration) and with the exception of the 20- and 40-min samples, were treated as follows. Triplicate subsamples of 40 µl were added to tubes containing ice-chilled 10% (w/v) TCA in PBS containing 0.25% BSA, vortexed, and left on ice. The remainder of the blood from the first hour of sampling, including the 20- and 40-min samples, was left at 4°C before measurement of the Evans Blue concentration. Any surplus blood from the 120- to 360-min samples inclusive was resuspended in Hartmann's saline and returned to the rat. The haematocrit was measured in the 0-, 60-, 180-, and 360-min blood samples.

Table 3.1 Timing and volumes of blood and wound fluid samples

	Sample (µl)	
Time (min)	**Blood (µl)**	**Wound fluid (µl)**
0	90	19
10	170	27
20	40	19
30	170	27
40	40	19
60	200	27
120	140	7.5
180	140	7.5
240	140	7.5
300	140	7.5
360	140	7.5
Total	**1410**	**175.5**

Immediately following the injection of the dye into the jugular vein, a bolus (40-70 µl) of 10^7 cpm of ^{125}I-labelled IGF-I (40-70 ng of peptide) or Na^{125}I and 25 µl of Evans Blue dye (30 mg/ml in 0.15 M NaCl) was injected into the lumen of the chamber. To ensure a rapid and even distribution of the radiolabel and marker dye within the chamber, the micro-syringe was flushed repetitively with wound fluid. The sample volumes of wound fluid, which occurred at the same intervals as those for blood, are listed in Table 3.1. The samples were also heparinised and triplicate subsamples of 2.5 µl were immediately added to 10% (w/v) TCA, vortexed, and left on ice. Together with the 20- and 40-min samples, the remaining wound fluid of the samples from the first hour were left at 4°C for measurement of the Evans Blue concentration.

Excretion of the radiolabelled tracer into urine was monitored by collecting urine hourly for the duration of the experiment with urine volumes being calculated from the weight increase of pre-weighed tubes. An equivalent volume of Hartmann's saline containing 10% (w/v) glucose was injected iv to help maintain the intravascular volume. Triplicate subsamples (50 µl) of the hourly urine collections were added to 10% (w/v) TCA, vortexed, and left on ice. The blood, wound fluid, and urine samples treated with TCA were kept on ice for at least 30 min, centrifuged, and the radioactivity in the TCA-soluble and TCA-insoluble fractions measured.

3.2.4 Trichloroacetic acid precipitation

Degradation of the tracers was determined by adding triplicate subsamples of plasma, wound fluid, or urine to ice-chilled 10% (w/v) TCA and 0.25% (w/v) BSA in PBS. Following incubation on ice for at least 30 min, the total amount of radioactivity contained within each

subsample was measured with a gamma counter. The tubes were then centrifuged at 4°C to separate the supernatant (TCA-soluble fractions) and the precipitate (TCA-insoluble fractions), representing degraded and undegraded peptide respectively, and the radioactivity within each measured. The percentage of intact peptide in each subsample, or TCA-precipitability, was calculated by the formula:

$$\frac{\text{TCA-insoluble cpm}}{(\text{TCA-insoluble cpm} + \text{TCA-soluble cpm})} \times 100$$

3.2.5 Measurement of blood and wound fluid volumes

To enable calculation the total amount of radioactivity within the circulation or in the wound fluid a measure of the volume of each was required. This was achieved using a dye marker dilution technique (Wang, 1959; Kaufman *et al.*, 1981; Kaufman, 1991) adapted for use with 96-well microplates. The possibility of interference of readings due to haemolysis or turbidity of samples was excluded by measuring the absorbances of heparinised plasma (10 IU/ml), wound fluid, or haemolysed red blood cells between 488 and 800 nm on a spectrophotometer. Haemolysed red blood cells were obtained by lysing 300 µl of blood with 200 µl of fluorescence-activated cell sorter lysing solution (FACS®; Becton Dickinson, Sydney, Australia). Additionally, the wavelength corresponding to the peak absorbance of a solution of Evans Blue dye in wound fluid was also determined across the same spectrum.

Samples of plasma or wound fluid collected during the first hour after injection of the respective dye solutions were prepared for analysis of the dye concentration by centrifuging at 10 000 x*g* and 4°C for 10 min to sediment any cells or cellular debris. From the supernatant, triplicate subsamples of 5 µl were diluted in 95 µl of 0.15 M NaCl and the absorbances measured at 600 nm using a microplate reader. A standard curve was constructed for

comparing the plasma or wound fluid readings. The stocks of Evans Blue dye for plasma (10 mg/ml) or wound fluid (30 mg/ml) for injection into the rats were used. In the case of plasma, 60 µl of 10 mg/ml Evans Blue was added to 1.14 ml of plasma that had been pooled from rats of the same age and sex, and serially diluted. The wound fluid standard curve was constructed in the same manner except that 80 µl of 30 mg/ml Evans Blue was added to 1.12 ml of pooled wound fluid (final concentration 0.27 mg/ml). Plasma and wound fluid samples were collected prior to dye injection to serve as blanks in the assay. The time zero concentrations of Evans Blue were derived by extrapolation of a line fitted to a semilogarithmic plot of the readings. Each volume was calculated by the formula:

$$\text{volume (ml)} = \frac{\text{mass of dye injected (mg)}}{\text{dye concentration at time zero (mg/ml)}}$$

Total blood volume was calculated from the plasma volume and haematocrit according to the method of Wang (1959):

$$\text{Total Blood Volume (ml)} = \text{Plasma Volume (ml)} \times \left(\frac{100}{100 - (0.95 \times \text{Haematocrit})} \right)$$

3.2.6 Neutral-gel chromatography

Subsamples of wound fluid (200 µl) collected from each rat at the completion of the experiment were thawed, Freon extracted, centrifuged, and the supernatant subjected to size-exclusion chromatography as previously described in section 2.2.7. The column was calibrated with molecular mass standards of 150 kDa (human IgG), 66 kDa (BSA), 30 kDa (carbonic anhydrase), and 12.5 kDa (cytochrome-C) as well as radiolabelled IGF-I (7.5 kDa).

3.2.7 Recovery and distribution of radioactivity

At the completion of the experiment, the anaesthetised rats were killed and weighed after which the wound fluid remaining in the chamber was aspirated and measured. This volume was used to calculate the total amount of radioactivity remaining in the chamber fluid. The chamber and tissue immediately surrounding the chamber were dissected free and added to 25 ml of 0.5 M NaOH. The rat carcasses, less catheters, were homogenised and added to 1 litre of 0.5 M NaOH. After digestion at room temperature for several days, the carcass homogenates were made up to 2 litres. Quadruplicate subsamples (0.5 ml) of tissue or carcass digestions were then made and the total radioactivity in each determined.

3.2.8 Data analysis

All radioactivity measurements were corrected for radioactive decay back to the day the experiment was performed. The amount of TCA-precipitable radioactivity remaining in the wound fluid at each time point was expressed as a percentage of the initial amount of TCA-precipitable radioactivity injected into the chamber. Data from the IGF-I rats only were then fitted to a biexponential equation (Bastian *et al.*, 1993) using a non-linear curve-fitting program (TableCurve 2D version 3.0, Jandel Scientific, Corte Madeira, CA). As there were insufficient data collected within the first hour of the experiment, elimination rate constants (k_d) of the slow phase of the curves only (120 to 360 min data) were calculated. The half-lives of decay for each curve were calculated from the equation: $t_{1/2} = \frac{\ln 2}{kd}$, where $t_{1/2}$ is half-life and k_d is the elimination rate constant. The elimination rate constants are presented as fractions per hour (hr^{-1}). All data are presented as means ± SEM.

3.3 RESULTS

3.3.1 Measurement of blood and wound fluid volumes

One Hunt-Schilling chamber was subcutaneously implanted in adult male Sprague-Dawley rats. Fourteen days later the rats were anaesthetised, a bolus of radiolabelled peptide injected into the lumen of the chamber and the amount of radioactivity remaining in the chamber measured over time. Calculation of the amount of radioactivity present in blood or wound fluid at any time was dependent on a precise estimate of the volume of each. Furthermore, the volume of wound fluid within each chamber varied between rats necessitating establishment of these volumes for each rat (see section 2.3.1). To this end, a dye marker dilution technique was chosen and adapted for reading on a 96-well microplate reader. Earlier reports had indicated possible interference of readings due to haemolysis or turbidity in samples. To resolve whether such conditions existed in this experiment and to establish the wavelength at which the peak absorbance of Evans Blue occurred, the absorbances of plasma, wound fluid, plasma containing haemolysed red blood cells, or plasma containing Evans Blue were measured between 488 and 800 nm (Figure 3.1). Neither plasma nor wound fluid absorbed light to any extent within this spectrum of wavelengths. The peak absorbance of Evans Blue in plasma was 600nm, whereas the absorbance measured at this wavelength in haemolysed red blood cells was negligible. On this basis, all future readings of samples were made at 600nm.

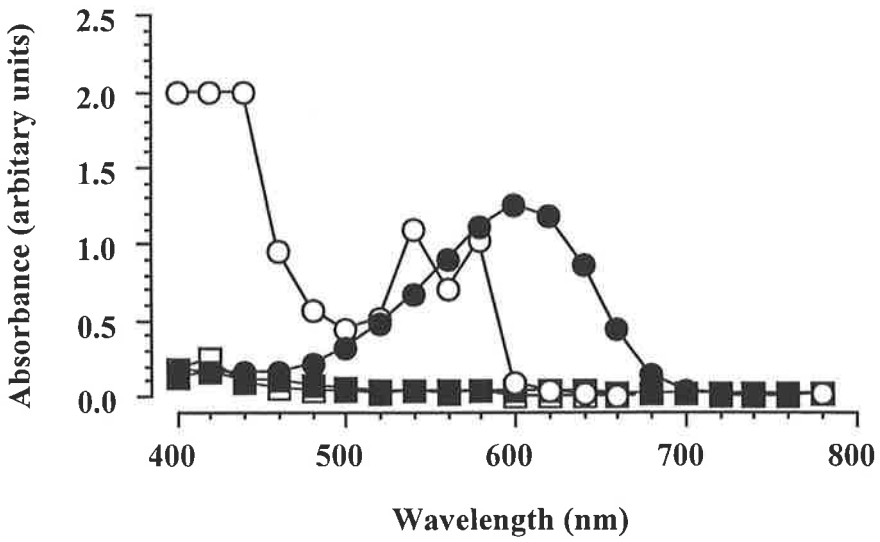

Figure 3.1 Absorbance spectra

The absorbances of Evans Blue in wound fluid (●), haemoglobin from red blood cells (○), wound fluid (■), or plasma (□) were measured across a spectrum of wavelengths to determine whether haemolysis of red blood cells interfered with readings.

Verification of the technique using Evans Blue for estimation of the wound fluid volumes was achieved by collecting wound fluid at the end of the experiment. The values obtained by this method were added to the volume of wound fluid removed by sampling and compared to the volumes obtained by the Evans Blue dye dilution assay (Table 3.2). As the two procedures gave similar results, subsequent calculations of the amount of tracer in the chamber were made using the volume calculated by the Evans Blue method.

Table 3.2 Comparison of wound fluid volumes

Rat	Method		Evans Blue minus aspirated (ml)
	Evans Blue (ml)	Aspirated (ml)	
1	1.16	1.39	-0.23
2	0.93	1.25	-0.32
3	1.20	1.27	-0.07
4	1.32	1.23	+0.09
5	1.11	1.24	-0.13
6	1.31	1.21	+0.10
7	1.72	1.49	+0.23
8	1.70	1.45	+0.25
9	1.26	1.12	+0.14
Mean	**1.30**	**1.30**	
SEM	**0.08**	**0.04**	

The wound fluid volume collected at the end of the experiment was added to the volume removed by sampling and compared with that calculated by the Evans blue dye dilution method.

3.3.2 Clearance of ^{125}I-labelled IGF-I and ^{125}I from wound fluid

Preliminary data on the decay profiles of ^{125}I-labelled IGF-I or ^{125}I in wound fluid were derived from an experiment in which tracer was injected into the chamber and the elimination of radioactivity from the wound fluid monitored. In this experiment, the haematocrit of the IGF-I-infused rats increased marginally from a mean of 41.6% at the start of the experiment to 41.8% at 6 h whereas the Na^{125}I-infused rats declined slightly from 41.8% to 41.2%. For both

groups, the results at 1 h and 3 h were similar to those obtained at the initial readings. The bodyweights of the IGF-I-infused rats declined by an average of 4.4 g or 1.2% of initial bodyweight. A similar result was obtained for the $Na^{125}I$-infused rats (3.7 g and 1.0%, respectively).

After injection of tracer into the chamber, the radioactivity remaining in the wound fluid was measured over time and expressed as the percentage of the administered dose. The use of radiolabelled peptides with the TCA-precipitation assay provided a simple and rapid method for measuring total and degraded peptides. Sampling volumes were also minimised. The amount of intact (TCA-insoluble) radiolabelled IGF-I present in the chamber decreased over time (Figure 3.2A) so that after the first hour approximately 22% of the original amount of peptide had been eliminated. However, from 1 h until the end of the experiment, the rate of elimination of intact labelled IGF-I from wound fluid was slower with a decline of approximately 20% occurring between 1 and 6 h. The slopes of the TCA-insoluble decay curves were used to calculate an elimination rate constant. Rate constants were not calculated for the fast phase of decay, as there were too few data points to enable accurate analysis. The mean elimination rate constant of the slow phase (120-360 min) was calculated to be 0.064 ± 0.005 hr^{-1} from which the mean half-life was 680 min.

In contrast to labelled IGF-I, ^{125}I was rapidly eliminated from the chamber (Figure 3.2A), such that by one hour after injection only 45% of the original material was present. Further, the rapid rate of elimination of ^{125}I continued after 1 h with less than 5% of the administered dose remaining after 6 h.

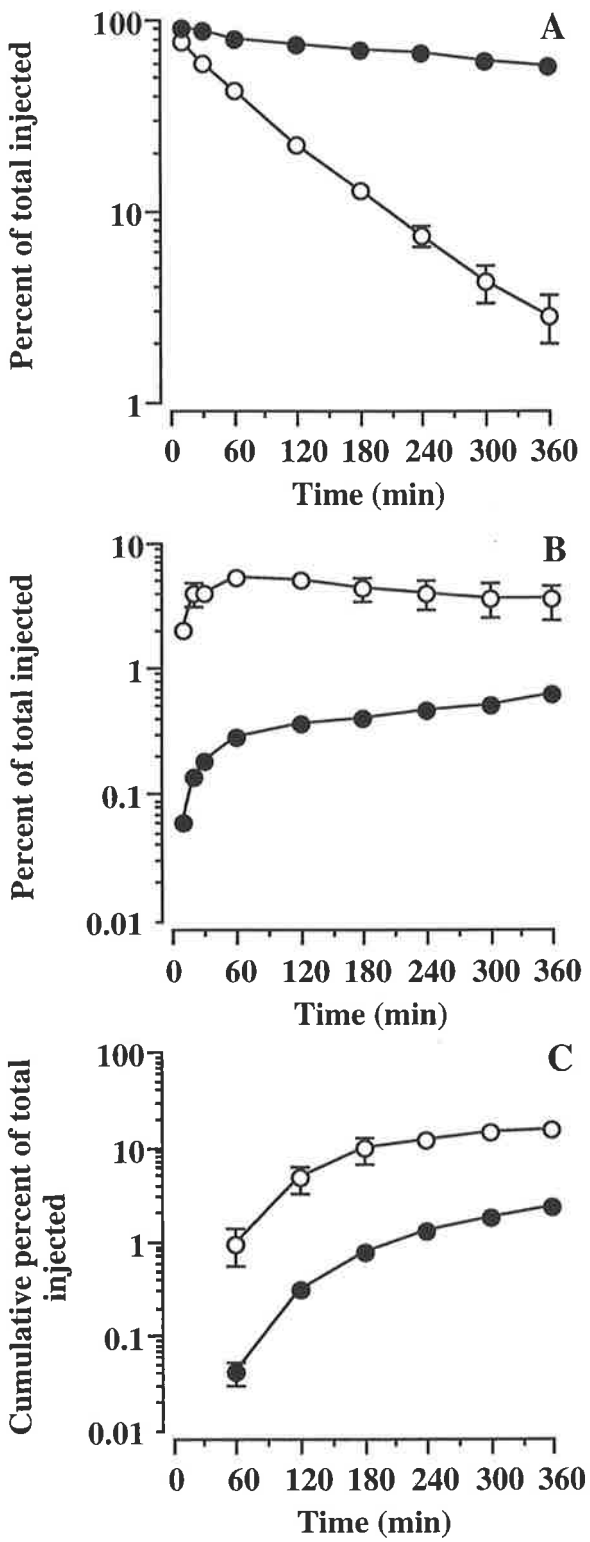

Figure 3.2 Clearance of tracer from wound fluid

TCA-insoluble ^{125}I-labelled IGF-I (●; n = 9 rats) or total ^{125}I (○; n = 3 rats) radioactivity was measured in wound fluid (A), blood (B), and urine (C) over 6 h after injection into the chamber. The amount of radioactivity remaining was calculated as a percentage of that injected into the chamber. All values are means ± SEM.

3.3.3 Appearance of radioactivity blood and urine

Subsamples of blood and urine were taken throughout the experiment and the radioactivity measured. The amount of intact IGF-I peptide in the circulation was at all times very low compared to the amount injected into the chamber (Figure 3.2B). Thus, 10 minutes after injection less than 0.1% of that injected was present in blood and by 6 h this had risen to only 0.6%. By comparison, ^{125}I radioactivity in the circulation had risen to approximately 5% of the administered dose at 1 h, but by the end of the experiment this had fallen to 3.5%. Similarly, the accumulation of IGF-I radioactivity in urine over the duration of the experiment was less than 3% of the total radioactivity injected (Figure 3.2C), whereas approximately 16% of the ^{125}I radioactivity had accumulated in the urine by 6 h.

3.3.4 Interaction of radiolabelled IGF-I with wound fluid IGFBPs

Wound fluid was collected at end of the experiment after which the association of [^{125}I]IGF-I with wound fluid IGFBPs was examined by size-exclusion chromatography at pH 7.2. The elution profile of wound fluid from IGF-I-infused rats is shown in Figure 3.3. Peaks of radioactivity were observed at approximately 150 kDa, 30-50 kDa, and a third peak, corresponding to free IGF-I, at 7.5 kDa. This elution profile is similar to that seen after incubation of wound fluid with radiolabelled IGF-I *in vitro* (see section 2.3.4).

