Transcellular transport of Insulin-like growth factor-I (IGF-I)

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ABSTRACT

The insulin-like growth factors (IGFs) are potent mitogens of growth and differentiation, which are found in blood and body fluids associated with high affinity specific IGF binding proteins (IGFBPs). IGFs are thought to act in a paracrine / autocrine as well as an endocrine manner. To achieve the latter, IGFs in circulation must first cross the endothelial cell barrier. However, the pathway(s) by which IGFs are transported across endothelial cells and the role of IGFBPs in this transfer are unknown. To examine these issues, a well characterised *in vitro* model of an endothelial cell barrier using human umbilical vein endothelial (HUVE) cells was utilised. Studies demonstrated that HUVE cells possess both type I and II IGF receptors and secrete IGFBP-2, -3 and -4. HUVE cells grown on microporous filters in a bi-chamber system produced confluent monolayers which displayed tight junctions. When compared to filters devoid of cells, HUVE cell monolayers restricted the passage of ^{125}I-IGF-I. This transport was not inhibited by either excess unlabelled IGF-I nor a monoclonal antibody to the type I IGF receptor, indicating that the movement of free IGF-I across HUVE cell monolayers occurs by a paracellular route and not by a receptor mediated transcellular pathway. When ^{125}I-IGF-I transport across HUVE cells was examined in the presence of either IGFBP-1 to -6 or IGFBP-3 and the acid labile subunit, transport was decreased. In addition, HUVE cell derived extracellular matrix was found to contain IGFBP-5 and an unidentified IGFBP of 24 kDa. When compared to filters alone , filters coated with HUVE cell ECM significantly restricted the transport of ^{125}I-IGF-I, which implicates the ECM as a potential co-regulator of IGF-I flux. LR^{3}IGF-I is an analogue of IGF-I with a weak affinity for IGFBPs. Since IGFBPs inhibited the transendothelial migration of IGF-I but not of LR^{3}IGF-I across HUVE cell monolayers, it was hypothesised that the transfer of IGF-I from blood to extracellular fluid sites would be inhibited by IGFBPs. Studies in rats found that ^{125}I-LR^{3}IGF-I transfer from blood to extravascular wound fluid accumulation sites was

greater than ^{125}I-IGF-I which confirmed this postulate. These results show that the IGFBPs inhibit transfer from blood to extracellular fluid sites.

STATEMENT

This work contains no material which has been accepted for the award of any other degree or diploma in any other University or other tertiary institution and, to the best of my belief and knowledge, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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COMMONLY USED ABBREVIATIONS

ALS	acid labile subunit
ANOVA	analysis of variance
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
ci	chamber implanted
cpm	counts per minute
DAB	diaminobenzidine
DMEM	Dulbecco's modified Eagle's medium
ECGS	endothelial cell growth supplement
ECM	extracellular matrix
FBS	foetal bovine serum
FPLC	fast performance liquid chromatography
GH	growth hormone
[^{3}H]-	tritiated-
HBSS	Hanks balanced salt solution
HUVE	human umbilical vein endothelial
IEC6	intestinal epithelial cells-6
IGF	insulin-like growth factor
IGFs	insulin-like growth factors
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor -II
IGFBP	insulin-like growth factor binding protein
IgG	immunoglobulin G
LR^{3}IGF-I	Long Arg^3 insulin-like growth factor-I
MDBK	Madin Darby Bovine Kidney epithelial cells
MDCK	Madin Darby Canine Kidney epithelial cells
Mv1Lu	Normal mink lung epithelial cells
NHS	Normal horse serum
P	probability
PBS	phosphate buffered saline
sc	sham control
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sem	standard error of the mean
SEM	scanning electron microscopy
TCA	trichloroacetic acid
TEM	transmission electron microscopy
TER	transendothelial or transepithelial electrical resistance
wlb	Western ligand blot

CHAPTER: 1 LITERATURE REVIEW

1.1 Overview

It is now firmly established that a group of macromolecules, the insulin-like growth factors (IGF-I and IGF-II) are potent stimulators of growth. These protein mitogens are structurally homologous to proinsulin, insulin and relaxin, and have proliferative as well as differentiative effects in a wide variety of organs and cell types. Receptors for the IGFs have been identified, with varying degrees of abundance, in all major tissues examined. The type 1 IGF receptor binds both these peptides with high affinity, and it is now apparent that these effects are mediated through this receptor. Another receptor, the type 2 IGF receptor, only binds IGF-II and is identical to the cation-independent mannose-6 phosphate receptor. Its role in IGF physiology is not well defined.

The IGFs also specifically bind with high affinity to a family of six IGF-binding proteins (IGFBPs). These binding proteins are believed to control the bioactivity and prolong the half-life of free plasma IGFs. Further investigations suggest that the IGFBPs also inhibit or enhance IGF action, modulate tissue distribution of the IGFs and control extracellular targeting of IGFs.

At the time the present study was commenced, the roles of the different components of the IGF system in the transfer of IGF-I from blood to tissues was not well defined. The objective of the current study was to determine the pathway of transfer of IGF-I from the circulation to tissues at the cellular level and investigate the roles of the IGFBPs in this process *in vitro*, as well as *in vivo*.

This literature review examines aspects of 1) the discovery, structure and chemistry of IGFs, along with their distribution, 2) the two forms of IGF receptors, 3) the biological actions

of the IGFs, 4) the IGFBPs, including their postulated roles and modulation of IGF action, 5) an introduction to the IGF analogues and 6) the transfer of IGFs from blood to tissues.

This review cites work published prior to 1993, the time of the commencement of experiments designed to study IGF-I transfer. Information relevant to this thesis published since 1993 is incorporated in the appropriate sections of the remaining chapters.

1.2 Discovery of IGFs

Three separate biological activities observed in serum, led to the discovery of IGFs. Salmon and Daughaday (1957), first reported that the growth promoting action of growth hormone (GH) was mediated by "sulfation factors", (later renamed somatomedins). These researchers noted that the uptake of ^{35}S into cartilage was stimulated by serum of normal rats. Serum of hypothysectomized rats was devoid of this activity, even upon the addition of GH. However, this activity in serum reappeared with treatment of hypothysectomized rats with GH. At around the same time, another research group realised that the content of insulin in serum could not entirely account for serum's insulin-like effects. In addition, the insulin-like activity in serum could not be totally suppressed with anti-insulin antibodies (Froesch *et al.*, 1963). This insulin-like activity was denoted non-suppressible insulin-like activity (NSILA). The third line of research leading to the discovery of IGFs stemmed from the search for factors in serum which had the ability to stimulate cell proliferation *in vitro*. Multiplication - stimulating activity (MSA) was one such factor, extracted from calf serum by Pierson and Temin (1972). The same activity was later to be identified in the conditioned medium of rat liver cells (Dulak and Temin, 1973). As sulfation activity could not be separated from MSA and NSILA, a joint decision by these independent research groups led to the origin of the term somatomedins. This denoted substances able to transmit the GH signal in the stimulation of somatic growth and display insulin-like activity. Upon determination of the primary

sequences of these different factors, it was apparent that these factors were the same peptides (Klapper *et al.* 1983; Marquardt *et al.* 1981; Rinderknecht and Humbel, 1978*a* and 1978*b*). Analyses of NSILA in human serum led to the elucidation of the primary structures of two peptides named human IGF-I (Rinderknecht and Humbel, 1978*a*) and human IGF-II (Rinderknecht and Humbel, 1978*b*), due to their structural similarity to proinsulin. Two forms of somatomedins were purified from human serum, somatomedin-C (Van Wyk *et al.*, 1974) and somatomedin-A (Hall, 1972). Somatomedin-C was later shown to be structurally identical to IGF-I (Klapper *et al.*, 1983) and somatomedin-A a mixture of IGF-I and IGF-II (Spencer *et al.*, 1983). MSA was purified from serum-free medium conditioned by the Buffalo rat liver cell line BRL-3A and characterised as the rat counterpart of human IGF-II (Marquardt *et al.*, 1981). Since IGF-I, and not IGF-II was shown to be primarily regulated by GH, the term somatomedin was no longer adequate to describe the two homologues. Thus, by convention the terms IGF-I and IGF-II were adopted for these peptides (Daughaday *et al.*, 1987).

1.3 Structure and synthesis of IGFs

The insulin-like growth factors (IGF-I and IGF-II) and proinsulin are all common to an ancient family of polypeptides which probably diverged from a common precursor gene some 600 million years ago (Blundell and Humbel, 1980). Divergence between IGF-I and IGF-II is considered to have occurred some 300 million years later (Froesch *et al.*, 1985). IGF-I and IGF-II are single chain polypeptides with three intrachain disulphides, containing 70 amino acids [7,649 kD] and 67 amino acids [7,471 kD], respectively (Rinderknecht and Humbel, 1978*a* and 1978*b*). These peptides and the insulin precursor, proinsulin, exhibit approximately 60% sequence identity, with IGF-I and IGF-II sharing identical amino acids in 45 positions (Rinderknecht and Humbel, 1978*a* and 1978*b*). IGF-I, IGF-II and proinsulin all

contain an N-terminal B domain, a connecting C domain and an A domain. Unlike proinsulin, where the C domain is cleaved during processing to form the mature insulin molecule, the IGFs do not have their C domain cleaved. IGF-I and IGF-II also contain a fourth region, the C-terminal D domain. The A and B domains of IGF-I are 75% and 72% homologous with IGF-II. However, the C domain homology between IGF-I and IGF-II is only 25%. The A domain of insulin exhibits 48% and 40% sequence identity with IGF-I and IGF-II, respectively, while the B domain of insulin is 52% and 57% homologous with the corresponding domain in IGF-I and IGF-II (Rinderknecht and Humbel, 1978*b*). Based on the known tertiary structure of insulin and the similarities between the A and B domains of insulin and the IGFs, Blundell (1972) predicted the corresponding tertiary structures for IGF-I and -II. Three-dimensional models for IGF-I and IGF-II were later constructed using X-ray crystallography (Blundell *et al.*, 1978). Most recently, the secondary structure of human IGF-I in solution has been shown to be consistent with that of insulin in the crystalline state using two-dimensional ^{1}H-NMR spectroscopy (Sato *et al.*, 1992).

The primary structures of IGF-I and IGF-II have been determined for human, rat, mouse, bovine, ovine, porcine and chicken forms of the molecule (Walton *et al.*, 1990). Sequence homology is highly conserved across species, suggesting that IGFs are functionally important peptides. Human, bovine and porcine IGF-I are identical (Rinderknecht and Humbel, 1978a; Francis *et al.*, 1988a; and Tavakkol *et al.*, 1988), and their IGF-II peptides differ by only three amino acids (Daughaday and Rotwein, 1989). Rat and mouse IGF-I differ by only one amino acid and from human by three and four amino acids, respectively (Sara and Hall, 1990). Rat and mouse IGF-II differ from each other by only two amino acids (Sara and Hall, 1990), while they differ from human by four and six amino acids, respectively. Furthermore, species as distinct from mammals such as insects have immunoreactive IGFs.

1.4 IGF genes, mRNA transcripts, precursors and posttranslational processing

IGF-I is a single gene product, expressed in a tissue and developmentally specific manner. In humans, the IGF-I gene is located on the long arm of chromosome 12 (Brissenden *et al.*, 1984). In both humans and rats, IGF-I genes are relatively large, being more than 80 kb of genomic DNA, containing 6 exons and numerous introns that encode large precursor proteins (Daughaday and Rotwein, 1989; Rotwein *et al.*, 1986). As a result, the release of the complete IGF-I molecule requires extensive posttranslational processing of the precursors (Sussenbach *et al.*, 1991). The mature IGF-I protein is coded by exons 3 and 4, and alternate splicing of exon 1, 2, 5 and 6 produces 4 potentially different sequences that lie both 5' and 3' to the IGF-I coding sequence (Lund *et al.*, 1989). IGF-I mRNAs have recently been designated either Class 1 Ea, Class 1 Eb, Class 2 Ea or Class 2 Eb mRNAs (Holthuizen *et al.*, 1991). In man, mRNA sequence variations 3' to the IGF-I coding site arise from alternate exon splicing mechanisms or alternate polyadenylation signals (Daughaday and Rotwein, 1989, Humbel, 1990). These resultant mRNAs encode precursor molecules (Ea / Eb) with distinct carboxyl-termini (Simmons *et al.*, 1993). Class 1 and class 2 mRNAs are specified by alternate splicing at the 5' end of the IGF-I gene to exon 3.

Interestingly, there is widespread expression of class 1 Ea precursors in non-hepatic tissue (Hoyt *et al.*, 1988; Adamo *et al.*, 1989), which supports a role for the Ea-type prohormone in local IGF-I action. Both foetal and adult liver express class 1 Ea precursor mRNAs, but post-natally, there is mainly expression of class 1 Eb mRNA, which is preferentially sensitive to GH treatment of hypothysectomised rats, which could possibly indicate a role for Eb precursor peptides in targeting IGF-I to the circulation (Lowe *et al.*, 1987 and 1988; Hoyt *et al.*, 1988).

Like IGF-I, IGF-II is also a single gene product. In humans, the IGF-II gene is found on the short arm of chromosome 11, contiguous with the gene for insulin (Tricoli *et al.*, 1984).

It is a large gene comprised of 9 exons, spanning 30 kb of genomic DNA (Bell *et al.*, 1985; de Pägter Holthuizen *et al.*, 1988). Although similar in organisation to the human IGF-II gene, the rat counterpart has only 6 exons (Frunzio *et al.*, 1986). The IGF-II gene occurs in imprinted regions of the mouse and human genomes. In humans, monoallelic expression of the paternally derived gene copy has been shown for adult kidney, placenta, foetal muscle and kidney (Kalscheuer *et al.*, 1993). It is possible that differential imprinting of the IGF-II gene has important functional consequences for foetal growth and development, as well as the development of cancer.

All IGF-II mRNAs are specified by the 3' exons, 7, 8 and 9, whereas alternate splicing of the first 6 exons specifies the 5' untranslated sequence, producing the different IGF-II mRNA species (Nielsen *et al.*, 1990). In rats exons 4, 5 and 6 are alternately spliced giving rise to different IGF-II mRNA species (Soares *et al.*, 1986). In both humans and rats, different polyadenylation sites in the 3' untranslated region give rise to IGF-II mRNAs of different size (de Pagter Hothuizen *et al.*, 1988; Soares *et al.*, 1986).

The encoding sequences of human, rat and mouse IGF-II cDNAs specify a 22 kDa prepro-IGF-II precursor molecule composed of an amino-terminal signal peptide, mature IGF-II and a carboxyl-terminal extension (E domain or trailer peptide) (Gammeltoft *et al.*, 1991). Hence, proteolytic processing at both ends of prepro-IGF-II is required to produce the mature IGF-II peptide. It is proposed that the prepro-IGF-II (22 kDa) is initially cleaved at an alanine residue to remove the signal peptide. The resultant pro-IGF-II (20 kDa) is sequentially cleaved by enzymes at sites of basic residue pairs in the E domain to yield intermediate forms of pro-IGF-II. Finally, cleavage at a single basic residue produces mature IGF-II. Various pro-IGF-II molecules ranging in Mr from 9000 - 19000 have been reported in cultured media of BRL-3A rat liver cells (Yang *et al.*, 1985), serum (Zumstein *et al.*, 1985) and cerebrospinal fluid (Haselbacher and Humbel, 1982). It is not clear whether the pro-IGF-IIs or the E-fragments have specific functions or exert specific effects.

1.5 Distribution of IGFs in tissues and body fluids

The dual effector theory was proposed by Green *et al.*(1985), in which IGF-I has both paracrine / autocrine and endocrine functions. It is unlikely that these two modes of action are mutually exclusive, with the emphasis on either varying during development and with the physiological or pathophysiological state and tissue target. Evidence exists which implicates the liver as the major source of circulating IGF (Baxter, 1986). IGF-I levels in the circulation reflect the developmental pattern of the IGF-I gene expression in human livers (Adamo *et al.*, 1989), and in the adult rat, IGF-I mRNA is 50 times more abundant in liver than in other tissues (Lund *et al.*, 1986). Calculations based on the studies of perfused livers concluded that more than 90 % of total IGF is secreted by the liver (Schwander *et al.*, 1983). Hepatocyte cultures from adult liver produce IGF-I (Spencer, 1979; Scott *et al.*, 1985) and cirrhotic patients have low levels of IGF-I (Schimpff *et al.*, 1977). Production of IGFs by non-hepatic tissues prompts the question of the role of circulating IGFs. As a result, it has been proposed that the liver acts as a endocrine source of IGFs for distant tissue targets (Baxter, 1986). Evidence has been accumulating to suggest that IGF-I of endocrine origin may reach and act on several tissues to promote skeletal, renal, thymic, lung and splenic growth (Schoenle *et al.*, 1982; Smeets and van Buul-Offers., 1983; Zapf *et al.*, 1985; Skottner *et al.*, 1987; Guler *et al.*, 1988; Skottner *et al.*, 1989 and Glassock *et al.*, 1991). In addition, IGF-I acting in the classical endocrine fashion promotes formation and repair of bone (Ebeling *et al.*, 1993), stimulates body protein and glucose metabolism (Jacob *et al.*, 1989) and increases glomerular filtration rate and renal plasma flow (Hirschberg *et al.*, 1991; Guler *et al.*, 1989b; Hirschberg and Kopple, 1989). However, both IGF expression and local production have since been demonstrated in a number of tissues of many species, supporting a proposed paracrine /

autocrine role for IGFs in addition to their endocrine function (D'Ercole *et al.*, 1980; 1984 and 1986; Lund *et al.*, 1986; Murphy *et al.*, 1987; Underwood *et al.*, 1986). Antibodies directed against IGF-I, -II or the type I IGF receptor have been shown to block bioactivity in various *in vitro* systems. For example, proliferation of tumour cell lines known to synthesise IGFs and IGFBPs and possess IGF receptors can be inhibited by these antibodies (Macaulay, 1992), further supporting the IGF paracrine / autocrine regulation of growth.

In addition to their presence in serum and tissues, IGFs are also found in several body fluids, including lymph (Cohen and Nissley, 1972), spinal fluid (Hasselbacher and Humbel, 1982), urine (Hizuka *et al.*, 1987), saliva (Costigan *et al.*, 1988), ovarian follicular fluid (Adashi *et al.*, 1985), milk (Baxter *et al.*, 1984a), semen (Baxter *et al.*, 1984b), amniotic fluid (Chochinov *et al.*, 1977), ocular fluids (Arnold *et al.*, 1993) and the extracellular fluid compartment of the brain (Yamaguchi *et al.*, 1990).

1.6 IGF Receptors

IGFs, like other growth factors, exert their biological actions by binding to specific target cell surface receptors. IGFs interact with three receptor species; the insulin receptor, the type-I (IGF-I) receptor and the type-II (IGF-II) receptor. The type-I and insulin receptors are structurally and functionally related, whereas the type II receptor is distinct (Massague and Czech, 1982; Rechler and Nissley, 1985; Czech, 1989).

The type-I IGF receptor preferentially binds IGF-I. IGF-II also cross reacts with the type-I IGF receptor to a similar or lesser extent depending on the cell type and insulin is bound with a 500 - 1000 times lower affinity, thus explaining in part the role of insulin as a growth factor at high concentrations (Massague and Czech, 1982). Placenta cDNAs of the type-I receptor gene permitted the characterisation of its primary structure. It is a glycoprotein, structured as a $\alpha_2\beta_2$ heterotetrameric complex, with a molecular mass of

approximately 300 - 350 kDa (Czech, 1985; Rechler and Nissley, 1985). The complex is comprised of two disulphide-linked α-subunits (130 kDa), containing the extracellular ligand binding site and two β-subunits (95 kDa), which form the transcellular domain and ATP binding site and the intracellular domain which displays intrinsic tyrosine kinase activity (Jacobs *et al.*, 1983; Morgan *et al.*, 1986). Like the insulin receptor, each α-subunit is linked via disulphide bonds to the extracellular domain of a β-subunit.

Certain monoclonal human anti-insulin receptor antibodies cross-react with the type I receptor, suggesting the presence of common epitopes (Ullrich *et al.*, 1986). Further confirmation of the structural similarity between the type I and insulin receptors has come from cloning and sequencing the respective cDNAs. Generally, there is 41 - 48% amino acid sequence identity, the greatest similarity being found in the tyrosine kinase domain of the β-subunit of the two receptors, where the homology reaches 84% (Ullrich *et al.*, 1986). Ligand binding leads to autophosphorylation of tyrosine residues within the intracellular domain of the β-subunit (Jacobs *et al.*, 1983; Morgan *et al.*, 1986), resulting in enhanced phosphorylation of cellular proteins considered to be essential intermediates in many or all actions of IGF-I (Nissley and Lopacynski, 1991). The type I receptor is thought to mediate the growth promoting effects of both IGF-I and IGF-II (Rechler and Nissley, 1985; Ullrich *et al.*, 1986). Variant species of the type I receptor have been identified on membranes of rat and human brains (Gammeltoft *et al.*, 1985; Roth *et al.*, 1987). This neuronal type I receptor subtype is characterised by a smaller α-subunit, which is due to differences in glycosylation patterns (Heidenreich *et al.*, 1986). Sara and Carlsson-Skwirut (1988) propose that the functional significance of such a subtype in the brain relates to the presence of truncated IGF-I in the CNS. In addition, hybrid insulin / IGF receptors comprised of insulin α/β and type I α/β half receptors have been identified (Jacobs and Moxham, 1991; Treadway *et al.*, 1991). The functional significance of these hybrid receptors is still to be defined, yet they might enhance the molecular repertoire of the cell to respond to IGFs and to insulin.

The type II receptor is structurally quite different to the type I and insulin receptors, and does not contain tyrosine kinase activity. It is a transmembrane monomer of molecular mass 270 kDa that is composed of a large extracellular domain that contains 15 repeat sequences of cysteine residues and accounts for approximately 93% of the receptor protein, plus a relatively short cytoplasmic domain (Massague and Czech, 1982; Sara and Hall, 1990). The type II receptor is able to bind both IGF-II and mannose 6-phosphate at distinct binding sites (Braulke *et al.*, 1988). Analysis of amino acid sequences has established that the type II receptor is homologous with the cation-independent mannose 6-phosphate receptor (Morgan *et al.*, 1987; Oshima *et al.*, 1988), and are now known to be the same protein. As well as binding to IGF-II with high affinity, this receptor functions to target lysosomal enzymes bearing the mannose 6-phosphate recognition markers to lysosomes (Nissley and Keiss, 1991). To date, almost all mammalian cells described express type II receptors with this bifunctional property. However, in amphibian and avian cells, the cation-independent mannose 6-phosphate receptor does not bind IGF-II (Clairmont and Czech, 1989; Yang *et al.*, 1991). The mammalian type II receptor binds IGF-II, although does not bind insulin and has little affinity, if any for IGF-I (Rechler and Nissley, 1985; Ballard *et al.*, 1988).

Until now the transmembrane signalling role of the type II receptor was questioned. It was widely thought that IGF-II action was mediated by the type I receptor. However, some studies suggest a direct signalling function through the type II receptor, including specific cellular responses such as Ca^{++} influx in fibroblasts (Nishimoto *et al.*, 1987), glycogen synthesis in hepatoma cells (Hari *et al.*, 1987) or glucose uptake by chondrocytes (Bhaumick and Bala, 1991). Additionally, interaction of IGF-II with the type II receptor results in activation of a GTP-binding protein by a G-protein activating cascade in the cytoplasmic domain of the receptor (Okamoto *et al.*, 1990). Finally, other studies suggest that the IGF-II / type II receptor interaction may be permitted to activate the G-protein due to interaction with other GFs, such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF),

resulting in IGF-II stimulating progression of cells through the cell cycle (Okamoto *et al.*, 1991).

A soluble form of the type II receptor lacking the intracellular domain has also been demonstrated in rat, monkey, sheep and human serum (Kiess *et al.*, 1987; Gelato *et al.*, 1988; 1989 and Causin *et al.*, 1988). Type II receptor fragments have also been found in urine (Causin *et al.*, 1988). The function of the soluble receptor form has not been determined, although it has been suggested that release of the extracellular part of the receptor represents the major degradative pathway of the receptor. It may also function as a transport protein for IGF-II.

IGF receptors are widely distributed throughout the body. They have been detected in a number of foetal and adult tissues, including gut, heart, lungs, muscle, kidneys, brain, arteries and tissue capillaries (Nissley *et al.*, 1993; Cui *et al.*, 1993; Guse *et al.*, 1992; Adams *et al.*, 1983; Rosenfeld *et al.*, 1982; Lowe and LeRoith, 1986; Baskin *et al.*, 1988; Bornfeldt *et al.*, 1988; Bar and Boes, 1984; Bar *et al.*, 1988).

1.7 Biological actions of IGFs

In vitro actions: The two major biological actions of IGFs *in vitro* are i) acute anabolic effects on protein and carbohydrate metabolism and ii) longer term effects on cell replication and differentiation. The *in vitro* actions of the IGFs have been well established in a wide variety of cells. The acute effects include stimulation of glucose and amino acid uptake and metabolism, glycogen, lipid and protein synthesis and inhibition of protein breakdown and lipolysis (Froesch *et al.*, 1985; Humbel, 1990). Acute effects on adipose tissue apparently occur via the insulin receptor, unlike skeletal muscle where these effects are mediated by both insulin and type I receptors (Froesch *et al.*, 1985). Long term effects of the IGFs are mediated by the type I receptor (Baxter, 1988a). By far the most widely studied long term effects of

IGFs *in vitro* are the stimulation of DNA synthesis and cell proliferation. These effects have been demonstrated in multiple cell types, which have been reviewed in detail by Van Wyk (1984), Froesch *et al.* (1985), Guidice (1992), Mc Cauley (1992) and Cohick and Clemmons (1993). More recently, increased proliferation of primary cultures of chick chondrocytes and human keratinocytes in response to IGF have been reported (Bohme *et al.*, 1992; Barreca *et al.*. 1992). DNA synthesis has been found to be stimulated by IGF-I in cultures of seminiferous tubules, rat granulosa cells, thymic epithelial cells and astroglial cells (Soder *et al.*, 1992; Bley *et al.*, 1992; Timsit *et al.*, 1992 and Han *et al.*, 1992)

Stiles and co-workers (1979) hypothesised that IGFs are "progression" factors, whereas PDGF, and basic fibroblast growth factor (bFGF) render cells competent to respond to the "progression" factors. Treatment of cells with only one of these factors results in arrest in G1. Treatment of the BALB/c3T3 cells with IGF-I in addition to one of the competence factors allows progression through G1 and continuation through the cell cycle, resulting in DNA synthesis and cell proliferation. New developments in this area are intriguing. For example, over expression of IGF-I receptors in BALB/c-3T3 cells abrogates the requirement of a competence factor, and the cells will proliferate in response to IGF-I alone (Pietrzkowski *et al.*, 1992). Other long term effects of IGFs include the stimulation of cellular differentiation, RNA and protein synthesis and inhibition of protein breakdown (Sara and Hall, 1990; Ballard *et al.*, 1986).

In vivo actions: The relatively recent availability of large amounts of recombinant IGFs has allowed investigation of the effects of the administration of IGFs *in vivo*. Such studies have demonstrated that these peptides have both an acute insulin-like action along with a chronic growth-promoting effect. In normal and hypophysectomized rats, intravenous bolus injection of both IGF-I and less effectively IGF-II causes hypoglycaemia, primarily by inducing peripheral glucose uptake with a minimal effect on suppressing hepatic glucose production (Zapf *et al.*, 1986; Jacob *et al.*, 1989; Rossetti *et al.*, 1991). The effects of IGF-I

infusion in normal human subjects are comparable to the effects observed in rats. A single bolus injection rapidly causes symptomatic hypoglycaemia (Guler *et al.*, 1987). The effects of IGFs are dependent upon the method of administration. Following a bolus injection, IGF peptide in excess of IGF binding protein capacity induces insulin-like effects. After long-term subcutaneous administration, IGFs associate with the IGFBPs, affording protection against acute metabolic effects.