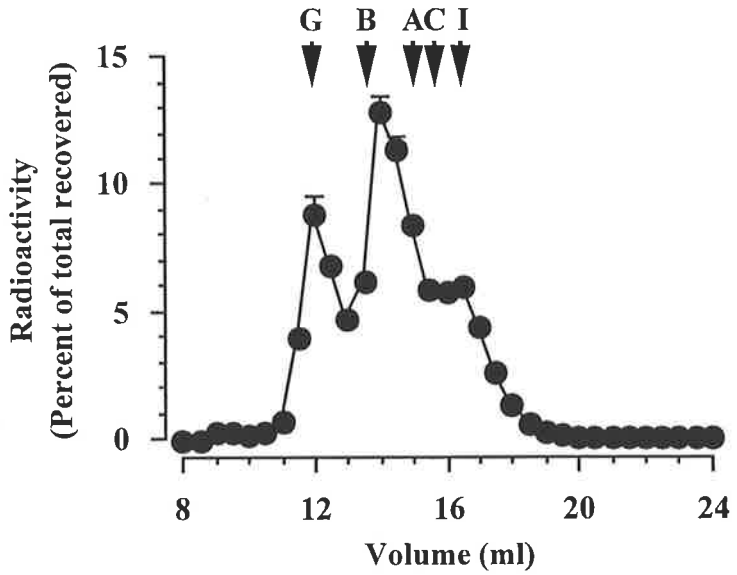

Figure 3.3 Association of [^{125}I]IGF-I with wound fluid IGFBPs

Wound fluid collected at the end of the experiment (200 µl) was subjected to size-exclusion chromatography at pH 7.2. The samples were eluted at 0.5 ml/min and the radioactivity determined in 0.5-ml fractions. The column was standardised with molecular weight markers of 150 kDa (human IgG, G), 66 kDa (BSA, B), 30 kDa (carbonic anhydrase, A), and 12.5 kDa (cytochrome-C, C), as well as ^{125}I-labelled IGF-I (7.5 kDa, I). Values are means ± SEM for n = 9 rats.

3.3.5 Recovery and distribution of infused [^{125}I]IGF-I

At the end of the experiment, the wound fluid of the IGF-I-infused rats was removed from the chambers and measured. The carcasses and chambers were digested in 0.5 M NaOH and the radioactivity contained within each measured (Table 3.3). Additionally, the radioactivity present in urine as well as that removed by sampling of blood and wound fluid was measured to complete the mass balance. Each measurement was corrected for radioactive decay to the day of the experiment and calculated as a percentage of that injected. More than 95% of the radioactive dose was recovered. The largest proportion of radioactivity (61%) remained in the wound fluid at 6 h. Of the remaining radioactivity, the greatest proportions were recovered from the chamber-associated tissue (16.3%) and the carcass (15%) whereas smaller amounts

were measured in blood and wound fluid samples or urine (0.025%, 3.9%, and 2.5% respectively).

Table 3.3 Recovery of [^{125}I]IGF-I radioactivity

Source	Percent recovered
Rat Digestion	15.1 ± 1.0
Chamber Digestion	16.3 ± 0.7
Wound fluid at 6 h	61.0 ± 1.3
Wound fluid sampling	3.9 ± 0.3
Blood sampling	0.025 ± 0.002
Urine excretion	2.5 ± 0.2
Total recovered	**95.3 ± 3.2**

The proportions of radioactivity in different sampling components were evaluated at the end of the experiment as described in section 3.2.7. Values are the means ± SEM for 9 rats and are expressed as a percentage of the radioactivity recovered.

3.4 DISCUSSION

In the previous chapter, IGFBP-3 was proteolysed in wound fluid. This observation suggests that a mechanism for increasing the bioavailability of IGF–I may exist during wound repair. The increased proportion of radiolabelled IGF-I associated with low molecular weight IGFBPs also supports this notion since these IGFBPs may play an important role in the transfer of IGF–I from the circulation to the tissues (Bar *et al.*, 1990c; Boes *et al.*, 1992). Given the increased clearance of IGF-I from plasma when associated with the low molecular weight IGFBPs (Lewitt *et al.*, 1993a), it may be expected that clearance of IGF-I from the wound site may also be increased when the growth factor is present in the lower molecular weight complexes. In this chapter I describe the development of an *in vivo* model that allows the measurement of IGFs clearance from the wound site.

My earlier studies with Hunt-Schilling chambers (see Chapter 2) provided the foundation for the development of the model in this chapter. This model has been employed many times for the study of growth factor actions and its biochemical and histological characteristics are well-documented (Schilling *et al.*, 1959; Hunt *et al.*, 1967). Furthermore, an extensive picture of the IGF:IGFBP profiles of plasma and wound fluid collected from rats implanted with the chambers were obtained in the previous chapter.

An important aspect of the study was the need for precise determinations of the plasma and wound fluid volumes. This was especially acute for wound fluid, as small errors in the estimations will lead to large errors in calculating the amount of radioactivity present. Furthermore, the volume of wound fluid varies between chambers. Although collection of wound fluid at the end of the experiments allows measurement of the wound fluid volume, it would be impossible to ensure removal all of the fluid leading to under-estimation of the true

volume. As such, an indicator dilution technique was adopted. Traditional dilution methods for measuring plasma or total blood volume include radiolabelled erythrocytes or albumin, carbon monoxide gas, and Evans Blue (T-1824) dye. A preferred indicator for plasma volume measurement is [^{131}I]albumin (Boyd, 1967), but the short half-life of this isotope (8.5 days) would have made measurement of the radioactivity more difficult. Alternatively, the use of a dye such as Evans Blue, enables samples to be read easily on a spectrophotometer although potential problems with this technique may arise if the samples contain haemoglobin or are excessively turbid (Chinard, 1951; Boyd, 1967). Haemolysis of red blood cells was avoided by adding heparin to the samples, careful handling of the samples and using physiological saline to dilute the dye prior to injection (Boyd, 1967). In agreement with other reports, the maximum absorbance of Evans Blue in plasma was recorded at 600 nm, a wavelength at which little absorbance was measured in haemolysed samples (Figure 3.1). Turbidity of samples such as that arising from lipaemia, nephrotic syndrome, or diabetic acidosis (Chinard, 1951) was not encountered because the rats were clinically normal and fed a standard rat chow. Additionally, all samples were clarified by high-speed centrifugation before assaying.

Calculation of the amount of radioactivity contained within blood or wound fluid was dependent on the assumption that the volume of each did not significantly alter throughout the experiment. This issue was addressed in several ways. Firstly, the sizes of the samples taken were minimised to less than 10% of the total volumes and unused portions of samples were returned to the rat. Secondly, physiological saline containing glucose was systemically infused at volumes equivalent to the urine output. Although the haematocrit was constant throughout the experiment, there is some suggestion that the fluid balance was not maintained. The bodyweights of the [^{125}I]IGF-I and Na^{125}I-infused rats fell approximately 1% during the 6 h experiment (3 to 4 g) and may represent a loss of blood, extravascular, or extracellular

volume, or a combination of all. It should be noted that this figure does not include the amount of blood removed by sampling (approximately 1.3 ml). Nevertheless, the concentration of radioactivity in the blood was at all times low (less than 5% in rats receiving peptides) and errors in this measurement would not impact greatly on the results. In contrast, the sample volumes of wound fluid plus the volume of wound fluid collected at the end of the experiment were similar to the time zero volume determined by the Evans Blue method suggesting that the wound fluid volume remained relatively constant throughout.

The long duration of anaesthesia in this study should be considered when examining the results. The anaesthetic drug, Inactin™, is an ultra-long acting barbiturate that has been commonly used in animal experiments requiring prolonged anaesthesia. Thus, it was the preferred anaesthetic agent for a single ip dose gives 3 to 4 h of anaesthesia before additional drug is required. The shorter duration of action of other commonly used agents such as pentobarbitone or xylazine/ketamine mixes, meant that more frequent administration would have been required. Such "topping-up" greatly increases the risk of anaesthetic over-dose. Inactin™ anaesthesia was also chosen in preference inhalation anaesthesia on the basis of simplicity of administration and the reported changes to plasma insulin and glucose levels that occur with halothane anaesthesia in sheep (Lord *et al.*, 1994b). In contrast, glucose levels in rats anaesthetised with Inactin™ are similar to those of conscious rats (Hindlyecke and Jansson, 1992).

Physiological parameters are reported to be more stable during Inactin™ anaesthesia than with other injectable anaesthetics (Buelke-Sam *et al.*, 1978; Hindlyecke and Jansson, 1992). Nevertheless, plasma IGFs and IGFBPs may be altered by fasting and nutrition (Busby *et al.*, 1988a; Ketelslegers *et al.*, 1996). In halothane anaesthetised sheep, a rise in circulating IGFBP-1 and declining levels of IGF-I and -II has been reported (Lord *et al.*, 1994b). Similar

findings were reported in pentobarbital/ketamine anaesthetised rats (Lewitt *et al.*, 1992). Although this study did not specifically investigate the possibility of such changes in plasma or wound fluid during prolonged anaesthesia, assessment of the distribution of radiolabelled IGF-I in wound fluid by neutral-gel chromatography revealed a profile similar to that seen in wound fluid incubated with radiolabelled IGF-I *in vitro* (see section 2.3.4). The wound fluid, in that instance, was collected within minutes of the induction of anaesthesia so that the IGFBP changes would be less marked. Hypoxia may also alter plasma IGFBP profiles (Kamada *et al.*, 1992; McLellan *et al.*, 1992), a concern addressed by supplementing the rats with oxygen.

Radiolabelled IGF-I was cleared from the chamber in two distinct phases. An initial rapid phase occurred in the first hour after infusion followed by a second slower phase from 1 h to 6 h. The decay profile was best described by a two-compartment model in which the wound chamber may represent one compartment (Gibaldi, 1991). The first phase likely represents distribution and mixing of the tracer within the chamber. It is within this period that equilibration of labelled IGF-I with the wound fluid IGFBPs would occur. Due to the relatively static nature of the accumulated wound fluid, this period of equilibration differs from that which may be seen following intravascular infusion. A mixing period following injection of the tracer was employed to ensure rapid and even distribution of the tracer in the chamber. Nevertheless, the duration of this first phase was much longer than that following intravascular infusion, which is in the order of 5 to 10 mins for labelled IGF-I in normal sheep or rats (Hodgkinson *et al.*, 1987; Gillespie *et al.*, 1990; Prosser *et al.*, 1992; Bastian *et al.*, 1993), and may be more akin to the kinetics of absorbtion following intramuscular of subcutaneous injection (Gibaldi, 1991).

At the time of iodination, only those fractions containing greater than 95% TCA-precipitable radioactivity were pooled for use in the clearance study. Intact IGF-I would be expected to form the bulk of these fractions, which were used in the experiment within a few weeks of iodination. However, the tracer preparation may contain impurities in the forms of free ^{125}I, ^{125}I-tyrosine and small peptide fragments containing ^{125}I-tyrosine. As suggested by the decay curve of ^{125}I, these impurities would be expected to be cleared from the wound fluid quite rapidly. Nevertheless, it would be desirable to assess the proportion of radioactivity present in the lower molecular weight forms in the tracer at the time of use. The existence of multiple forms of ^{125}I also alludes to the possibility that a third decay phase may occur within the first hour of the clearance experiment. Additional sampling times would be required to ascertain this possibility, although such sampling may confound the data by increasing the volume of wound fluid removed from the chamber. Furthermore, I considered that the acquisition of such data would be of questionable relevance to the assessment of the effects that IGFBP interactions have on the clearance of IGFs.

In contrast to the first equilibration phase, the second phase of decay from 1 h to 6 h is of more relevance in assessing any effect that IGFBP interactions may have on IGF-I clearance. This phase may be complex with radiolabelled IGF-I interacting not only with wound fluid and tissue-associated IGFBPs, but also with IGF receptors present in the granulation tissue that has grown into the chamber. Intact IGF-I may also exit the chamber without interacting with either the wound IGFBPs or IGF receptors.

Size-exclusion chromatography revealed that radiolabelled IGF-I had interacted with the IGFBPs in wound fluid and the distributed amongst the IGFBP pools in a similar pattern to that seen after incubation of wound fluid with radiolabelled tracer at 4°C *in vitro* (refer section 2.3.4). Similar, but not exact, profiles have been observed in goat (Prosser *et al.*, 1992), lamb

(Lord *et al.*, 1991), sheep (Davis *et al.*, 1992) and human lymph (Binoux and Hossenlopp, 1988).

It may be expected that the existence of relatively large binary and ternary complexes of IGF-I and IGFBPs in wound fluid would impede the movement of IGF-I from the wound fluid to the bloodstream. In contrast, uncomplexed IGF-I may be more able to leave the wound chamber or interact with IGF receptors in the tissue of the chamber. In the latter case, bound IGF-I would be internalised and degraded (Banskota *et al.*, 1986; Bar *et al.*, 1986). In this sense, the abundance of endothelial cells proliferating in the granulation tissue may be important in eliminating labelled IGF-I from wound fluid. These cells have been shown to possess both type 1 and type 2 IGF receptors *in vivo* (Bar and Boes, 1984; Jialal *et al.*, 1985; King *et al.*, 1985) and may express many IGFBPs, some of which are cell-surface associated (Bar *et al.*, 1987; Moser *et al.*, 1992). Additionally, the relatively large proportion of radioactivity recovered from this component suggests some evidence of the radiolabelled IGF-I interacting with the tissue. Degradation products may then be released into the circulation or back into the chamber, which may possibly result in a rise in the proportion of degraded or TCA-soluble radioactivity in the wound fluid. In future experiments, some indication of the occurrence of this process may be gained from measurement of the TCA-precipitability of the wound fluid radioactivity.

Given that radiolabelled IGF-I was bound by wound fluid IGFBPs, it may be hypothesised that those factors that alter the affinity of IGFs for the wound fluid IGFBPs may alter the rate of IGF clearance. Thus, the IGFBPs may serve to physically retain IGFs in the wound fluid by virtue of the physical size of the IGF:IGFBP complexes, or they may protect IGFs from proteolytic activity, the products of which would be cleared more quickly than intact IGF-I. In this sense, limited proteolysis of IGFBPs resulting in reduced affinity for IGFs, such as that

observed for IGFBP-3 in the preceding chapter, may increase clearance of IGF-I from the wound site. Similarly, IGF analogues that bind IGFBPs poorly may be cleared more quickly than IGF-I. Bastian *et al.* (1993) have shown not only that these analogues are cleared from the plasma of normal rats more quickly than IGF-I, but also that radiolabelled IGF-I is cleared more rapidly in pregnant rats than in normal rats. Similar observations in extravascular wound fluid results would provide useful insight into the extent that such interactions regulate IGF clearance, not only in wounds, but also at extravascular sites in general. This issue is addressed in next chapter.

My research on the methods that are the topic of this chapter have been published in an abbreviated form in a paper entitled "Clearance of IGFs and insulin from wounds: effect of IGF-binding protein interactions" by J.G. Robertson, D.A. Belford and F.J. Ballard in the *American Journal of Physiology (Endocrinology and Metabolism)* **276**: E663-E671, 1999.

Chapter Four

Clearance of Insulin-like Growth Factors and Insulin from Wounds

4 Clearance of Insulin-like Growth Factors and Insulin from Wounds

4.1 INTRODUCTION

In the previous chapter, an *in vivo* model of wound repair was established to enable measurement of the rate of elimination of IGFs from the wound site as well as to obtain preliminary data on the interaction of IGF-I with wound fluid IGFBPs and the clearance rate of IGF-I. This chapter describes the use of the model to examine in detail the effects that interactions with IGFBPs have on the elimination and degradation of IGFs.

For the experiment described in this chapter I have compared IGF-I with an IGF-I analogue, IGF-II and insulin. Analogues of IGF-I with differing affinities for IGFBPs are useful tools for examining the effects that interaction with IGFBPs have on the clearance of IGF-I. Moreover, while IGF-II is structurally similar to IGF-I, sharing 65% sequence homology (Rinderknecht and Humbel, 1978b), it binds to some IGFBPs in preference to IGF-I and in plasma binds unoccupied sites of the 150-kDa complex without displacing IGF-I from the complex (Lee and Rechler, 1995). Insulin shows structural homology to IGF-I and IGF-II with the A- and B-chains being 40-60% homologous to the same domains of the IGFs (Rinderknecht and Humbel, 1978b; Rinderknecht and Humbel, 1978a). However, insulin has no known affinity for the IGFBPs and only binds to the type 1 receptor at high concentrations (Czech, 1985; Chaiken *et al.*, 1986; Steele-Perkins *et al.*, 1988). The analogue, LR^3IGF-I, has a thirteen amino acid N-terminal extension and a substitution of Arg for Glu at position 3, modifications that greatly reduce affinities for all known IGFBPs (Lord *et al.*, 1994c), both *in vitro* and *in vivo*. LR^3IGF-I has increased biological potency despite possessing only one third the affinity for the type 1 receptor of native IGF-I (Francis *et al.*, 1992; Tomas *et al.*, 1992; Lemmey *et*

al., 1994). In rats it is cleared from the circulation more rapidly and is degraded to a greater extent than IGF-I (Gillespie *et al.*, 1990; Bastian *et al.*, 1993). Similar observations have been made with other analogues (Cascieri *et al.*, 1988; Francis *et al.*, 1988a; Ballard *et al.*, 1991). Consequently, the accepted role of IGFBPs in plasma, particularly the 150-kDa complex, is not only to inhibit the transfer of IGFs to the tissues but also to protect the IGFs from degradation. However, it is unclear whether such a mechanism may be present in the extravascular tissues. Thus, the aims of the work described in this chapter are firstly, to confirm the rate of elimination of radiolabelled IGF-I measured in the previous chapter and secondly, to relate the IGFBP affinity of labelled IGF-II, LR^3IGF-I and insulin to their rate of clearance from wound fluid. In this way it may be possible to draw conclusions as to the effect that IGFBPs may have on the extravascular actions of IGFs.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

Recombinant human IGF-I, IGF-II, and LR^3IGF-I were obtained from GroPep Pty Ltd (Adelaide, Australia) and recombinant human insulin from Sigma Chemical Co. (St Louis, Missouri, USA). Each peptide was ^{125}I-labelled using the chloramine-T method (Ballard *et al.*, 1991) to a specific activity of 75-85 μCi/μg and stored at -20°C (see section 2.2.1). Recombinant monoiodinated [A^{14}]-insulin was purchased from Amersham Pharmacia Biotech (Castle Hill, Australia). Polyacrylamide gels were purchased from Novex Experimental Technology (San Diego, CA, USA) and X-ray film from Eastman Kodak (Biomax MR; Rochester, NY, USA). Ultra-low range molecular weight markers were purchased from Sigma. All other reagents used in this chapter are as described in section 3.2.1.