In 1982, the first study to demonstrate IGF growth effects *in vivo* was reported using hypophysectomized rats subcutaneously infused with IGF-I for 6 days (Schoenle *et al.*, 1982). Similar increases in somatic growth have been observed in normal rats (Thissen *et al.*, 1991), postweening rats (Hizuka *et al.*, 1986), neonatal and preweening rats (Phillips *et al.*, 1988) and Snell dwarf mice (Smeets and van Buul-Offers, 1983). IGF-I anabolic effects on protein synthesis and nitrogen balance in animals have been reported using models of catabolism stimulated by corticosteroids and dietary protein restriction (Tomas *et al.*, 1992; Thissen *et al.*, 1991). While carcass weight was not increased by IGF-I, weights of spleen, kidneys and gut were increased. Although both IGF-I and GH stimulate growth in growth hormone deficient animals, the quantitative effect of GH is greater on total body weight gain and growth, while the growth effect of IGF-I is selectively greater on spleen, thymus and kidney (Schoenle *et al.*, 1982; Skottner *et al.*, 1989; Pell and Bates 1992). These differences may arise from a combination of a direct stimulation of GH on target organs, plus increases in local tissue concentrations of IGF-I resulting from GH treatment and GH-stimulated hepatic production of circulating endogenous IGF-I, as opposed to direct effects on target organs by infusion of endogenous IGF-I. The above studies demonstrating growth after systemic IGF-I administration clearly support an endocrine function for IGF-I. IGF-II had only weak growth promoting activity in hypophysectomized rats (Schoenle *et al.*, 1985), and IGF-II secreting tumours transplanted into nude mice did not support somatic growth of these animals (Wilson *et al.*, 1987).

Transgenic animals over-expressing the gene for GH demonstrated dramatic postnatal growth to approximately twice the normal body weight (Palmiter *et al.*, 1982; 1983). This accelerated growth started after the onset of induction of endogenous IGF-I expression, 2-3 weeks postnatally, indicating that IGF-I gene expression was unresponsive to GH until this time and that the growth promoting properties of the GH transgene were largely due to induction of IGF-I expression (Mathews *et al.*, 1988a). Over-expression of human IGF-I in transgenic mice resulted in an approximately 30% increase in body weight over control mice (Mathews *et al.*, 1988b). Circulating IGF-I levels were elevated, but not to the extent of that in GH transgenic mice, which may explain the relatively low growth rate of the IGF-I transgenic mice.

Other *in vivo* effects of IGF-I have been demonstrated. Infusion of IGF-I improves wound healing in a wound chamber model (Suh *et al.*, 1992), has a direct trophic action on gut epithelium when delivered directly to the ileal lumen (Olanrewaju *et al.*, 1992), and increases IGFBP-3 levels to normal in hypophysectomized and protein-restricted rats (Clemmons *et al.*, 1989a).

1.8 Insulin-like growth factor binding proteins

Insulin-like growth factor binding proteins (IGFBPs), in addition to IGFs and IGF receptors, represent the third component of the IGF system. In blood, lymph and other extracellular fluids, plus medium conditioned by cells in culture, IGFs are found complexed to IGFBPs (Hossenlopp *et al.*, 1987; Ooi and Herrington, 1988). These binding proteins determine IGF bio-availability and modulate IGF biological activities.

The first indication of the existence of IGFBPs came from incubation of radiolabelled IGF-I in plasma, followed by neutral gel-filtration chromatography, which revealed IGF binding activity at two Mr regions of 150 kDa and 50 kDa (Kaufmann *et al.*, 1977; Hintz and

Liu, 1977; Hintz *et al.*, 1981). With the introduction of the ligand blotting technique, which involves SDS-polyacrylamide gel electrophoresis (SDS-PAGE), protein transfer to nitrocellulose, followed by detection of IGFBPs immobilised on the nitrocellulose using radiolabelled IGFs, at least five IGFBPs in the range of 24 to 42 kDa were identified in plasma and lymph (Hardouin *et al.*, 1987; Hossenlopp *et al.*, 1987; Binoux and Hossenlopp, 1988). The cDNA clones and full primary sequences for six IGF binding proteins have been published (Binkert *et al.*, 1989; Brown *et al.*, 1989; Lee *et al.*, 1988; Murphy *et al.*, 1990; Shimasaki *et al.*, 1989; 1990; 1991a, b, c and d; Wood *et al.*, 1988). These developments have led to our current understanding that six distinct, but related classes of IGFBPs exist.

In 1989, during a workshop on IGF binding proteins held in Vancouver, Canada, a proposal for the terminology of the binding proteins was adopted to designate the binding proteins "IGFBP" followed by an arabic numeral. A single letter prefix indicates species. In 1991, participants of the Second International IGF Symposium in San Francisco met to assign nomenclature to three more IGFBPs discovered in the intervening two years. As a result the six known IGFBPs are designated IGFBP-1 through IGFBP-6.

This heterogeneous group of binding proteins bind IGF-I and IGF-II reversibly and with high affinity, but do not bind insulin (Clemmons, 1990). They have been shown to have both inhibitory and stimulatory effects on IGF actions in different model systems *in vitro* (Baxter and Martin, 1989b). The definition of the roles of the IGFBPs remains an active area of research endeavour. Within species, IGFBP sequences are greater than 50% homologous. For each individual IGFBP across species, considerable sequence homology exists, reaching up to 70%. The amino and carboxyl termini amino acids are highly conserved, including the positions of the cysteine residues, indicating a high degree of similarity in tertiary structure which would be required for these binding proteins to interact with IGFs or cell surfaces (Lamson *et al.*, 1991; Clemmons *et al.*, 1991).

IGFBP-1

Characterisation of IGFBP-1 resulted from the studies of at least four proteins that were thought to be distinct. IGFBP-1 was initially detected in and purified from amniotic fluid (Chochinov *et al.*, 1977; Drop *et al.*, 1979; Povoa *et al.*, 1984). IGFBP-1 has been purified from Hep G2-human hepatoma cell conditioned media and shown to be identical to amniotic IGFBP (Moses *et al.*, 1983; Povoa *et al.*, 1985). IGFBP-1 has also been isolated from human placenta (placental protein 12) (Koistinen *et al.*, 1986), and endometrium (pregnancy-associated endometrial α1-globulin) (Bell and Keyte, 1988).

IGFBP-1 is a 234 amino acid residue protein, with a predicted Mr of 25.2 kDa and appears at approximately 28 kDa on non-reduced SDS-PAGE (Lee *et al.*, 1988). The mature protein contains 18 cysteines, and no N-glycosylation sites (Lee *et al.*, 1988). The cysteine rich N-terminus region is followed by a region where the amino acids Pro, Ser and Thr are prevalent. The latter is normally found in proteins with a short intracellular half-life and rapid turnover rates (Holly, 1991). IGFBP-1 also contains an Arg-Gly-Asp (RGD) sequence, which is commonly found in matrix proteins involved in cell surface attachment via receptors of the integrin family (Hynes, 1987).

The gene for human IGFBP-1 spans 5.2 kb, contains four protein encoding exons and has been localised to chromosome 7 at the p12-p13 site (Brinkman *et al.*, 1988; Shimasaki *et al.*, 1991a). In competitive binding studies, IGFBP-1 has a slightly higher affinity for IGF-I than IGF-II (Forbes *et al.*, 1988).

The liver is considered the major source of IGFBP-1 found in the circulation. It has been identified in foetal as well as adult livers, and is released *in vitro* from liver cell lines (Lewitt and Baxter, 1989). IGFBP-1 is a minor IGFBP in serum, and contains the unsaturated serum IGF-binding sites. Circulating levels of IGFBP-1 are developmentally regulated in humans, with high levels at birth, falling steadily to the lowest levels at puberty and slight increases with increasing post-pubertal age (Hall *et al.*, 1988). Nutritional status affects

IGFBP-1 levels in blood, with fasting producing a 5-8 fold increase (Baxter and Cowell, 1987) and feeding causing an 80% decrease (Busby *et al.*, 1988). During pregnancy, there is a rise of IGFBP-1 (and a concomitant rise of IGF-I), where it is found in serum at several hundred μg/L and amniotic fluid levels are up to 1000-fold higher than those in serum (Rutanen *et al.*, 1982 and 1984). In humans, serum IGFBP-1 shows a distinct diurnal rhythm, with a nocturnal peak and a nadir during the day, which has been suggested to be either GH independent (Baxter and Cowell, 1987; Cotterill *et al.*, 1988) or inversely related to GH (Hall *et al.*, 1988; Tapanainen *et al.*, 1991). An inverse relationship between GH and IGFBP-1 exists, which has been most clearly demonstrated in patients with acromegaly in whom plasma IGFBP-1 levels are low (Hall *et al.*, 1988; Holly *et al.*, 1991). Conversly IGFBP-1 levels are high in GH-deficient patients (Degerblad *et al.*, 1989). However, it is considered that a direct effect of GH on IGFBP-1 concentrations is not likely, and that the IGFBP-1 levels are mediated by insulin (Hall *et al.*, 1991). Indeed several lines of evidence would suggest a role for IGFBP-1 in glucose counterregulation.

Regulation patterns of IGFBP-1 are similar to that of known glucose counter-regulators such as glucagon. The diurnal rhythm characteristic for IGFBP-1 is metabolically regulated, such that carbohydrate intake suppresses IGFBP-1 levels, while IGFBP-1 levels are inversely related to insulin levels (Cotterill *et al.*, 1988; Suikkari *et al.*, 1988). Yeoh and Baxter (1988) reported a rise in IGFBP-1 levels approximately 1-2 hours after insulin administration and resulting hypoglycaemia. This led to the postulate that glucose rather than insulin, might suppress IGFBP-1 levels. However, this hypoglycaemia-associated rise in IGFBP-1 levels may be due to a secondary suppression of insulin secretion into the hepatic portal circulation (Lee *et al.*, 1993). *In vitro* studies provide further evidence for a role of IGFBP-1 in glucose homeostasis. Hepatocytes derived from diabetic and insulin treated rats displayed increased IGFBP-1 levels (Scott and Baxter, 1986). Glucose concentration in the

medium of human foetal liver explants was inversely related to IGFBP-1, an effect which could be blocked by the addition of insulin to the medium (Lewitt and Baxter, 1989).

IGFBP-1 appears to be able to both inhibit and enhance IGF-I actions *in vitro* (Liu *et al.*, 1991; Elgin *et al.*, 1987). IGFBP-1 is present in plasma at concentrations sufficient to alter IGF action, but its role in doing so is incompletely defined. Data indicates that IGFBP-1 may play a role in regulating the metabolic actions of the free IGF fraction. This contention is supported by recent infusion studies, where human IGFBP-1 was shown to be able to block the hypoglycaemic activity of exogenous administration of IGF-I in rats (Lewitt *et al.*, 1991). Infusion of IGFBP-1 into isolated rat hearts has demonstrated that IGFBP-1 is able to cross intact endothelial barriers, and insulin accelerates its transcapillary passage (Bar *et al.*, 1990c). IGFBP-1 in circulation preferentially localises to muscle as opposed to connective tissue (Bar *et al.*, 1990b), implying that IGFBP-1 might act as a "shuttle" of IGFs from the vascular compartment to specific target tissues. Finally, the presence of the RGD sequence in IGFBP-1 implies that IGFBP-1 may be a key factor in channelling IGFs to specific cell types within a given tissue. The RGD sequence may also be related to the ability of IGFBP-1 to attach to the cell surface and potentiate the subsequent cellular response to IGF-I (Holly *et al.*, 1991).

IGFBP-2

IGFBP-2 was originally isolated from culture media conditioned by rat BRL-3A cells (Mottola *et al.*, 1986) and bovine kidney (MDBK) cells (Szabo *et al.*, 1988). The complete primary structure was derived from human, rat and bovine cDNAs isolated from human foetal livers (Binkert *et al.*, 1989), human Hep G2 hepatocarcinoma cells (Zapf *et al.*, 1990), rat BRL-3A cells (Brown *et al.*, 1989), adult rat liver (Margot *et al.*, 1989) and bovine MDBK cells (Upton *et al.*, 1990), which exhibit extensive sequence homology across these animal species. The mature hIGFBP-2 protein has 289 amino acid residues, with a predicted molecular mass of 31.3 kDa, contains 18 conserved cysteine residues, lacks N-glycosylation

sites (Binkert *et al.*, 1989) and contains a RGD sequence near the carboxyl terminus (Ooi, 1990). The IGFBP-2 gene has a total length of 32 kb, organised into four exons, each encoding protein (Ehrenborg *et al.*, 1991). The gene is located to region q33-q34 of chromosome 2 in the human genome (Shimasaki *et al.*, 1991a). In competitive binding studies, IGFBP-2 has approximately equal affinity for both IGF-I and IGF-II when radiolabelled IGF-I is the radioligand, but exhibits a marked preference for IGF-II when the radioligand is IGF-II (Forbes *et al.*, 1988).

IGFBP-2 has been isolated from human serum (Zapf *et al.*, 1990), and is the major binding protein in serum of pre- and perinatal rats (Glassock *et al.*, 1991; Babajko *et al.*, 1993). Although plasma levels in adult humans and rats are low, IGFBP-2 is the predominant IGFBP in human and rat cerebrospinal fluid (CSF) (Romanus *et al.*, 1989; Lamson *et al.*, 1989), rat amniotic fluid (Lamson *et al.*, 1989), vitreous fluid (Ocrant *et al.*, 1991) and urine (Hasegawa *et al.*, 1992). IGFBP-2 has also been detected in rat milk (Donovan *et al.*, 1991b).

IGFBP-2 is developmentally regulated, with high levels detectable in foetal rat serum, which decrease rapidly with postnatal life (Ooi, 1990). Circulating levels of IGFBP-2 are less acutely regulated by hormonal and nutritional factors than IGFBP-1, and show less diurnal variation (Clemmons *et al.*, 1991). Serum IGFBP-2 levels are increased in hypophysectomized, diabetic and fasted rats (Bach and Rechler, 1992; Yang *et al.*, 1989). In man, levels are increased under reduced ternary complex concentrations, during fasting, in non-islet cell tumour hypoglycaemia and following IGF-I infusion (Clemmons *et al.*, 1991; Zapf *et al.*, 1990; Thrailkill *et al.*, 1990). Plasma IGFBP-2 in humans is also increased by GH deficiency (Hardouin *et al.*, 1989). Infusion of IGF-I did not increase IGFBP-2 when insulin was held constant, but did when insulin was allowed to decrease (Schwander and Mary, 1991), indicating that insulin may be the major regulator of IGFBP-2 in humans. In rats, IGFBP-2 levels are increased in GH-deficiency (Yang *et al.*, 1989) and are believed to be GH

dependent since they are reduced by GH treatment. Yet, GH treatment of hypophysectomized rats does not normalise plasma IGFBP-2 levels (Margot *et al.*, 1989). This GH-dependency could be mediated via IGF-I or insulin, since IGF-I infusion into rats increases blood IGFBP-2 and decreases insulin levels (Zapf *et al.*, 1989).

Baxter (1993) has proposed that while IGFBP-3 is thought to carry approximately 75 % of circulating IGFs, IGFBP-2 may act as a significant carrier of IGF in the circulation. This is because IGFBP-2 levels increase under conditions where there is not sufficient IGFBP-3 to bind IGFs, such as IGF excess, caloric restriction and hypopituitarism in humans (Clemmons *et al.*, 1991) or in hypothyroidism in rats (Näntö-Salonen and Rutanen, 1992).

IGFBP-3

In postnatal life, IGFBP-3 is the predominant circulating binding protein, and represents the IGF-binding component of the 150 kDa ternary complex in blood (Martin and Baxter, 1986). This large molecular weight complex is composed of three subunits: an acid-labile subunit (α-subunit or ALS), which is a glycoprotein of approximately 85 kDa, an acid-stable IGF-binding subunit (β-subunit or IGFBP-3) of 47-53 kDa and an IGF polypeptide (γ-subunit) of approximately 7.5 kDa (Baxter, 1988b). Formation of the 150 kDa complex first requires IGFBP-3 to bind IGF to form a binary complex, before binding of ALS can occur to achieve formation of the ternary complex (Baxter and Martin, 1989). Since IGFs are stabilised by binding to IGFBP-3, and ALS circulates at a concentration much greater than IGFBP-3, it may be assumed that IGFBP-3 is a significant player in determining the total concentration of circulating IGFs (Baxter, 1993). IGFBP-3 from human serum is a glycosylated protein that resolves into two bands of approximately 47 and 53 kDa upon SDS-PAGE (Martin and Baxter, 1986), which represent N-glycosylated variants of the core protein of 29 kDa (Wood *et al.*, 1988). Mature human and rat IGFBP-3 consist of 264 and 265 amino acid residues, respectively, contain 18 conserved cysteines (Wood *et al.*, 1988; Shimasaki *et*

al., 1989) and lack RGD sequences (Rechler, 1993). The IGFBP-3 gene comprises five exons, is 8.9 kb in length (see review, Martin and Baxter, 1992a) and is located on chromosome 7 (Shimasaki *et al.*, 1991a). Competitive binding experiments have indicated that this IGFBP has a slightly higher affinity for IGF-II than IGF-I (Martin and Baxter, 1986; Forbes *et al.*, 1988).

Circulating levels of IGFBP-3 are approximately 10 fold greater than the other measurable IGFBPs and approximately 75% of the IGFs in plasma are bound to this carrier protein (Baxter, 1993; Gargosky *et al.*, 1990a). Shimasaki *et al.* (1989) have shown that rat IGFBP-3 mRNA is predominantly expressed in the liver, kidneys and stomach. In adult humans IGFBP-3 mRNA expression is highest in liver, ovaries, spleen and prostate (Naya *et al.*, 1991). Since the major site of IGFBP-3 synthesis is the liver, this supports the belief that hepatic synthesis is the main contributor to circulating levels of IGFBP-3. Plasma IGFBP-3 levels do not exhibit diurnal variation (Baxter and Cowell, 1987; Walton and Etherton, 1989). Human IGFBP-3 levels are clearly developmentally regulated, increasing rapidly after birth, peaking at puberty and gradually declining to low levels with age (Baxter and Martin, 1989b; Blum *et al.*, 1990). A similar developmental pattern of IGFBP-3 regulation has been reported in the rat and pig (Donovan *et al.*, 1989; Lee *et al.*, 1991). IGFBP-3 concentration in plasma is also strongly GH-dependent. Using radioimmunoassays, it has been determined that IGFBP-3 levels are increased approximately twofold in patients with acromegaly and reduced between 50-80 % in plasma from GH-deficient humans (Baxter and Martin, 1986). IGFBP-3 levels are stimulated twofold in GH-treated pigs (Walton and Etherton, 1989), decreased after hypophysectomy in rats (Zapf *et al.*, 1989) and decreased in GH-deficient transgenic mice (Comacho-Hubner *et al.*, 1991a). Levels of IGFBP-3 were restored by GH treatment and infusion of IGF-I in hypophysectomised rats (Zapf *et al.*, 1989) or by over expression of IGF-I in GH-deficient transgenic mice (Comacho-Hubner *et al.*, 1991a). These results suggest that in rats and mice IGF-I, rather than GH, may be the primary regulator of IGFBP-3 production.

Nutrition also appears to play a role in IGFBP-3 regulation, with decreased levels reported in humans after food restriction (Blum and Ranke, 1990), in patients in negative nitrogen balance following surgery (Baxter, 1993) and in rats on low protein diets (Tomas *et al.*, 1991a). IGFBP-3 was also decreased in rats rendered diabetic with streptozotocin treatment (Zapf *et al.*, 1989). When circulating IGFBPs were analysed in pregnant rats and humans using Western-ligand blotting, circulating IGFBP-3 was undetectable (Gargosky *et al.*, 1990a and b; Davenport *et al.*, 1990). In contrast, immunoreactive IGFBP-3 in human plasma during pregnancy increases (Baxter and Martin, 1986). The concentration of IGF-I also increases in pregnancy plasma, and most IGF-I is found in the 150 kDa fraction indicating that IGFBP-3 has not lost its ability to bind IGF-I and stabilise it in the ternary complex (Hall *et al.*, 1984; 1986; Gargosky *et al.*, 1991; 1990b). In addition, pregnancy IGFBP-3 is able to re-form the ternary complex *in vitro* (Suikkari and Baxter, 1992). The apparent decrease in IGFBP-3 found upon Western ligand blot analysis appears to be due to proteolytic enzyme activity within pregnant serum (Guidice *et al.*, 1990; Binoux *et al.*, 1991), that cleaves IGFBP-3, preventing binding to ^{125}I-labelled IGF-I tracer. It is possible that proteolysis of IGFBP-3 during pregnancy may reduce its affinity for IGFs, resulting in variations in IGF tissue availability. However, the physiological importance of IGFBP-3 proteolysis in pregnancy remains speculative.

As part of the 150 kDa complex, IGFBP-3 is postulated to protect against the hypoglycaemic action of IGFs, yet the exact role of IGFBP-3 remains incompletely defined. However, IGFBP-3 prolongs the biological half-life of IGFs from just under 10 minutes (Hodgkinson *et al.*, 1989a, Davis *et al.*, 1989) to 12 to 15 hours (Guler *et al.*, 1989a). IGF variants with reduced affinity for IGFBP-3 have shorter serum half-lives than IGF-I (Ballard *et al.*, 1991; Cascieri *et al.*, 1988; Francis *et al.*, 1988b) implying that IGFBP-3 may also act as a reservoir for IGFs.

IGFBP-4

IGFBP-4 has been isolated from rat and human serum (Shimonaka *et al.*, 1989; Kiefer *et al.*, 1991b) and ovine plasma (Walton *et al.*, 1991). IGFBP-4 is also produced by various cell lines including; TE89 human osteosarcoma (Mohan *et al.*, 1989), HT29 human colon carcinoma (Culouscou and Shoyab, 1991), T98G human glioblastoma (Camacho-Hubner *et al.*, 1992) and rat B104 neuroblastoma (Ceda *et al.*, 1991; Cheung *et al.*, 1991). The complete sequence of this protein has been determined from cDNAs isolated from libraries of human placenta and rat liver (Shimasaki *et al.*, 1990), a human osteosarcoma cell line (La Tour *et al.*, 1990) and bovine pulmonary artery (Moser *et al.*, 1992). The mature human IGFBP-4 protein consists of 237 amino acid residues (Shimasaki *et al.*, 1991b), and appears as 24 and 30 kDa bands on non-reduced SDS-PAGE gels, the latter being a N-glycosylated variant (Kiefer *et al.*, 1991a). Glycosylated IGFBP-4 levels increase during gestation in mice (Fielder *et al.*, 1990), but its physiological significance is not known. IGFBP-4 also contains the 18 conserved cysteine residues seen in IGFBPs -1 to -6, plus two additional cysteines that are not present in the other IGFBPs (Shimasaki *et al.*, 1990). The IGFBP-4 gene spans 12 kb and contains 4 exons (Gao *et al.*, 1993), and is located on chromosome 17 in the human genome (Shimasaki *et al.*, 1991a) at region q21-q21.1 (Bajalica *et al.*, 1992). IGFBP-4 has similar affinities for IGF-I and IGF-II irrespective of whether IGF-I or IGF-II is the radioligand (Mohan *et al.*, 1989; Kiefer *et al.*, 1992). IGFBP-4 is the second most abundant IGFBP in rat serum (Shimonaka *et al.*, 1989), but the contribution by various tissues to circulating levels of IGFBP-4 is at present unknown. It is also found in lymph, porcine follicular fluid, human seminal plasma and milk (Shimasaki *et al.*, 1991a; Rosenfeld *et al.*, 1990; Donovan *et al.*, 1991). An IGFBP-4 mRNA of 2.6 kb is expressed predominantly in the liver, and to a lesser extent in adrenal, testis, spleen, heart, lung, kidney, stomach, hypothalamus and brain cortex of six week old rats (Shimasaki *et al.*, 1990). Kiefer *et al.* (1991b) demonstrated positive hybridisation of RNA probes to adult human liver, embryonic liver and brain.

Little is known about regulation of circulating IGFBP-4. In age-related osteoporosis, circulating levels of a 24 kDa IGFBP presumed to be IGFBP-4 are increased in elderly women with fractures, and correlate with parathyroid hormone levels (Rosen *et al.*, 1992). Although IGFBP-4 preferentially localises in connective tissue in perfused heart studies (Boes *et al.*, 1992), there is no evidence of association of IGFBP-4 with cell surfaces, which suggest that IGFBP-4 exists solely as a soluble, extracellular IGFBP.

IGFBP-5

Another structurally distinct IGFBP was isolated from medium conditioned by a human osteosarcoma cell line (Kiefer *et al.*, 1991b), adult rat serum (Shimasaki *et al.*, 1991a), porcine follicular fluid (Shimasaki *et al.*, 1991b) and human CSF (Binoux *et al.*, 1991). Based on its unique sequence deduced from cDNAs isolated from rat ovary and human placenta libraries (Shimasaki *et al.*, 1991a), this protein was designated IGFBP-5. The mature protein contains 252 amino acid residues, and has a molecular weight of approximately 28.5 kDa (Shimasaki *et al.*, 1991a). IGFBP-5 contains 18 cysteines but no N-glycosylation sites or RGD sequences (Shimasaki *et al.*, 1991a). The IGFBP-5 gene has been localised to chromosome 5 (Shimasaki *et al.*, 1991a). IGFBP-5 binds IGF-II with greater affinity than IGF-I (Bautista *et al.*, 1991; Kiefer *et al.*, 1991b). IGFBP-5 has not yet been clearly identified in human serum. Northern blot analysis of young rat tissues detected an intense band of 6 kb in all tissues examined including; testis, intestine, adrenals, stomach, spleen, heart, lung, brain and liver. However, IGFBP-5 mRNA is predominantly found in the kidney (as opposed to the liver like other IGFBPs), implying a role in IGF action and renal function (Shimasaki *et al.*, 1991a, b). Jones *et al.* (1993a) have shown that IGFBP-5 is incorporated intact into extracellular matrix (ECM) of cultured human foetal fibroblasts, and that ECM-associated IGFBP-5 has an eight-fold decrease in affinity for IGF-I compared to soluble IGFBP-5. They

also found that IGFBP-5 present in fibroblast ECM is protected from degradation and can potentiate the biologic actions of IGF-I.

IGFBP-6

The most recently discovered member of IGFBP, IGFBP-6, was isolated from human CSF (Roghani *et al.*, 1989), human serum (Zapf *et al.*, 1990), conditioned media of He(39)L human lung fibroblast and SV40 transformed human fibroblast cell lines (Forbes *et al.*, 1990; Martin *et al.*, 1990), rat serum (Shimasaki *et al.*, 1991a) and porcine follicular fluid (Shimasaki *et al.*, 1991b). Mature rat and human IGFBP-6 contain 201 and 216 amino acid residues respectively, and have a molecular mass of approximately 21.5 and 22.8 kDa (Shimasaki *et al.*, 1991c). Rat and human IGFBP-6 have 14 and 16 of the 18 cysteine residues conserved in IGFBPs 1-5, respectively (Shimasaki *et al.*, 1991c). Human IGFBP-6 contains a potential N-glycosylation site (Shimasaki *et al.*, 1991c) and a RGD sequence (Rechler, 1993). The gene for human IGFBP-6 has been localised to chromosome 12 following analysis of human-hamster somatic cell hybrids (Shimasaki *et al.*, 1991c). IGFBP-6 exhibits a marked preferential affinity for IGF-II over IGF-I (Martin *et al.*, 1990). Northern analysis has detected the presence of a 1.3 kb mRNA of IGFBP-6 in testis, spleen, intestine, adrenal, kidney, stomach, heart, lung, brain and liver rat tissues (Shimasaki *et al.*, 1991c). IGFBP-6 is also present in human amniotic fluid (Baxter and Saunders, 1992) and bovine vitreous humor (Waldbillig *et al.*, 1991).