4.2.2 Comparison of the rates of clearance of IGF-I, IGF-II, LR^3IGF-I, and insulin from wound fluid

In this study, two separate experiments were conducted. In an initial experiment (*Experiment 1*), the rates of elimination of ^{125}I-labelled IGF-II (n = 2 rats), LR^3IGF-I (n = 3 rats), and insulin (n = 3 rats) from wound fluid were compared. The aim of this experiment was to ensure that the duration of the experiment was adequate to estimate the rate of decay of these peptides in wound fluid. In the second experiment (*Experiment 2*), the rate of clearance of radiolabelled IGF-I from wound fluid, which was also measured earlier in Chapter 3, was compared to those of radiolabelled IGF-II, LR^3-IGF-I, and insulin (n = 8 rats per treatment). In both experiments male Sprague-Dawley rats (175-200 g) were implanted with Hunt-Schilling

chambers as described in section 3.2.2. The housing of the rats, the surgical preparation, the experimental procedures and the collection and analysis of samples are as described in Chapter 3.

4.2.3 Suitability of the chloramine-T technique for the radioiodination of insulin

Radiolabelled insulin used in the clearance studies was iodinated using the chloramine-T method (Hunter and Greenwood, 1962). Although insulin prepared in this manner has been used routinely in studies of insulin binding (Francis *et al.*, 1992; Francis *et al.*, 1993), potential damage to the insulin molecule may occur due to the oxidising conditions of this technique such that the stability of the molecule may be reduced. Additionally, the presence of the large iodine atom on the tyrosine residues, of which there are four, may alter the affinity of insulin for the insulin receptor to varying degrees (Garratt *et al.*, 1972). Consequently, the ability of the radiolabelled insulin used in these experiments to bind insulin receptors *in vitro* was first compared with that of commercially derived monoiodinated [A^{14}]-insulin. This product is also prepared by the chloramine-T technique, but is purified by high-performance liquid chromatography so that only insulin labelled at tyrosine A^{14} is present. This isomer is reported to have an affinity for the insulin receptor that is similar to unlabelled insulin (Gliemann *et al.*, 1979).

To evaluate receptor binding, rat H35 hepatoma monolayers were grown to near confluence and tracer binding to the monolayers at 4°C was measured in the presence of increasing concentrations of unlabelled insulin (Ross *et al.*, 1989; Francis *et al.*, 1993). Binding was expressed as a percentage of that bound in the absence of competing ligand. The concentration of cold insulin required to give half-maximal competition of the binding of monoiodinated

[A^{14}]-insulin was approximately 0.5 nM (Figure 4.1). A similar value, approximately 1 nM, was recorded for the labelled insulin preparation used in the clearance experiments.

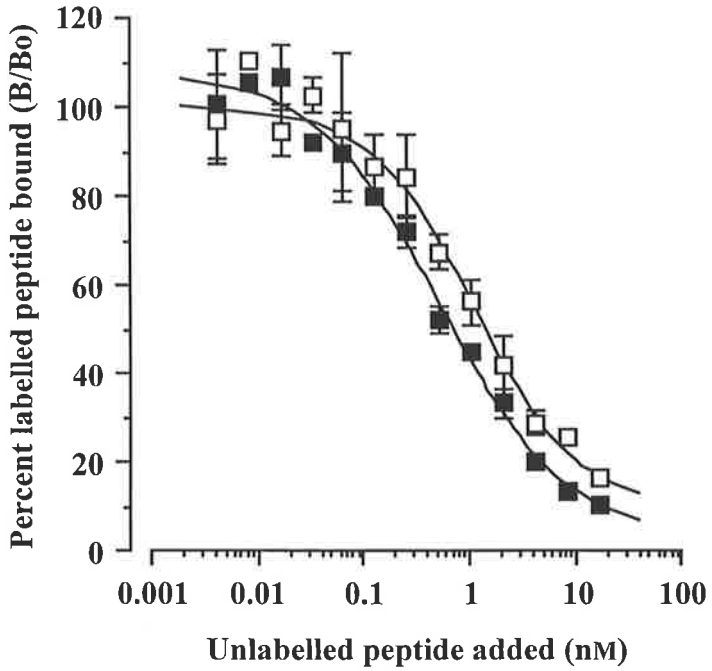

Figure 4.1 Binding of insulin tracers to insulin receptors

Competition for binding of ^{125}I-labelled insulin (□) or mono-iodinated [A^{14}]-insulin (■) for insulin receptors in rat H35 hepatoma cells. Values are means ± SEM of triplicate determinations at each concentration of peptide.

The stability of the iodinated insulin prepared for the clearance experiments was compared to that of monoiodinated [A^{14}]-insulin. Radiolabelled IGF-I, which was iodinated by the chloramine-T method for use in the clearance studies, was also assessed. The tracers were stored at -20°C and were analysed within 4 weeks of iodination. Subsamples of iodinated peptides were diluted in PBS or wound fluid to give approximately 50 000 cpm per 10 µl (approximately 0.35 ng of peptide). The mixtures were incubated for 24 h at 37°C with subsamples withdrawn at 0, 6, and 24 h and stored at -80°C. The subsamples, together with molecular weight markers, were analysed by electrophoresis on high-density [16% (w/v)] tris/tricine SDS polyacrylamide gels (Schagger and von Jagow, 1987). Following

electrophoresis, the gels were immediately fixed in freshly prepared 5% (w/v) gluteraldehyde for 1 h and then 20% (w/v) methanol for 2 h before drying and exposure to X-ray film in the presence of intensifying screens (Cronex Hi-Plus; Du Pont, Wilmington, MA, USA). Measurements of the intensity of the bands were made by laser scanning of phosphor images (Molecular Dynamics, Sunnyvale, CA, USA). The autoradiographs are shown in Figure 4.2. The intensity of the radiolabelled IGF-I band did not decline in either PBS or wound fluid after 24 h of incubation at 37°C (Figure 4.2; *upper* and *lower panels*, respectively). Radiolabelled insulin prepared for use in the infusion experiments also appeared to show little degradation after 6 h of incubation in PBS, a situation that also applied to the monoiodinated [A^{14}]-insulin. However, the intensities of the bands of both insulins were decreased after 2 h of incubation in wound fluid with further reductions were seen in the 6- and 24-h bands (Figure 4.2; *lower panel*).

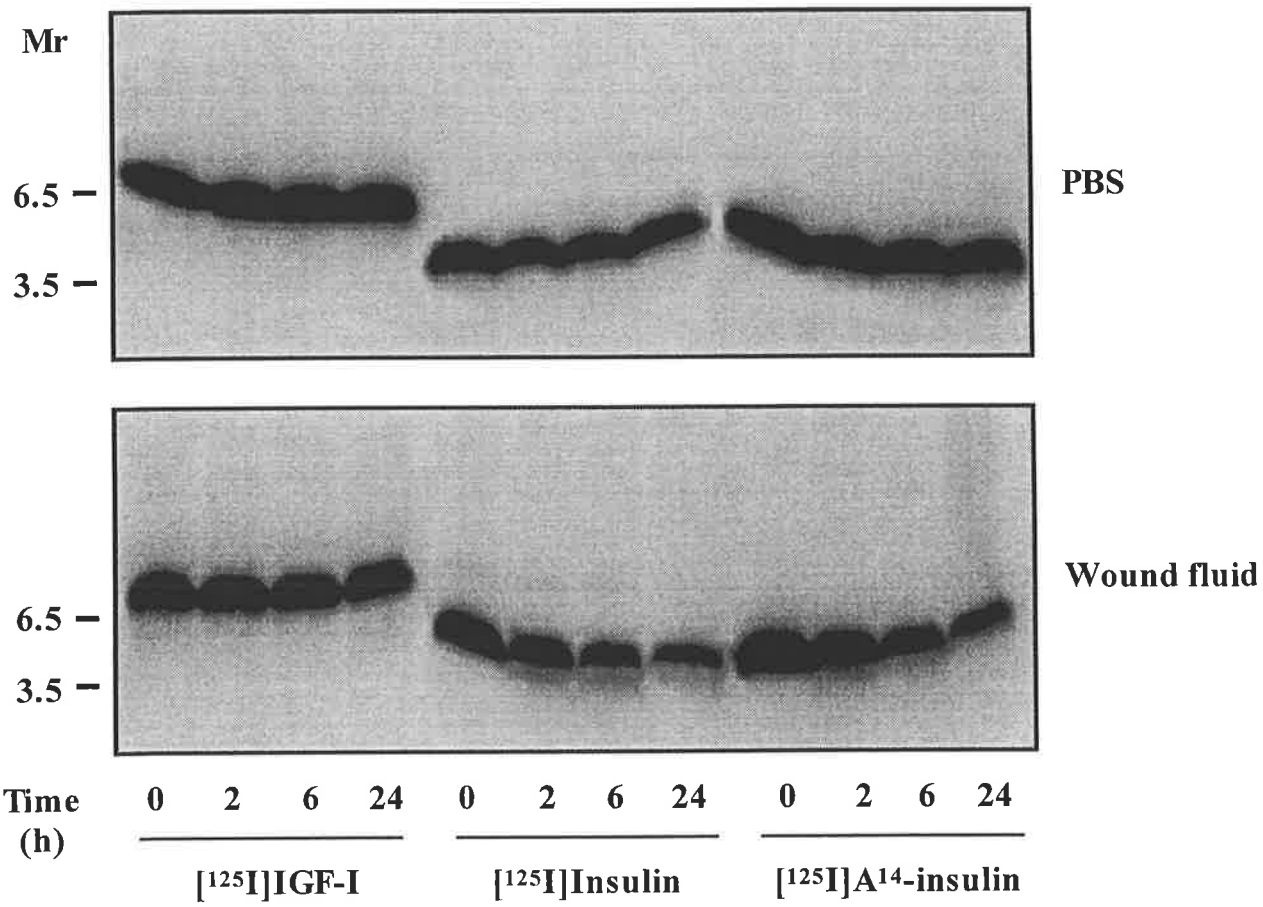

Figure 4.2 Degradation of labelled insulins by wound fluid *in vitro*

Radiolabelled proteins were incubated in PBS (*upper panel*) and in pools of day 14 wound fluid (*lower panel*) at 37°C for 24 h. Subsamples were withdrawn at 0, 2, 6 and 24 h, diluted in SDS-PAGE sample buffer and subjected to electrophoresis. The gels were fixed and dried as described above and autoradiographs produced by exposing to X-ray film for 6 h. The approximate positions of SDS-PAGE molecular weight markers ($x10^{-3}$) are shown on the left.

The results of these two assays suggest that, based on receptor binding or stability of the insulin molecule *in vitro*, the radiolabelled insulin prepared for use in this manner does not appear to be adversely affected by the radiolabelling process. These conclusions are supported by the work of others (Schneider *et al.*, 1976; Woltanski *et al.*, 1990; Francis *et al.*, 1993). Accordingly, insulin used in the following clearance experiments was iodinated as described in section 4.2.1.

4.2.4 Data analysis

All radioactivity measurements were corrected for radioactive decay back to the day the experiment was performed. The amount of TCA-precipitable peptide remaining in the wound fluid at each time point was expressed as a percentage of the initial amount of TCA-precipitable peptide injected into the chamber after which the data were analysed and elimination rate constants and half-lives calculated according to the technique described in section 3.2.8. All data are presented as means ± SEM. Comparison of the elimination rate constant of IGF-I obtained in this study and that in Chapter 3 was made using the Mann-Whitney Rank Sum test. Analysis of the wound fluid decay rate constants and the distribution of radioactivity was achieved by one-way ANOVA, while post hoc pair-wise multiple comparisons were made with Fisher's protected least squares differences procedure (SuperANOVA, 1989, Abacus Concepts, Berkeley, CA). Differences which gave $P < 0.05$ were considered to be statistically significant.

4.3 RESULTS

4.3.1 *Experiment 1*: Preliminary comparison of the rates of clearance of IGF-II, LR^3IGF-I, and insulin from wound fluid

In an initial experiment, the clearance profiles of radiolabelled IGF-II, LR^3IGF-I, and insulin from wound fluid were investigated. The aim of this experiment was to gain preliminary information on the rate of decay of these peptides in wound fluid. In IGF-II-treated rats, approximately 83% of the TCA-insoluble dose remained 1 h after infusion and 72% of the dose remained at 6 h (Figure 4.3A). In contrast, LR^3IGF-I-infused rats showed greater changes in the amounts of TCA-insoluble material present in the wound fluid so that 1 h after infusion 77% of the dose remained and at 6 h approximately 45% of the dose endured. Rats receiving insulin showed similar changes in the TCA-insoluble material to the rats receiving LR^3IGF-I, having approximately 65% and 30% of the dose remaining at 1 h and 6 h respectively.

The amount of TCA-insoluble radioactivity appearing in the circulation was monitored throughout the experiment (Figure 4.3B). In IGF-II-infused rats, TCA-precipitable radioactivity could be detected in the circulation, but no more than 0.3% of the original radioactivity was present at any time during the experiment. In contrast, greater amounts of TCA-insoluble radioactivity were measured in the blood of LR^3IGF-I-infused rats. Thus, ten minutes after infusion, 0.1% of the dose was measured in the circulation and by 6 h more than 1% was present. A similar result was observed for insulin.

The accumulation of radioactivity in urine was measured hourly throughout the experiment (Figure 4.3C). In rats administered IGF-II, 2.2 ± 0.5% of the radioactive dose was recovered in

the urine. A slightly larger amount was recovered in rats receiving $LR^3IGF\text{-}I$ (3.4 ± 0.9%) but the largest amount of radioactivity was recovered from rats receiving insulin (5.9 ± 1.9%).

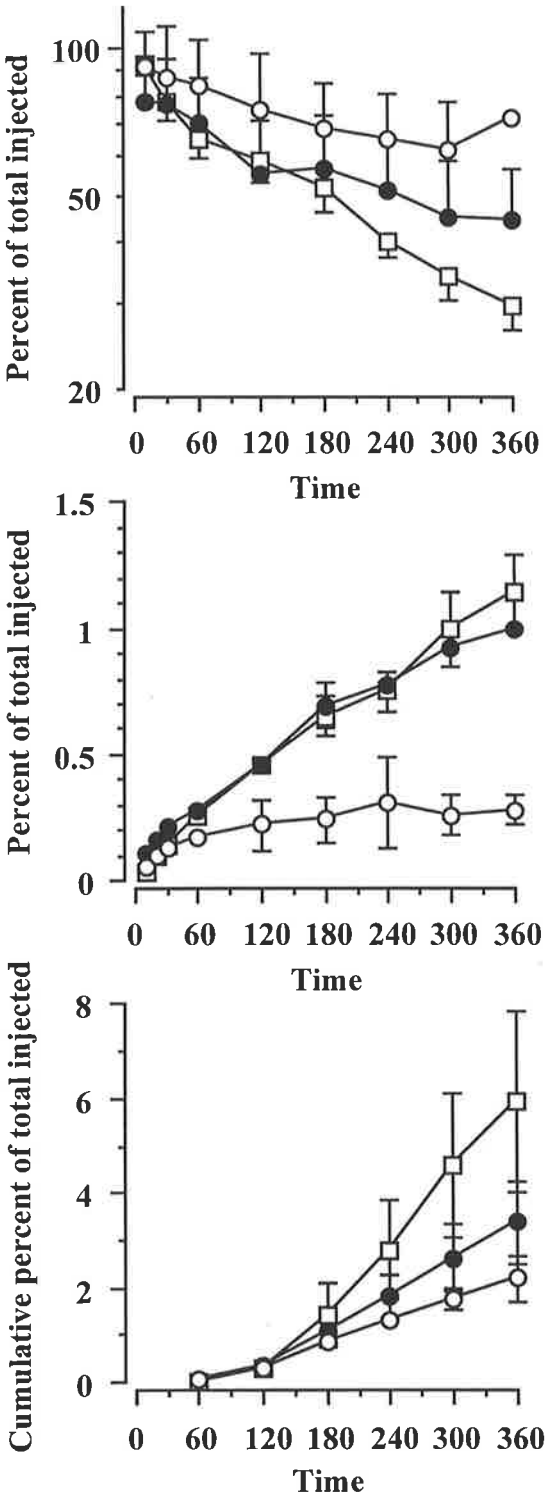

Figure 4.3 ***Experiment 1*****: Clearance of IGFs and insulin from wound fluid**

Radioactivity was measured in wound fluid (A), blood (B), and urine (C) over 6 h after injection of IGF-II (●; n = 2 rats), $LR^3IGF\text{-}I$ (○; n = 3 rats), or insulin (□; n = 3 rats) into the chambers. The amount of radioactivity remaining was calculated as a percentage of that injected into the chamber and all values are means ± SEM.

4.3.2 *Experiment 2*: Clearance of IGF peptides and insulin from wound fluid

Comparison of the rates of elimination of different peptides from wound fluid

Following establishment of the wound fluid decay profile of IGF-I in the previous chapter and the preliminary comparison with other peptides, a complete experiment was performed to compare the rate of elimination of IGF-I with those of IGF-II, LR^3IGF-I, and insulin. The experiment included insulin, which is of a similar molecular weight to the IGFs but does not interact with the IGFBPs, to establish whether the rate of elimination from the wound fluid was a function of molecular size.

The decline of the total amount of radioactivity present in wound fluid was biphasic for each peptide (Figure 4.4A). In the first hour after injection, approximately 20% of the radioactivity of labelled IGF-I or IGF-II was eliminated, a lesser amount than the approximately 30% observed with LR^3IGF-I or insulin. A slow phase of elimination then occurred from 1 h until the end of the experiment by which time approximately a further 15% of the radioactivity of the IGF peptides had been eliminated from the wound fluid. Total insulin radioactivity declined faster with approximately 28% removed between 1 h to 6 h (Figure 4.4).

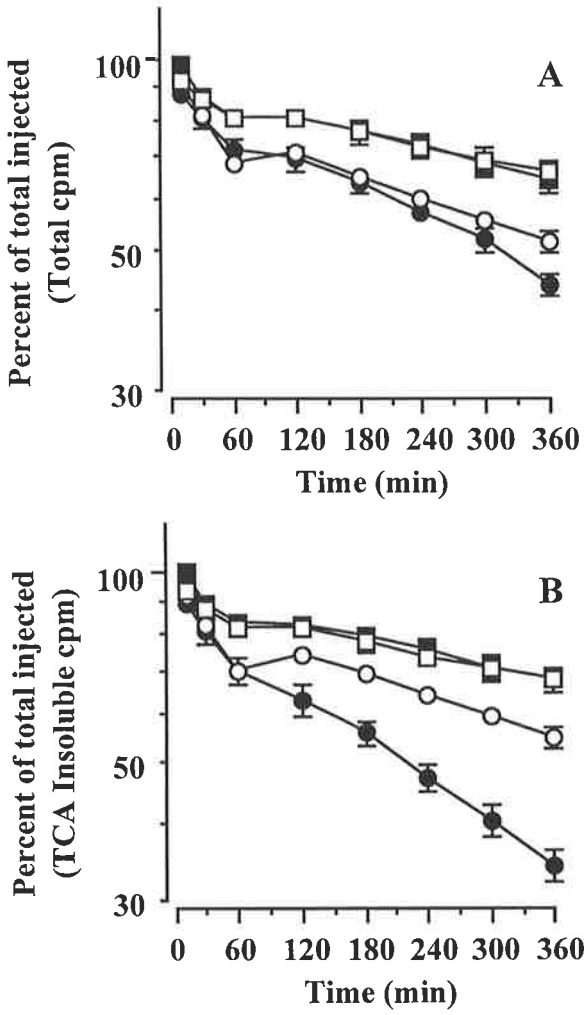

Figure 4.4 *Experiment 2*: **Clearance of ^{125}I-labelled peptides from wound fluid.**

Radiolabelled IGF-I (□), IGF-II (■), LR^{3}IGF-I (○), or insulin (●) was injected into the chambers and the total radioactivity (A) or the TCA-insoluble radioactivity (B) was measured over a 6 h period. Radioactivity is expressed as a percentage of that injected. Values are means ± SEM for n = 8 rats per treatment. (See Table 4.2 for statistical summary.)

The amount of intact IGF-I present in wound fluid declined in a biphasic manner (Figure 4.4B). TCA-insoluble radioactivity for IGF-II and LR^{3}IGF-I (Figure 4.4B) showed a similar biphasic pattern of decay to IGF-I, although the amount of intact LR^{3}IGF-I remaining after 1 h was 70% of the injected dose compared to 82% for IGF-I. By 6 h this difference was greater with 55% of intact LR^{3}IGF-I remaining compared to 68% of intact IGF-I. Insulin showed the fastest decline of TCA-insoluble radioactivity remaining in the chamber declining rapidly to 70% in the first hour and to 35% at 6 h (Figure 4.4B).

The slopes of the slow phases of the TCA-insoluble curves were used to calculate the elimination rate constants (k_d) and half-lives for each peptide (Table 4.1). The k_d obtained for IGF-I did not significantly differ ($P > 0.05$) from that calculated in the previous chapter (see section 3.3.2). Additionally the rate constant of IGF-I did not differ to that of IGF-II ($P > 0.05$). This was in contrast to LR^3IGF-I or insulin which had significantly greater rates of elimination than IGF-I ($P < 0.05$ and $P < 0.001$ respectively).

Table 4.1 ***Experiment 2*: Elimination rate constants of intact peptides in wound fluid**

Peptide	Elimination rate constant (hr^{-1})	Half-life (min)
IGF-I	0.049 ± 0.003	872
IGF-II	0.053 ± 0.009	861
LR^3IGF-I	0.076 ± 0.006*	563
Insulin	0.141 ± 0.013†	324

The amount of intact peptide remaining in the chamber wound fluid (Figure 4.4) was expressed as a percentage of that injected. The slopes of the slow phase of the curves were used to calculate elimination rate constants and half-lives as described. The data represent means ± SEM for 8 rats per treatment. *, $P < 0.05$; †, $P < 0.001$ vs. IGF-I by one-way ANOVA.

TCA-precipitability of radioactivity in wound fluid

Degradation of the labelled peptides in wound fluid was evaluated by measuring the proportion of intact material present over time (Figure 4.5). Each of the IGF peptides remained greater than 89% intact in the wound fluid throughout the experiment, whereas insulin, being 94% TCA-insoluble at the beginning of the experiment, declined to be only 73% intact by 6 h. In accordance with this observation, there was an increased rate of elimination of the TCA-insoluble radioactivity in wound fluid (Figure 4.4B) compared with the rate of elimination of the total radioactivity (Figure 4.4A). In contrast, the rates of elimination of the TCA-insoluble

material and total radioactivity for LR^{3}IGF-I were more similar (Figure 4.4A and Figure 4.4B). It should be noted that the TCA-precipitation assay might underestimate tracer degradation since some peptide fragments may be precipitable yet functionally inactive (Duckworth, 1988). This assay was chosen in preference to other techniques, such as radioimmunoassay, which may not only not distinguish between active and inactive fragments, but would also be incapable of distinguishing added from endogenous peptides and may suffer interference from IGFBPs.

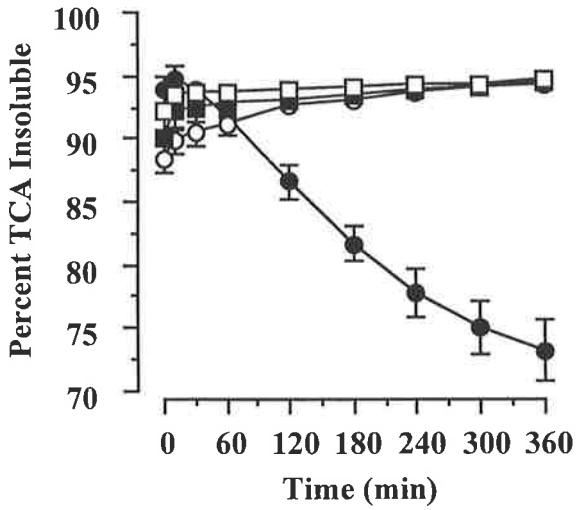

Figure 4.5 ***Experiment 2*: TCA-insolubility of radiolabelled peptides in wound fluid**

The proportion of intact radiolabelled IGF-I (□), IGF-II (■), LR^{3}IGF-I (○), or insulin (●) was measured over time using the TCA precipitation method (see section 3.2.4). Values are means ± SEM for n = 8 rats per treatment.

Association of radioligands with wound fluid IGFBPs

Samples of wound fluid collected from each rat at the end of the experiment were chromatographed under neutral conditions to evaluate the capacity of the respective labelled peptides to bind IGFBPs. IGF-I eluted in 3 regions corresponding to 150, 30-50, and 7.5 kDa

respectively (Figure 4.6A), a profile that was similar to that seen after incubating wound fluid with [^{125}I]IGF-I *in vitro* at 4°C (see section 2.3.4). IGF-II showed a similar pattern of elution to IGF-I, although the relative amount of radioactivity recovered in the 150-kDa region was substantially greater, and there was a corresponding reduction in the 30-/50- and 7.5-kDa peaks (Figure 4.6B). Chromatography of LR^{3}IGF-I or insulin revealed two small peaks of radioactivity at approximately 50-70 kDa (Figure 4.6C and Figure 4.6D), but the greatest amounts, representing unbound peptide, were recovered in each case as a single peak at 5-7 kDa.

Whilst the IGFBP- and receptor-binding abilities of the tracers after incubation in wound fluid was not assessed, the binding properties of similarly prepared labelled peptides have been described previously (Francis *et al.*, 1992; Francis *et al.*, 1993; Lord *et al.*, 1994c). The neutral-gel filtration chromatography profile also suggests that the endogenous IGF binding capacity of wound fluid were not exceeded by the amount of tracer added, which was calculated to be less than 10% of the total IGF-I present (Robertson *et al.*, 1996; Upton *et al.*, 1996).

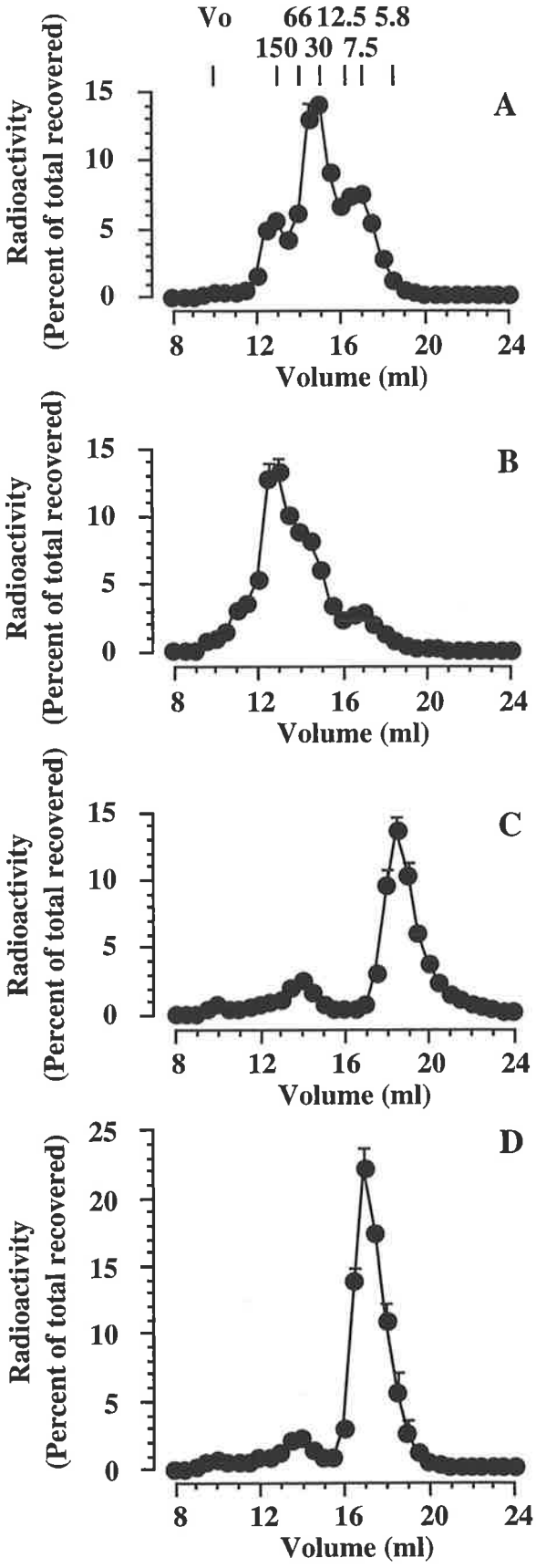

Figure 4.6 ***Experiment 2*: Association of the radiolabelled peptides with IGFBPs**

Wound fluid containing labelled IGF-I (A), IGF-II (B), insulin (C), or LR^3IGF-I (D) was collected at the end of the experiment and applied to a Superose-12 column equilibrated in PBS at pH 7.2. The samples were eluted at 0.5 ml/min with the same buffer and the radioactivity determined in 0.5-ml fractions. The column was standardised with molecular weight markers of 150 kDa (human IgG), 66 kDa (BSA), 30 kDa (carbonic anhydrase), and 12.5 kDa (cytochrome-C), as well as the ^{125}I-labelled peptides (IGF-I and IGF-II, 7.5 kDa; LR^3IGF-I, 9.1 kDa; and insulin, 5.8 kDa). Values are means ± SEM for n = 8 rats per treatment.

Radioactivity in blood and urine

Radioactivity in blood and urine was measured to determine whether the increased rates of clearance from the wound fluid were reflected in the amount of each appearing in these fluids (Figure 4.7 and Figure 4.8, respectively). LR^3IGF-I and insulin showed similar and increasing amounts of intact peptide in blood throughout the experiment (Figure 4.7B). This was in contrast to the IGF-I or IGF-II radioactivity that was lower and constant from 120 min onwards. For IGF-I, 45% of the total radioactivity present in the blood throughout the experiment was TCA-insoluble. Approximately similar values were recorded for LR^3IGF-I, IGF-II, and insulin.

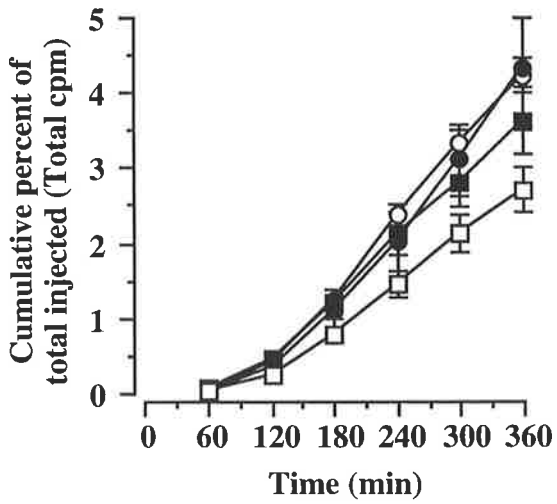

Figure 4.7 ***Experiment 2*: Radioactivity in blood.**

Total (A) or TCA-insoluble (B) radioactivity in blood after injection of labelled IGF-I (□), IGF-II (■), LR^3IGF-I (○), or insulin (●) into the chambers. Radioactivity is expressed as a percentage of that injected. Values are means ± SEM for n = 8 rats per treatment.

Accumulation of radioactivity in urine (Figure 4.8) showed similar patterns of appearance as for blood in that by the end of the trial more LR^3IGF-I or insulin radioactivity accumulated than that observed with IGF-I or IGF-II. However, for the duration of the experiment no more

than 5% of the total radioactivity of each peptide injected into the chamber was excreted into the urine.

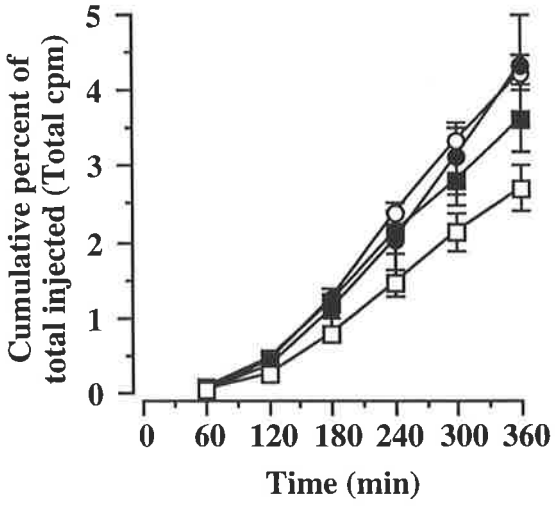

Figure 4.8 ***Experiment 2*: Cumulative radioactivity in urine.**

Radiolabelled IGF-I (□), IGF-II (■), LR^3IGF-I (○), or insulin (●) were injected into the chambers and total radioactivity measured in hourly collections of urine. The cumulative radioactivity is expressed as a percentage of that injected. Values are means ± SEM for n = 8 rats per treatment.

Recovery and distribution of radioactivity

At completion of the experiment, radioactivity present in the wound fluid, chamber and associated tissue, and rat carcass was measured (Table 4.2). Radioactivity present in urine or that removed by blood or wound fluid sampling was also measured to complete the mass balance. For each of the peptide treatments, greater than 92% of the administered dose was recovered with no significant difference occurring between treatments ($P > 0.05$). Additionally, no significant differences in the tracer distribution were found between rats injected with either labelled IGF-I or IGF-II. Compared with the IGF-I group, there was significantly more radioactivity recovered in the carcasses of LR^3IGF-I or insulin-treated rats

($P < 0.0001$). In contrast, the amount of radioactivity recovered from tissue associated with the chambers of insulin-treated rats was approximately half that recovered for the other treatments ($P < 0.0001$). As inferred from the rates of elimination of tracer from wound fluid, at 6 h significantly more radioactivity was present in the wound fluid of rats injected with IGF-I than those injected with LR^{3}IGF-I or insulin ($P < 0.0001$). Similarly, more radioactivity was recovered from the urine of LR^{3}IGF-I or insulin-treated rats than IGF-I-treated rats ($P < 0.01$ and $P < 0.0001$ respectively).

Table 4.2 ***Experiment 2*: Recovery of radioactivity for each peptide in body compartments**

Source	IGF-I	IGF-II	LR^{3}IGF-I	Insulin
Rat Digestion	17.5 ± 1.0	20.9 ± 1.6	30.2 ± 1.1*	43.7 ± 1.8*
Chamber Digestion	11.3 ± 0.8	10.7 ± 1.2	11.1 ± 0.4	5.3 ± 0.4*
Wound fluid at 6 h	64.9 ± 1.8	61.2 ± 2.3	50.6 ± 1.3*	42.7 ± 2.1*
Wound fluid	3.4 ± 0.2	3.5 ± 0.4	3.8 ± 0.2	2.8 ± 0.1‡
Blood sampling	0.025 ± 0.003	0.030 ± 0.004	0.040 ± 0.002†	0.054 ± 0.004*
Urine excretion	2.8 ± 0.3	3.8 ± 0.5	4.3 ± 0.2†	5.4 ± 0.4*
Percent recovered	**95.4 ± 1.3**	**96.7 ± 1.5**	**97.2 ± 0.7**	**92.6 ± 2.9**

The proportion of radioactivity throughout the rats at the completion of the experiment was evaluated as described in section 3.2.7. Values are the means ± SEM for 8 rats per treatment and represent a percentage of the radioactivity recovered. *, P < 0.0001 and †, P < 0.01 vs. IGF-I; ‡, P < 0.01 vs. LR3IGF-I by one-way ANOVA.

4.4 DISCUSSION

Few studies have investigated the role IGFBPs have in regulating the actions of IGFs in the extravascular environment. As such the behaviour of IGF peptides is mostly derived from *in vitro* or circulatory studies. In the present study, the Hunt-Schilling chamber model of wound repair (Schilling *et al.*, 1959; Hunt *et al.*, 1967) has been adapted to allow repeated sampling of extravascular wound fluid from the same chamber with minimal risk of contamination with plasma. The results imply that interactions with IGFBPs retarded the elimination from the wound environment of IGF-I and IGF-II, since both interact with IGFBPs, compared with an analogue of IGF-I, LR^3IGF-I, which binds very weakly to IGFBPs.