Baxter and Saunders (1992) have shown that serum IGFBP-6 levels are lower in women than in men, and are further reduced during pregnancy. They also reported a 50 % reduction in samples of patients with acromegaly, suggesting that IGFBP-6 may be inversely regulated by GH.

1.9 Modulation of IGF bioactivity by IGFBPs

IGFBPs may both inhibit or augment the metabolic and mitogenic effects of IGFs at the cellular level (Baxter and Martin, 1989b). It appears that this inhibition or enhancement of IGF action depends upon i) the type of IGFBP, ii) the specific cell type, iii) the complement of IGFBPs that is in the extracellular compartment in the immediate vicinity of the cell surface, iv) whether the IGFBPs within the microenvironment exist freely in solution or are bound to cell surfaces or ECM and v) if they are proteolysed, polymerised, phosphorylated or non-phosphorylated.

Excess IGFBP-1 from amniotic fluid inhibited IGF stimulated cell growth and DNA synthesis in chick embryo fibroblasts (Liu *et al.*, 1991). IGF-I stimulated thymidine incorporation by JMGE-3 human osteosarcoma cells was inhibited by IGFBP-1, while stimulation by des(1-3)IGF-I (which binds IGFBPs weakly) was not inhibited by IGFBP-1 (Campbell and Novak, 1991). IGFBP-1 inhibited glucose incorporation by BALB/c3T3 fibroblasts in both the presence and absence of exogenous IGF-I, suggesting the cells were secreting an IGF or that IGFBP-1 itself directly inhibited glucose uptake (Okajima *et al.*, 1993). Even at concentrations lower than that of IGF-I, IGFBP-1 inhibited IGF-I stimulated growth of chick embryo pelvic cartilage (Burch *et al.*, 1990). Inhibition of IGF action by IGFBP-1 is thought to be the result of the formation of IGF:IGFBP complexes that are unable to interact with cell surface receptors (Rechler and Nissley, 1990). In contrast, in the presence of low amounts of platelet-poor plasma, IGFBP-1 from human amniotic fluid has been shown to markedly augment the mitogenic effect of IGF-I on porcine aortic smooth muscle cells (Elgin *et al.*, 1987). IGFBP-1 binding to IGF-I was shown to be required for potentiation by IGFBP-1 on MDA-231 breast carcinoma cells (Comacho-Hubner *et al.*, 1991b). In addition, it has been demonstrated that IGF-I binding to the type I IGF receptor is necessary for optimal growth stimulation, since an IGF-I analogue with a reduced affinity for the type I receptor was

less active than native IGF-I in the presence of IGFBP-1 (Clemmons *et al.*, 1990). Two isomers of IGFBP-1 that differ in their ability to bind to cell surfaces and to form multimers, have been isolated from amniotic fluid (Busby *et al.*, 1989). Subsequently, it was reported that isomers of IGFBP-1 that are able to form disulfide-linked multimers and bind cell surfaces via integrin receptors, are able to potentiate IGF-I stimulated DNA synthesis (Busby *et al.*, 1989; Clemmons, 1989), while IGF-I stimulated DNA synthesis is inhibited by the isoform that could not form multimers or bind cell surfaces. These data imply that cell surface association and / or multimer formation is required if IGFBP-1 is to enhance IGF action. Phosphorylation may also be a mechanism by which IGFBP-1 activity is regulated, since non-phosphorylated IGFBP-1 potentiates effects of IGF-I, while the phosphorylated form inhibits IGF-I activity (Frost and Tseng, 1991). Non-phosphorylated IGFBP-1 has a 4-6 fold lower affinity for IGF-I than the phosphorylated form (Jones *et al.*, 1991). This would favour sequestration of IGF-I by the phosphorylated IGFBP-1, and release of IGF-I by the non-phosphorylated IGFBP-I, the latter resulting in greater exposure of IGF-I to receptors.

IGFBP-2 has been less intensely studied compared with IGFBP-1. IGFBP-2 isolated from bovine kidney epithelial (MDBK) cells inhibits IGF-I and -II effects on DNA synthesis, protein accumulation and protein breakdown in chick embryo fibroblasts (Ross *et al.*, 1989). In the same system, des (1-3) IGF-I was observed to stimulate DNA synthesis more potently than native IGF-I, implying that IGFBP-2 binding to IGF-I or IGF-II was necessary for inhibition. In the presence of platelet-poor plasma, IGFBP-2 inhibited IGF-I stimulated DNA synthesis in porcine aortic smooth muscle cells (Bourner *et al.*, 1992). Feyden *et al.* (1991) demonstrated that IGFBP-2 containing a cysteine residue at position 281, which does not alter its biological activity or binding to IGF-I and IGF-II, inhibited IGF-I stimulated DNA and collagen synthesis in foetal rat calvaria. Stimulatory effects of IGFBP-2 have also been observed. Microvascular endothelial cells display increased uptake of α-aminoisobutyrate

and glucose transport in serum-free medium containing IGF-I and partially purified IGFBP-2 (Bar *et al.*, 1989).

Numerous studies have demonstrated the inhibitory effect of IGFBP-3 on IGF action. Zapf *et al.*(1979) found that serum fractions of IGF binding activity enriched with IGFBP-3 inhibited glucose incorporation by fat cells. An excess of purified IGFBP-3 when added in combination with IGF-I significantly inhibited IGF-I stimulated DNA synthesis in human skin fibroblasts (DeMellow and Baxter, 1988). Most recently, excess IGFBP-3 was shown to inhibit glucose incorporation in BALB / c3T3 cells, but had no effect on insulin stimulated incorporation (Okajima *et al.*, 1993). This suggests that the prevention of IGF-I binding to its receptor is the probable inhibitory mechanism of IGFBP-3. There is also some evidence to indicate that IGFBP-3 might have inhibitory potential independent of its capacity to bind IGFs. IGFBP-3 inhibited DNA synthesis in mouse embryo fibroblasts that had been exposed to 1% serum or FGF (Villaudy *et al.*, 1991). This finding was intriguing because it was the first report that IGFBP-3 may inhibit responsiveness to other growth factors. However, the likelihood that FGF stimulated local synthesis of IGF that could in turn be bound by IGFBP-3 was not ruled out. Oh *et al.* (1993) showed that in incubation medium containing calcium chloride and magnesium chloride, recombinant IGFBP-3 bound to HS578T human breast cancer cells and inhibited cell growth in a dose dependent manner, with 60% inhibition of cell growth apparent at 20 nM IGFBP-3. However, although no IGF was added to the cells, the possibility that they secreted IGFs was not totally excluded. The inhibitory effect of IGFBP-3 could be partially attenuated by incubation with IGF-I, but not by IGF analogues with reduced affinity for IGFBP-3.

DeMellow and Baxter (1988) reported conflicting results with respect to the effect of IGFBP-3 on IGF-I stimulated DNA synthesis in human skin fibroblasts. In contrast to co-incubation, if these cells were pre-incubated with IGFBP-3 followed by its removal, enhancement of the effect of IGF-I on DNA synthesis was noted. Conover *et al.* (1990)

similarly found that bovine IGFBP-3 co-incubated with increasing concentrations of IGF-I produced a dose dependent inhibition of aminoisobutyrate (AIB) uptake by cultured fibroblasts. However, when these cells were pre-incubated for 24 hours with bovine IGFBP-3 followed by its removal, there was a 32 to 86% dose dependent increase of the AIB uptake response to IGF-I. IGFBP-3 associated with cell surfaces has a lower affinity for IGF-I and -II than IGFBP-3 in solution (McCusker *et al.*, 1990). Therefore, any cell surface associated IGFBP-3 remaining during the experimental period may be capable of modulating the IGF receptor interaction such that IGFs become more readily available to bind receptors.

Using the Hunt-Schilling wound healing model, Sommer *et al.* (1991) and Hamon *et al.* (1993) showed that in contrast to IGF-I alone, systemic administration of equimolar amounts of IGFBP-3 and IGF-I results in accelerated wound healing and enhanced wound tissue deposition in both normal and impaired wound healing models in rats. Clark *et al.* (1993) subcutaneously injected a combination of human IGFBP-3 and IGF-I to GH deficient rats, which resulted in a two-fold increase in weight gain and a significant increase in epiphyseal width compared to subcutaneous injection of IGF-I alone.

IGFBP-4 appears to inhibit IGF bioactivity in most *in vitro* systems. IGFBP-4 inhibited IGF-II stimulated bone cell proliferation (Mohan *et al.*, 1989). In B104 neuroblastoma cells, IGF-I stimulated thymidine incorporation was inhibited when rat IGFBP-4 was present in excess (Cheung *et al.*, 1991). Both IGF-I and IGF-II stimulated DNA and glycogen synthesis were inhibited by excess recombinant human IGFBP-4 in cultures of human osteosarcoma cells (Kiefer *et al.*, 1992). It has been proposed that proteolysis of IGFBP-4 may free cells from a growth arrested state. IGFBP-4 incubated with porcine aortic smooth muscle cells gave rise to 14 and 18 kDa fragments which were unable to bind IGF, and had lost the ability to inhibit IGF-I stimulated DNA synthesis (Cohick *et al.*, 1993).

Studies indicating that IGFBP-5 has an inhibitory function are limited. Kiefer *et al.* (1992) found that excess recombinant IGFBP-5 could inhibit IGF-I stimulated DNA and glycogen synthesis in human osteosarcoma cells, whereas stimulation by IGF-II was inhibited by IGFBP-5 at concentrations lower than those of IGF-II. Co-incubation of mouse MC3T3-E1 osteoblasts with equimolar concentrations of IGF-II and IGFBP-5 potentiated the proliferative action of IGF-II (Bautista *et al.*, 1991). IGF-I stimulated DNA synthesis by mouse osteoblasts was enhanced by a mixture of IGFBP-5 and IGFBP-6 (Andress and Birnbaum, 1991). Most recently, a 23 kDa fragment of IGFBP-5 has been shown to potentiate the mitogenic effect of IGF-I and -II in mouse osteoblasts (Andress and Birnbaum, 1992). The authors suggested that since the affinity of the IGFBP-5 fragment for IGF-I was greatly reduced compared with intact IGFBP-5, proteolysis may be an important mechanism in allowing IGFBP-5 to augment IGF action (Andress *et al.*, 1993). Similarly, the affinity for IGF-I by ECM associated IGFBP-5 is reduced, which may result in IGF-I being presented to IGF receptors more readily. GM10 human foetal fibroblasts secrete IGFBP-5 which localises at the ECM (Jones *et al.*, 1993a). Fibroblasts subsequently grown on this ECM displayed increased growth in response to IGF-I. In contrast, IGFBP-5 added to the medium supporting these cells was degraded and did not augment the IGF-I growth response.

IGFBP-6 was capable of inhibiting IGF-II stimulation of osteosarcoma DNA and glycogen synthesis, even when IGF-II was in excess. In contrast, IGF-I stimulation was not inhibited even when IGFBP-6 was in molar excess, possibly reflecting the greater affinity of this IGFBP for IGF-II (Kiefer *et al.*, 1992).

1.10 Postulated roles of IGFBPs

Maintenance of a circulating IGF pool

A proposed role for the IGFBPs, in particular for the ternary complex, is to prolong the half-lives of IGFs and regulate their metabolic clearance. An estimated 75 % of plasma

IGF-I and -II is carried by the ternary complex (see section 1.7). The half-life of free IGF-I in plasma has been determined to be approximately 10 min (Hodgkinson *et al.*, 1989a, Davis *et al.*, 1989). However, when associated with the 150 kDa complex the IGFs have prolonged half-lives of 12 - 15 hours (Guler *et al.*, 1989a). Furthermore, a more rapid clearance of IGF-I has been reported in states where circulating IGFBP-3 levels or its affinity for IGF-I are reduced, such as in hypophysectomized rats (Cohen and Nissley, 1976), protein-restricted rats (Thissen *et al.*, 1992) and pregnancy (Davenport *et al.*, 1990). Circulating half-lives of IGF-I analogues with reduced affinities for IGFBPs are shorter than IGF-I, further suggesting that IGF-I bound to IGFBPs are cleared more slowly than free peptide (Ballard *et al.*, 1991; Cascieri *et al.*, 1988; Francis *et al.*, 1988b).

The specific function of the reservoir of IGF bound in the ternary complex is not clear. Some investigators have postulated that it may act as a functional store of IGF that becomes readily available under stress. The existence of several proteases for IGFBP-3 (Davenport *et al.*, 1990; Giudice *et al.*, 1990; Hossenlopp *et al.*, 1990), including increased activity of a stress related protease that may cleave IGFBP-3 and increase the bioavailability of IGF-I (Davenport *et al.*, 1992a), supports this hypothesis.

Suppression of IGF insulin-like effects

In humans, the total plasma concentration of IGF-I and IGF-II is about 1000 times greater than the insulin concentration (Baxter, 1986). Although IGFs in plasma are present in sufficient amounts to cause hypoglycaemia (Guler *et al.*, 1987), hypoglycaemia does not normally occur, presumably because the activity of IGFs is quelled as a result of binding to the IGFBPs. Based on the premise that a component of the IGF pool circulates free *in vivo*, one possible role of IGFBP-1 is that it may neutralise undesirable insulin-like effects of free IGF in the circulation. In support of this hypothesis, Lewitt *et al.* (1991) demonstrated that infusion of IGFBP-1 results in a state of hyperglycaemia. In addition, IGF-I infusion results

in an insulin-like decrease in blood glucose concentration that can be suppressed by co-infusion of equimolar amounts of IGFBP-1 (Lewitt *et al.*, 1991).

Transport of IGFs from the vasculature to tissues

The majority of circulating IGFs are contained either within the 150 kDa complex with the remainder complexed in 50 kDa binary complexes or circulating as free peptide. Using ^{125}I-IGF-I, Binoux and Hossenlopp (1988) compared the molecular weight size distribution of IGF-I both in serum and human lymph on a gel-filtration column under neutral conditions. Unlike serum, greater than 90% of IGF-I eluted with the material in the lower molecular weight region. Western ligand blot analysis showed that in contrast to serum, the smaller IGFBP species in lymph were more abundant than IGFBP-3 as a proportion of total IGFBPs. These data suggest that most of the 150 kDa complex is retained in plasma and does not cross the endothelial barrier, while the 50 kDa binary complexes are capable of leaving the circulation. Thus, a potential role for lower molecular weight IGFBPs is that they may 'shuttle' the IGFs out of the vascular compartment (Sara and Hall, 1990). In support of this notion, IGFBP-1 and IGFBP-2 have been shown to cross both intact endothelium and capillaries (Bar *et al.*, 1990b and 1990c). Furthermore, IGF-I bound to an endothelial cell derived IGFBP, identified as IGFBP-4, has been reported to cross capillary boundaries (Bar *et al.*, 1990a). However, some studies have reported the detection of the 150 kDa IGF-IGFBP complex in ovine mammary lymph and follicular fluid (Hodgkinson *et al.*, 1989b) and human peritoneal fluid (Bowsher *et al.*, 1991). The origin of these complexes is uncertain. As the concentration of ALS in circulation is 2- to 3-fold greater than the other constituents of the ternary complex (Baxter, 1988b and 1990), ALS may cross the capillary barrier and bind IGF-IGFBP-3 binary complexes in extravascular fluid, resulting in the formation of the 150 kDa complex. Alternatively, it is possible that ALS is produced locally, as ALS has been reported in amniotic fluid, CSF and seminal plasma in amounts less than 0.5 % of serum levels

(Baxter, 1990) and is expressed in rat brain, kidney, heart, lung, spleen, muscle and liver (Dai and Baxter, 1992).

Tissue targeting

Earlier studies indicated that IGFBPs were capable of specifically binding to certain target cells, thus facilitating the delivery of IGFs to adjacent IGF receptors (Clemmons *et al.*, 1986; De Vroede *et al.*, 1986). Both IGFBP-1 and IGFBP-2 contain a RGD sequence (see section 1.7), which in the case of IGFBP-1 has been shown to mediate binding to $\alpha 5\beta 1$ integrin receptors (Jones *et al.*, 1993b), and which in turn may be involved in targeting IGFBP-1 to tissues expressing this receptor type. Demonstration that cellular association of IGFBPs may serve a role in regulating IGF activity has been proposed by Busby *et al.* (1988; 1989), who reported that IGFBP-1 isoforms able to bind cell surfaces were capable of augmenting IGF-I stimulation of DNA synthesis, while IGFBP-1 forms unable to associate with cell surfaces inhibited IGF-I activity. Taken together these data imply a role for the IGFBP RGD site in regulating IGF access to cellular receptors.

IGF-independent intrinsic IGFBP activity

The conventional view that IGFBPs simply modulate IGF action and act as transport proteins is being challenged by evidence that IGFBPs posses intrinsic biological activity themselves. For example, Andress and Birnbaum (1992) demonstrated that IGFBP-5 derived from osteoblasts stimulated mitogenesis of normal mouse osteoblast-like cells in the absence of IGFs. Furthermore, they suggested that stimulation of mitogenesis was a result of association of the IGFBP with the osteoblast surface (Andress and Birnbaum, 1992). IGFBP-1 contains an RGD integrin recognition sequence and is capable of binding to cell surfaces (see section 1.7). In vitro mutagenesis was used to alter this RGD sequence to Trp-Gly-Asp

(WGD) in Chinese Hamster Ovary (CHO) cells (Jones *et al.*, 1993b). Migration of CHO cells expressing the wild type protein was three fold greater compared with cells expressing the mutant form of IGFBP-1 and independent of IGF-I. IGFBP-3 displays specific binding to the cell surface and can inhibit growth of Hs578T human breast cancer cells (Oh *et al.*, 1993). In addition, IGF-I and -II can abrogate the inhibitory effect of IGFBP-3, by preventing cell surface binding of IGFBP-3 due to formation of IGF-IGFBP-3 complexes.

1.11 Proteolysis of IGFBPs

IGFBP-3 is highly sensitive to proteolysis. In rats and humans, IGFBP-3 as determined by Western ligand blotting declines dramatically in late pregnancy (Gargosky *et al.*, 1990a and b; Guidice *et al.*, 1990), yet IGFBP-3 concentrations are generally unchanged or increased when measured by radioimmunoassay (Baxter and Martin, 1986). These observations have been explained by the presence of IGFBP-3 proteases in pregnancy plasma (Binoux *et al.*, 1991b; Guidice *et al.*, 1990; Hossenlopp *et al.*, 1990), which produce IGFBP-3 fragments which still bind native IGF-I but cannot bind to ^{125}I-IGF-I (Suikkari and Baxter, 1991). Preliminary characterisation of the IGFBP-3 pregnancy associated protease activity in humans and rats suggests that as yet unidentified cation dependent serine proteases are responsible for the limited proteolysis of IGFBP-3 in the serum (Binoux *et al.*, 1991b; Davenport *et al.*, 1992b; Davenport *et al.*, 1990; Guidice *et al.*, 1990). Other than late term pregnancy, proteolytic breakdown of IGFBP-3 is increased under other catabolic conditions including post-surgery (Davenport *et al.*, 1992a) and cancer (Frost *et al.*, 1993). IGFBP-3 proteases are also produced *in vitro* by a variety of cultured human cell lines (Frost *et al.*, 1993). In addition, Lalou and Binoux (1993) have recently presented evidence of limited proteolysis of IGFBP-3 in lymph of normal animals. The affinity of IGFBP-3 for binding to IGF is at least equal to the affinity of the type I IGF receptor for IGF, which enables

sequestration of IGFs by IGFBP-3 and prevention of IGF association with target cell receptors. Generally, cleavage of IGFBP-3 results in the appearance of a 31 kDa fragment that displays a 50-fold reduction in its affinity for IGF-I or IGF-II (Clemmons, 1993). Despite its reduction in affinity, this fragment is still able to bind native IGF-I and form the ternary complex (Suikkari and Baxter, 1991; Suikkari and Baxter, 1992). Since IGFBP-3 acts as a reserve for IGF-I in the vascular compartment, an increase in enzymatically processed IGFBP-3 with a weaker affinity for IGF-I than intact IGFBP-3 could increase the tissue bioavailability of IGF-I (Holly *et al.*, 1993; Lamson *et al.*, 1993). In line with this proposal, IGF-I has been reported to be more rapidly cleared from the serum in pregnant rats (Davenport *et al.*, 1990). Some IGF-I stimulated processes have been demonstrated to be inhibited due to binding of IGF-I to IGFBP-3 (see section 1.8). Since a reduction in IGFBP-3 affinity may potentiate cellular responses to IGFs, proteolysis may also represent a specific mechanism that regulates IGFBP modulation of IGF action. Proteolytic activity has also been described for IGFBP-2 (McCusker *et al.*, 1991a), IGFBP-4 (Fowlkes and Freemark, 1992; Conover *et al.*, 1993) and IGFBP-5 (Camacho-Hubner *et al.*, 1992). Generally, the fragments generated have greatly reduced affinity for the IGFs, which raises the possibility that proteases may function to release IGFs form IGFBPs, making them available to bind to IGF receptors.

1.12 IGF-I analogues that bind poorly to IGFBPs

An N-terminal tripeptide truncated derivative of IGF-I, known as des(1-3)IGF-I, has been isolated from a variety of sources that includes bovine colostrum (Francis *et al.*, 1986), human brain (Sara *et al.*, 1986; Carlsson-Skwirut *et al.*, 1986), porcine uterus (Ogasawara *et al.*, 1989) and human platelet lysates (Karey *et al.*, 1989; Karey and Sirbasku, 1989). Des(1-3)IGF-I is structurally identical to IGF-I, except for the absence of the first three amino acids

Gly-Pro-Glu present in IGF-I. It has a 5-10 fold greater potency than IGF-I in stimulating DNA and protein synthesis and in inhibiting protein breakdown in most cultured cells, including L6 myoblasts (Ross *et al.*, 1989). This enhanced potency is due to a substantially reduced affinity of this analogue for IGFBPs produced by cultured cells (Ballard *et al.*, 1989). The fact that the amino terminus of IGF-I is crucial in its interaction with IGFBPs was first demonstrated by Szabo *et al.* (1988), who found that des(1-3)IGF-I was unable to compete with IGF-I or IGF-II for binding to IGFBP-2. In structure function studies using N-terminally modified synthetic variants of IGF-I, Bagley *et al.* (1989) demonstrated that the third amino acid residue Glu, is critical for IGF-I binding to IGFBP-2, as removal of either Gly or Gly-Pro did not alter the affinity for IGFBP-2, whereas the absence of Gly-Pro-Glu virtually eliminated its capacity to bind IGFBP-2. Using purified IGFBP-1, -2 and -3, Forbes *et al.* (1988) showed that des(1-3)IGF-I exhibited about 3-fold reduced binding for IGFBP-3, and did not significantly bind IGFBP-2 or IGFBP-1.

Removal or substitution of the Glu^3 residue with a residue of different charge, such as Gly or Arg, substantially reduces binding to IGFBPs (King *et al.*, 1992). This latter finding has led to the generation of another IGF-I analogue known as long (Arg^3) IGF-I (LR^3IGF-I). This peptide differs from IGF-I not only by the substitution of Arg for Glu at the third residue, but it also contains a thirteen amino acid N-terminal extension that includes an eleven amino acid fragment of methionyl porcine GH (met-pGH (1-11)) (Francis *et al.*, 1991). Despite possessing about one third the affinity for the type I receptor as native IGF-I, LR^3 IGF-I is approximately twice as potent as des(1-3)IGF-I and ten times more so than IGF-I in L6 myoblast protein synthesis assays (Francis *et al.*, 1992). Both des(1-3)IGF-I and LR^3 IGF-I are also more potent *in vivo*. These peptides were 3-5 fold more potent than IGF-I in stimulating growth and nitrogen accretion in rats rendered catabolic by dexamethasone treatment or nitrogen restriction (Tomas *et al.*, 1992; Tomas *et al.*, 1991a and b) and more potent than IGF-I in stimulating gut growth in rats following intestinal resection (Lemmey *et*

al., 1991). It is considered that this enhanced potency *in vivo* occurs as a result of the reduced affinity of these peptides for IGFBPs in fluids and tissues. These potent analogues provide valuable tools for elucidating the effect of reduced IGFBP interaction on transfer of plasma IGF-I to tissues and extracellular fluid compartments. The utilisation of recombinant DNA techniques to produce these analogues in E. coli has provided sufficient quantities of these peptides to conduct animal studies (King *et al.*, 1992).

1.13 Transfer of IGF-I from the vascular space and the role of IGFBPs

The majority of IGFs circulate in plasma with a high molecular weight binding protein and ALS to form the 150 kDa complex (Baxter and Martin, 1989a and 1989b). The remaining circulating IGF is associated in complexes with several smaller binding proteins, of approximately 40 kDa, including IGFBP-1, -2 and -4 (Binoux and Hossenlopp, 1988; Shimonaka *et al.*, 1989). To act *in vivo*, circulating IGFs must traverse the capillary barrier between the vascular compartment and target tissues. IGFs area able to cross the capillary boundaries, as numerous studies *in vivo* have directly demonstrated transfer of circulating plasma IGFs into tissues (Cascieri, *et al.*, 1988; Hodgkinson *et al.*, 1991; Ballard *et al.*, 1991), lymph (Prosser *et al.*, 1992) and milk (Prosser *et al.*, 1991; Prosser and Fleet, 1992). Bar *et al.* (1985; 1988) have utilized a non-recirculating perfusion model in the isolated beating rat heart to study transfer of radiolabelled IGF from the arterial blood supply to cardiac tissue. Radiolabelled IGF-I dissolved in an oxygenated nutrient buffer was infused into the coronary artery, and efferent flow sampled using a slit in the right ventricle, after which, tissue sections were prepared and autoradiographed to determine tissue distribution of the infused labelled peptide. Subsequently, Bar *et al.* (1990b) showed that 3 - 5 % of perfused ^{125}I-IGF-I left the vasculature and localized in heart tissue.

It has been postulated that the 150 kDa complex acts as a reservoir of readily releasable IGFs in plasma. However, Sara and Hall (1990) hypothesized that the low molecular weight IGFBPs act as transporting proteins to 'shuttle' IGFs out of the vascular space into tissues. In support of this, human amniotic IGFBP-1, bovine IGFBP-2, recombinant IGFBP-3 and bovine microvessel IGFBP-4 have all displayed the ability to traverse the capillary barrier in the perfused isolated beating rat heart (Bar *et al.*, 1990b; 1990c; Boes *et al.*, 1992). IGFBP-1 also left the vascular compartment and localized in cardiac tissue in intact animals (Bar *et al.*, 1990b). Furthermore, Bar and colleagues went on to demonstrate that the transcapillary movement of IGFBP-1 was increased, and IGFBP-4 decreased by insulin (Bar *et al.*, 1990c). Moreover, IGF-I covalently cross-linked to IGFBP-4 was able to cross capillary boundaries (Bar *et al.*, 1990a). Taken together, these data infer that a sophisticated, nutrient dependent mechanism is responsible for delivering IGFs from the vascular compartment to extracellular and tissue sites. In addition, different binding proteins localize within different tissue compartments. For example, IGFBP-1, -2, and -3 along with IGF-I preferentially localize within cardiac muscle, whereas glycosylated and non-glycosylated IGFBP-4 primarily distribute in subendothelial connective tissue (Boes *et al.*, 1992). Yet, when IGFBP-4 was associated with IGF-I, it localized to cardiac muscle, but when cross-linked with an IGF-I analogue lacking the type I IGF receptor domain, it localized to connective tissue, suggesting that following transcapillary passage of IGF-I-IGFBP-4, tissue distribution is mediated by the IGF-I molecule (Bar *et al.*, 1990c). More recently, radiolabelled recombinant human IGFBP-3 unassociated with the ternary complex has been shown to rapidly cross capillary endothelia from blood to extravascular sites including kidney, liver and gastrointestinal tissues, supporting a role for IGFBP-3 in the delivery of IGFs to peripheral tissues (Arany *et al.*, 1993). Whether IGFs may leave the vasculature in an unbound form is unknown.