All the peptides tested were eliminated from the chamber in a biphasic manner with an initial rapid phase in the first hour followed by a slower phase for the remainder of the experiment. Although few studies have attempted to measure clearance of growth factors from extravascular sites, the biphasic patterns of elimination in this study are consistent with those observations. Thus radiolabelled insulin injected into the wound fluid of Hunt-Schilling chambers that had been implanted in diabetic rats was cleared in a similar manner to the peptides of this study (Goodson *et al.*, 1980). It has also been reported that ^{125}I-labelled epidermal growth factor rapidly disappeared from implanted sponges with only 10% of the radioactivity remaining after 4 h (Buckley *et al.*, 1985). In another study, radiolabelled transforming growth factor-β, platelet-derived growth factor, or fibroblast growth factor-2 was emulsified in collagen and the clearance of each from the chambers over 10 days examined (Sprugel *et al.*, 1988). A biphasic clearance of the growth factors was observed, with approximately 30-35% of the peptides remaining after 6 h. Furthermore, the initial phase of decay lasted up to 24 h and some radioactivity was recoverable after 10 days. The long

duration of that study may be due to the chambers being filled with collagen, which could have impeded clearance of the growth factors, and as such the results may not be directly comparable.

Interaction and binding to components of the extracellular matrix, which may occur with growth factors, would be one mechanism by which the clearance of growth factors could be regulated. However, the results of Sprugel *et al.* (1988) with fibroblast growth factor-2 and platelet-derived growth factor, both of which may bind glycosaminoglycans, suggested that such interactions may not be sufficient to increase the tissue half-lives of exogenous growth factors. In contrast, my experiments suggest the presence of a pool of specific carrier proteins, in the form of the IGFBPs in wound fluid, is sufficient to alter the kinetics of an exogenous IGF provided it is capable of interacting with the IGFBPs. I evaluated the clearance of LR^3IGF-I, an analogue of IGF-I with a 13 amino acid N-terminal extension and a substitution of Arg for the Glu at position 3 (Francis *et al.*, 1992). Its affinity for IGFBPs, compared to IGF-I, is greatly reduced, a property that increases its potency *in vitro* and *in vivo* (Francis *et al.*, 1992; Tomas *et al.*, 1993). In an earlier study, it was observed that LR^3IGF-I was cleared from plasma more rapidly than IGF-I (Bastian *et al.*, 1993), a finding consistent with the present results. This study also demonstrates that, in contrast to IGF-I and IGF-II, LR^3IGF-I was not able to significantly interact with IGFBPs in wound fluid, as chromatographed samples of wound fluid containing LR^3IGF-I eluted as a single peak in the region expected for unbound peptide (Lord *et al.*, 1994c).

Given the *in vitro* IGFBP binding characteristics of LR^3IGF-I, it may also be expected that this peptide would also interact to a lesser extent with tissue- or matrix-associated IGFBPs than IGF-I or IGF-II. However, the amount of radioactivity present in the chamber tissue at the end of the experiment did not differ between the IGF peptides, whereas the amount of radioactivity

found in the carcass was greater for LR^3IGF-I. This observation is consistent with a more rapid transfer of this peptide from the chamber wound fluid to other tissues compared to IGF-I and IGF-II. Although the actual sites of binding were not determined in this study, the data suggest that despite LR^3IGF-I being more rapidly cleared from the chamber, this peptide was still able to interact with wound tissue to the same extent as IGF-I and IGF-II. The result is perhaps not unexpected, as LR^3IGF-I exhibits significant binding to plasma IGFBPs in other species (Lord *et al.*, 1994c).

It has been reported that, as determined by binding-inhibition studies, IGF receptor numbers are increased during wound repair (Hakim *et al.*, 1995). Moreover, the concentration of labelled LR^3IGF-I required to prevent 50% of the receptor binding was similar to labelled IGF-I. Such results are intriguing as the affinity of LR^3IGF-I for the type 1 IGF receptor is about one third that of IGF-I and the affinity for IGFBPs is about 1000-fold less (Francis *et al.*, 1992). However, the report does offer a plausible explanation for the recovery of similar amounts of radiolabelled IGFs in the chamber tissue; LR^3IGF-I may bind to IGF receptors *in vivo* with similar affinity to IGF-I but not to IGFBPs. More detailed examination of the tracer-binding sites in chamber tissue, perhaps by autoradiography, would be required to give a definitive response to this paradox.

IGF-I present in the 150-kDa ternary complex is cleared more slowly from plasma than that present in the 30-/50-kDa forms (Lewitt *et al.*, 1993a). Additionally, IGFBP-3 in wound fluid is proteolysed after which it exhibits a reduced ability to bind radiolabelled IGF-I (Blat *et al.*, 1994; Robertson *et al.*, 1996), leading to a greater amount of the labelled IGF-I associating with low molecular weight binding proteins. As an extension of this finding, it might be expected that IGF-II would exhibit a lower rate of elimination from wound fluid than IGF-I since a greater proportion of IGF-II was present in the 150-kDa complex compared to IGF-I.

That finding is consistent with the preferential binding of IGF-II to the unoccupied, proteolytically nicked IGFBP-3 subunit of the ternary complex (Lee and Rechler, 1995). Why no difference was observed is not obvious from the data, but may in part be due to the increased permeability of the capillary barrier in granulation tissue. Nevertheless, I was not able to demonstrate any significant difference between the clearances of IGF-I and IGF-II.

Insulin, which has only a slightly smaller molecular weight than the IGFs and like LR^3IGF-I does not associate with binding proteins, was eliminated more rapidly from wound fluid. However, the process of insulin elimination was complex due to degradation of the hormone in wound fluid. In other experiments the half-life of intact radiolabelled insulin in 14-day wounds of diabetic rats was calculated to be 150 min (Goodson *et al.*, 1980). The smaller estimate, compared to my data, is reasonable given the well-described disturbances to healing that occur in diabetic rats (Goodson *et al.*, 1980). The degradation of insulin in wound fluid has been attributed to the action of a specific extracellular protease which, in accordance with these results, was also shown not to proteolyse either IGF-I or IGF-II (Shearer *et al.*, 1997). However, another study showed that insulin-degrading enzyme degraded IGF-I slightly *in vitro*, as assessed by either TCA precipitation or by the loss of ability to bind type 1 IGF receptors (Roth *et al.*, 1984). Furthermore, Roth *et al.* (1984) observed that IGF-II was more susceptible than IGF-I to proteolysis of the IGFs by the human insulin-degrading enzyme. It is plausible that some degradation of the IGF peptides may have occurred in my experiments, but no breakdown products accumulated because of rapid clearance of such material.

Although rates of elimination of the degraded (TCA-soluble) fractions could not be calculated in this experiment, I had shown previously that $Na^{125}I$ was rapidly cleared from the chamber such that by 6 h less than 5% of the original material remained (see section 3.3.2). The labelled IGFs were 88-92% intact before injection into the chambers with the range of values

attributed to varying specific activities. The TCA-insolubility of the IGF peptides in wound fluid increased with time, suggesting that any TCA-soluble impurities are cleared rapidly from wound fluid. Although it is conceivable that radioactivity could re-enter the wound chamber from the circulation, no allowance for this possibility was made in the model nor in my calculations of elimination rate constants. However, in earlier work by co-workers in my laboratory, less than 0.1% of an intravenously administered bolus of IGF-I was detected in the wound after 4 h (S.E. Bastian *et al.*, in press). This result, together with the observation that $Na^{125}I$ is rapidly cleared from the chamber, supports the view that re-entry contributes little to the total chamber radioactivity. Against the view that IGFBPs protect IGFs from proteolytic action, LR^3IGF-I did not appear to be significantly degraded in wound fluid. Interestingly, the greater breakdown of insulin in wound fluid was not reflected in an increase in the proportion of TCA-soluble radioactivity in blood. Presumably, this is due to the rapid removal of these products from the blood, which was reflected by the higher accumulation of insulin radioactivity in urine.

Constitutive expression of IGFs and most IGFBPs by cells of the repair response suggests a role for IGFs in wound repair (Gartner *et al.*, 1992; Tavakkol *et al.*, 1992; Steenfos, 1994). Furthermore, the potent *in vitro* stimulatory activity of IGF analogues with low IGFBP affinity on cells that are principal components of the repair process enhances the prospect of IGFs as agents that may have therapeutic value in wound healing. Yet, positive responses to exogenous IGF-I have been equivocal and generally limited to those studies where an IGFBP was administered conjointly (Hamon *et al.*, 1993; Jyung *et al.*, 1994; Tsuboi *et al.*, 1995), pertubations of the GH/IGF/IGFBP system occurred (Suh *et al.*, 1992; Tsuboi *et al.*, 1995), or where the IGF was present at increased levels (Buckley *et al.*, 1985; Suh *et al.*, 1992; Bitar, 1997).

The roles of the IGFBPs that are present during wound repair and the parts they play in regulating IGF bioavailability are unclear. Nevertheless, some insight has been gained from observations of the effects that application of IGFBPs with IGF-I to wounds. Thus IGFBP-1, in the phosphorylated form, has been shown to enhance wound epithelialisation and granulation tissue formation when administered with IGF-I (Jyung *et al.*, 1994; Tsuboi *et al.*, 1995), an effect that has been attributed to the ability of this IGFBP to associate with tissue integrins (Galiano *et al.*, 1996). In addition, proteolytic fragments of IGFBP-3 that have reduced affinity for IGF-I may enhance IGF-I bioactivity by bringing IGF-I in close contact with the cell membrane (Oh *et al.*, 1992; Lalou *et al.*, 1996). This process has yet to be confirmed *in vivo*, although the proteolysis of IGFBP-3 in wound fluid suggests that such a mechanism for increasing IGF-I bioactivity does exist during wound repair (see Chapter 2). A role for other IGFBPs in dermal wound repair has also been postulated based on their expression by components of normal skin (Batch *et al.*, 1994), and the presence of glycosaminoglycan-binding domains in IGFBP sequences (Hodgkinson *et al.*, 1994). However, not all IGFBPs are expressed in skin and the expression of each IGFBP is localised to different components of the integument (Batch *et al.*, 1994) indicating that the activity of IGFs may be regulated according to the target cells involved.

In conclusion, the observations that an IGF analogue and insulin, which have reduced affinity for the IGFBPs or do not bind to IGFBPs at all, are cleared from wound fluid at a greater rate than native IGF-I or IGF-II emphasises the importance of IGFBPs in maintaining a pool of IGF at the wound site. Furthermore, the clearance experiments may serve as a general model for measuring the regulation of the bioactivity and transfer of growth factors and other bioactive molecules from one extravascular compartment to another. Nevertheless, whether altered IGF/IGFBP associations affects the amount of IGF-I available to bind the type 1

receptor and whether this can be used as a means to alter the healing processes in wounds has yet to be determined

My research described in this chapter has been published in a paper entitled entitled "Clearance of IGFs and insulin from wounds: effect of IGF-binding protein interactions" by J.G. Robertson, D.A. Belford and F.J. Ballard in the *American Journal of Physiology (Endocrinology and Metabolism)* **276**: E663-E671, 1999.

Chapter Five

Bioavailability of IGF-I in rat wound fluid

5 Bioavailability of IGF-I in rat wound fluid

5.1 INTRODUCTION

It has been suggested that limited proteolysis of IGFBPs may be a general mechanism for increasing the availability of IGFs during times of increased physiological demand (Lamson *et al.*, 1993). Thus in humans during pregnancy, IGFBP-3, which is the predominant plasma IGFBP and carrier protein for IGFs, is subjected to proteolytic modification (Giudice *et al.*, 1990; Hossenlopp *et al.*, 1990). This phenomenon has also been shown to occur during pregnancy in rodents (Davenport *et al.*, 1990; Fielder *et al.*, 1990) and other pathophysiological conditions (Davies *et al.*, 1991; Cwyfan-Hughes *et al.*, 1992; Davenport *et al.*, 1992b; Cohen *et al.*, 1994a; Giudice *et al.*, 1994; Lee *et al.*, 1994a; Lee *et al.*, 1994b) as well as in the normal state (Lalou and Binoux, 1993). Such modification of IGFBP-3 by proteases results in reduced affinity for radioiodinated IGFs (Suikkari and Baxter, 1991; Baxter and Skriver, 1993), perhaps explaining the apparent discrepancy between levels of IGFBP-3 determined by Western-ligand blotting and radioimmunoassay (Gargosky *et al.*, 1992). Although the exact purpose of IGFBP-3 proteolysis is unclear, Davenport *et al.* (1990) noted that whilst the 150-kDa ternary complex was still present in human pregnancy plasma the half-life of circulating IGF-I was decreased. This implies an increased turnover of IGF-I during pregnancy, perhaps in response to an increased physiological demand during gestation.

The possibility that the physiological demand for IGF-I is increased during pregnancy has been partially substantiated by Binoux and co-workers. In an important study by them, stimulation of DNA synthesis by chicken embryo fibroblasts by late-pregnancy serum was shown to be twice that attained with normal serum (Blat *et al.*, 1994). Moreover, the effect of

both sera was inhibited by an excess of either recombinant human IGFBP-3 or a monoclonal antibody to the IGFs, demonstrating that the IGFs accounted for most of the stimulation of DNA synthesis. However, whether this result can be extrapolated to other instances where IGFBP-3 undergoes proteolysis, such as in the wound, is unknown.

Further support of the notion that limited proteolysis of IGFBPs leads to increased IGF bioavailability has been given by other *in vitro* studies. Thus, the addition of IGFBP-3 to prostatic epithelial cells inhibited the stimulatory actions of IGF-I, whereas the addition of prostate-specific antigen, a kallekrein-like enzyme found in human seminal plasma (Watt *et al.*, 1986; Cohen *et al.*, 1992), reversed the inhibitory effect of IGFBP-3 (Cohen *et al.*, 1994b). Likewise, Conover (1992) noted that the *in vitro* co-incubation of IGF-I and IGFBP-3 was inhibitory to the actions of IGF-I on bovine fibroblasts. However, if the exogenous IGFBP-3 was pre-incubated for 24 h or more with the cell monolayers then the inhibitory effects of IGF-I were reversed. Further investigation revealed that this response is dependent on the processing of IGFBP-3 to lower molecular weight forms that have a 10-fold reduced affinity for IGF-I (Conover, 1991). Thus, the reduction in affinity of IGFBP-3 for IGF-I is an important process for increasing IGF availability, a feature that also appears to be a characteristic of pregnancy-associated IGFBP-3 proteolysis (Baxter and Skriver, 1993; Lassarre and Binoux, 1994).

In the preceding two chapters, I showed that the rates of clearance from the wound of IGF analogues with reduced affinity for IGFBPs were greater than native IGF-I. These data inferred that the IGFBPs are important for maintaining a pool of IGFs at the wound site. However, in Chapter 2 I described the action of proteases in wound fluid that led to the diminished appearance of IGFBP-3 on Western-ligand blots. I hypothesised that the existence of IGFBP-3 proteases in the wound environment may be a response to a heightened metabolic

demand for IGF-I, similar to that which occurs during pregnancy. Together these observations suggest that not only are the IGFBPs important regulators of IGF action at the wound site, but that the action of proteases may offer a further level to the regulation of IGF-I. Thus, it may be expected that IGF-I within wound fluid may show increased interaction with the type 1 IGF receptor. Although several studies have proposed that limited proteolysis of IGFBP-3 increases the availability of IGF-I in the plasma of pregnant humans (Holly *et al.*, 1993; Blat *et al.*, 1994), the question of whether a similar mechanism for increasing IGF-I availability exists in the wound has yet to be addressed.

In this chapter, I aim to address the hypothesis that proteases in wound fluid act to increase the availability of IGF-I. To do this I am employing an *in vitro* assay that allows measurement of IGF binding to cell monolayers. The principal of the assay is that unlabelled IGFs present in the wound fluid, are added in increasing concentrations in the presence of a fixed concentration of radiolabelled IGF tracer. Thus, increased binding of the added IGFs to the cell monolayers would result in decreased binding of the tracer. Although it will not be possible to draw conclusions from the results of the binding of IGF-I in wound fluid alone, comparison with the binding achieved by IGF-I in plasma should provide some indication of whether wound fluid IGF-I is more available to interact with cellular receptors.

5.2 MATERIALS AND METHODS

5.2.1 Peptides

Sodium iodide ($Na^{125}I$) was obtained from Amersham Australia (Castle Hill, Australia). Recombinant rat IGF-I, recombinant human (rh) IGF-I, rhIGF-II, and LR^3IGF-I were obtained from GroPep (Adelaide, Australia). As described in section 2.2.1, the peptides were ^{125}I-labelled to a specific activity of 75-85 μCi/μg using the chloramine-T method (Ballard *et al.*, 1991) before storage at -20°C.

5.2.2 Plasma and wound fluid collection

Rat plasma and wound fluid were collected from Hunt-Schilling chambers that had been subcutaneously implanted in adult male Sprague-Dawley rats (250-300g) as described in section 2.2.3. The chambers were left *in situ* for 14 days at which time the rats were anaesthetised and plasma and wound fluid samples were collected. Human plasma samples were withdrawn from healthy normal and pregnant female volunteers. All samples were heparinised such that the final concentration of heparin was 10 IU/ml. The samples were then processed and stored as described in section 2.2.4.

5.2.3 IGF-I binding to the Type 1 IGF receptor

Cell culture

Rat L6 myoblast cells were grown as monolayers at 37°C under an atmosphere of 5% CO_2 and humidified air in Dulbecco's-modified Eagle's Minimal Essential Medium (Trace Biosciences, Castle Hill, Australia) supplemented with 6.0 mM L-glutamine, 100 U/ml penicillin G, 0.1 mg dihydro-streptomycin and 10% (v/v) foetal bovine serum (pH 7.2). When confluence was reached, the monolayers were trypsinised and seeded onto 24-well multiwell plates. Cells from passages 11 to 14 were used for the experiments within 24 h of the cells becoming confluent.