1.14 Statement of the problem

A review of the literature reveals that IGF activity has been detected in the circulation as well as in tissue extracts and conditioned media of many cells. As a result, in recent years investigations have focussed on the possible paracrine / autocrine nature of IGFs. However, it is stressed that these growth factors do have an endocrine function. In the circulation, bioactive IGFs are stored at high concentrations as IGFBP-associated peptides, which are potentially able to promote growth of tissues outside the vasculature, and represent a large pool of readily accessible IGF activity. In addition, systemic IGF-I administration to GH-deficient along with other animal models, results in increased growth and long term anabolic effects which clearly support an endocrine role for IGF-I.

At the commencement of my PhD candidature, no information concerning the pathway of transfer of IGF-I across endothelial barriers was known. Although the literature review revealed that IGFBPs may inhibit or potentiate the effect of IGFs *in vitro*, and that IGFBPs alone or complexed to IGF-I were able to cross the capillary boundary and localise in specific tissues, a general role for plasma IGFBPs in regulating the egress of IGF-I from plasma to tissues had not been elucidated. Since certain IGF-I variants bind poorly to IGFBPs, I hypothesised that transfer of the variants would differ to that of IGF-I both *in vitro* and *in vivo*, inferring regulation by IGFBPs.

As a result the specific aims of this thesis were:

- to characterise the association of IGF-I and LR^3IGF-I with IGFBPs *in vitro* and determine the tissue distribution of IGF-I and LR^3IGF-I in rats with normal and perturbed IGFBP profiles,
- to develop and validate an *in vitro* endothelial barrier model that would enable the elucidation of the pathway of IGF-I transfer across endothelial cells and permit

examination of the effects of IGFBPs on this process using both IGFBPs in association with radiolabelled IGF-I and radiolabelled IGF-I variants with reduced affinities for IGFBPs,

- utilise this model to determine the pathway of IGF-I transfer across epithelial cell monolayers, which like endothelial cells function as a barrier and share similaritires with respect to junctional complexes between adjacent cells,
- examine the transfer of IGF-I and LR^3IGF-I from blood to extracellular fluid sites *in vivo* using a well characterised wound healing model.

CHAPTER: 2 TISSUE DISTRIBUTION OF CIRCULATING LABELLED INSULIN-LIKE GROWTH FACTOR-I (IGF-I) AND AN ANALOGUE LR^{3}IGF-I IN PREGNANT RATS.

2.1 PROLOGUE

This chapter examines the association of IGF-I and LR^{3}IGF-I with rat and human plasma binding proteins and the tissue distribution of systemically administered ^{125}I-labelled IGF-I and LR^{3}IGF-I in pregnant and non-pregnant rats. This study was conducted at the commencement of my PhD candidature to complete research initiated in my honours degree, which described both the *in vitro* interaction of IGF-I and LR^{3}IGF-I with rat IGFBPs and the pharmacokinetic parameters of radiolabelled IGF-I and LR^{3}IGF-I in pregnant rats. Data arising from these studies are now published in Bastian *et al.*, (1993) and Lord *et al.*, (1994).

2.2 INTRODUCTION

The IGFs bind avidly and specifically to IGFBPs in blood and other body fluids (Ooi and Herrington, 1988; Hossenlopp *et al.*, 1987), however, our knowledge of the resultant biological effects of the interaction between IGFs and IGFBPs *in vivo* remains limited. In various *in vitro* models, IGFBPs have been reported to inhibit or enhance the action of IGF-I (Baxter and Martin, 1989b) and *in vivo*, increase the serum half-life of IGF-I (Thissen *et al.*, 1992; Ballard *et al.*, 1991; Davenport *et al.*, 1990; Cascieri *et al.*, 1988; Francis *et al.*, 1988b; Zapf *et al.*, 1986; Cohen and Nissley, 1976). From these reports it has been postulated that the roles of IGFBPs are to maintain a circulating reservoir of IGFs, suppress insulin-like effects of IGFs, modulate IGF transport in the vascular and tissue compartments, target IGFs in extracellular fluid to tissues and regulate IGFs' interaction with IGF receptors (see sections

1.8 and 1.9). In addition, some IGFBPs have shown that they possess intrinsic activity independent of the IGFs (see section 1.9).

In the vascular compartment, the majority of IGF is carried by the 150 kDa complex (Baxter, 1988b), which consists of IGFBP-3, an IGF molecule and the acid labile subunit. In rats, plasma IGFBP-3, measured by Western ligand blotting, declines dramatically in late pregnancy (Gargosky, *et al.* 1990b), yet IGFBP-3 concentrations are generally unchanged or increased when measured by radioimmunoassay (Baxter and Martin, 1986). These observations have been explained by the presence of IGFBP-3 proteases in plasma of pregnant animals (Binoux *et al.*, 1991b; Guidice *et al.*, 1990; Hossenlopp *et al.* 1990). Generally, cleavage of IGFBP-3 results in the appearance of a 31 kDa fragment that displays a 50-fold reduction in its affinity for IGF-I or IGF-II (Clemmons, 1993). Despite its reduction in affinity, this fragment is prevented from binding iodo-IGF-I but is still able to bind native IGF-I and form the ternary complex (Suikkari and Baxter, 1991; Suikkari and Baxter, 1992). It is believed that an increase in enzymatically processed IGFBP-3 with a weaker affinity for IGF-I than intact IGFBP-3 could increase the tissue bioavailability of IGF-I (Holly *et al.*, 1993; Lamson *et al.*, 1993). We have confirmed previous studies by Davenport *et al.* (1990), that a more rapid clearance of IGF-I occurs in pregnant rats (Bastian *et al.*, 1993).

LR^3IGF-I is a recombinant human IGF-I analogue which has Glu^3 replaced with Arg^3 resulting in a change of charge at position three (Bagley *et al.*, 1989). In addition, LR^3IGF-I contains a 13-amino acid N-terminal extension originating from porcine growth hormone, comprising of Met-Phe-Pro-Ala-Met-Pro-Leu-Ser-Ser-Leu-Phe-Val-Asn (Francis *et al.*, 1992). LR^3IGF-I has several fold higher potencies than IGF-I in stimulating protein synthesis and inhibiting protein breakdown in rat L6 myoblasts, H35 hepatoma cells and chicken embryo fibroblasts (Francis *et al.*, 1992). The increased potency of LR^3IGF-I is attributed to its lower affinity for the IGFBPs. This analogue has been employed in numerous *in vitro* (Francis *et al.*, 1992; King *et al.*, 1992; Bagley *et al.*, 1989; Ballard *et al.*, 1989; Francis *et al.*,

1988b; Ballard *et al.*, 1986) and *in vivo* (Bird *et al.*, 1994; Read *et al.*, 1992; Tomas *et al.*, 1992; Lemmey *et al.*, 1991) studies. Clearance of LR^{3}IGF-I was 11-fold higher than IGF-I in virgin animals, and 3-fold higher than IGF-I in pregnant animals (Bastian *et al.* 1993), in accordance with its poor association with IGFBPs (Francis *et al.*, 1992). Further to this, the clearance rate of IGF-I from the circulation in pregnant animals was increased 3-fold compared with virgin animals, producing a decay curve resembling more closely that of LR^{3}IGF-I (Bastian *et al.* 1993).

Future transport experiments within this thesis were designed to investigate the effects of IGFBPs on IGF-I transmigration across endothelial cell monolayers. To achieve this, I wanted to compare the transport of recombinant human ^{125}I-LR^{3}IGF-I and ^{125}I-IGF-I across endothelial cell monolayers in the presence of human IGFBPs. Although it was known that recombinant human ^{125}I-LR^{3}IGF-I has a poor affinity for rat plasma IGFBPs, it had not been demonstrated that this IGF-I analogue also had a reduced affinity for human IGFBPs. As a result, the studies reported in this chapter aimed firstly to examine the association of IGF-I and LR^{3}IGF-I with rat and human plasma IGFBPs *in vitro*. The second objective was to examine the tissue distribution of IGF-I and LR^{3}IGF-I in normal rats and in rats with abnormally low circulating IGFBP levels. Ultimately, pregnancy was chosen as the experimental condition, since the IGFBPs in pregnancy have a markedly reduced affinity for IGF-I. This would allow us to examine how changes in IGFBP status affects the targeting of circulating IGFs to maternal or foetal tissues and contribute to the current knowledge on the functional relevance of the dramatic reductions in IGFBP affinity for IGF-I that occurs during late pregnancy. Comparison of the amino acid sequences of recombinant human IGF-I and rat IGF-I indicates a high degree of similarity, differing by only three amino acids (Ballard *et al.* 1989). Prior to this study, rat IGF-I was not available, but based on their amino-acid sequences it was expected that infusion of recombinant human IGF-I into rats would display similar biological activities to rat IGF-I. In addition, LR^{3}IGF-I was chosen because it has a

low affinity for rat IGFBPs (Lord *et al.*, 1994), providing a useful complimentary tool for defining the roles of IGFBPs *in vivo*.

Rats were the favoured animal model for the tissue distribution study for the following reasons: 1) they are inexpensive to purchase and house, 2) the peptide requirements are minimised, 3) they have a relatively short gestation period and 4) they have been used extensively by us and in independent IGF-I pharmacokinetic and tissue distribution studies (Thissen *et al.*, 1992; Ballard *et al.*, 1991; Davenport *et al.*, 1990; Cascieri *et al.*, 1988; Francis *et al.*, 1988b; Zapf *et al.*, 1986; Cohen and Nissley, 1976)

2.3 MATERIALS AND METHODS

2.3.1 Materials

Sodium iodide ($Na^{125}I$) was obtained from Amersham International (Bucks, U.K). Methohexitone sodium was from Eli Lilly and Co. (West Ryde, Australia). Pentobarbitone sodium was obtained from Boehringer Ingleheim Pty. Ltd. (Smithfield, Australia). Xylazine was from Bayer Australia Limited (Botany, Australia). Atropine sulphate was purchased from Apex Laboratories (St Mary's, Australia) and ketamine hydrochloride came from Troy Laboratories Pty. Ltd (Smithfield, Australia). Heparin was obtained from Commonwealth Serum Laboratories, Melbourne, Australia. Sodium dihydrogen orthophosphate, trichloroacetic acid (TCA) and sodium chloride were purchased from Ajax Chemicals, Auburn, Australia. Bovine serum albumin (BSA; RIA grade), protamine sulphate, sodium azide and activated charcoal were from Sigma Chemical Co., St Louis, USA.

2.3.2 Recombinant IGF peptides

Recombinant human IGF-I (rhIGF-I) and the recombinant human variant LR^3IGF-I were supplied by GroPep Pty. Ltd., Adelaide, Australia. rhIGF peptides were synthesised in *Eschericia coli*, and exhibited > 95 % purity. IGF-I and LR^3IGF-I peptides were iodinated with carrier free $Na^{125}I$ to specific activities of 35 μCi / μg and 31 μCi / μg respectively, using the chloramine T method as described in Gargosky *et al.* (1990b). Biological activity of the labelled peptides was confirmed as described by Ballard *et al.*, (1986).

2.3.3 Plasma collection

Pooled blood from 10 Sprague-Dawley rats and from 2 human subjects were collected in chilled tubes containing heparin at a final concentration of 10 U / ml. Following centrifugation at 1800 g for 20 min at 4°C, plasma was aspirated and stored at -70°C for competition binding experiments.

2.3.4 Competitive binding experiments

The binding characteristics of rat plasma relative to human plasma were investigated by assessing the ability of IGF-I and LR^3IGF-I to compete with binding of radiolabelled IGF-I in an equilibrium binding assay. Initially, rat and human plasma were titrated against ^{125}I-IGF-I . Increasing amounts of rat and human plasma were incubated with radiolabelled IGF-I (10,000 cpm, 0.1 ml) in 0.3 ml of assay buffer (0.05M NaH_2PO_4 / l, 0.25 % (w / v) BSA, 0.02 % (w / v) sodium azide, pH 6.5) in triplicate tubes. Total counts (radiolabelled IGF-I only) and non-specific binding (NSB, radiolabelled IGF-I and assay buffer only) tubes were also set up in triplicate. All tubes were vortexed and incubated at 4°C for 18 h. Free and bound ^{125}I-IGF-I were separated by the addition of 1 ml of 0.5 % activated charcoal and 0.02 % protamine sulphate diluted in the assay buffer, incubation for 35 min at 4°C, and

centrifugation at 2,000 g for 15 min at 4°C. Radioactivity was measured in a portion of the supernatant (0.65 ml) using a gamma counter (LKB, 1261 Multi-Gamma). The radioactivity due to non-specific binding was subtracted from each test measurement. The percentage of added radioactivity bound to rat or human plasma IGFBPs in the supernatant was calculated, using the following equation;

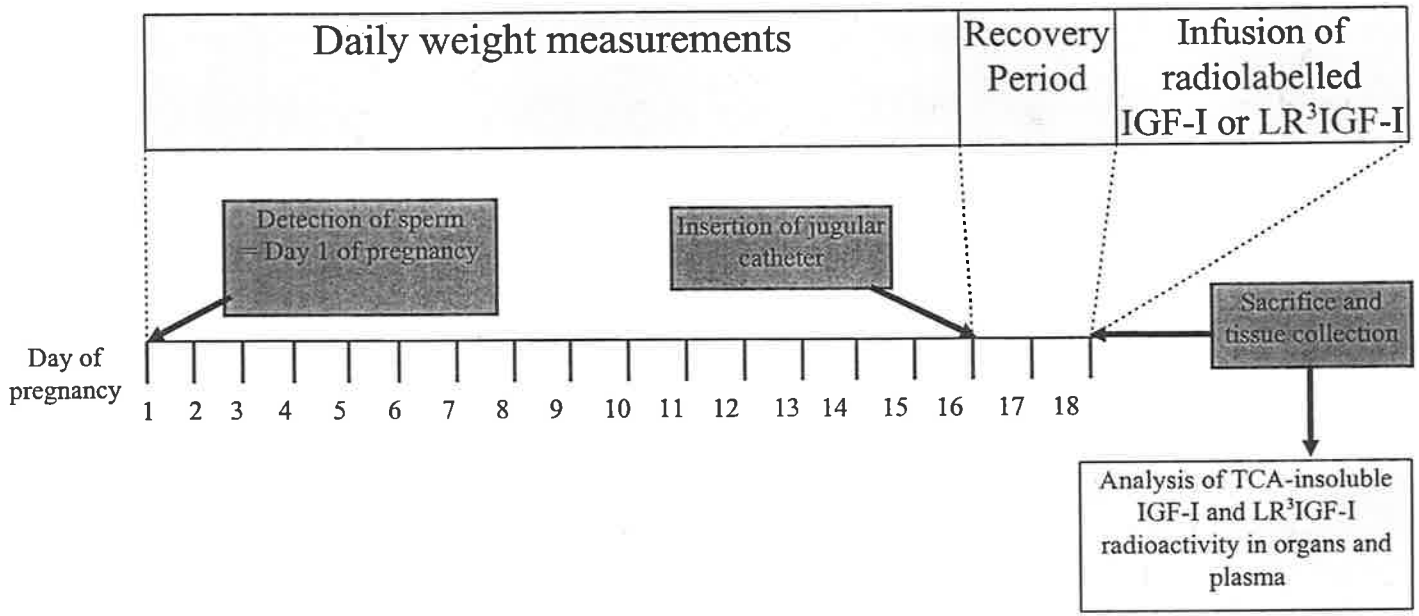
$$\% \text{ COUNT BOUND} = \frac{(\text{MEAN cpm} - \text{MEAN NSB cpm}) \times 2}{\text{MEAN TOTAL COUNTS cpm}} \times 100$$

Competition studies were performed by the addition of increasing amounts of either IGF-I or LR^3IGF-I to a fixed amount of rat (5 µl) or human (30 µl) plasma and radiolabelled IGF-I. Separation was achieved as above. Binding of IGF to plasma IGFBPs was calculated as the percentage of radioactivity bound relative to binding in the absence of competing peptide. Binding data were analysed by fitting a 4-parameter, symmetrical dose response curve, using the Tablecurve software program (Jandel Scientific, CA, USA).

2.3.5 Animal care and surgery

Sixteen female Sprague-Dawley rats bred in a specific pathogen free environment, were purchased from the CSIRO animal house (Adelaide, Australia). Rats were mated and first detection of sperm by vaginal smear was taken as day 1 of pregnancy. Pregnancy was monitored daily by weight measurements up until day 18 of pregnancy. On day 18 the 8 pregnant rats had a mean weight ± sem of 308 ± 10 g and the 8 non-pregnant age matched controls weighed 233 ± 5 g. The experimental protocol for the tissue distribution study was approved by the Animal Care and Ethics Committee of the CSIRO, Division of Human Nutrition, and is outlined in Fig 2.1. Non-pregnant rats were anaesthetised by an i.p. injection of a 9:1 solution of methohexitone sodium and pentobarbitone sodium (3.75 ml / kg body weight) and pregnant rats received an analgesic and pre-anaesthetic by s.c. injection of

Figure 2.1 Experimental protocol for the tissue distribution study



xylazine (5 mg / kg body weight) plus atropine sulphate (0.5 mg / kg body weight), prior to being anaesthetised with an i.m. injection of ketamine hydrochloride (45 mg / kg body weight) before insertion of a jugular catheter. After insertion, the animals were kept warm until conscious, and allowed free access to feed and water, and left to recover for two days prior to experimentation.

2.3.6 Labelled IGF peptide infusion and tissue collection

Eight pregnant rats and eight non-pregnant rats were randomly assigned to one of two peptide treatment groups. On day 18 of pregnancy, each rat was infused with an i.v. bolus (100 μl) of either ^{125}I-IGF-I or ^{125}I-LR^{3}IGF-I (10 x 10^{6} cpm) in saline and 0.01 % (w / v) BSA. The catheter line was immediately flushed with saline and then filled with an heparin / saline solution (1000 U / ml). Exactly 15 min after infusion, the rats were stunned, killed by decapitation and trunk bled. Blood was collected in tubes containing heparin and centrifuged at 1400 *g* for 10 min. The plasma supernatant was removed, immediately frozen in liquid nitrogen and placed at -20°C until subsequent analysis. In the meantime, brain, liver, spleen, adrenals, kidneys, ovaries, uterus, thymus, heart, lungs, stomach, small intestine, large intestine, caecum, pelt and gastrocnemius muscle were rapidly removed. Gut contents were removed and each organ weighed and placed on a glass slab on ice. Subsamples of each tissue were weighed, frozen in liquid nitrogen and stored at -20°C for further analysis. In addition, placenta and foetuses were removed from pregnant animals and weighed. One foetus was weighed and frozen for later analysis, while the remainder were decapitated and their blood collected and processed similarly to the adult blood.

2.3.7 Determination of intact (TCA-insoluble) and degraded (TCA-soluble) label in plasma and tissues.

Duplicate portions (25 μl) of the plasma sample from each rat and foetus was mixed with ice cold 10 % (w / v) TCA (475 μl), incubated for 1h on ice, then centrifuged at 1400 *g* for 10 min. The supernatant was removed, and the radioactivity in both the supernatant (TCA-soluble portion) and pellet (TCA-insoluble potion) determined with a gamma counter for 10 min. Subsamples of tissues and foetuses were thawed and homogenised in 10 % (w / v) TCA (1 ml). The tissue was incubated for 1 h on ice, centrifuged for 10 min at 1400 *g* at 4°C, the supernatant removed and both the supernatant and pellet radioactivity counted for 10 min with a gamma counter. These measurements were subsequently used to calculate the intact amount of labelled IGF peptide / mg of tissue.

2.3.8 Statistical analysis

Tissue distribution data were analysed by independent Student's t-test or by two-way analysis of variance with replications, where $P < 0.05$ was considered significant. Values are means ± sem for four animals per group. Statistical analyses were performed using the Sigma Stat software package (Jandel Scientific, CA, USA).

2.4 RESULTS

2.4.1 Binding of ^{125}I-IGF-I to increasing quantities of rat and human plasma

To date, all data generated in our laboratory regarding IGF-I and LR^{3}IGF-I interaction with IGFBPs had been obtained from studies utilizing IGFBPs from rats. Consequently, I wanted to compare the relative binding of ^{125}I-IGF-I and ^{125}I-LR^{3}IGF-I to rat and human

plasma IGFBPs, to determine whether differences existed which would assist in future interpretation of results. To achieve this, IGF-I and LR^{3}IGF-I were used as competing ligands for the binding of ^{125}I-IGF-I to plasma *in vitro*. Initially, the amount of each plasma required to produce a relatively constant proportion of bound to free radioligand was determined. These experiments showed, that to obtain approximately 30 % binding of ^{125}I-IGF-I to rat and human plasma, 5 and 30 µl respectively of each would be required (Fig 2.2). These amounts were subsequently used in competitive binding experiments.

2.4.2 Competitive binding experiments

When IGF-I was the competing ligand for ^{125}I-IGF-I, approximately 5 ng was able to produce half-maximal inhibition in plasma of both species (Fig 2.3). However, no competition for ^{125}I-IGF-I binding to rat or human plasma IGFBPs was observed when LR^{3}IGF-I was the competing ligand (Fig 2.3). Thus, we can conclude from these results that LR^{3}IGF-I has diminished affinities for rat and human plasma. Scatchard analysis was not performed as it could not adequately account for the poor competition obtained with LR^{3}IGF-I as the competing ligand.

2.4.3 Distribution of radioactive IGF peptides in tissues

To examine the fate of labelled IGFs when metabolic clearance rate is increased during pregnancy or following administration of LR^{3}IGF-I, the amount of ^{125}I-IGF-I and ^{125}I-LR^{3}IGF-I radioactivity in tissues 15 min after administration was determined. This data has been expressed as TCA-insoluble radioactivity / mg organ (Fig 2.4). With both tracers, radioactivity appeared in the kidneys to a greater extent than in any other organ. In confirmation of previous clearance results (Bastian *et al.*, 1993), IGF-I radioactivity was significantly higher in plasma of virgin animals compared with pregnant animals, while

Figure 2.2: Binding of ^{125}I-IGF-I to increasing quantities of rat (squares) and human (circles) plasma. Values are means ± sems of triplicate estimates from a representative experiment. Plasma concentrations are μl / tube in a 0.3 ml incubation volume.

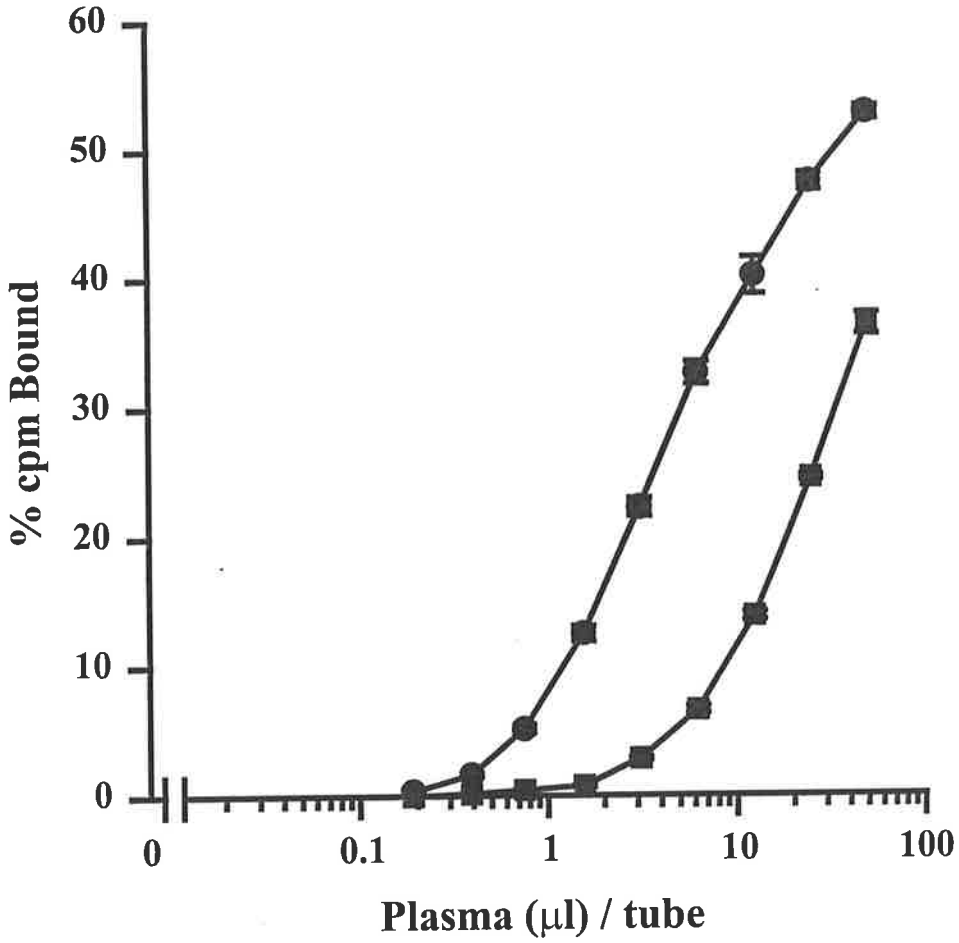

60
50
40
30
20
10
0
% cpm Bound
0
0.1
1
10
100
Plasma (μl) / tube

Figure 2.3: Competition for the binding of labelled IGF-I in rat and human plasma by unlabelled IGF-I (closed circles) and LR^3 IGF-I (open squares). Values represent the mean ± sem of triplicate experiments and are expressed as the fraction of binding in the absence of competing ligand.