Binding assay

The IGF binding assay was performed using L6 myoblasts according the technique of Ross *et al.* (1989). Upon reaching confluence, the growth medium was removed from the multiwell plates and the monolayers washed twice in HEPES-buffered saline (0.1 M HEPES, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM D-glucose and 0.5% (w/v) BSA, pH 7.6). The cells were then incubated at 4°C in air for 2 h after which 20 000 cpm of radiolabelled IGF and increasing concentrations of plasma, wound fluid or unlabelled IGF in the same buffer were added to each well. The plates were then incubated for 18 h at 4°C before washing twice with Hanks buffered salts (5 mM KCl, 0.3 mM K_2HPO_4, 0.8 mM $MgSO_4$, 137 mM NaCl, 0.4 mM NaH_2PO_4, 1.7 mM $CaCl_2$, 4.2 mM $NaHCO_3$, 5.5 mM D-glucose, pH 7.25; ICN Biomedicals, Costa Mesa, CA, USA). Finally, the monolayers were dissolved in 0.5 M NaOH containing 0.1% (w/v) Triton-X-100 and the bound radioactivity within each well measured using a

gamma counter. All wash steps were performed on ice. Binding was expressed as the percentage of that present in the absence of added unlabelled ligand.

5.2.4 IGF assays and Western-ligand blotting

Procedures for the measurement of IGF-I in plasma and wound fluid are described in section 2.2.6. In these experiments rat IGF-I was used as the competing ligand and the rat samples were compared to a rat IGF-I reference standard (Upton *et al.*, 1996), whereas the IGF concentrations of human samples were measured by comparison with the respective human homologues. The procedures for visualising IGFBPs in plasma or wound fluid samples by Western-ligand blotting are described in section 2.2.8. However, the nitrocellulose filters were exposed to phosphor screens and images of the plasma IGFBP bands obtained by laser scanning densitometry.

5.2.5 Data analysis

Data derived from the radioimmunoassay of plasma and wound fluid samples were analysed by one-way ANOVA followed by pair-wise multiple comparisons using Tukey's test when a significant F-ratio was present. A significant effect was deemed present for values of P that were 0.05 or less.

The binding data for the rat fluids are representative of three separate experiments, whereas the human plasma data represent the mean of four separate individuals for either normal or pregnancy plasma. Triplicate determinations were made of each sample with corrections to the proportion of bound radioactivity made to allow for different IGF-I concentrations in the

plasma and wound fluid samples. The corrected data were fitted to a four-parameter equation with the aid of a non-linear curve-fitting program (Tablecurve; Jandel Scientific, San Rafael, CA, USA), and the ED_{50} values determined from the fitted curves.

5.3 RESULTS

5.3.1 Measurement of IGF-I in rat plasma and wound fluid

Following gel-permeation chromatography at pH 2.5 to separate IGF-I from IGFBPs in samples of rat plasma and wound fluid, fractions were pooled as described in section 2.2.6 and IGF-I measured using a homologous rat RIA. Although the assay used a polyclonal antibody raised against human IGF-I, recombinant rat IGF-I was used both as the competing radioligand and as the reference standard (Upton *et al.*, 1996): the use of a homologous reference standard gives a better estimation of the true IGF-I concentration.

The amounts of IGF-I measured in plasma and wound fluid collected from rats 14 days after implantation with Hunt-Schilling chambers are given in Table 5.1. Wound fluid contained less than half the amount of IGF-I found in plasma from the same animals ($P < 0.05$). The IGF-I concentrations of plasma collected from rats of the same sex and age but without chambers were similar to rats that had chambers.

Table 5.1 Concentrations of IGF-I in rat plasma and wound fluid

	Normal rats	Wounded rats	
	plasma (ng/ml)	plasma (ng/ml)	wound fluid (ng/ml)
	2129	2554	998
	2475	1575	913
	2240	2125	1091
	2172	2474	972
Mean	2254	2182	993
SEM	89	257	43

Plasma and wound fluid were collected from four rats 14 days after implantation with Hunt-Schilling chambers or from four age- and sex-matched control rats and subjected to gel-permeation chromatography at pH 2.5 to separate IGF-I from IGFBPs. IGF-I concentrations were determined by comparison with a rat IGF-I reference standard. Values represent the means of triplicate determinations for each animal.

5.3.2 Availability of IGF-I in rat plasma and wound fluid

The ability of IGF-I in pooled day 14 rat wound fluid samples to bind IGF receptors on L6 myoblasts was compared to that of IGF-I contained within rat plasma. Plasma samples were obtained from the same animals that the wound fluid was collected from as well as control rats that were matched for age and sex.

The results of an assay using ^{125}I-labelled IGF-I as the competing ligand are shown in Figure 5.1. Approximately 13.2% of the radiolabelled peptide was bound to the cell monolayers in the absence of either the unlabelled peptide or the sample fluids. Unlabelled recombinant human IGF-I exhibited binding competition with half-maximal responses achieved at an

approximate concentration of 0.26 nM. Although the binding competition curves of pools of plasma or wound fluid were slightly different to that of unlabelled IGF-I, similar values for 50% inhibition of binding in all fluids were obtained after correction for the IGF-I content. Thus, half-maximal binding was calculated to occur at approximately 0.87 nM, 0.76 nM and 0.58 nM for control plasma, plasma from chamber-implanted rats and wound fluid, respectively.

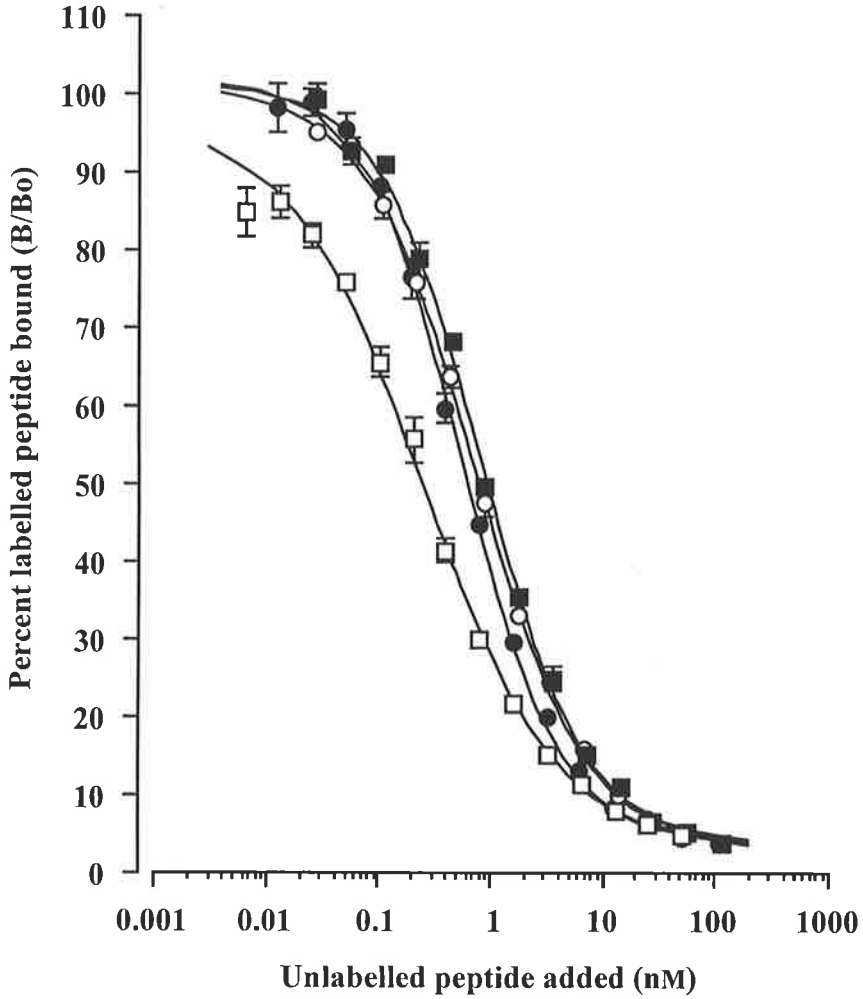

Figure 5.1 Binding of radiolabelled rhIGF-I to L6 myoblasts

Unlabelled rhIGF-I (□), control plasma (■), or plasma (○) and wound fluid (●) collected from rats 14 days after implantation with Hunt-Schilling chambers were added in increasing amounts to cell monolayers in the presence of radiolabelled rhIGF-I. The plotted values are the means of triplicate determinations at each peptide concentration and are expressed as a percentage of the radioactivity in the absence of unlabelled peptide or sample fluid. The error bars indicate SEMs when they are larger than the symbols.

Given the similar values for half-maximal binding of IGF-I in the rat plasma and wound fluid when ^{125}I-labelled IGF-I was used as a competing ligand, it was considered that IGFBPs other than IGFBP-3 may have bound and sequestered the IGF-I tracer . As such, a further experiment utilising radiolabelled LR^{3}IGF-I as the competing ligand was conducted; LR^{3}IGF-I

has an almost 1000-fold reduced affinity for IGFBPs with only a modest reduction in affinity for the type 1 IGF receptor (Francis *et al.*, 1992). Figure 5.2 shows a representative experiment in which LR^3IGF-I was the radioligand. In the absence of unlabelled peptide or test samples, approximately 3.6% of the labelled peptide was bound to the cell monolayers. Unlabelled rhIGF-I produced 50% inhibition of binding at an approximate concentration of 0.24 nM, similar to that achieved when radiolabelled rhIGF-I was used as the radioligand. Values of 1.5 nM, 3.1 nM, and 0.92 nM were calculated as giving half-maximal binding for control plasma, plasma from rats with chambers and wound fluid, respectively.

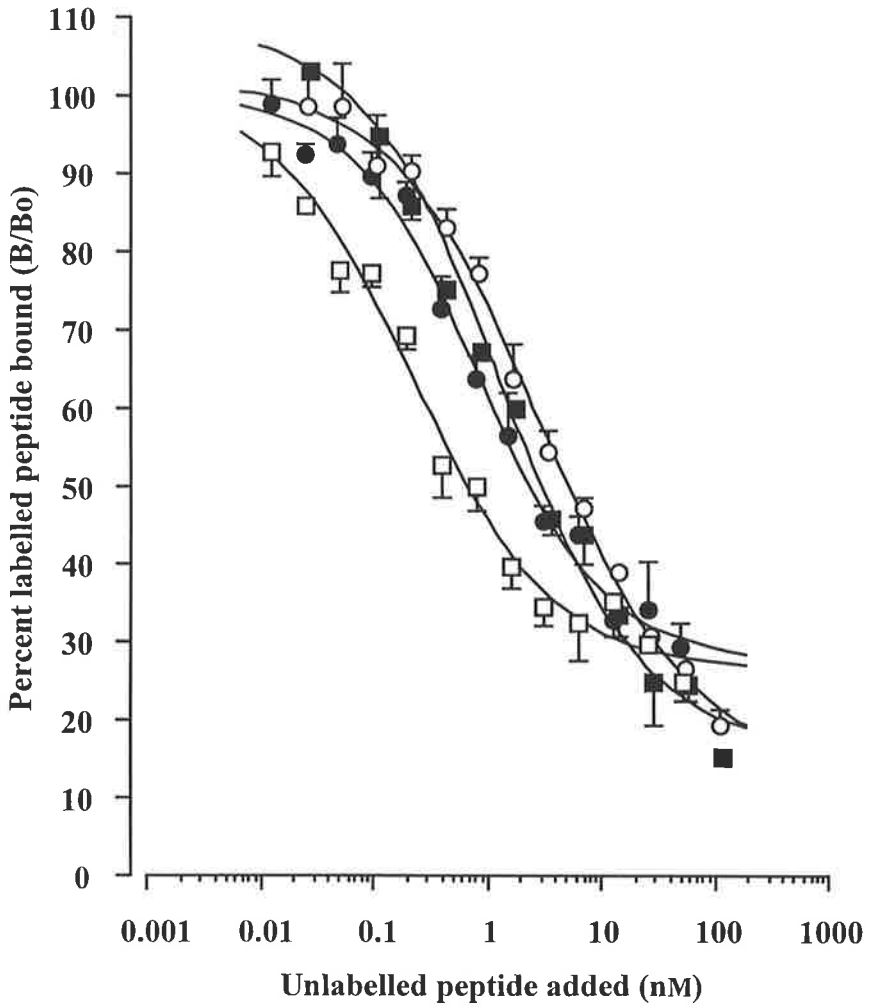

Figure 5.2 Binding of radiolabelled LR^{3}IGF-I to L6 myoblasts

Unlabelled rhIGF-I (□), control plasma (■), or plasma (○), and wound fluid (●) collected from rats 14 days after implantation with Hunt-Schilling chambers were added in increasing amounts to cell monolayers in the presence of radiolabelled LR^{3}IGF-I. The plotted values are the means of triplicate determinations at each peptide concentration and are expressed as a percentage of the radioactivity in the absence of unlabelled peptide or sample fluid. The error bars indicate SEMs when they are larger than the symbols.

5.3.3 Availability of IGFs in human pregnancy plasma

At this point, the failure of IGF-I in wound fluid to show an increased potency of binding to L6 myoblasts relative to that achieved by plasma IGF-I of either control or wounded rats was perplexing. Indeed, it was unclear whether the competitive binding assays were capable of detecting differences in the binding of IGF-I in complex samples such as plasma. As such, a further experiment was conducted to verify the methodology.

Limited proteolysis of plasma IGFBP-3 was first observed *in vivo* during pregnancy and has been since observed in a range of other pathophysiological conditions. As the affinity of proteolysed IGFBP-3 for IGF-I is decreased, this process has been proposed as a means to increase the bioavailability of IGF-I. Accordingly, human pregnancy plasma was collected from four women during the last trimester of gestation and the availability of IGF-I in this fluid was compared to that of normal human plasma. This approach was used to repeat the experiment reported by Blat *et al.* (1994) in which DNA synthesis was stimulated to a greater extent *in vitro* by human pregnancy serum than normal human serum.

Radioimmunoassay of IGF-I and IGF-II content and visualisation of plasma IGFBPs by Western-ligand blotting

The plasma was first assayed for IGF-I and IGF-II content (Table 5.2). No significant differences were present for either IGF-I or IGF-II in normal or pregnancy plasma ($P = 0.14$ and $P = 0.85$, respectively). IGF-II was also measured for completeness, although the values were not used to correct the binding data since the presence of type 2 IGF receptors in L6 myoblasts has been reported to create a "sink" for IGF-II (Beguinot *et al.*, 1985; Francis *et al.*,

1993). Furthermore, an earlier study using this cell line showed that only small amounts of IGF-II compete for the type 1 IGF receptor in the presence of IGF-I (Ballard *et al.*, 1986).

Table 5.2 Concentrations of IGF-I or IGF-II in normal or pregnancy human plasma

	Normal		Pregnancy	
	IGF-I (ng/ml)	**IGF-II (ng/ml)**	**IGF-I (ng/ml)**	**IGF-II (ng/ml)**
	87	677	154	633
	153	869	190	731
	122	789	112	1065
	128	926	206	918
Mean	205	815	277	837
SEM	26	62	40	111

Samples of pregnancy plasma (n = 4) were collected during the last trimester of gestation, whilst normal plasma was collected from healthy female volunteers (n = 4). After gel-permeation chromatography at pH 2.5 to remove binding proteins and pooling of the fractions, triplicate determinations were made on each sample. In different assays, IGF-I and IGF-II concentrations were determined by comparison to reference standards of each human peptide, respectively.

Plasma samples from each normal or pregnant volunteer were subjected to electrophoretic analysis on a polyacrylamide gel (Figure 5.4). Following Western-ligand blotting, intense bands were seen in normal plasma at approximately 40-46 kDa, corresponding to IGFBP-3 (lanes 1-4). Fainter bands were also observed between 30 and 40 kDa, at 30 kDa and at approximately 24 kDa. In contrast, the 40-/46-kDa bands of pregnancy plasma had disappeared, a feature which is characteristic of this situation (lanes 5-8). The other IGFBP bands were also very faint.

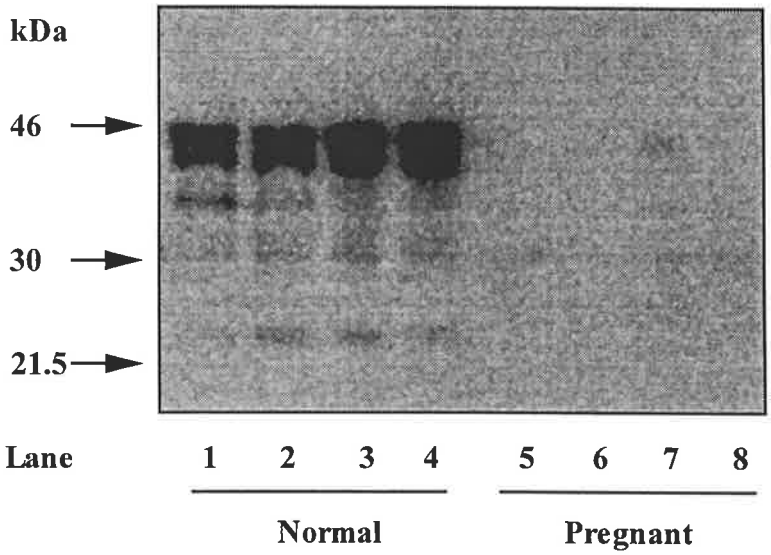

Figure 5.3 Western-ligand blot of normal and pregnant plasma

Samples of normal or pregnancy plasma (all 1 µl) were added to sample buffer and separated on 12% SDS-PAGE gels under non-reducing conditions. The proteins were transferred to nitrocellulose filters and incubated with ^{125}I-labelled IGF-II before washing, drying and exposure to phosphor screens for 6 days and visualisation by laser-scanning densitometry. The positions of molecular weight markers (kDa) are shown.

Binding of IGF-I in human pregnancy plasma to L6 myoblasts

A competition binding experiment was performed to compare the ability of pregnancy plasma IGF-I to bind IGF receptors with that of normal plasma in the presence of radiolabelled LR^{3}IGF-I. The experimental conditions were the same as in the wound fluid IGF competition assays (see section 5.3.2) except that triplicate determinations were made on individual rather than pooled samples. Thus, the data represent the mean of four individuals for either pregnancy or normal plasma.

Approximately 2.7% of the ^{125}I-labelled LR^{3}IGF-I bound the L6 myoblasts in the absence of unlabelled IGF-I. Half-maximal binding of unlabelled rhIGF-I was achieved at approximately 0.31 nM. Pregnancy plasma produced a similar value in the presence of radiolabelled

$LR^3IGF–I$ (0.18 nM), though it should be noted that even at the highest concentration of added sample there was only 40-50% inhibition of binding. Normal human plasma inhibited binding much less such that at the highest concentrations of IGF-I only 40% inhibition of binding was achieved. By extrapolation, the ED_{50} of normal plasma was calculated to be approximately 75 nM. Similar results were obtained when the experiment was repeated.