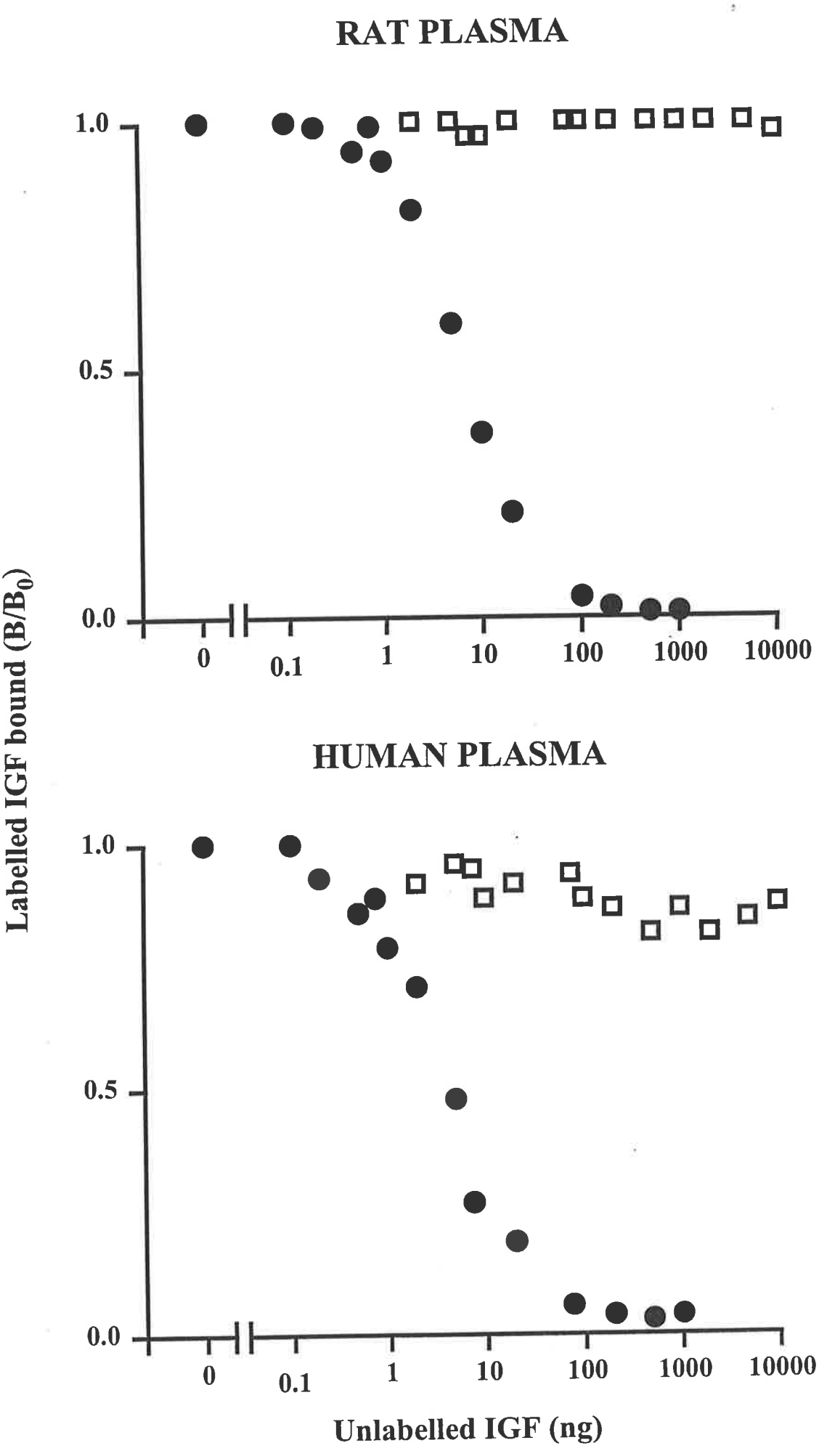
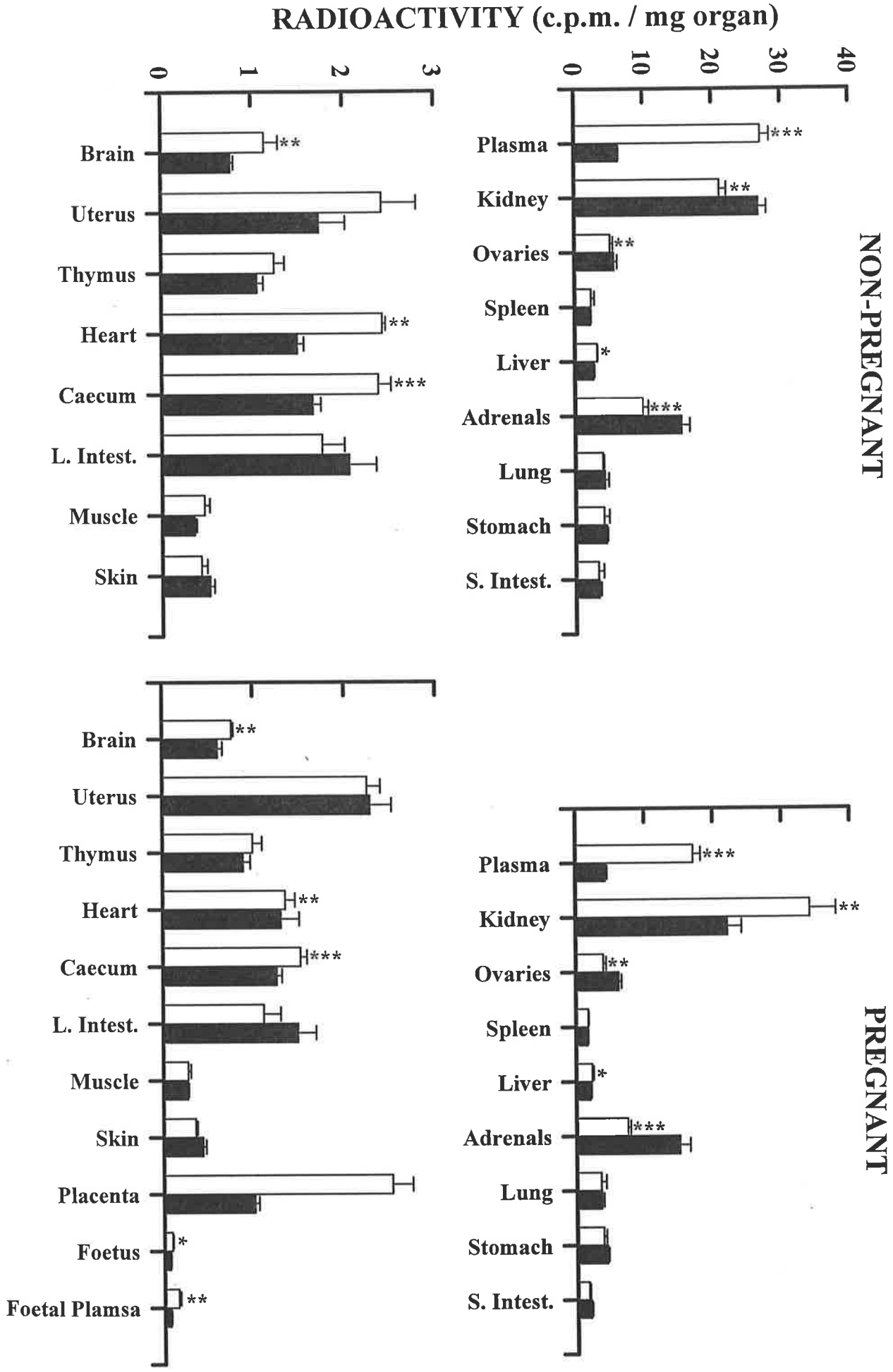

RAT PLASMA
1.0
0.5
0.0
0
0.1
1
10
100
1000
10000
HUMAN PLASMA
1.0
0.5
0.0
0
0.1
1
10
100
1000
10000
Labelled IGF bound (B/B0)
Unlabelled IGF (ng)

Figure 2.4: Uptake of ^{125}I-labelled IGF-I or LR^3 IGF-I radioactivity (c.p.m. / mg) by organs of non-pregnant and pregnant rats 15 min after administration of labelled peptide. Values are means ± sems for four animals in each group. Open bars represent radioactive IGF-I and solid bars radioactive LR^3 IGF-I. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$, for two-way analysis of variance determining significant differences between amounts of IGF-I or LR^3 IGF-I tracer in specific tissues. Independent Student's *t*-test was carried out for placenta, foetus and foetal plasma.


RADIOACTIVITY (c.p.m. / mg organ)
0
1
2
3
Brain
Uterus
Thymus
Heart
Caecum
L. Intest.
Muscle
Skin
**
**
***
0
10
20
30
40
Plasma
Kidney
Ovaries
Spleen
Liver
Adrenals
Lung
Stomach
S. Intest.
***
**
**
*
***
NON-PREGNANT
Brain
Uterus
Thymus
Heart
Caecum
L. Intest.
Muscle
Skin
Placenta
Foetus
Foetal Plamsa
**
**
***
*
**
Plasma
Kidney
Ovaries
Spleen
Liver
Adrenals
Lung
Stomach
S. Intest.
***
**
**
*
***
PREGNANT

LR^3IGF-I specific radioactivity in plasma was much lower than IGF-I in both virgin and pregnant animals ($P \leq 0.001$).

The distribution pattern of labelled IGF-I differed in pregnant relative to virgin animals, with more radioactive IGF-I in kidneys, and less in the small intestine, caecum, liver, heart, spleen and muscle ($P \leq 0.001$). LR^3IGF-I radioactivity was also lower in the small intestine, caecum, spleen and muscle of pregnant animals ($P \leq 0.001$). Significant differences also existed in amounts of radioactive IGF-I compared with LR^3IGF-I as indicated in Fig 2.4. In virgin animals, LR^3IGF-I radioactivity was higher than that of IGF-I in the kidneys, ovaries and adrenals, but lower in caecum, brain, liver and heart. In pregnant rats LR^3IGF-I radioactivity was higher than IGF-I in the ovaries and adrenals but lower in the kidneys, caecum, brain, liver, heart, placenta, foetus and foetal plasma.

2.5 DISCUSSION

Production of biologically active IGF-I fusion peptide analogues provides useful reagents for the investigation of IGF-I action. One such molecule, LR^3IGF-I, is known to bind more weakly than IGF-I to IGFBPs in rat L6 myoblast conditioned media (Francis *et al.*, 1992). The same molecule also displays increased biological potency relative to authentic IGF-I in rat L6 myoblasts, yet is less potent than IGF-I in chicken embryo fibroblasts, a cell line unable to secrete detectable IGFBPs (Francis *et al.*, 1992). These findings reinforce the conclusion that the increased biological potency of LR^3IGF-I is due to its inability to bind IGFBPs.

The current study is the first to contrast the affinities of IGF-I and LR^3IGF-I for unfractionated rat or human plasma IGFBPs. A substantial difference in affinity between IGF-I and LR^3IGF-I for rat or human plasma IGFBPs was measured, with the affinity of LR^3IGF-I being notably weaker. Previous findings have shown that IGF-I analogues in which Glu^3 is substituted by Arg^3 reduces the binding to IGF binding proteins (King *et al.*, 1992).

Furthermore, the addition of a 13 amino acid N-terminal extension, as for Long IGF-I and LR^{3}IGF-I, significantly reduces binding to IGFBPs (Francis *et al.*, 1992).

Competition assays showed that there are essentially no binding sites for LR^{3}IGF-I in both human and rat plasma. In contrast, weak competition was displayed by LR^{3}IGF-I for the binding of labelled IGF-I to rat IGFBP-3 or human IGFBP-3 (Lord *et al.*, 1994). This may indicate that the available IGFBP-3 sites in plasma may be saturated with endogenous IGF which would minimise competition by LR^{3}IGF-I. Several studies have shown that LR^{3}IGF-I is a more potent anabolic agent than IGF-I in rats (see section 1.11). This reduced affinity of LR^{3}IGF-I for the IGFBPs, may enable more 'free' LR^{3}IGF-I to act on target organs in these animals. The reduced association of LR^{3}IGF-I with human plasma IGFBPs observed in this study suggests that comparable *in vivo* human studies may produce results similar to those of rats.

As the half-life of free IGF-I has been estimated to be approximately 10 min (Davis *et al.*, 1989; Hodgkinson *et al.*, 1989a), I examined the distribution of labelled IGF-I and LR^{3}IGF-I 15 min following bolus administration into key tissues. In corroboration with other studies, the kidney, which is a major site of IGF-I degradation and excretion, contained the highest proportion of intact ^{125}I-IGF-I and ^{125}I-LR^{3}IGF-I of all tissues (Hill *et al.*, 1997; Ballard *et al.*, 1991). The pregnant rats had increased kidney intact ^{125}I-IGF-I compared with non-pregnant rats, which may account for the increased extent and rate of degradation of ^{125}I-IGF-I in these animals. In support of this finding, Hill *et al.* (1997) reported decreased kidney ^{125}I-IGF-I in rats treated with anti-IGF-I IgG, which prolongs the circulating half-life of ^{125}I-IGF-I, and reduced rates of IGF-I degradation compared to control animals. Indeed the kidney may be a specific target organ of circulating IGF-I peptides, as systemic administration of IGF-I has been shown to result in stimulated renal growth, increased glomerular filtration rate and increased renal blood flow (Glassock *et al.*, 1991; Guler *et al.*, 1989b).

Since the LR^{3}IGF-I clearance rate was higher than that of IGF-I in virgin animals, it could be assumed that more LR^{3}IGF-I would be located in tissues relative to IGF-I. In general this was not the case. For example, IGF-I radioactivity was significantly greater than that of LR^{3}IGF-I in caecum, brain, liver and heart. It is possible this effect is a result of the more rapid degradation of LR^{3}IGF-I relative to IGF-I in the tissue. In contrast, LR^{3}IGF-I selectively targeted the adrenals and ovaries. This pattern of tissue distribution between the two peptides was also observed in pregnant animals. Although the reason for the differences in the tissue specificities between IGF-I and LR^{3}IGF-I is unclear, it is plausible that the tissue translocation process may be related to the abilities of these peptides to bind to the IGFBPs. The radioactivity of both peptides was lower in the small intestine, caecum, spleen and muscle of pregnant rats compared with virgin animals, suggesting that the growth factors were diverted to other organs which are known to display remarkable growth rates in pregnant animals, namely the placenta and mammary glands.

Interestingly, less LR^{3}IGF-I radioactivity was observed in the placenta, foetus and foetal plasma compared with IGF-I at 15 min. Thus, it seems that the ability of the IGFs to bind the IGFBPs during pregnancy may actually enhance uptake by the placenta and conceptus. This could be explained by the observation that LR^{3}IGF-I binds to the type I IGF receptor with three- to fourfold lower affinity than IGF-I (Francis *et al.*, 1992). Davenport *et al.* (1990) concluded that labelled IGF-I did not cross the placenta intact since they did not detect TCA-precipitable counts in foetal serum. In our studies, 37% of the counts were TCA-precipitable in foetal plasma and 38% in whole foetuses, indicating that some labelled IGF-I crossed the placenta intact. Similar values were obtained with LR^{3}IGF-I.

Decreased association of labelled IGF-I with the 150 kDa IGFBP complex might be of physiological importance during pregnancy. It is possible that association with only the lower molecular weight IGFBPs may help target IGF-I from the vascular compartment to the tissues (Binoux and Hossenlopp, 1988). Studies by Gargosky *et al.* (1991) found that administration

of IGF-I to pregnant rats stimulates maternal weight gain, but has no effect on foetal growth, suggesting that the reduction in IGFBP-3 affinity for IGFs may provide "free" growth factor to allow adaptation by the mother.

In summary, the present study has allowed us to understand how IGF-I and LR^3IGF-I interact with rat and human plasma IGFBPs *in vitro*. Furthermore, an *in vivo* model was utilised in which IGFBP affinity for IGF-I was greatly reduced, with the intention of using such a model to investigate the uptake of exogenously administered IGF tracers by various organs. Specifically, this has given us a more precise idea about tissue targeting by these peptides. Although IGF-I and LR^3IGF-I are capable of leaving the systemic circulation to appear in various organs intact, the mechanism of how IGFs might traverse the endothelial barrier is not known. While blood binding proteins alter overall clearance of IGF-I, it is not known whether they increase, decrease or have any effect on the delivery of IGF-I from blood to its sites of action at cell receptors. The next chapter outlines the development and validation of an *in vitro* model of an endothelial cell barrier, which will be utilised to determine the transcellular pathway of IGF-I across the vascular wall and the effects of IGFBPs on this transport.

CHAPTER: 3 DEVELOPMENT OF AN *IN VITRO* ENDOTHELIAL CELL MONOLAYER MODEL FOR THE STUDY OF IGF-I TRANSPORT FROM BLOOD TO EXTRACELLULAR SITES.

3.1 INTRODUCTION

IGF-I is thought to act in both a paracrine / autocrine and endocrine manner (Green *et al.*, 1985). For IGF-I to exert its action in an endocrine fashion, it must first leave the blood by traversing the endothelial cell barrier. Results of chapter 2 confirmed earlier studies suggesting that circulating IGF-I is distributed outside the vascular space into peripheral tissues (Hill *et al.*,1997; Ballard *et al.*, 1991; Cascieri *et al.*, 1988). However, the mechanism by which IGF-I traverses the endothelial cell barrier at present has not been elucidated. In this chapter, a well characterised *in vitro* model of the blood vessel wall is described and validated as a means to examine the transendothelial migration of IGF-I peptides.

The vascular endothelium is a cell monolayer lining the blood vessels, and represents an active interface between blood and tissue. Among the important functions of the vascular endothelium is the control of the exchange of fluid, macromolecules and cells between the blood and the extravascular tissue beds. Over the past two decades, considerable effort has gone into the development of techniques which allow isolation and culture of intact endothelial monolayers from human tissue. Human umbilical vein endothelial (HUVE) cells have been the most common human endothelial cells isolated, mainly because of the ease with which the umbilici may be obtained. As a result numerous studies have been conducted with a model blood vessel wall, consisting of a monolayer of HUVE cells cultured on permeable filters (Larson and Sheridan, 1982; Casnocha *et al.* 1989; Garcia *et al.*, 1993 and Westendorp *et al.*, 1994). Endothelial cells grown on filters permit use of radiolabelled tracers, facilitating

the study of barrier function concurrently with the morphological appearance of the same endothelial layer.

The methods for isolation, growth and maintenance of HUVE cells and the subsequent use of primary cultures of these cells in transport studies required considerable development. The aim of this chapter is to describe the development of methodologies to study the transport pathway of IGF-I across an endothelial cell barrier. To achieve this, a number of general parameters had to be investigated and achieved, in particular; (i) successful isolation of HUVE cells from human umbilical veins and their positive identification as endothelial cells, (ii) determination of the most appropriate growth media and growth factor supplements for growth, maintenance and experimentation, (iii) determination of the optimal substratum and the most appropriate passage number of cells for use in experiments, (iv) development of an appropriate serum-free media, (v) examination of the best method to monitor the integrity of HUVE cell monolayers grown on filters and finally, (vi) examination of the ability of the HUVE cell monolayer model to transport test molecules.

3.2 MATERIALS AND METHODS

3.2.1 Materials

RPMI 1640 was purchased from Gibco Laboratories (Grand Island, USA). HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]), EDTA (ethylenediamine tetraacetic acid), TCA (trichloroacetic acid), sodium chloride, potassium chloride, potassium dihydrogen orthophosphate, disodium hydrogen orthophosphate, disodium tetraborate and Dulbecco's phosophate buffered saline (PBS) without calcium or magnesium, glycerin, methanol and ethanol came from BDH Chemicals (Victoria, Australia). Collagenase Type I was from Worthington Biochemicals Corporation (Freehold, USA). Hanks' balanced salts

solution (HBSS) was from ICN Biomedicals (Costa Mesa, USA). Syringes and three way taps were from Terumo (Melbourne, Australia) and conical centrifuge tubes from Beckton Dickson Labware (Lincoln Park, USA). M199 with Earle's salts, sodium pyruvate, L-glutamine, non-essential amino acids and foetal bovine serum (FBS) were all from Cytosystems (Castle Hill, Australia). Penicillin, trypsin and Fraction V bovine serum albumin (BSA) were obtained from Commonwealth Serum Laboratories (Parkville, Australia). BSA was iodinated with carrier free $Na^{125}I$ from Amersham (Buckinghamshire, UK) to a specific activity of 60 μCi / μg, using a modification of the chloramine T method (Gargosky *et al.*, 1990b). Streptomycin was purchased from Jurox (Silverwater, Australia). All tissue culture flasks, dishes and Transwells (6.5 mm in diameter and of 0.4 μm pore size) were from Costar (Cambridge, USA). Gelatin was from Eastman Fine Chemicals (Rochester, USA). Human fibronectin was obtained from Boehringer Mannheim (Mannheim, Germany). L6 rat myoblasts (CRL 1458, passage 13) were from American Type Culture Collection (Maryland, USA). Dulbecco's modified Eagle's essential medium (DMEM) was purchased from Flow Laboratories (Irvine, Scotland). Endothelial cell growth supplement (ECGS), primary rabbit anti-human Factor VIII related antigen (von Willebrand Factor), goat anti-rabbit IgG (whole molecule) - fluorescein isothiocyanate conjugate - (FITC), biotinylated goat anti-rabbit IgG (whole molecule), 3,3'-diaminobenzidine tetrahydrochloride (DAB), Harris haematoxylin solution, heparin, sodium chloride, sodium bicarbonate and methylene blue were from Sigma (St Louis, USA). Normal horse serum, and the Vectastain Elite ABC (avidin-biotinylated horseradish peroxidase complex) were from Vector (Burlingame, USA). Recombinant human basic fibroblast growth factor (bFGF) was from Austral Biologicals (San Ramon, USA). The Millicell-ERS (electrical resistance system) voltohmmeter was purchased from Millipore Corporation (Bedford, USA).

3.2.2 Isolation of human umbilical vein endothelial cells

HUVE cells were isolated from the umbilical vein of 12 -24 h old umbilical cords by collagenase digestion using an adaptation (Smith *et al.*, 1991), of the methods of Jaffe *et al.* (1973) and Gimbrone *et al.* (1974). Once severed from the placenta just after birth, the cord was stored at 4°C in a sterile container. A sterile technique was used for all subsequent manipulations of the cord. The cord was wiped of blood, then placed in a glass petri dish containing RPMI 1640 plus 10 mM HEPES (cord buffer). The umbilical vein was cannulated using a metal adaptor with a luer lock, which was secured with string. A three way tap was inserted into the metal adaptor and using a 20 ml syringe, the vein washed of blood 2-3 times using cord buffer. Another metal adaptor, 3-way tap and 20 ml syringe were attached to the other end of the vein. With both taps open, the vein was filled with 0.2 % collagenase Type I in HBSS containing calcium and magnesium from one end, and the first 2 ml collected and removed from the opposite end to remove collagenase solution diluted by residual cord buffer. Air bubbles were removed from the vein, and the cord incubated in a beaker containing 0.9 % NaCl solution, in a water bath at 37°C for 12 min. Incubation time varied between 10 -15 min depending on the batch of collagenase used. Following incubation, the collagenase solution containing endothelial cells was chased from the cord by perfusion with 20 ml of cord buffer into two sterile 50 ml conical centrifuge tubes, leaving approximately 5 ml within the vein. The cord was gently massaged, and washed twice more with 20 ml of cord buffer, collecting the effluent into the 50 ml conical tubes after each wash. The cells were pelleted at 250 g for 5 min at room temperature. Cell pellets in each 50 ml tube were resuspended in HUVE wash (M199 with Earle's salts containing 2 % FBS and 20 mM HEPES pH 7) and pooled. Cells were pelleted again, resuspended in fresh HUVE wash, pelleted and resuspended in 10 ml of HUVE medium (M199 with Earle's salts containing 20 % FBS, 20 mM HEPES, 3 % sodium bicarbonate, 2 mM L-glutamine and non-essential amino acids, 1 mM sodium pyruvate, 200 μg / ml penicillin and 200 μg / ml streptomycin, pH 7). Endothelial cell suspension was

equally distributed between two sterile gelatin coated T25 tissue culture flasks and incubated at 37°C under 5 % CO_2. The following day, the cells were washed with HUVE medium to remove any debris or red blood cells, and fed with a fresh change of HUVE medium, and then every second day until they were confluent. Initially, cells obtained from the cords appeared as either single cells or clusters of cells which rapidly attached. The cells grew out from small confluent aggregates and were confluent between 2-7 days. Flasks of cells not confluent by 7 days were discarded.

To subculture HUVE cells, confluent flasks of primary cultures were washed with Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium, and harvested with a brief exposure, approximately 60 sec to 1.25 % trypsin / 0.5 mM EDTA. Detached cells (usually > 95 % of original cells) were suspended in fresh HUVE medium, split 1:2 and finally plated in gelatin-coated T80 tissue culture flasks. The low split ratio was necessary as HUVE cells only grow at high cell seed densities (Maciag *et al*,. 1984a). All tissue culture flasks were gelatin-coated by adding 3 ml of 0.2 % gelatin in HBSS containing 3 % FBS for 5 min. The gelatin was removed just prior to using the flasks.

3.2.3 Identification of HUVE cells as endothelial cells by light microscopy

For identification studies, HUVE cells were seeded on T-80 flasks, coated with human fibronectin (50 μg / ml) in sterile deionised water for 30 min at room temperature. Cells were grown in HUVE medium plus 20 % FBS containing 25 μg / ml of both ECGS and heparin at 37 °C under 5 % CO_2 until confluent. Confluent flasks of HUVE cells were viewed by light microscopy and photographed.

3.2.4 Detection of factor VIII related antigen in primary HUVE cell cultures

HUVE cells were assayed for the presence of Factor VIII antigen, which was detected either by indirect immunofluorescent staining, using modifications of the method of Hyroyoshi and Mc Keehan (1984) or indirect immunoperoxidase staining. Primary cultures of HUVE cells and passage 13 L6 rat myoblasts were grown on 19 mm round glass coverslips, in fibronectin-coated plastic 12 well multiplace plates. HUVE cells were grown in HUVE medium (see section 3.2.2) and the L6 myoblasts were grown in DMEM containing 10 % FBS (pH 7.2) until confluent. The cells were washed four times for 5 min in PBS pH 7.2, fixed for 10 min at 4°C with cold methanol, washed three times with PBS and non-specific binding sites blocked by incubating the cells with 10 % normal horse serum (NHS) in PBS at room temperature for 20 min. The coverslips were removed and glued to glass slides before being incubated with either a 1:1000 dilution of primary rabbit anti-human Factor VIII related antigen in PBS and 1 % NHS or a 1:1000 dilution of normal rabbit serum in PBS and 1 % NHS for 12 h at 4°C. Following three additional washes in PBS, immunofluorescent detection was achieved by incubating the cells with a 1:200 dilution of FITC-labelled goat anti-rabbit IgG in PBS for 35 min at 37°C. The slides were washed five times in PBS before being mounted in glycerin and photographed using a Leitz Wetzlar orthoplan fluorescence microscope. For the immunoperoxidase detection method, following incubation with the primary antibody and three washes in PBS, the cells were incubated with a 1:200 dilution of biotinylated goat anti-rabbit IgG in PBS for 30 min at room temperature. The cells were washed three times in PBS before being incubated with avidin-biotinylated horseradish peroxidase complex in PBS for 30 min. Finally, the cells were incubated with the peroxidase substrate, DAB for 30 min, counterstained in 1:4 Harris haematoxylin, then mounted and viewed by light microscopy.

3.2.5 Determination of serum-free media culture conditions

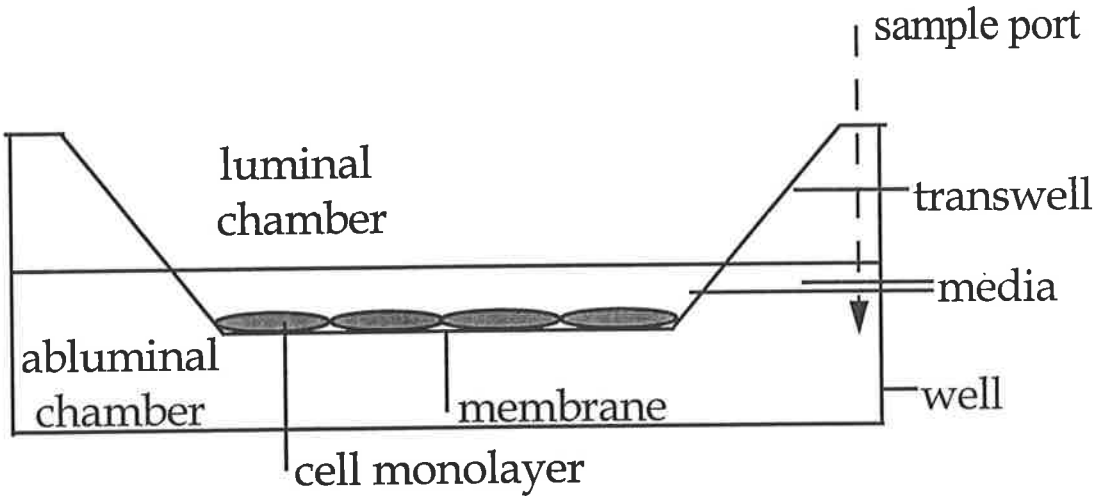
To define serum-free media conditions for HUVE cells, confluent monolayers of HUVE cells grown in fibronectin-coated 96 well multiwell plates were maintained in either HUVE medium without FBS or growth factors or in HUVE medium without FBS but containing 25 μg / ml heparin plus recombinant human bFGF at either 1, 2, 6, 12, 25, 50 or 100 ng / ml for three days with daily changes of media and growth factors. The morphology of these monolayers was compared to that of monolayers that had been maintained in HUVE medium supplemented with 20 % FBS, ECGS and heparin.

3.2.6 Monitoring the integrity of confluent HUVE cell monolayers on Transwell filters

3.2.6.1 Visualisation by light microscopy

A schematic diagram of the Transwell apparatus is shown in Fig. 3.1. Several preliminary experiments were conducted to determine optimal growth conditions for HUVE cells cultured on Transwells. HUVE cells were seeded at 20 x 10^3 cells / well on fibronectin-coated Transwells, and grown in HUVE medium supplemented with 20 % FBS plus 25 μg / ml ECGS and heparin. Daily media and growth factor changes were required to support the culture of HUVE cell monolayers on Transwells. Experiments were conducted three days post-seeding because after this the integrity of the monolayers began to regress. To view confluent monolayers of HUVE cells on Transwells by light microscopy, growth media was removed and the cells washed twice in 0.15M NaCl, fixed in methanol for 15 min and stained with 1 % methylene blue in 0.01M disodium tetraborate buffer for 15 min. Excess stain was removed by washing the cells five times with disodium tetraborate buffer. Monolayers were then visualised under a Wild M32 dissecting microscope (Heerbrugg, Switzerland) and photographed.

Figure 3.1: Schematic diagram of the Transwell apparatus. Placement of the Transwell in the well of a 24 place multi-well plate, results in the formation of a luminal and abluminal chamber. The cells are seeded on the 0.4 μm polycarbonate filter membrane. Samples may be taken directly from the luminal chamber or from the abluminal chamber via the sample ports at the top of the Transwell.


sample port
luminal
chamber
transwell
media
abluminal
chamber
membrane
well
cell monolayer

3.2.6.2 Visualisation by scanning and transmission electron microscopy

Three day post-seeding, confluent HUVE cell monolayers on Transwell filters were excised from the Transwell apparatus and fixed for 6h in 4 % paraformaldehyde / 1.25 % glutaraldehyde in PBS containing 4 % sucrose, pH 7.2, washed twice for 30 min in PBS containing 4 % sucrose, then post-fixed in 1 % osmium tetraoxide in PBS for 1-2 h. Monolayers were dehydrated with two 30 min washes in a series of graded ethanol solutions, ranging from 70 - 100 % ethanol. For transmission electron microscopy, monolayers were left for 12 h in 50 % ethanol / 50 % Spur's low viscosity embedding resin, then underwent three 8 h changes in 100 % resin. The monolayer was embedded in resin and polymerised in an oven at 60 °C. Sections of 80 - 90 nm (silver / pale gold) were cut on an Ultracut E ultramicrotome (Leica, Austria) then viewed by TEM (Phillips, CM100).

To prepare HUVE cell monolayers for scanning electron microscopy, monolayers on Transwell filters were excised from the chamber and fixed, washed, post-fixed and dehydrated as described for transmission electron microscopy. Following the final dehydration step in 100 % ethanol, cells were immersed in a solution of 50 % ethanol and 50 % Peldri II for 1h, then in 100 % Peldri II for 1h at 27°C. The Peldri II solution was allowed to cool and solidify, then was removed by evaporation for 3h in a vacuum desiccator. Finally, the sample was coated with gold / palladium and viewed (Phillips, XL20 SEM).

3.2.6.3 Measurement of transendothelial electrical resistance

HUVE cells were plated on Transwells as described (see section 3.2.6.1). Three fibronectin-coated Transwells which had not been seeded with cells were used as blank controls. Electrical resistance was measured using the Millicell-ERS voltohmmeter. The electrodes were sterilised in 70 % ethanol for 15 min, air dried and then equilibrated in HUVE medium plus 20 % FBS for 15 min. The voltage was checked for drift, and the electrical

resistance of the blank control Transwells measured, by placing one electrode in the luminal (upper) chamber and the other electrode in the abluminal (lower) chamber. The voltage drift was corrected for again and the electrical resistance of the HUVE cell monolayers measured. The voltage drift was monitored again before taking a second measurement of the blank control Transwells. To calculate the transendothelial electrical resistance of HUVE cell monolayers, the mean electrical resistance value of the blank Transwell filters was subtracted from the electrical resistance value of each HUVE cell monolayer. This value was corrected for the effective area of the Transwell filter (0.33 cm^2). Values were expressed as $\Omega.cm^2$.

3.2.7 Transendothelial migration of bovine serum albumin using the HUVE cell monolayer model

Fibronectin-coated Transwells were seeded with 20 x 10^3 cells / well in HUVE medium, with 25 µg / ml of both ECGS and heparin and cultured for three days at 37° C, 5 % CO_2. HUVE medium, ECGS and heparin were replaced daily, and prior to each experiment, control Transwells were fixed with methanol, stained with 1 % methylene blue in 0.01M di-sodium tetraborate and visualised by light microscopy to monitor confluency (see section 3.2.6.1). Other control Transwells were fixed for 6 h in phosphate buffered saline containing 4 % paraformaldehyde, 1.25 % gluteraldehyde plus 4 % sucrose (pH 7.2) and processed for electron microscopy (see section 3.2.6.2).

To avoid any interference of hydrostatic pressure differences across the monolayer, during transport experiments, the luminal (upper) chamber contained 150 µl and the abluminal (lower) chamber 700 µl of HUVE medium, as recommended by the manufacturers. Prior to each experiment, both the cell monolayers and the wells of the 24 well plate that the experiment would be performed in were washed three times with serum-free HUVE medium plus 0.1 % BSA to reduce non-specific binding of radiolabelled BSA tracer to the tissue culture plastic. The Transwell was then transferred to the pre-washed well containing 700 µl

of fresh serum-free HUVE medium plus 100 ng / ml bFGF and 25 μg / ml heparin (see section 3.2.5). The medium from the luminal chamber was removed and 150 μl of serum-free HUVE medium plus bFGF (100 ng / ml), 25 μg / ml heparin and ^{125}I-BSA (1 ng) was added. Following a 5 min incubation at 37° C, 5 % CO_2, the abluminal chamber was triturated, an aliquot (100 μl) collected and the chamber immediately replenished with the same amount of fresh media. Further aliquots were taken at 30 min, 1 h and 2 h. At 2 h, each Transwell was fixed, stained for light microscopy and the confluency of the monolayer examined (see section 3.2.6.1).

^{125}I-BSA was measured as trichloroacetic acid (TCA)-insoluble radioactivity. Portions of medium from the abluminal chamber at the various time points, the luminal chamber at 2 h, as well as tracer mix added to the luminal chamber at time zero were each mixed with ice-cold TCA to a final concentration of 10 % TCA, incubated for 30 min at 4° C and centrifuged at 3500 g. The radioactivity in both the TCA-soluble and TCA-insoluble portions was measured. Transendothelial migration of intact ^{125}I-BSA was expressed as a percentage of total intact ^{125}I-BSA added to the luminal chamber at time zero. Transendothelial migration of ^{125}I-BSA was also carried out in control Transwells not seeded with HUVE cells.

3.2.8 Statistical analyses

Transmigration data were analysed by one-way analysis of variance with all pairwise multiple comparisons using Student-Newman-Keuls method. $P < 0.05$ was considered significant. All statistical analyses were performed using SigmaStat statistical software from Jandel Scientific (San Rafael, CA).

3.3 RESULTS

3.3.1 Isolation and maintenance of primary HUVE cell cultures

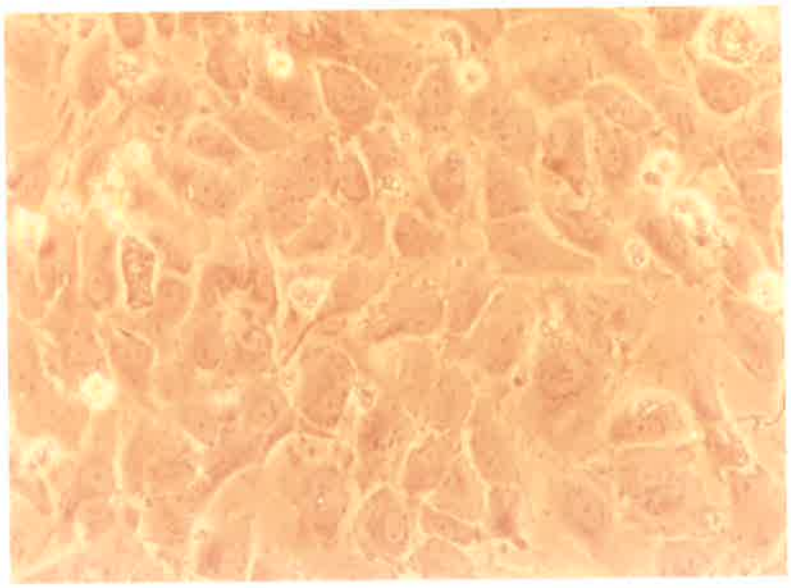
HUVE cells were isolated from 12 - 24 h old human umbilical cord veins by collagenase digestion and maintained in HUVE medium (see section 3.2.2). Initially, cells obtained from the cords appeared as either single elongated cells or clusters of cells, typical of subconfluent primary isolates of HUVE cells, which rapidly attached to tissue culture flasks (Jaffe, 1984). The cells grew out from these small aggregates and completely covered the flasks by 2 - 7 days, at which time they produced a single monolayer of closely packed, uniform epithelioid cells exhibiting polygonal morphology typical of HUVE cells at confluence (Gimbrone *et al.*, 1974). Following isolation, both primary cultures and the subsequent subcultures were maintained in HUVE media supplemented with 20 % FBS (Fig. 3.2A). FBS levels as low as 10 % and serum-free media produced HUVE cell monolayers with endothelial cells that became elongated and detached from the tissue culture plate (Fig. 3.2B and C).

3.3.2 Positive identification of isolated HUVE cells as endothelial

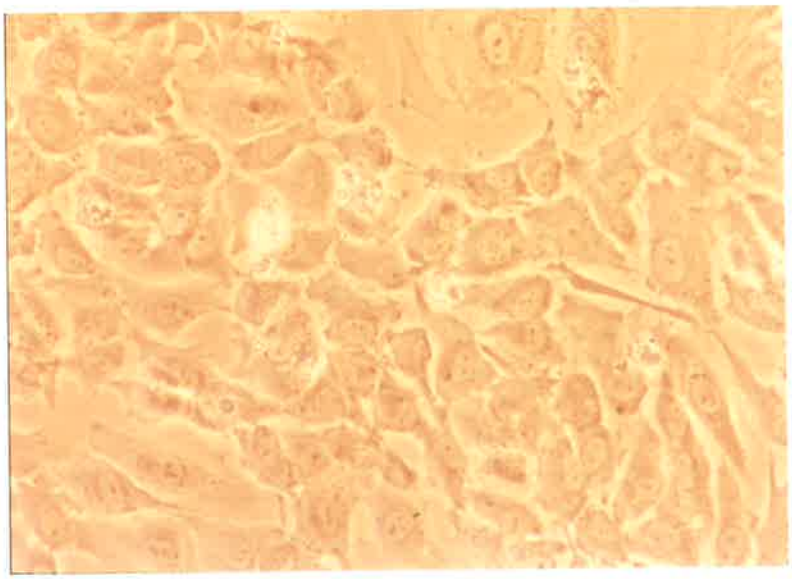
Isolated cells were positively identified as endothelial cells by two methods; (i) by displaying 'cobblestone' morphology and (ii) by the presence of Factor VIII related antigen. Under optimal conditions in HUVE medium supplemented with 20 % FBS plus 25 μg / ml of both ECGS and heparin, on gelatin or human fibronectin coated plastic, monolayers of passage 3 up to passage 6 HUVE cells grew with a characteristic "cobblestone" morphology (Fig. 3.3). Further to this, indirect antibody immunoassays for human Factor VIII related antigen resulted in perinuclear, cytoplasmic immunofluorescent staining of HUVE cells (Fig 3.4A). Immunofluorescent staining was not observed with HUVE cells incubated with

Figure 3.2: Light microscopy of HUVE cells grown in 96 well multi-place plates. HUVE cells were seeded in fibronectin-coated wells in HUVE medium containing 25 μg / ml of both ECGS and heparin supplemented with either 20 % FBS (A) or 10 % FBS (B) or serum-free HUVE medium (C). The media was changed daily. On day 3 post-seeding, the cells were viewed by light microscopy (x 250) and photographed.

A



B



C

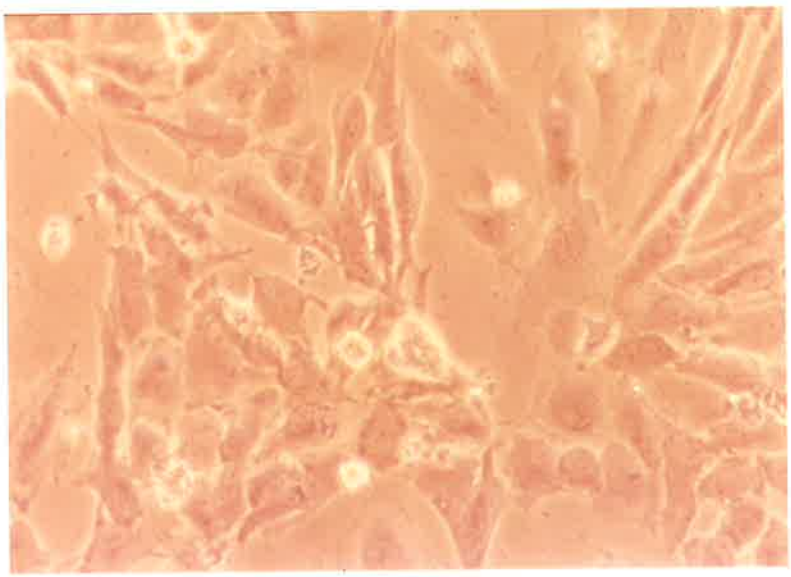


Figure 3.3: Light microscopy of HUVE cells grown in fibronectin-coated tissue culture flasks. Confluent monolayers of HUVE cells grown in HUVE medium supplemented with 20 % FBS, ECGS and heparin displayed normal "cobblestone" morphology.

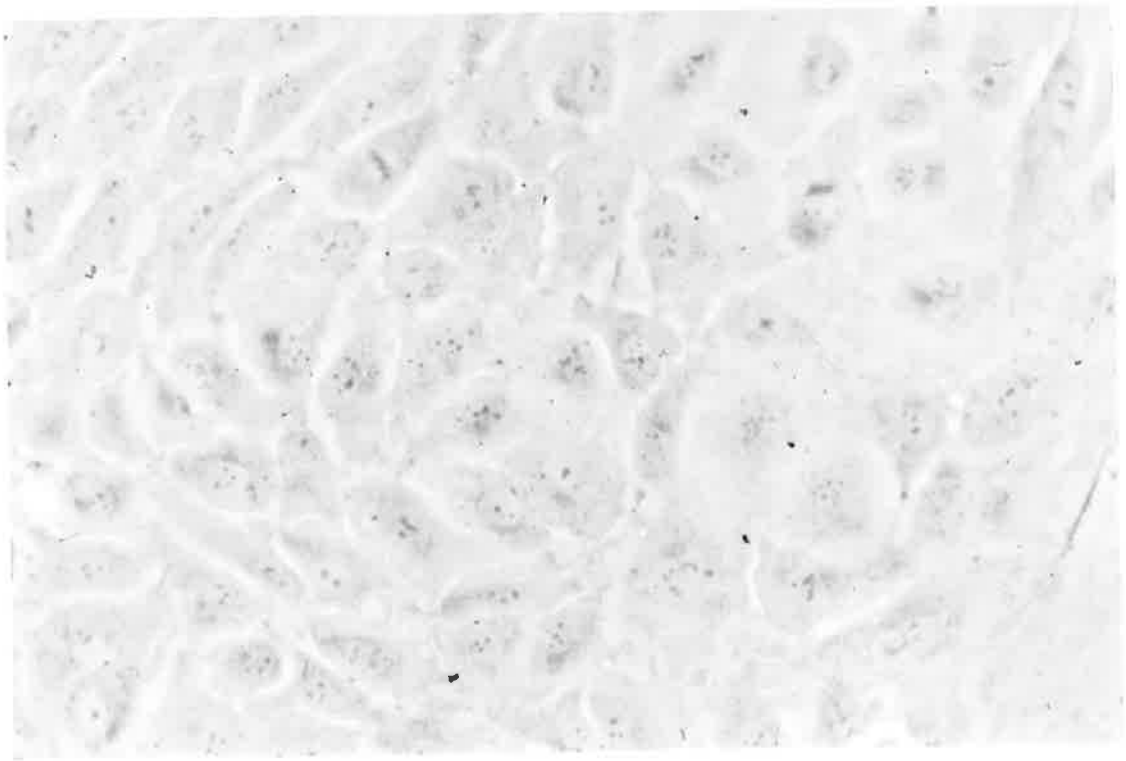
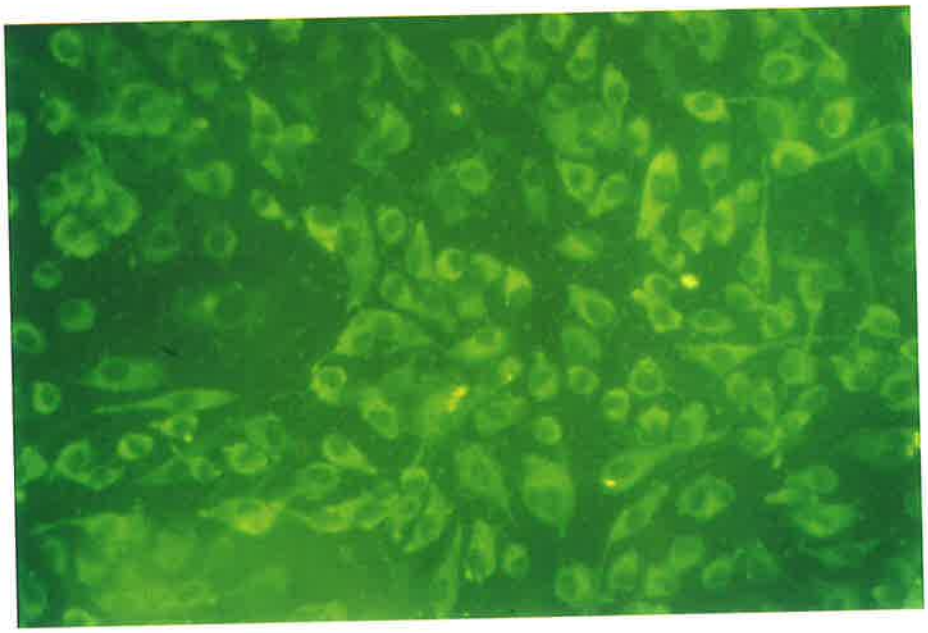


Figure 3.4: Presence of Factor VIII related antigen in HUVE cells. HUVE cells and L6 rat myoblasts were grown on microscope slide coverslips, fixed and incubated with primary rabbit anti-human Factor VIII related antigen or normal rabbit serum, followed by incubation with fluorescein-conjugated goat anti-rabbit IgG. HUVE cells incubated with anti-Factor VIII (A), HUVE cells incubated with normal rabbit serum (B) and L6 rat myoblasts incubated with anti-Factor VIII (C) were viewed by fluorescence microscopy (x 400) and photographed.

A



B

C

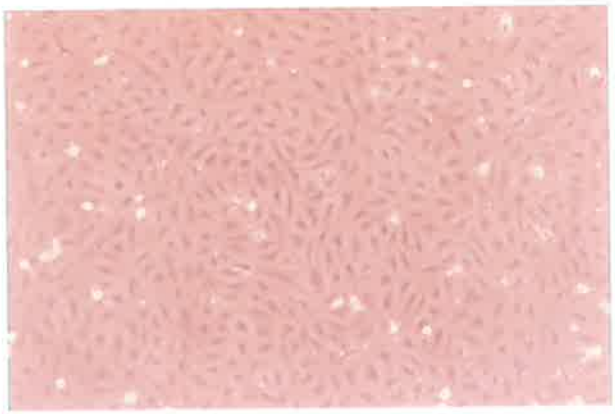
normal rabbit serum, nor for the L6 rat myoblasts incubated with the primary anti-human Factor VIII related antigen (Fig. 3.4B and C). This positively confirmed that the cells isolated from human umbilical veins were endothelial. Similar data were obtained using the immunperoxidase detection system.

3.3.3 Culture of HUVE cells in serum-free media

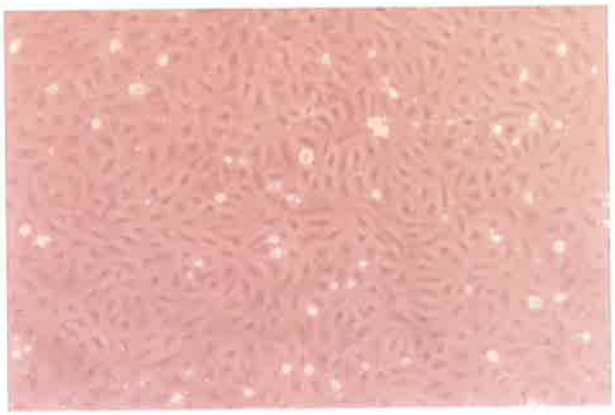
Serum is known to contain IGFs and IGFBPs. To study the transendothelial migration of IGF-I across HUVE cell monolayers, experiments would have to be conducted in the absence of serum due to the possible interference of serum IGFs and IGFBPs on this transport. As a result, a serum-free growth media for the HUVE cells was formulated. The results of these studies are visually demonstrated in Fig. 3.5. HUVE cells grew poorly in serum-free HUVE media (Fig. 3.2C) compared to cells cultured in HUVE medium supplemented with 20 % FBS, ECGS and heparin (Fig. 3.2A and Fig. 3.3). Although addition of bFGF and heparin maintained healthy cultures of HUVE cells, less cell death was apparent at the two highest concentrations (Fig. 3.5C and D). HUVE cells cultured in serum-free HUVE medium with 100 ng / ml bFGF and 25 μg / ml of heparin exhibited the same "cobblestone" morphology as seen with 20 % FBS, ECGS and heparin supplemented cultures, enabled HUVE cells to express Factor VIII related antigen, was able to maintain a monolayer with tight junctions on Transwells when viewed by SEM and TEM, and could successfully maintain primary isolates of HUVE cells along with subsequent passages as well as HUVE medium supplemented with 20 % FBS and growth factors (results not shown).

Figure 3.5: Culture of HUVE cells in serum-free HUVE medium supplemented with bFGF and heparin. HUVE cells were cultured in fibronectin-coated wells of 96 place multi-well plates, and maintained for three days with daily media changes in serum-free HUVE medium containing either 1 (A), 12 (B), 50 (C), or 100 (D) ng / ml recombinant human bFGF plus 25 μg / ml of heparin.

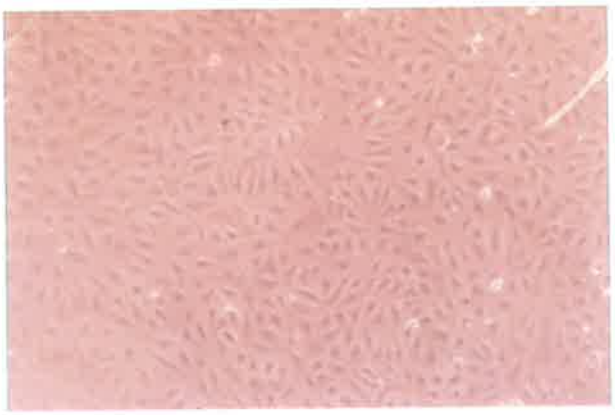
A



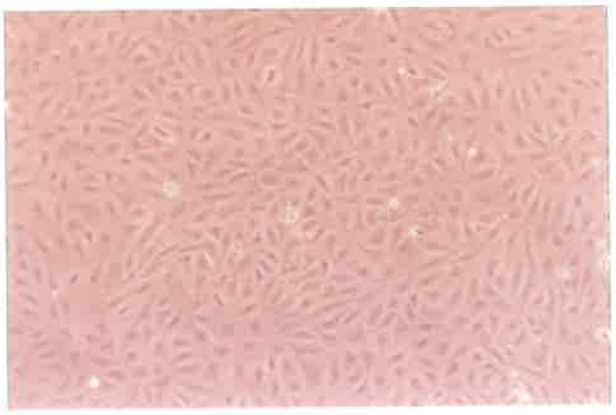
B



C



D



3.3.4 Confirmation of the integrity of HUVE cell monolayers on Transwells by light and electron microscopy

HUVE cells were plated on Transwells as described in section 3.2.6.1. At day 4 post-seeding at 20 x 10^3 cells per Transwell, it was consistently found that the monolayers began to regress. Thus, all experiments involving the HUVE cell monolayers on Transwells were carried out on day 3 post-seeding. Since cells grown on Transwell filters can not be visualised directly as the Transwell filter is opaque, the integrity of HUVE cell monolayers was monitored by staining and light microscopy and by both transmission and scanning electron microscopy. Each method confirmed that a monolayer of closely opposed HUVE cells had grown on the Transwell filter (Fig. 3.6 A, B and C). Transmission electron microscopy also indicated the presence of tight junctions between adjacent cells (Fig. 3.7, tight junction indicated by an arrow).

3.3.5 Transendothelial electrical resistance of HUVE cell monolayers

Transendothelial electrical resistance values of HUVE cell monolayers grown on Transwells were measured for 5 days after seeding (Fig. 3.8). On days 1 and 2 post-seeding, it was consistently shown that HUVE cell monolayers did not generate resistance values greater than those obtained with fibronectin-coated Transwell filters. On day 3 post-seeding, a peak electrical resistance was reached with a mean value of 3.8 ± 0.1 $\Omega.cm^2$ (n=9 from 3 separate experiments). However, this value was not significantly greater than the values obtained for filters alone. On day 4 and 5 post-seeding the resistance values decreased and were again no greater than those obtained with Transwell filters alone.

Figure 3.6: Light microscopy and transmission and scanning electron microscopy of HUVE cell monolayers cultured on Transwell inserts. HUVE cells were seeded on fibronectin-coated Transwells in HUVE medium plus 20 % FBS, ECGS and heparin and the media changed daily. On day 3 post-seeding Transwells, the cells were appropriately fixed and stained for either light microscopy (x 400) (A), transmission electron microscopy (x 2200) (B) and scanning electron microscopy (x 336) (C).