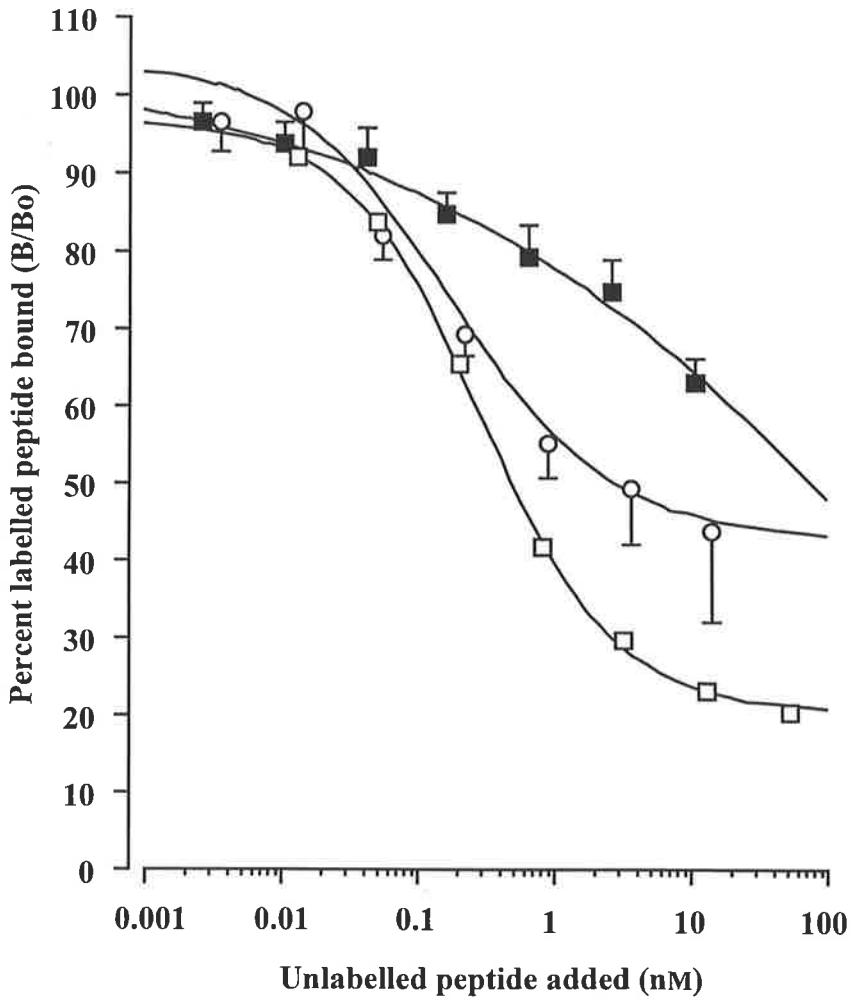

Figure 5.4 Binding of IGF-I in human normal or pregnancy plasma to L6 myoblasts

Unlabelled rhIGF-I (□), normal plasma (■), or pregnancy plasma (○) collected during the last trimester of gestation were added in increasing amounts to cell monolayers in the presence of radiolabelled LR^3IGF-I. Each point is expressed as a percentage of the radioactivity in the absence of unlabelled peptide or sample fluid and represents the mean of four individuals with triplicate determinations at each peptide concentration. The error bars indicate the SEMs when they are larger than the symbols.

5.4 DISCUSSION

IGFBP-3, present in plasma predominantly as part of a ternary complex that contains approximately 75% of the circulating IGFs (Baxter and Martin, 1989b; Gargosky *et al.*, 1991; Baxter, 1993), is functionally altered during pregnancy and other pathological conditions (Davies *et al.*, 1991; Cwyfan-Hughes *et al.*, 1992; Baxter and Skriver, 1993; Giudice *et al.*, 1994; Lassarre and Binoux, 1994; Lee *et al.*, 1994a), possibly in response to a heightened metabolic demand for IGF-I. Consequently, binding to IGFs is reduced compared to the intact form resulting in increased IGF availability (Blat *et al.*, 1994). In Chapter 2, I showed that both IGFBP-3 and the 150-kDa ternary complex were present in wound fluid, yet Western-ligand blotting revealed that IGFBP-3 was much less visible in wound fluid than in plasma. Further investigation showed that this was due to the presence of proteases in wound fluid and led me to speculate that proteases might act on IGFBP-3 in wound fluid to increase the bioavailability of IGF-I at the wound site in the same manner as limited proteolysis has been reported to do so in pregnancy plasma.

The results of the investigations in this chapter showed that for equal concentrations, IGF-I in rat wound fluid did not bind to cells more readily than that in rat plasma. Indeed the receptor availability of IGF-I in rat wound fluid and in rat plasma was only modestly lower than that of pure IGF-I. In contrast to these results, the ED_{50} of IGF-I in human plasma was approximately 100-fold higher, whereas IGF-I in human pregnancy plasma exhibited an ED_{50} similar to that of rat plasma or rat wound fluid.

In the competitive binding experiments, L6 myoblast monolayers were incubated in the presence of decreasing concentrations of rat wound fluid or plasma with corrections made to the binding data to allow for the different amounts of IGF-I in each sample. This was an

important aspect of the study as the concentration of IGF-I in rat wound fluid is only approximately 45% that of plasma (Table 1). Thus, unlike Blat *et al.* (1994), who were able to mix normal human or pregnancy serum until each pool contained approximately equal amounts of IGF-I and IGF-II, it would have been impossible to create a pool of wound fluid with an IGF-I concentration similar to that of plasma unless the plasma was diluted or modified in some way or, conversely, the wound fluid was concentrated. However, as the aim of these experiments was to replicate the *in vivo* situation as near as possible, I considered that these approaches would unreasonably increase the risk of artifacts.

An explanation for the failure to show increased binding of IGF-I in wound fluid is not immediately clear. I considered initially that IGFBPs other than IGFBP-3 within wound may not have been susceptible to proteolysis and retained an affinity for IGFs that was greater than that of the IGF receptors. Some evidence that these IGFBPs could possibly sequester IGFs was gained in the results obtained in Chapter 2. There, the intensity of the 24-kDa band on Western-ligand blots of day 14 wound fluid was approximately 50% of that observed in plasma, whereas the IGFBP-3 band was not more than 15% of the equivalent band in plasma. Pregnancy plasma also appears to spare the 24-kDa band (Davenport *et al.*, 1990; Giudice *et al.*, 1990), yet others have been still able to demonstrate increased biological responses with this plasma. I was able to confirm those results by demonstrating increased binding to IGF receptors.

The possibility of sequestration of IGF-I by IGFBPs other than IGFBP-3 was addressed through the utilisation of LR^3IGF-I as a competing radioligand in the competitive binding assays. The affinity of this analogue for IGFBPs other than IGFBP-3 is approximately 1000-times less than that of IGF-I (Francis *et al.*, 1992; Lord *et al.*, 1994c). Thus, far less interference by IGFBPs would be expected in these assays. However, it also should be noted

that LR^3IGF-I also has an affinity for the type 1 IGF receptor that is approximately one third that of IGF-I (Francis *et al.*, 1992), as was evident in the reduced binding of this analogue in the absence of unlabelled IGF compared to radiolabelled IGF-I (3.6% and 13.2% respectively). Nonetheless, binding of IGFs in the presence of radiolabelled LR^3IGF-I was clearly not increased in wound fluid relative to plasma, leading me to conclude that interference by IGFBPs was not a reason for the failure to show increased potency of IGF-I in wound fluid.

Although the availability of IGF-I in rat plasma was not compared to that of normal human plasma in the same assay, there were very marked differences between the two plasmas. Thus, the IGF-I in rat plasma showed approximately 100-fold lower binding in the receptor assays than that in normal human plasma. Indeed the potency of rat plasma IGF-I was equal to that of rat wound fluid, even though the IGFBP-3 in wound fluid has a binding deficit for IGFs. Hence, it is tempting to speculate that rat plasma IGFBP-3 also suffers a similar binding deficit to that in wound fluid. While limited proteolysis of IGFBP-3 was first observed during pregnancy, there is some evidence to suggest that this phenomenon does occur normally in plasma (Lee and Rechler, 1995; Lee and Rechler, 1996; Kubler *et al.*, 1998). Moreover, in Chapter 2, a 30% decline in the intensity of the plasma IGFBP-3 band was observed after incubation at 37°C for 6 h (see section 2.3.5). Taken together with the competitive binding data in this chapter, it perhaps would be more accurate to state that rat plasma IGF-I is as available as rat wound fluid IGF-I, rather than to argue that wound fluid IGF-I is not more available than plasma IGF-I.

Another possibility that may help to explain the similar availabilities of IGF-I in rat wound fluid and plasma may be that plasma IGFBP-3 in rats implanted with chambers might be susceptible to damage by proteases that leak from the wound chamber into the bloodstream. It

is suspected that major pregnancy IGFBP-3 proteases produced in the maternal reproductive tissues for placental tissue growth and extracellular matrix turnover are released into the bloodstream (Bang *et al.*, 1994c; Fowlkes *et al.*, 1994b; Vettraino *et al.*, 1996). Such an explanation is not inconsistent with my finding that binding of IGF-I in the wounded rats was also equal to that of wound fluid. However, the availability of IGF-I in the plasma of control rats, which did not have chambers, was also equal to that of IGF-I in wound fluid. Thus, the most logical explanation may be that IGF availability is greater in the normal rat than in the non-pregnant human. In support of this notion, Frystyk *et al.* (1998) reported that the levels of plasma free IGF-I in rats were several-fold higher than those measured in human plasma. As the half-life of uncomplexed IGF-I is very short (Guler *et al.*, 1989), these data imply that there is a more rapid turnover of IGF-I in rats. This scenario is consistent with my observations that IGF-I in rat plasma is highly available.

Another intriguing possibility is that the relatively high availability of IGF-I in rat plasma versus human plasma may be related to the observation that rats continue to grow throughout their life while humans do not. This concept could perhaps be tested by comparing plasmas from growing children with those from rats. In this context, it would also be of interest to compare the availability of IGFs in wound fluid obtained from children, adults and elderly individuals.

Although the primary aim of the experiments described in this chapter was to attribute limited proteolysis of IGFBP-3 in rat wound fluid to a heightened availability of IGF-I, they also yielded support for earlier studies that demonstrated stimulatory effects of human pregnancy plasma (Holly *et al.*, 1993; Blat *et al.*, 1994). However, a potential criticism of these earlier studies is that the assays were conducted at 37°C for long periods, allowing the possibility that the conditions of the assays may have been altered by the cultured cells during the incubation.

For example, Blat *et al.* (1994) added human pregnancy sera to chicken embryo fibroblasts and found it was up to twice as potent as normal sera at equivalent concentrations (0.05% to 0.4% serum), yet a 32-kDa IGFBP appeared in the media of the cells after 24 h of incubation. As this band is likely to have been IGFBP-2, which has a preferential affinity for IGF-II and has been shown to bind extracellular matrix in the presence of IGF-I or IGF-II (Arai *et al.*, 1996; Khosla *et al.*, 1998), possible sequestration of IGF-II may have occurred. Although such a mechanism ought to be consistent between the normal and pregnancy sera, it does raise the possibility of an unequal response contributing to the observed differences. In my study, the assays were conducted at 4°C to minimise the possibility that the assay conditions could be modified through the secretion by the cells of IGFBPs or proteases.

In summary, the bioavailability IGF-I in rat wound fluid does not appear to be enhanced compared to that of IGF-I in plasma from the same rats or from age- and sex-matched controls that did not have wound chambers. In contrast, IGFs in human pregnancy plasma showed markedly increased potency in a binding assay over normal plasma from healthy volunteers. While these results do not indicate a precise role for IGFBP-3 proteases in rat wound fluid, they do suggest that substantial species differences exist and that conclusions regarding the regulation of IGFs in one species cannot necessarily be extrapolated to another. Nevertheless, the data do indicate that IGF-I in rat wound fluid and plasma is readily available to cell receptors, possibly reflecting the high rate of metabolism in rats. Furthermore, the differences may help explain the responsiveness of rats to exogenous IGFs that has been demonstrated in numerous *in vivo* studies, while applications of IGFs in human subjects have generally been disappointing. As a final speculation, the low availability of IGF-I in human plasma to receptors may indicate that IGF analogues with reduced affinities for IGFBPs may have therapeutic potential in humans.

Chapter Six

General Discussion

and

Future Directions

6 General Discussion

Most of the IGF-I present in the circulation is bound to IGFBP-3 and an acid-labile subunit to form a ternary complex of approximately 150 kDa (Zapf *et al.*, 1975; Baxter and Martin, 1989b; Gargosky *et al.*, 1991; Baxter, 1993). This complex is thought to confine IGF-I to the vascular space, prolonging its half-life and protecting it from degradation. IGF-I can also be found in extravascular fluids including wound fluid, often at concentrations approaching that found in plasma (Steenfos *et al.*, 1989; Suh *et al.*, 1992). Several other lines of evidence support a role for IGF-I in wound repair such as its identification in many regenerating tissues (Hansson *et al.*, 1986; Hansson *et al.*, 1987; Jennische and Hansson, 1987; Hansson *et al.*, 1988; Edwall *et al.*, 1989), its release by platelets during the clotting process (Karey *et al.*, 1989; Karey and Sirbasku, 1989) and its expression and secretion by many cells that participate in the wound repair process (Rappolee *et al.*, 1988; Gartner *et al.*, 1992; Taylor and Alexander, 1993). Furthermore, compromised wounds often have decreased levels of IGF-I (Suh *et al.*, 1992; Bitar, 1997). However, the roles this growth factor fulfils during acute wound repair and how the IGFBPs, the principal IGF-regulatory proteins, modulate its interactions with cellular receptors in the wound are unclear.

While much of the extravascular behaviour of the IGFs and IGFBPs may be extrapolated from intravascular studies, characterisation of extravascular fluids such as lymph (Binoux and Hossenlopp, 1988) reveals qualitative and quantitative differences to plasma, suggesting that the regulatory mechanisms for IGF-I in the extravascular space may differ from those in the circulation. Indeed, results of studies designed to examine the effect of administration of IGF-I to wounds have been conflicting. Thus, local infusion of IGF-I into wounds has been shown to increase DNA and hydroxyproline content, whereas topical or systemic administration has

been less rewarding (Lynch *et al.*, 1989; Skarulis and Bondy, 1994) except when added conjointly with an IGFBP (Sommer *et al.*, 1991; Jyung *et al.*, 1994; Mueller *et al.*, 1994; Tsuboi *et al.*, 1995; Zhao *et al.*, 1995). The investigations reported in this thesis seek to explore the possible reasons for these discrepancies by addressing the roles IGFBPs may have regulating IGF actions in the acute wound, particularly with respect to their functions as carrier proteins and the prevention of IGF degradation. I hypothesised that the IGFBPs act to limit clearance and degradation in the wound environment. Thus, my aims were:

a) to measure the amounts of IGFs and determine the forms of the IGFBPs that may be present in the milieu of an acute wound;

b) to develop a model of wound repair that would allow measurement of the rate of clearance of IGF-I from the wound site; and

c) to compare the rate of clearance of IGF-I from the wound with those of an IGF analogue with reduced affinity for IGFBPs and IGF-II, which has an affinity for some IGFBPs that is greater than IGF-I.

In the second chapter of this thesis, I employed a rodent model of acute wound repair and collected wound fluid over a three-week period following wounding. Examination of wound fluid is commonly undertaken, as this fluid is believed to closely reflect the environment in which the wound repair cells exist. However, it is difficult to determine whether the composition of wound fluid is primarily due to contributions from plasma or wound tissue. Thus, wound fibroblasts and macrophages (Clemmons, 1984; Gartner *et al.*, 1992), may produce for IGF-I or IGFBPs, whereas the acid-labile subunit, of which evidence of its presence in wound fluid was gained in Chapter 2 and which is not thought to be produced extra-hepatically (Baxter *et al.*, 1994), indicates contributions from plasma. Yet, if the

composition of wound fluid was largely determined by contributions from plasma, it may be argued that levels of IGF-I in wound fluid may be more similar to those in plasma. Measurement of IGF-I in wound fluid revealed this not to be the case with levels falling to approximately half those of plasma seven days after wounding with similar discrepancies observed for the IGFBPs, as determined by Western-ligand blotting, appearing between plasma and wound fluid. Moreover, total protein and albumin levels were approximately two-thirds those of plasma. These data collectively suggest that the endothelial barrier of the wounds is actively involved in regulating the transfer of macromolecules between plasma and wound fluid. Although some investigation of the nature of IGF transfer into the extravascular space has occurred (Bar and Boes, 1984; Dernovsek *et al.*, 1984; Dernovsek and Bar, 1985; Bar *et al.*, 1990a; Bastian *et al.*, 1997), it still needs to be established whether the 150-kDa complex passes into wound fluid as a whole or as individual constituents that reassemble in the wound.

Proteases for IGFBP-3 have been described in many biological systems and are commonly associated with physiological states in which there is an increased metabolic load. Many proteases are also present in wounds, being required for cell migration and the extensive tissue remodelling that occurs. Although the identities of the wound fluid proteases were not established in my studies, several proteases known to be involved in wound repair have also been identified as IGFBP proteases. These include, but are not limited to cathepsin-D (Frost *et al.*, 1993), plasmin (Campbell *et al.*, 1992; Campbell *et al.*, 1993; Booth *et al.*, 1996), kallikreins (Conover and De Leon, 1994; Rajah *et al.*, 1995) and matrix metalloproteinases (Fowlkes, 1994; Fowlkes *et al.*, 1994a). Of these, plasmin is particularly interesting as it is generated from plasminogen during the clotting process and has recently been shown to bind IGFBP-3 (Campbell *et al.*, 1998a). The functions that the plasminogen, IGFBP-3 and IGF-I

complexes may have are unclear. However, release of plasminogen activators upon injury may result in the conversion of plasminogen to plasmin. In turn, plasmin may proteolyse IGFBP-3 to form fragments with reduced affinities for IGF-I (Campbell *et al.*, 1992; Angelloz-Nicoud and Binoux, 1995; Campbell *et al.*, 1998a), thus making IGF-I more bioavailable. The possibility of this hypothesised mechanism of IGF regulation is further indicated by recent observations that plasminogen/IGFBP-3/IGF-I complexes may reversibly bind fibrinogen and fibrin in the clot and that IGFBP-3 was subjected to proteolysis even when bound to fibrin *in vitro* (Campbell *et al.*, 1998b). Moreover, complexes of plasminogen, IGFBP-3 and IGF-I are detectable in normal human serum and plasma and may possibly explain the low level of IGFBP-3 proteolytic activity seen in normal rat plasma (Chapter 2).