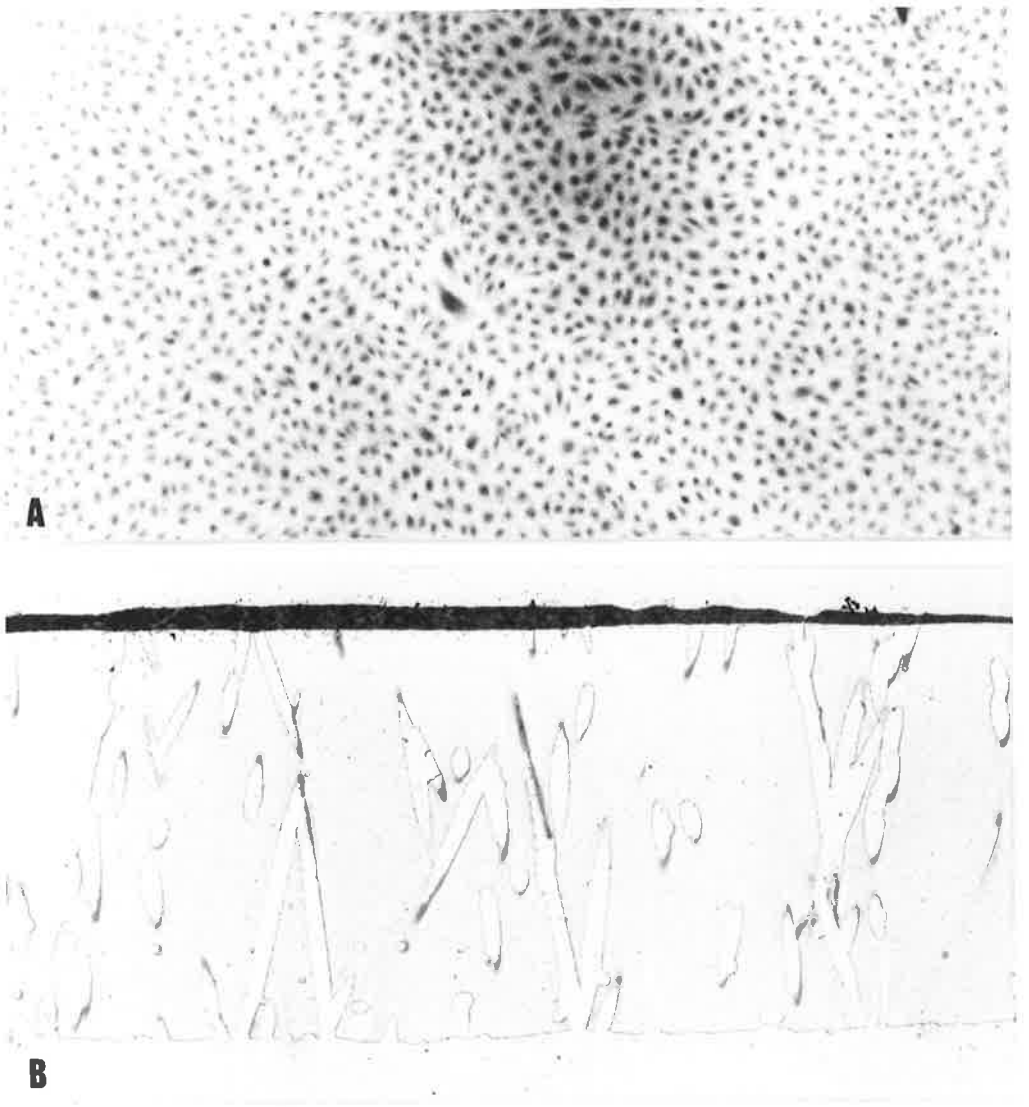

A
B
5 µm
C
100 µm

Figure 3.7: Transmission electron microscopy of confluent monolayers of HUVE cells cultured on Transwells displaying a tight junction between adjacent cells (indicated by an arrow x 28 500, f = filter of Transwell).

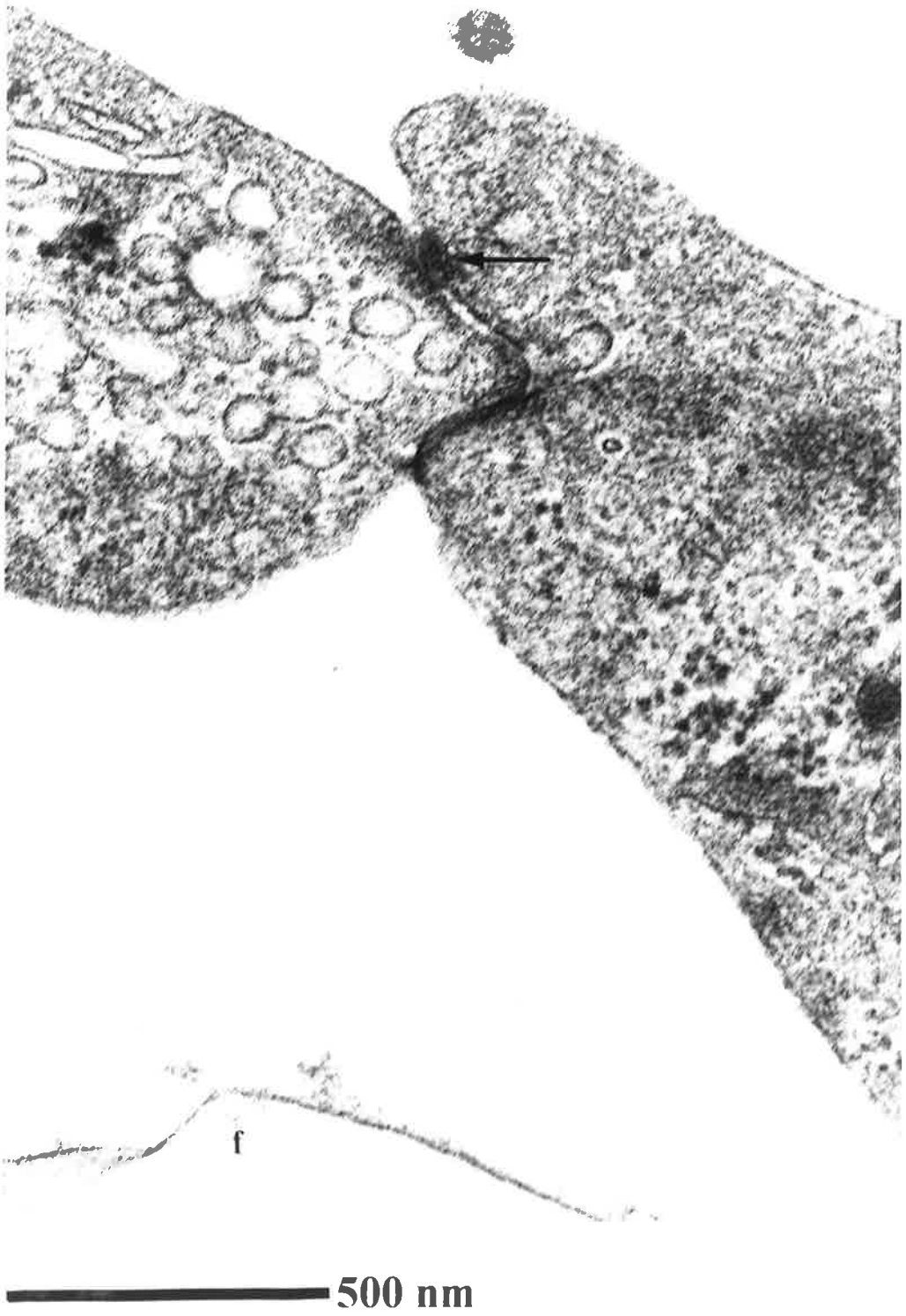
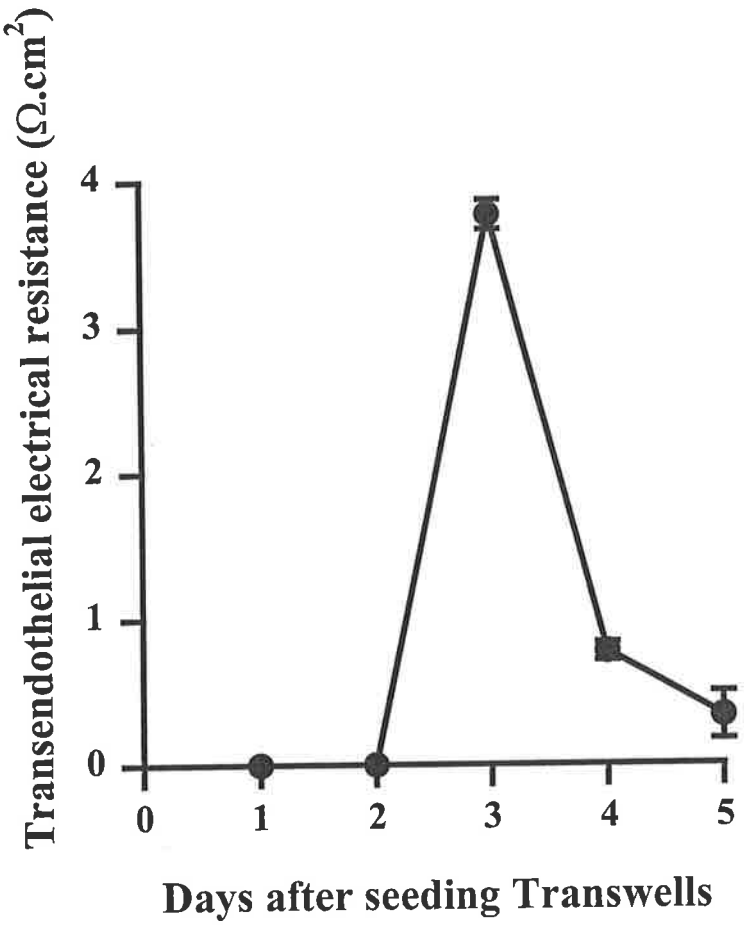

f
500 nm

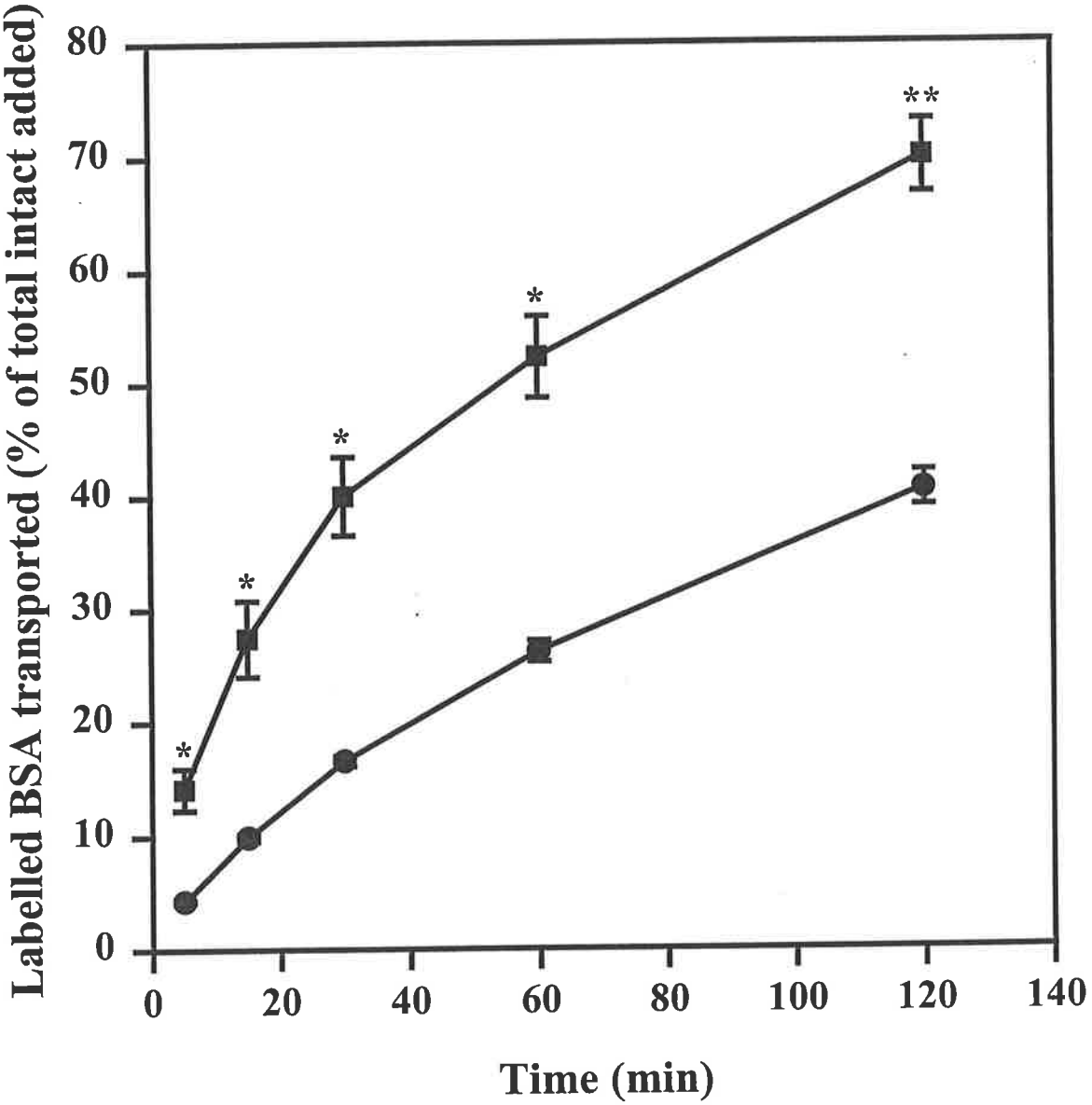
Figure 3.8: Transendothelial electrical resistance measurements of HUVE cell monolayers. HUVE cells were seeded on Transwells in HUVE medium plus 20 % FBS, ECGS and heparin at 20 x 10^3 cells per well. Electrical resistance values were measured every 24 h. Each value represents the mean ± sem of 9 Transwells.


Transendothelial electrical resistance (Ω.cm²)
4
3
2
1
0
0
1
2
3
4
5
Days after seeding Transwells

3.3.6 Transendothelial transport of ^{125}I-bovine serum albumin across HUVE cell monolayers

To test the validity of the HUVE cell monolayer model, its ability to transport albumin, a protein which is known to be transported by other endothelial cells (Antohe *et al.*, 1993), was examined. HUVE cell monolayers grown on Transwell filters were exposed to ^{125}I-BSA at the luminal surface at 37° C. Analysis of the aliquots collected from the abluminal chambers at the different time intervals revealed that the radiolabelled ligand appeared in the abluminal compartment in concentrations that increased with time (Fig. 3.9). At the 5 min time point, 4.3 ± 0.1 % of intact ^{125}I-BSA was transported from the luminal to abluminal chamber, while at 2 h, 40.6 ± 1.5% had appeared in the abluminal chamber. The movement of intact ^{125}I-BSA across filters without cell monolayers was significantly greater, with 14.2 ± 1.8% (n=9, $P<0.05$) and 70.0 ± 3.2% (n=9, $P<0.01$) of intact ^{125}I-BSA appearing in the abluminal chamber at the 5 min and 2 h time points, respectively, indicating that the endothelial cells had excluded substantially the passage of ^{125}I-BSA. TCA precipitability of the ^{125}I-BSA tracer in the luminal chamber at time zero was 88.2 ± 0.4% (n=9). TCA precipitability of ^{125}I-BSA in the abluminal chambers gradually increased from 80.3 ± 3.6 % at 5 min up to 88.9 ± 1.9% (n=9) by 2 h, indicating a preferential movement of free ^{125}I- at the early time points.

Figure 3.9: ^{125}I-BSA transport across HUVE cell monolayers. Transport of ^{125}I-BSA across HUVE cell monolayers and Transwell filters without cells is indicated by circles and squares, respectively. ^{125}I-BSA transport is expressed as a percentage of total intact ^{125}I-BSA added to the luminal chamber at time zero. Data are the pooled means ± sems of triplicate Transwells from three experiments. Asterisks represent significant differences in the transport of ^{125}I-BSA across HUVE cell monolayers compared to transport across Transwell filters alone (*P<0.05, **P<0.01).


Labelled BSA transported (% of total intact added)
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
Time (min)
*
*
*
*
**

3.4 DISCUSSION

Human vascular endothelial cells have consistently proven difficult to propagate *in vitro*, due to their fastidious nutritional requirements. It has previously been reported that HUVE cells require rich media such as M199 containing glutamine to support growth (Gimbrone *et al.*, 1974). Jaffe (1984) is in agreeance, having tested many other commercial cell culture media. In addition, for optimal growth, it was reported that original isolates require medium supplemented with 20 % FBS, but only 10 % serum for subcultures (Gimbrone *et al.*, 1974). However, my studies indicated that HUVE cells grown in media supplemented with less than 20 % FBS became elongated, resulting in a non-contiguous monolayer of cells that would not be appropriate to study transport of proteins. Jaffe (1984) states that growth of HUVE cells was supported better with media supplemented with 35 - 50 % human serum, however this would involve consumption of too much serum and time. HUVE cells grew well and reached confluence rapidly in media containing commercially available FBS, especially when further supplemented with growth factors. In support of this, Maciag *et al.* (1984a) reported that the FBS requirements could be reduced in the presence of endothelial cell growth factor. Each new batch of FBS was tested before use to ensure optimal growth conditions.

After isolating a pure culture of HUVE cells, the main problem is then to stimulate cell growth. The endothelium is known to be an extremely quiescent tissue (D'Amore, 1992). Although it is well known that serum provides a number of hormones and mitogenic factors for mammalian cell growth *in vitro* (Freshney, 1983), consistent with the nature of endothelial cells *in vivo*, the human vascular endothelial cell *in vitro* is unique since it responds poorly to the mitogenic effects of serum (Gimbrone *et al.*, 1974). Without addition of extra growth factors, the low mitotic index of human endothelial cells renders long term culture impossible. Maciag *et al.* (1979) purified a growth factor derived from bovine brain and hypothalami which stimulated HUVE cell growth *in vitro* in the presence of FBS. They named the growth

promoting activity in this extract Endothelial Cell Growth Factor (ECGF) and characterised it as an acid and heat labile protein of 75 kDa which is identical to aFGF. The ability of the fibroblast growth factors, particularly FGF-1 (aFGF) and FGF-2 (bFGF), in either crude (ECGS) or purified form to promote the prolifierative capacity and lifespan of HUVE cells is well described (Rosengart *et al.*, 1988; Gimenez-Gallego *et al.*, 1986; Maciag *et al.*, 1984b; Thornton *et al.*, 1983; Maciag *et al.*, 1979) and not covered in more detail here. Beekhuizen and van Furth (1994) reported that stimulation of HUVE cells by ECGF alone was less effective than ECGF in combination with heparin. Potentiation of aFGF by soluble heparin is thought to result from binding of heparin to the growth factor. This interaction causes a conformational change in the protein (Schreiber *et al.*, 1985), protects the molecule from proteolytic attack and heat denaturation (Rosengart *et al.*, 1988; Lobb, 1988), increases the *in vitro* half-life of the factor (Damon *et al.*, 1989) and potentiates binding to the cell surface receptor (Schreiber *et al.*, 1985). A similar potentiation of activity has been reported for bFGF (Gimenez-Gallego *et al*, 1986).

ECGF is commercially available from Sigma (St Louis, MO) under the trade name Endothelial Cell Growth Supplement (ECGS) which is a crude mixture of aFGF and bFGF. Primary cultures and HUVE cells at passage 1 and 2 were not given ECGS, except at the time of passaging (personal communication, Dr Jenny Gamble). Jaffe (1984) stated that endothelial cells cultured without special growth factors can be passaged from 3 - 5 times, after which they degenerate. However, I routinely supplemented the HUVE media of cells of passage 3 and higher with 25 µg / ml of both ECGS and heparin, as I found that HUVE cells became disorganised after only a couple of passages. Disorganised monolayers are characterised by the appearance of 'sprouting' endothelial cells under the monolayer, which have been shown to be directly related to the lack of ECGS (van Hinsbergh, 1990).

To permit permeability studies using endothelial cells *in vitro*, optimal culture conditions are required. However, such studies may be hampered due to the fact that

endothelial cells cultured on cell culture plastic may differ from endothelial cells *in vivo*, which grow on an extracellular matrix comprised of fibronectin, collagen type IV and V, laminin and proteoglycans (Timpl and Dziadek, 1986). Cultures grown on plastic alone often display defects within the monolayer even when confluent, which renders them unusable for permeability studies (Haudenschild, 1984). As a result, artificial substrates have been utilised to circumvent this problem. Commonly, gelatin or fibronectin have been used to provide an artificial substrate to which HUVE cells can adhere. Beekhuizen and van Furth (1994) reported that for HUVE cells, optimal growth and formation of a monolayer of closely associated cells was observed when HUVE cells were cultured on fibronectin or gelatin substrates. However, fibronectin was the best substrate with regard to the induction of proliferation. Simiarly, Maciag *et al.* (1984a) carried out numerous studies examining a large variety of substrates, and found the most effective to be human fibronectin. Also relevant is the demonstration that other cell contaminants such as fibroblasts and smooth muscle cells are less readily able to bind it when compared with gelatin (Scott and Bicknell, 1993).

In vivo tight junctions regulate the passage of certain macromolecules across endothelial cell barriers. Therefore, formation of tight junctions is an essential requirement of an *in vitro* endothelial cell monolayer model designed for permeability studies. PECAM-1 is a plasmalemmal glycoprotein, whose presence at intercellular junctions of endothelial cells is considered indicative of close contact between adjacent cells. When grown on gelatin or fibronectin coated plastic, HUVE cell monolayers displayed enrichment of PECAM-1 at intercellular junctions (Smeets *et al.*, 1992). In large blood vessels *in vivo*, silver staining results in the precipitation of silver grains at intercellular junctions. Similarly, Smeets *et al.* (1992) demonstrated that HUVE monolayers grown on gelatin or fibronectin coatings produced monolayers that could also be stained with silver due to intercellular junctions, resembling their *in vivo* counterparts. Following close examination of the literature, and due to the expense of recombinant human fibronectin, it was decided that gelatin would be used as

the substrate for routine growth and culture of HUVE cells, while fibronectin would be used for all experimental work.

One important property of large vessel endothelial cells, including HUVE cells grown in culture, is that they resemble endothelial cells *in vivo* by growing as a monolayer (Jaffe *et al.*, 1973). However, an exception to this monolayer characteristic is observed in long-term cultures, where multiple layers pile up on each other, separated by extracellular matrix (Haudenschild, 1984). HUVE cells in culture also maintain both gap and tight junctions, although they are less complex and less frequent after multiple passaging (Larson and Sheridan, 1982). For these reasons and taking into account the number of isolations that would have to be performed to obtain enough cells for future experiments, only cells from passage 2 to 5 were used in all studies.

Once isolated, it is imperative that primary cultures of HUVE cells are identified positively as endothelial cells. The most common contaminants of HUVE cell cultures are 1) fibroblasts, which are easily recognised as long spindle-shaped cells that grow in parallel formation and multiple layers or 2) smooth muscle cells, which have a "hill and valley" appearance and when viewed using transmission electron microscopy contain large amounts of myofilaments (Jaffe *et al.*, 1973). However, no contaminants were observed, most probably because the isolation technique used has been considerably refined over the years, only requiring minimum incubation with collagenase. Since the first successful isolation of HUVE cells, a number of metabolic and ultrastructural markers have been routinely used to identify cultured endothelial cells derived from most species. Angiotensin converting enzyme (ACE), once thought to be specific only for endothelial cells and alveolar macrophages, has been utilised as an endothelial cell marker, however, it is also present in fibroblasts (Weinberg *et al.*, 1982). Other markers for endothelial cells include production of prostaglandin I_2 and the maintenance of a non-thrombogenic surface in tissue culture, both of which have been demonstrated for HUVE cells (Weksler *et al.*, 1977 and Curwen *et al.*, 1980). One of the

easiest ways to initially identify cells as endothelial cells is to examine the morphology by light microscopy. HUVE cells in culture typically grow as monolayers with a "cobblestone" appearance. Cells are closely apposed, homogeneous, large and polygonal shaped with an oval, centrally placed nucleus (Jaffe *et al.*, 1973 and Gimbrone *et al.*, 1974). The abundance of membrane bound organelles, vesicular and tubular membranous structures, and free ribosomes are also features that distinguish endothelial cells both in culture and *in vivo* from other vascular cells (Haudenschild, 1984). In 1964, Weibel and Palade described the presence of rod-shaped, cytoplasmic organelles (Weibel and Palade, 1964). Weibel Palade bodies have proven to be reliable markers of endothelial cells of some species and have been identified in HUVE cells (Haudenschild *et al.*, 1975). Probably the most commonly used and preferred method for identification of endothelial cells for most species is the presence of Factor VIII related (von Willebrand factor) antigen. Factor VIII related antigen is a 270 kDa multimeric plasma glycoprotein. It mediates platelet adhesion to injured vessel walls and serves as a carrier and stabiliser of Factor VIII for coagulation. Only endothelial cells, megakaryocytes and platelets contain Factor VIII related antigen and it has been detected in endothelial cells isolated from human umbilical vein (Jaffe, 1977). Having examined the literature it was decided to identify the cells isolated as endothelial cells, using both light microscopy and assaying for the presence of Factor VIII related antigen. The latter was observed using two different detection systems, indirect immunofluorescence and immunoperoxidase. The studies presented in this chapter confirmed that HUVE cell monolayers grew with a characteristic "cobblestone" morphology, as has been previously reported by Jaffe *et al.* (1973) and Gimbrone *et al.* (1974). In addition, their identity was further substantiated by identifying the presence of Factor VIII related antigen.

The next parameter that needed to be defined was the culture of HUVE cells in serum-free media. Most studies of endothelial cells *in vitro* have used serum-supplemented media. However, the use of sera in some experiments creates problems, such as batch performance

differences, extraneous effects due to undefined agents contained within sera and the possible masking of an effect or the detection of cell-derived factors which may be present at low concentrations. Future experiments planned for this investigation required serum-free conditions, chiefly because serum contains IGFs and IGFBPs. Firstly, to examine the IGFBPs secreted by HUVE cells, the presence of serum would not allow delineation of which IGFBPs arose from HUVE cells or which originated from serum. Secondly, to study the transport of IGF-I from blood to tissues, experiments would be conducted using a model blood vessel wall, consisting of HUVE cell monolayers on a polycarbonate filter in a bicameral chamber. Trace amounts of ^{125}I-IGF-I would be used as the test molecule. Thus, if carried out in media supplemented with serum, serum borne IGFs or IGFBPs could possibly alter the transport of ^{125}I-IGF-I. Berliner (1981) has described a medium containing hydrocortisone, transferrin, insulin and FGF which supports endothelial cell growth for short periods of time in the absence of serum. However, the influence of insulin in future transport studies of IGF-I could not be discounted. Endothelial serum-free media has also been described for the culture of non-human vascular endothelial cells (Gorfien *et al.*, 1993). However, this medium does not support growth of human endothelial cells without the addition of other supplements including crude bovine pituitary extract (Battista *et al.*, 1995), preparations which are known to contain IGFs and IGFBPs, thus rendering this serum-free media formulation inappropriate in this instance.

Fibroblast growth factor (FGF) is known to be a potent mitogen for growth of a number of mesoderm and ectoderm derived cells (Gospodarowicz *et al.*, 1976). More specifically, it has been reported that FGF is mitogenic for HUVE cells (Gospodarowicz *et al.*, 1978). ECGS is a tissue extract, which contains a number of growth factors including FGFs. With this knowledge and the concepts of other serum-free media, it was decided to attempt to formulate a serum-free media for HUVE cells, using HUVE medium without FBS or ECGS, but containing basic FGF (bFGF) and heparin. Studies documenting the growth and

appearance of HUVE cells in serum-free HUVE medium supplemented with bFGF and heparin produced subcultures that exhibited all the characteristics of healthy HUVE cells grown in optimal serum-supplemented media. The serum-free culture conditions described in these studies eliminated the problems associated with serum borne IGFs and IGFBPs for future experiments.

Another important aspect to consider was monitoring the integrity of the HUVE cell monolayers grown on Transwell filters. This simple *in vitro* model of the blood vessel wall has been used to examine the exchange of macromolecules, fluid and cells between the blood and the extravascular tissue. The fundamental requirements of such a model are a substrate upon which an intact monolayer of the cells, possessing intercellular junctions can be maintained in a well differentiated state. Growth of HUVE cells on Transwells does not permit viewing the monolayer by light microscopy as the filter is opaque. Thus, the presence of continuous HUVE cell monolayers on Transwells was confirmed by fixing and staining the cells and viewing each monolayer at the end of each experiment by light microscopy. In addition, selected control Transwells were viewed by light and electron microscopy prior to experiments. Another means to examine the integrity and confluence of a cell monolayer is to measure its ability to resist the passage of electrical current. Thus, the transendothelial electrical resistance of each monolayer was measured. On day 1, 2 and 4 post-seeding HUVE cells on Transwells, values were no higher than filters without cells. On day 3 post-seeding HUVE cells on Transwell filters the mean transendothelial electrical resistance value was 3.76 $\pm$ 1.00 $\Omega.cm^2$, but this value was still not significantly greater than the value for Transwell filters alone However, this value is well within the range of 1 to 25 $\Omega.cm^2$ that has been observed for other monolayers of systemic endothelial cells (Shasby, 1988). This low resistance value indicated that the monolayer presented no barrier to the passage of electrolytes. This is in agreeance with the growth studies, since monolayers were observed to regress after day 3 post-seeding the Transwell. Since HUVE cell monolayers did not produce

electrical resistance values significantly greater than those of Transwell filters without cells, it was decided that this method was not useful for confirming the integrity of the HUVE cell monolayers, and that this would be achieved by viewing the monolayers by light and electron microscopy only.