Whether the conversion of plasminogen to plasmin and subsequent degradation of IGFBP-3 represents a general mechanism for increasing IGF bioactivity is a not yet clear. However, kallikrein reportedly may also bind IGFBP-3 via its heparin-binding domain suggesting that this may be so (Campbell *et al.*, 1998a). It should also be noted that IGFBP-5, which has a heparin-binding domain similar to that of IGFBP-3, might also bind plasminogen (Campbell and Andress, 1997). While the IGFBPs in wound fluid were not identified in the study reported in Chapter 2, the presence of IGFBP-5 mRNA throughout the dermis of human skin (Batch *et al.*, 1994) and the discovery that IGFBP-5 may form an alternative ternary complex in human serum (Twigg and Baxter, 1998), suggest that a similar mechanism may also apply to other IGFBPs following injury. It would therefore be of interest to identify the IGFBPs in wound fluid and to determine whether complexes of plasminogen and IGFBPs or degradation products of IGFBPs other than IGFBP-3 can be found in the wound environment.

Plasminogen has a molecular weight similar to that of the acid-labile subunit, which is thought to bind IGFBP-3 in the same region of the molecule as plasminogen (Baxter and Firth, 1995;

Campbell *et al.*, 1998a). Consequently, in molecular size-separation studies at neutral pH, plasminogen may also elute with IGFBP-3 and IGF-I as an approximately 150-kDa ternary complex. As these complexes appear to occur normally in plasma (Campbell *et al.*, 1998a), the presence of 150-kDa complexes in wound fluid raises the possibility that plasminogen may also bind IGFBP-3 in the extravascular space. Although the presence of the acid-labile subunit in wound fluid was not conclusively ascertained, it would be of interest to do so as it is possible that a proportion of the 150-kDa complexes in wound fluid will contain plasminogen. Such investigations could form the basis of future studies investigating the role of the plasminogen-plasmin system in regulating IGF bioavailability in the wound.

As the factors that regulate IGFBP protease activity are yet to be fully defined, it is tempting to speculate that IGF actions at the wound site may be regulated indirectly by factors that co-ordinate protease expression and function separate to traditionally recognised IGF regulators. Indeed, it has been recently reported that GH status did not seem to have a major impact upon IGFBP-3 protease activity in human serum (Skjaerbaek *et al.*, 1998b). Although this study appears not to reconcile with earlier studies (Rutishauser *et al.*, 1993; Lassarre *et al.*, 1994), suggestions that GH status, and possibly IGF-status, has no effect upon serum protease activity nonetheless add further complexity to the regulation of IGF actions in the wound.

Although proteolytic modification of IGFBP-3 in wound fluid may be deemed a significant regulatory factor of the actions of IGF-I, the roles of other IGFBPs must also be considered. IGF-I associated with low molecular weight IGFBPs in plasma is cleared at a greater rate than that present in the high molecular weight ternary complex (Young *et al.*, 1992; Lewitt *et al.*, 1993b). Furthermore, IGF analogues that have reduced affinities for IGFBPs have greater rates of clearance and degradation in plasma (Bastian *et al.*, 1993). The greater proportion of radiolabelled IGF-I accumulating in the low molecular weight complexes of wound fluid

suggested that similar dynamics might also occur in the wound environment and perhaps more generally in the extravascular space. However, few models have been available for examining the regulation of IGF kinetics in extravascular spaces. The establishment of an *in vivo* model that addressed this deficiency was described in Chapter 3 and the hypothesis that IGFBPs limit IGF-I clearance and degradation in the extravascular space was tested more rigorously in Chapter 4.

An understanding of the kinetics of growth factors in the wound environment is important for the design of appropriate therapies. From the clearance data derived in Chapters 3 and 4, it may be concluded that the IGFBPs act to sequester IGFs in the wound environment and have a general role in limiting clearance of IGF-I from wounds, observations consistent with those made of their function in the circulation. Thus, clearance of the IGFs from wound fluid was biphasic and was best described by a two compartment pharmacokinetic model with an initial rapid phase followed by a longer slower phase. A similar pattern of clearance from wound sites has also been observed with other growth factors (Goodson *et al.*, 1980; Buckley *et al.*, 1985). Although the data suggested that one of the roles of the IGFBPs in wound fluid is to sequester IGFs, the roles of tissue-associated IGFBPs, whether in the extracellular matrix or on cell surfaces, should not be overlooked. In this regard, similar studies with models of wound repair that have a larger mass of wound tissue may give a better indication of the effect that these IGFBPs have upon IGF clearance.

In accordance with its intravascular behaviour (Bastian *et al.*, 1993), LR^3IGF-I was cleared from wound fluid at greater rate than IGF-I or IGF-II. This suggested not only that analogues such as LR^3IGF-I may be less potent than IGF-I when locally administered due to a more rapid clearance from the wound site, but also that complexing IGFs with an IGFBP may result in increased potency through prolongation of half-lives in the wound. Some support for this

latter prospect has already been gained from studies with IGFBP-1 and IGFBP-3 (Sommer *et al.*, 1991; Jyung *et al.*, 1994; Mueller *et al.*, 1994; Tsuboi *et al.*, 1995; Zhao *et al.*, 1995), although in these instances there appear to be specific mechanisms that promote the bioactivity of IGF-I. Thus, IGFBP-1 may bind integrin receptors via an RGD consensus sequence (Galiano *et al.*, 1996), while IGFBP-3 possibly binds glycosaminoglycans via a heparin-binding domain (Hodgkinson *et al.*, 1994). However, these data do not preclude the possibility that the IGFBPs act generally to increase wound half-lives and limit IGF-induced effects such as receptor down-regulation (Conover and Powell, 1991).

Notwithstanding the possibility that interactions with IGFBPs may be necessary for fulfilment of the bioactivity of IGF-I in tissue repair, it should also be noted that the accumulation of radiolabelled LR^3IGF-I in the chamber tissue was equal to that of the other IGFs. This finding should not be totally unexpected, as some earlier *in vivo* studies with this analogue have produced results that are not always consistent with its *in vitro* binding characteristics. Thus, in a plasma clearance study, Bastian *et al.* (1993) reported that LR^3IGF-I-associated radioactivity preferentially accumulated in some organs in rats, while Lord *et al.* (1994c) found significant amounts of radiolabelled LR^3IGF-I in high and low molecular-weight complexes in lamb plasma. Furthermore, a study of the competition for binding to sections of wound tissue by LR^3IGF-I was equal to IGF-I (Hakim *et al.*, 1995). Thus, interaction of the analogue with IGF receptors, extracellular matrix or cell membrane-bound IGFBPs *in vivo* may explain the higher than expected levels of radioactivity recovered in the chamber tissue. Although no attempt was made to distinguish between intracellular or extracellular radioactivity, future studies should examine the potential tissue-binding sites for LR^3IGF-I in more detail.

As one of the postulated roles of the IGFBPs is to protect IGFs from degradation, it was interesting to observe that none of the IGFs appeared to be degraded in wound fluid, either *in vitro* or *in vivo*. In contrast, insulin was rapidly degraded in wound fluid, an observation that was supported by a report that appeared in the literature around the time this experiment was performed (Shearer *et al.*, 1997). However, it is interesting to note that in plasma the TCA-insolubilities of the IGFs were similar to that of insulin. These data suggest that the site of degradation may be the chamber tissue, whereupon the IGFs are degraded upon egress from the chamber lumen. Likewise, Bastian and co-workers (1993) reported that LR^3IGF-I was degraded more in plasma than IGF-I and speculated that the site of degradation was in the extravascular tissues. In a further study by these investigators, this analogue appeared in wound fluid in degraded forms after intravenous administration (S.E. Bastian *et al*, in press). However, these studies do not rule out the possibility that such IGF degradation occurred in other tissues throughout the body. In this regard, estimation of the TCA-insolubility of the radioactivity in the chamber tissue may have given some indication as to whether this was the site of IGF degradation or not.

Whilst the IGF-infusion experiments described in this thesis have investigated some of the effects IGFBPs may have on the clearance and degradation of IGFs in the acute wound milieu, general conclusions of the effects that IGFBPs may have on the clearance and degradation of IGFs may be limited as the Hunt-Schilling chamber model does not include phases of contraction or re-epithelialisation. Furthermore, the model only permits estimates of rates of clearance to be made at a particular time after implantation in any single animal and it is conceivable that estimates of rates of IGF clearance may vary at other times following injury. Similarly, altered wound fluid IGFBP profiles that may accompany chronic wounds or systemic diseases such as diabetes may also give different estimates of clearance rates.

Limited proteolysis of IGFBP-3 has been observed in several extravascular fluids including synovial fluid (Fernihough *et al.*, 1996; Matsumoto *et al.*, 1996), peritoneal fluid (Koutsilieris *et al.*, 1995), human blister fluid (Xu *et al.*, 1995; Xu *et al.*, 1996) and ovarian follicular fluid (Cwyfan-Hughes *et al.*, 1997). Currently, it is thought that this phenomenon reduces the affinity of IGFBPs for IGFs making the IGFs more available to bind cell receptors (Holly *et al.*, 1993; Lamson *et al.*, 1993; Blat *et al.*, 1994). As rat wound fluid contained proteases that degraded IGFBP-3, the competitive binding studies of Chapter 5 were designed to determine whether IGF-I was more readily available in rat wound fluid than that present in rat plasma, which did not show significant IGFBP-3 protease activity.

The results of the binding assays showed little difference in the availability of IGF-I in either rat plasma or wound fluid suggesting that the proteolysis of IGFBP-3 does not act to increase the availability of IGF-I above that of rat plasma. As recent evidence has hinted that levels of free IGF-I are positively correlated with serum protease activity in humans following major surgery (Skjaerbaek *et al.*, 1998a), it was difficult to reconcile these observations. However, half-maximal binding of rat plasma and wound fluid was achieved at similar concentrations to that of pure IGF-I indicating that the IGF-I in both of these fluids is highly available. Although significance of the high availability of IGF-I in rats is not immediately obvious, the results may help to explain the increased potency of IGF-I in this species. Thus, exogenous IGF-I might be bound with less affinity in rats and be more readily available to IGF receptors. However, data from the clearance studies and the literature suggests that interaction with IGFBPs is necessary for a pool of IGF to be maintained at the wound site. Therefore, increased potency of IGF analogues with decreased affinity for IGFBPs may be countered by an increased clearance. Such a proposition may help to explain why local infusion of IGF-I

into the wound has been the only successful means of eliciting a vulnerary in response to treatment solely with IGF-I (Suh *et al.*, 1992).

Similar values for the availability of IGF-I to receptors were obtained for human pregnancy and rat plasma, whilst normal human plasma yielded an ED_{50} at IGF-I concentrations approximately two orders of magnitude higher. These results not only confirmed an earlier report of the increased bioavailability of IGF-I in human pregnancy plasma (Blat *et al.*, 1994), but were also consistent with the reported lower levels of free IGF-I in the circulation of normal humans compared to rats (Frystyk *et al.*, 1998). Notwithstanding that the plasma samples were collected from different species, it has been noted that plasma free IGF-I levels in humans appear to peak at puberty and decline thereafter (Juul *et al.*, 1996). Thus, it is plausible that the availability of IGF-I in rat plasma samples collected from older or geriatric rats may be more similar to that of adult human plasma. On the other hand, IGF availability may be increased in normal children that are actively growing. Similarly, it would be of interest to investigate the availability of IGF-I in human wound fluid. These aspects could be examined in future studies that examine the relationship of IGF availability and IGFBP-3 protease activity.

In summary, IGFBPs present in the acute wound milieu appear to have a significant role in regulating the clearance of IGFs from the wound site, while the presence of IGFBP-3 proteolytic activity in wound fluid points to the existence of a mechanism for increasing the availability of IGF-I during wound repair. However, IGF-I in rat wound fluid was no more available than that present in rat plasma, whereas comparison of human plasma from normal and pregnant individuals showed substantially increased IGF availability in the latter fluid. These observations may help to explain the particular responsiveness of rats to IGF-I and also suggest that the use of analogues with reduced affinities for IGFBPs, such as LR^3IGF-I, may

be of use in treating wounds in humans, which appear to have lower IGF availability. Together with knowledge gained from future studies of growth factor actions in wounds, this may help in the design of therapeutic applications for the IGFs.

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Errata and addenda

1. Page 6, line 21 – add IGF-I after "...limited to..."
2. Page 8, line9 – insert "*In vitro* studies suggest that ..." before "...the ability..."
3. Page 8, lines 15, 18 and 19 – insert "*in vitro*" after "...synthesis...", "...deposition..." and "...fibroblasts", respectively
4. Page 8, line 18 – insert the sentence "Additionally, several *in* vivo studies of compromised wound repair have indicated a role for IGF-I in the fibroplastic response (Leibovich and Ross, 1975; Spencer *et al.*, 1988; Steenfos *et al.*, 1989, Suh *et al.*, 1992)." before "Importantly,...".
5. Page9, line 7 – insert "IGF-I" after "...interleukin-1..." and add reference "Taylor and Alexander, 1993".
6. Page 10, line 3 – insert "including IGF-I (Bhora, F.Y., Dunkin, B.J., Batzri, S., Aly, H.M., Bass, B.L., Sidawy, A.N., Harmon, J.W. (1995) Effect of growth factors on cell proliferation and epithelialization in human skin. J. Surg. Res 95:236-244." after "...paracrine growth factors."
7. Page 11, line 3 - insert "including IGF-I" after "...growth factors..." and insert reference "(Tsuboi *et al.*, 1992)." at end of sentence.
8. Page 21, line 2 – delete "...500-..." and insert "...100-..." and add reference "(Jones and Clemmons, 1995)"
9. Page 26, line 13 – insert "in humans and pigs" after "...liver..."
10. Page 26, line 15 – delete Zapf *et al.*, 1989 reference.
11. Page 28, line 6 – delete "...an approximately 72% fall in plasma glucose levels..." and insert "...plasma glucose levels approximately 72% of controls..."
12. Pages 31, 48 and 54 – It should be noted that the studies cited in these sections mostly administered pharmacologic doses of IGF-I. As current literature also demonstrates that IGF-I may be produced and act at the cellular level, the relative contribution of endocrine IGF-I is difficult to assess.
13. Page 44, line 18 – the correct year of the reference is 1987.
14. Page 46, line 19 – delete "Mueller *et al.*, 1994" reference.
15. Page 48, line 16 – insert "pharmacologic doses of" after "...intravascular dynamics of..."
16. Page 54, line 11 – insert "exogenous" after "...in the transfer of..."
17. Page 63, line 4 – the correct MW for BSA is 66 kDa.
18. Page 64, section 2.2.9 – at the time of this study (c.1994) large quantities of IGFBP-3 were not available for *in vitro* co-incubation studies with wound fluid.
19. Page 69 and 70, Figure legends of Figs. 2.3 and 2.4 –after "RIA" add "...using an anti-human IGF-I antibody that has only approximately 25% cross-reactivity with rat IGF-I. Thus, true values are approximately 4-fold higher."
20. Page 79, line 2 – delete "...are present."

21. Page 90, Section 3.2.3 – The radioiodination technique employed in this thesis has been conducted at my laboratories for many years and only fractions containing high amounts of TCA-precipitable protein (> 95%) were employed in this study. That only small amounts of degraded material were present was confirmed by neutral-gel chromatography that showed that only small amounts of radioactivity eluted as free iodine and that a single peak of radioactivity corresponding to the MW of the intact peptide was evident. Similarly, electrophoresis of the prepared IGF-I tracer revealed only a single band (See Fig. 4.2). Furthermore, only intact protein was used for comparisons of clearance from the wound chambers. TCA precipitation is a commonly used technique for estimating the proportion of intact protein in samples. However, it is recognised that small inactive peptides or fragments of proteins may not be precipitated in some instances (Duckworth, W.C., 1988). With this in mind, all samples were diluted in BSA to aid precipitation. It should be noted that other techniques such as radioimmunoassay also may not distinguish between active and inactive fragments and may be subjected to interference by IGFBPs.

22. Page 100, Fig. 3.2 – insert "where larger than the symbols" after "mean ± SEM"

23. Page 119, line8 – add "…for non-normal data." after Mann-Whitney Rank Sum test."

24. Page 122, Fig 4.3 – The top, middle and bottom panels represent A, B and C while the correct symbols for IGF-II, LR^3IGF-I and insulin are ○, ●, and □, respectively.

25. Page 128, Fig 4.6 - The causes of the minor peaks present in the chromatography profiles of LR^3IGF-I and insulin were not investigated in this thesis. However, LR^3IGF-I is known to interact with plasma IGFBPs in some species (Lord *et al.*, 1994). In the case of insulin, polymerisation of insulin is the most likely explanation for the minor peak.

26. Page 131, Table 4.2 – insert "sampling" after "Wound fluid " on line 4.

27. Page 134, line 3 – delete "…data suggests…" and insert "recovery of tracer from the chamber tissue suggests"

28. Page 134, line 4-5 – delete "…was still able…" and insert "appeared"

29. Page 148, Table 5.1 - Upton *et al.* (1996) reported that plasma levels IGF-I levels of 130-175g rats were 1600 to 1800 ng/ml. As plasma IGF-I levels reportedly increase with age (Frystk *et al.*, 1998) the plasma IGF-I levels in this study are within expected ranges.

30. Pages 150, 152 and 156, Figs. 5.1, 5.2 and 5.4 – add "The plasma and wound fluid IGF-I concentrations are expressed on the basis their immunoreactive IGF-I content as determined in 5.3.1".

31. Page 156, Fig. 5.4 – Although human plasma contains high levels of IGF-II, it is unable to compete for the type 1 IGF receptor in the presence of IGF-I (Ross *et al.*, (1989). Thus, it was expected that IGF-II would have no effect in this assay.

32. Page 159, line10 - delete "…lower…" and insert "higher".