Finally, one important question regarding the validity of the HUVE cell monolayer model, was whether it was able to transport macromolecules known to be transported by other endothelia. Evidence has revealed that specific binding sites for albumin and more recently, albumin-binding proteins, are expressed on the luminal surface of endothelial cells (Schnitzer *et al.*, 1992; Antohe *et al.*, 1991; Schnitzer *et al.*, 1988; Milici *et al.*, 1987; Ghitescu *et al.*, 1986). Antohe *et al.* (1993) recently presented data suggesting the coexistence of a receptor-mediated and a receptor-independent transcytosis of albumin across monolayers of bovine aortic endothelial cells cultured on filters in a dual chamber system. Thus, the transport of albumin across HUVE cell monolayers in the bi-chamber system was examined as a validation of the model. Although the HUVE cell monolayers were unable to significantly occlude the passage of electrolytes, as indicated by the transendothelial electrical resistance values, it did restrict the passage of albumin. The value of 26.24 ± 0.93 % at the 1 h time point in these studies agrees with the data of Antohe *et al.* (1993) and Meyrich and Harris (1988), who found approximately 30 % and 20 % of radiolabelled albumin was transported from the upper to the lower compartment after 1 h in bovine aortic endothelial cell monolayers, respectively.

In conclusion, the present studies have led to the development of an experimental model of the blood vessel wall. HUVE cell isolates displayed histotypical 'cobblestone' morphology, were factor VIII positive as determined by anti-Factor VIII immunocytochemistry, and transmission microscopy revealed the presence of tight junctions. As the presence of FBS would have interfered both with the transport of ^{125}I-IGF-I across an endothelial monolayer and the detection of endothelial cell derived IGFBPs, several

preliminary studies were undertaken to determine optimal growth conditions for HUVE cells in serum-free medium. Although serum-free HUVE medium without ECGS and heparin was unable to maintain HUVE cells over the 2 h time course of the transport studies, the addition of bFGF plus heparin was able to support cultures on Transwells that displayed morphological and growth characteristics identical to those of cultures in the presence of HUVE medium supplemented with 20 % FBS, ECGS and heparin. The next chapter will address two main issues i) the IGF and IGFBP biology of HUVE cells, and ii) how IGF-I crosses the endothelial cell barrier in its egress from the vascular compartment to underlying subendothelial tissues.

CHAPTER: 4 PARACELLULAR TRANSPORT OF INSULIN-LIKE GROWTH FACTOR-I (IGF-I) ACROSS HUMAN UMBILICAL VEIN ENDOTHELIAL CELL MONOLAYERS.

4.1 INTRODUCTION

The IGFs are cell mitogens and growth promoters that circulate in human plasma in association with a specific group of high affinity IGF-binding proteins (Hossenlopp *et al.*, 1987). However, to exert growth stimulating effects on extravascular tissues, IGFs must first traverse the endothelial cell barrier, a cell monolayer which acts as a selectively permeable barrier to a wide range of molecules including ions, hormones, proteins and growth factors. Since IGF-I and the IGFBPs continually bathe the endothelium *in vivo*, it is likely that these cells regulate the transport of IGF-I from the vascular compartment to tissue sites of action. Transendothelial migration of macromolecules occurs via two separate pathways; (i) paracellular transport, involving diffusion at intercellular or transcellular junctions and (ii) vesicular transport, which is activated by a receptor-ligand interaction, or occurs by pinocytosis of macromolecules at the plasmalemmal surface. However, the process by which IGF-I is transported across endothelial cells is yet to be elucidated.

Endothelial cells isolated from blood vessels other than human umbilical veins are known to possess receptors for both IGF-I and IGF-II (Bar *et al.*, 1988; Jialal *et al.*, 1985; King *et al.*, 1985; Bar and Boes, 1984), and depending on their origin, secrete distinct profiles of IGFBPs (Yang *et al.*, 1993; Boes *et al.*, 1992; Moser *et al.*,1992; Bar *et al.*, 1987). More recently, endothelial cells have been shown to produce mRNAs for IGFBP-2 through to IGFBP-6 (Moser *et al.*,1992). Bar and his colleagues have demonstrated that IGF-I is capable of crossing capillary boundaries (Boes *et al.*, 1992; Bar *et al.*, 1990a,c). IGF-I also crosses monolayers of endothelial cells derived from porcine aorta (Taylor *et al.*, 1993).

Results of chapter 2 showed that IGF-I and LR^{3}IGF-I are able to leave the vascular compartment and distribute into many different tissues in rats. A number of studies have suggested that IGF-I of endocrine origin may reach and act on many tissues (Ebeling *et al.*, 1993; Glassock *et al.*, 1991; Guler *et al.*, 1989b; Jacob *et al.*, 1989; Skottner *et al.*, 1989; Guler *et al.*, 1988; Skottner *et al.*, 1987; Zapf *et al.*, 1985; Smeets and van Buul-Offers, 1983; Schoenle *et al.*, 1982). For this to occur IGF-I must first traverse the endothelial barrier. Chapter 3 resulted in the establishment and validation of a HUVE cell model to study the transport of macromolecules across an endothelial cell monolayer. The aim of the present chapter was to investigate the IGF and IGFBP physiology of HUVE cells and using this model, determine the pathway by which IGF-I moves across the endothelial barrier.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Recombinant human IGF-I and IGF-II were purchased from GroPep Pty. Ltd. (Adelaide, Australia). Anti-human IGF binding protein -1, -2, -3 and -5 were purchased from Upstate Biotechnology Incorporated (Lake Placid, USA). Horseradish peroxidase-conjugated anti-rabbit IgG was purchased from DAKO Corporation (Carpinteria, USA). Human IGFBP-1 was supplied by Diagnostic Systems Laboratories (Texas, USA). Human IGFBP-2 was generously supplied by Dr. E. Kuto (Basel, Switzerland). Human plasma IGFBP-3 was a kind gift from Dr. R. Baxter (Sydney, Australia). Human IGFBP-5 was donated by Dr. D. Andress (Seattle, USA). IGF-I and IGF-II were iodinated with carrier free Na^{125}I from Amersham (Buckinghamshire, UK), to a specific activity of 85 μCi/μg, using a modification of the chloramine T method (Gargosky *et al.*, 1990b). [^{3}H]-inulin (1.18 Ci/mmol) and [methyl-^{3}H]-thymidine (20 Ci/mmol) were also obtained from Amersham. Madin Darby canine kidney

epithelial cells (MDCK) were a gift from Arthur Webster Pty. Ltd. (Castle Hill, Australia). M199 with Earle's salts, fetal bovine serum (FBS), sodium pyruvate, L-glutamine and non-essential amino acids were purchased from Cytosystems (Castle Hill, Australia). RPMI 1640 was supplied by Gibco Laboratories (Grand Island, USA) and Hanks' balanced salts solution (HBSS) was purchased from ICN Biomedicals (Costa Mesa, USA). Dulbecco's modified Eagle's essential medium (DMEM) was purchased from Flow Laboratories (Irvine, Scotland). Collagenase type I was obtained from Worthington Biochemical Corporation (Freehold, USA). Basic fibroblast growth factor (bFGF) was from Austral Biologicals (San Ramon, USA). Endothelial cell growth supplement (ECGS), heparin, methylene blue, bovine serum albumin (BSA), sodium chloride, potassium chloride, magnesium sulphate, glucose, Coomassie Blue R-250 N,N'-methylene-bis-acrylamide, Tween-20, glycine-HCl and Tris (Tris[hydroxylmethyl]amino-methane) were from Sigma (St Louis, USA). HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]), EDTA (ethylenediamine tetraacetic acid), bromophenol blue, glacial acetic acid, methanol, ethanol and di-sodium tetraborate were from BDH Chemicals (Victoria, Australia). Sodium hydroxide, Triton X-100, SDS (sodium dodecyl sulphate), glycerol, TCA (trichloroacetic acid) sodium dihydrogen orthophosphate and di-sodium hydrogen orthophosphate were from Univar, Ajax Chemicals (Sydney, Australia). All transmigration experiments were carried out using transwells [6.5 mm diameter, 0.4 μm pore size] from Costar (Cambridge, USA), coated with human fibronectin (Boehringer Mannheim, Mannheim, Germany). Monoclonal mouse anti-IGF-I receptor (AB-1) antibody (clone αIR3) was from Oncogene Science (Uniondale, USA). Disuccinimidyl suberate (DSS) was obtained from ICN Biomedicals Australasia Pty. Ltd. (Seven Hills, Australia). The Hi Trap Protein G column and protein molecular weight standards were obtained from Pharmacia (Uppsala, Sweden). Nitrocellulose sheets were from Schleicher and Schull (Dassel, Germany).

4.2.2 Cell Culture

Human umbilical vein endothelial cells were isolated from the vein of 12-24 hour old umbilical cords, using a modification of the methods of Jaffe *et al.* (1973) and Gimbrone *et al.* (1974) (see section 3.2.2). HUVE cells were maintained in HUVE medium plus 20% FBS and 25 μg / ml each of ECGS and heparin (see section 3.2.2) in an humidified atmosphere at 37°C in 5% CO_2, and used between passages 2 and 6. MDCK cells were grown in DMEM media containing 10% FBS, and used in transmigration experiments at the ninety fifth subculture.

4.2.3 Radioreceptor Assays

Radioreceptor assays were undertaken according to the method of Ross *et al.* (1989). Briefly, HUVE cells were subcultured onto fibronectin-coated (see section 3.2.3) 24 place multiwell plates (Costar, Cambridge, USA), and grown to confluence. The cells were washed twice and incubated for 2 h at 4°C in HEPES-buffered saline (0.1 M HEPES, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$ and 8mM glucose, pH 7.6) plus 0.5% BSA. Cells were then incubated for 18 h at 4°C in 500 μl of the same buffer containing 2 x 10^4 cpm of ^{125}I-IGF-I or ^{125}I-IGF-II and the indicated amounts of unlabelled IGF-I or IGF-II. Monolayers were harvested by washing twice with HBSS at 4°C, followed by dissolution in 0.5 M NaOH containing 0.1 % Triton X-100. Bound radioactivity was determined using a gamma counter. Data was fitted to a four parameter equation with the aid of a non-linear curve fitting program (Tablecurve, Jandel Scientific, San Rafael, CA).

4.2.4 Affinity label cross-linking experiments

Cross-linking was performed as described by Kasuga et al. (1981). Confluent HUVE cell monolayers in fibronectin-coated 6-place multiwell plates were washed twice and

incubated at 4°C in serum-free HUVE medium plus 0.1 % BSA for 2 h. The medium was removed and the cells incubated for a further 4 h with 0.5 μCi (8 ng) of either ^{125}I-IGF-I or ^{125}I-IGF-II in the presence or absence of a 1,000 fold excess of IGF-I or IGF-II or a 10,000 fold excess of insulin. The medium was aspirated, the monolayers washed twice on ice with HBSS and bound IGFs chemically cross-linked by the addition of 0.5 mM DSS in serum-free medium for 20 min at 4°C. The reaction was terminated with the addition of 4.5 ml of 0.1 M Tris containing 1 mM EDTA at pH 7.4 for 20 min. The cells were solubilised in 0.1 M Tris containing 1 mM EDTA, 10% (w / v) SDS, 20% (v / v) β-mercaptoethanol and 0.04% (w / v) bromophenol blue at pH 8 for 30 min, scraped off using a rubber policeman and heated at 95°C for 10 min. Cell lysates were subjected to SDS-PAGE on 4 % stacking and 6 % separating acrylamide gels (Laemmli, 1970, see section 4.2.6). Gels were stained using 0.125% Coomassie Blue R-250 in 50% methanol / 10% acetic acid for 4 h, destained in 50% methanol / 10% acetic acid for 1h and in 5% methanol / 7% acetic acid for 12 h. The gel was equilibrated in 10% acetic acid / 10% glycerol for 1h before being dried for 1h at 80°C using a Hoefer Drygel slab gel dryer (Hoefer Scientific Instruments, San Francisco, USA). Labelled bands were visualised by exposure to X-ray film for 4 weeks.

4.2.5 DNA synthesis assays

Confluent monolayers of HUVE cells grown in fibronectin-coated 24-place multiwell plates were washed twice in serum-free HUVE medium plus 0.1 % (w / v) BSA for 30 min. Each monolayer was subsequently incubated for 18 h at 37°C / 5% CO_2, in either serum-free HUVE medium plus 1% (w / v) BSA and 1 ng / ml bFGF or 0.1 % (v / v) FBS containing IGF-I at 1, 10 or 100 ng / ml or serum-free HUVE medium with 1% (w / v) BSA alone and the same dilution series of IGF-I. A 10% FBS and serum-free control was also incorporated on each plate. During the last 6 h of this incubation, 1 μCi (5 nmol) of [^{3}H]-thymidine was

added to each well. Each monolayer was washed with HBSS, twice with 5% TCA and once with water. Cells were solubilised by trituration in 0.5 M NaOH containing 0.1% (w / v) Triton X-100, and incorporated [^{3}H]-thymidine quantified using a beta counter. Results are expressed as the percentage of DNA synthesis above that observed in control wells containing serum-free medium or serum free medium plus either 1 ng / ml bFGF or 0.1% FBS.

4.2.6 Western ligand blots and immunoblots

Confluent T75 flasks of HUVE cells were incubated for 24 h in serum-free HUVE medium without ECGS but with the addition of bFGF (100 ng/ml), 25 μg / ml heparin and 0.1 % (w / v) BSA. The conditioned media was collected, dialysed against 0.1 M acetic acid at 4° C for 72 h using Spectrapor 3 dialysis membrane tubing (Mr 3500 cut off, Spectrum, Houston, USA) and 1 ml aliquots vacuum dried. Conditioned media samples (1 ml equivalence), human plasma (2 μl equivalence) and hIGFBP-1, -2, -3 and -5 and oIGFBP-4 (100 ng of each) were incubated at 65°C for 15 min in SDS-loading buffer (0.25 M Tris, 0.3 M SDS, 2% (v / v) glycerol and 0.004% (w / v) bromophenol blue). Samples were subjected to discontinuous SDS-PAGE through a 4 % acrylamide stacking gel and a 12 % acrylamide separating gel in running buffer (0.25 mM Tris, 180 mM glycine and 3.5 mM SDS) using a 2050 Midget Electrophoresis Unit (LKB, Bromma, Sweden), (Laemmli, 1970). All gels were run at a constant current of 15 mA, until the dye front reached the bottom of the gel. Following electrophoresis, proteins were transferred to nitrocellulose using a Multiphor II NovaBlot electrophoretic unit, at a constant current of 37 mA per cm^2, following instructions from the manufacturer (Pharmacia, Uppsala, Sweden). Nitrocellulose sheets were treated in 1% (v / v) Triton X-100 for 30 min and 1% (w / v) BSA for 90 min, then washed in 0.1% (v / v) Tween-20 for 5 min. Each wash was performed in Western saline buffer (0.01 M Tris, 1.4 M NaCl). The treated nitrocellulose was further incubated in Western saline buffer containing 1% (w /

v) BSA, 0.1% Tween-20 and probed with 5 x 10^5 cpm. ^{125}I-IGF-II for 24 h. The nitrocellulose sheets were subsequently washed for 2 h in 0.1% (v / v) Tween-20, with changing every 15 min. Nitrocellulose sheets were air dried and exposed to X-ray film for 4 days at -80°C C, in X-ray cassettes with Cronex Hi-Plus intensifying screens (Du Pont, Wilmington, MA). X-ray films were developed, fixed and washed automatically (AGFA-GEVERT, West Germany).

The same nitrocellulose sheets were then cut into strips for immunoblotting. Nitrocellulose strips were blocked for 12h in Tris buffered saline (20 mM Tris, 137 mM NaCl, 3.8 mM HCl, pH 7.6) plus 3% (w / v) BSA, then rinsed twice and washed once for 15 min and twice for 5 min in Tris buffered saline containing 1% (w / v) BSA and 0.1% (v / v) Tween-20. Strips were then probed with one of four primary antibodies against human IGFBP-1, -2, -3 or -5 or antiserum raised against ovine IGFBP-4 (1:1000 dilutions) in Tris buffered saline containing 1% (w / v) BSA and 0.1% (v / v) Tween-20 at 4°C for 18 h. Following washing three times for 15 min in Tris buffered saline containing 1% (w / v) BSA and 0.1% (v / v) Tween-20, strips were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (1:5000) for 1 h at room temperature, then washed once for 15 min and four times for 5 min. Bound antibody was detected using enhanced chemiluminescence (ECL, Western Blot Detection System, Amersham Corporation, Arlington Heights, USA). Bands were detected by exposing the nitrocellulose strips briefly to X-ray film, which was developed as for Western blotting.

4.2.7 Affinity chromatography purification of mouse ascites fluid IgG 1

Mouse ascites fluid containing mouse myeloma protein IgG1, which has no known reactivity, was clarified by centrifuging at 10 000 g at 4°C for 2 min and then filtered through a 0.45 μm low protein binding filter. A Hi-trap protein G column was equilibrated with 0.02

M sodium phosphate, pH 7, at 1 ml / min for 20 min, and the collection of 2.5 ml fractions begun. Following equilibration, 2 ml of the clarified ascites fluid was pumped over the column at a flow rate of 1 ml / min. IgG was eluted from the column using 0.1M glycine-HCl at pH 2.7. The absorbance of each fraction was measured using a spectrophotometer at a wavelength of 280 nm. Two fractions corresponding to the IgG peak were pooled and a 4 µl subsample, along with a 2 µl subsample of the high molecular weight protein fraction were run on a 1% agarose gel along side a rat IgG subclass 1 control. The purified mouse ascites fluid IgG was neutralised to pH 7 with 1M Tris-HCl and the amount purified determined using Bradford protein estimation method (Bradford, 1976). This IgG was purified to act as a negative control in transmigration experiments.

4.2.8 Binding assays

Confluent monolayers of HUVE cells in fibronectin-coated 24 place multiwell plates, were pre-incubated for 30 min with either αIR-3 (10 µg / ml), the control antibody, IgG (10 µg / ml) or unlabelled IGF-I (8 µg) in serum-free HUVE medium for 2 h at 37° C / 5% CO_2. Following addition of ^{125}I-IGF-I (8 ng), the cells were further incubated for 2 h, washed in HBSS and the monolayers dissoluted with 0.1 % Triton X-100 / 0.5 N NaOH and the entire contents of the well counted in a gamma counter.

4.2.9 Transmigration experiments

Transwells were coated with human fibronectin (50 µg/ml) for 30 min at room temperature, and seeded with 2 x 10^4 HUVE cells in HUVE medium, placed in 24-well plates, and cultured for 3 days at 37°C, 5% CO_2. Medium was replaced daily and prior to each experiment control transwells were routinely fixed with methanol, stained with 1 % methylene blue in 0.01 M di-sodium tetraborate (pH 8.5) and visualised by light microscopy

to monitor confluency. Other control transwells were fixed for 6 h in phosphate buffered saline containing 4 % paraformaldehyde, 1.25 % gluteraldehyde plus 4 % sucrose, pH 7.2, and processed for electron microscopy by Dr. Marilyn Henderson (see section 3.2.6.2).

At time zero, the monolayers were washed with serum-free HUVE medium plus 0.1 % BSA and the transwells transferred to a new lower chamber containing 700 µl of fresh serum-free HUVE medium plus 0.1 % BSA and bFGF (100 ng/ml). The medium from the luminal (top) chamber was removed and 150 µl of serum-free HUVE medium plus 0.1% BSA, bFGF (100 ng/ml), heparin (25 µg/ml) and containing either ^{125}I-IGF-I (8 ng) or [^{3}H]-inulin (0.1 µg) was added separately to the luminal chamber. After a 5 min incubation at 37°C, 5% CO_2, the abluminal (bottom) chamber was triturated, a subsample (100 µl) taken, and the chamber immediately replenished with the same amount of fresh media. Further subsamples were collected at 30 min, 1h and 2h. At 2h, each transwell was fixed, stained for light microscopy and the confluency of the monolayer examined. Transmigrated ^{125}I-IGF-I was measured as trichloroacetic acid (TCA)-insoluble radioactivity. ^{125}I-IGF-I subsamples were counted in a gamma counter and [^{3}H]-inulin containing subsamples counted in a beta-counter. In all experiments, transmigration of each macromolecule was also carried out in control transwells without HUVE cell monolayers. Similar experiments were conducted using MDCK monolayers seeded at 2 x 10^4 cells / transwell. Transendothelial or transepithelial migration of each macromolecule was expressed as a percentage of the total of intact macromolecule added to the luminal chamber at time zero.

4.2.10 Statistical Analyses

Transmigration and binding data were analysed by one-way analysis of variance with all pairwise multiple comparisons using Student-Newman-Keuls method. $P < 0.05$ was considered significant.

4.3 RESULTS

4.3.1 HUVE cell culture conditions

For the studies conducted in this chapter, HUVE cells were isolated, routinely maintained and utilised for experiments as described in chapter 3.

4.3.2 Competitive-binding, ligand-receptor cross-linking and DNA synthesis experiments

Since several macromolecules have been shown to be transported across endothelial cells by a transcellular route mediated by a receptor-ligand interaction at the cell surface, the possible presence of receptors for IGF-I on HUVE cells was investigated using radioreceptor assays as well as affinity cross-linking and DNA synthesis experiments. HUVE cells bound 1.6% of ^{125}I-labelled IGF-I and 13.5% of ^{125}I-IGF-II when added in the absence of unlabelled ligand. Competition by cold ligand is shown in Figure 4.1. The concentrations of IGF-I and IGF-II required to reduce binding of ^{125}I-IGF-I to HUVE cells by 50% was 0.4 ng/ml and 1.7 ng/ml, respectively. IGF-I was unable to displace the binding of ^{125}I-IGF-II, although IGF-II displaced 50 % of ^{125}I-IGF-II at a concentration of 11.8 ng/ml. IGF-I was able to stimulate DNA synthesis by HUVE cells when in the presence of bFGF or FBS but not when alone (Fig. 4.2), indicating that HUVE cells possess functional IGF receptors.

To further characterize IGF binding sites on HUVE cells, ^{125}I-IGF-I or ^{125}I-IGF-II were cross-linked to cell surface receptors in the presence or absence of saturating concentrations of cold ligand and the receptor-ligand complexes identified by SDS-PAGE and autoradiography (Fig. 4.3). Cross-linking studies with ^{125}I-IGF-I revealed two bands with a molecular weight of 140 and greater than 200 kDa, consistent with the cross-linked type I IGF

Figure 4.1: IGF radioreceptor assays. Inhibition of ^{125}I-IGF-I (A) and ^{125}I-IGF-II (B) binding to HUVE cells by cold IGF-I (circles) or IGF-II (squares). Values are expressed as the fraction of binding in the absence of competing ligand. Each point is the mean ± sem of triplicate determinations from a representative experiment.

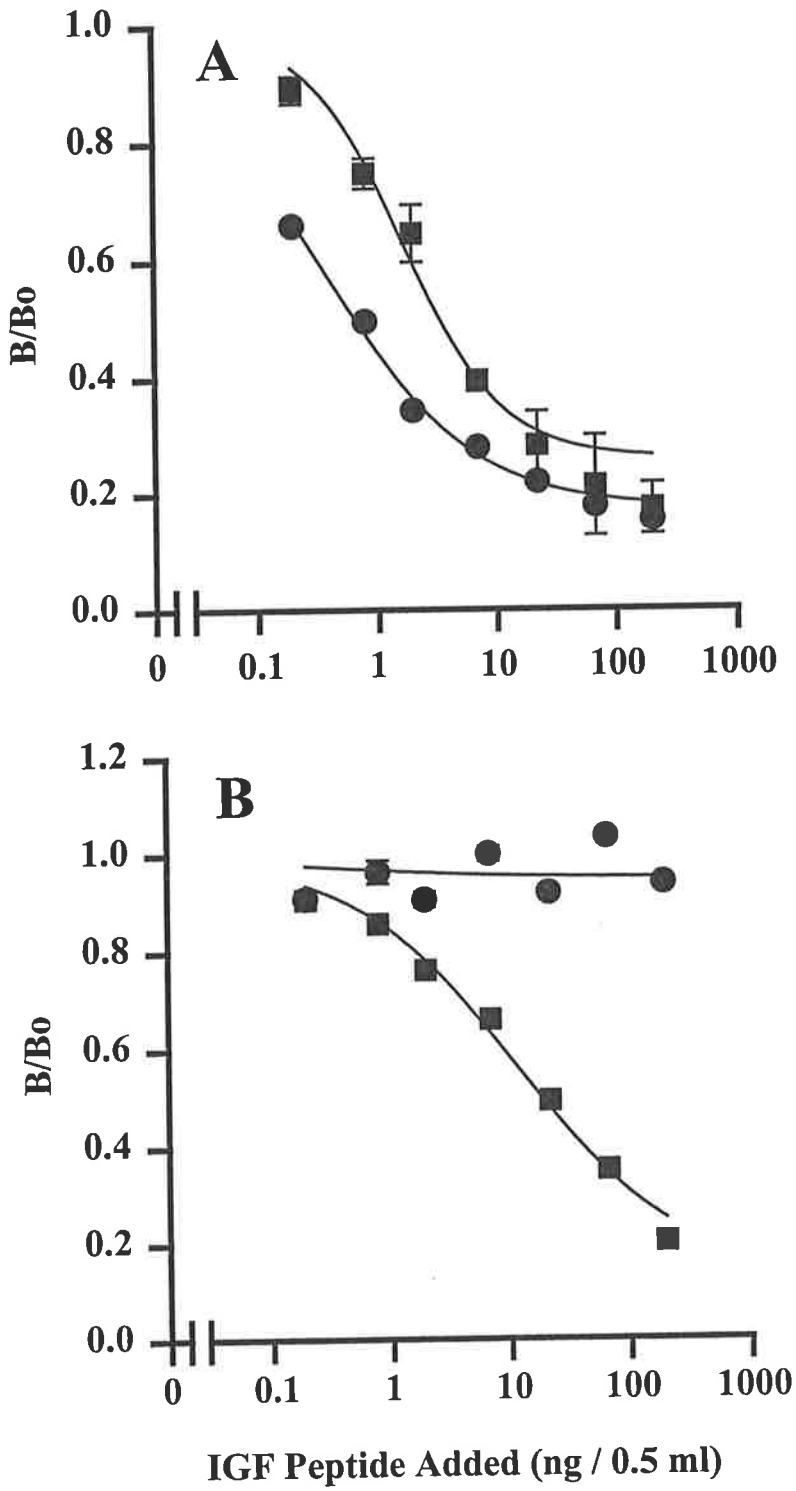

A
B
B/Bo
1.0
0.8
0.6
0.4
0.2
0.0
1.2
0
0.1
1
10
100
1000
IGF Peptide Added (ng / 0.5 ml)

Figure 4.2: [^{3}H]-thymidine incorporation into HUVE cells maintained in serum-free HUVE medium (circles), HUVE medium plus 0.1% FBS (squares) or HUVE medium plus 1 ng / ml bFGF (triangles) and treated with the indicated concentrations of IGF-I. Results are expressed as a percentage above incorporation observed under control conditions (means ± sem, n=3, $^*P<0.05$, $^{**}P<0.01$).

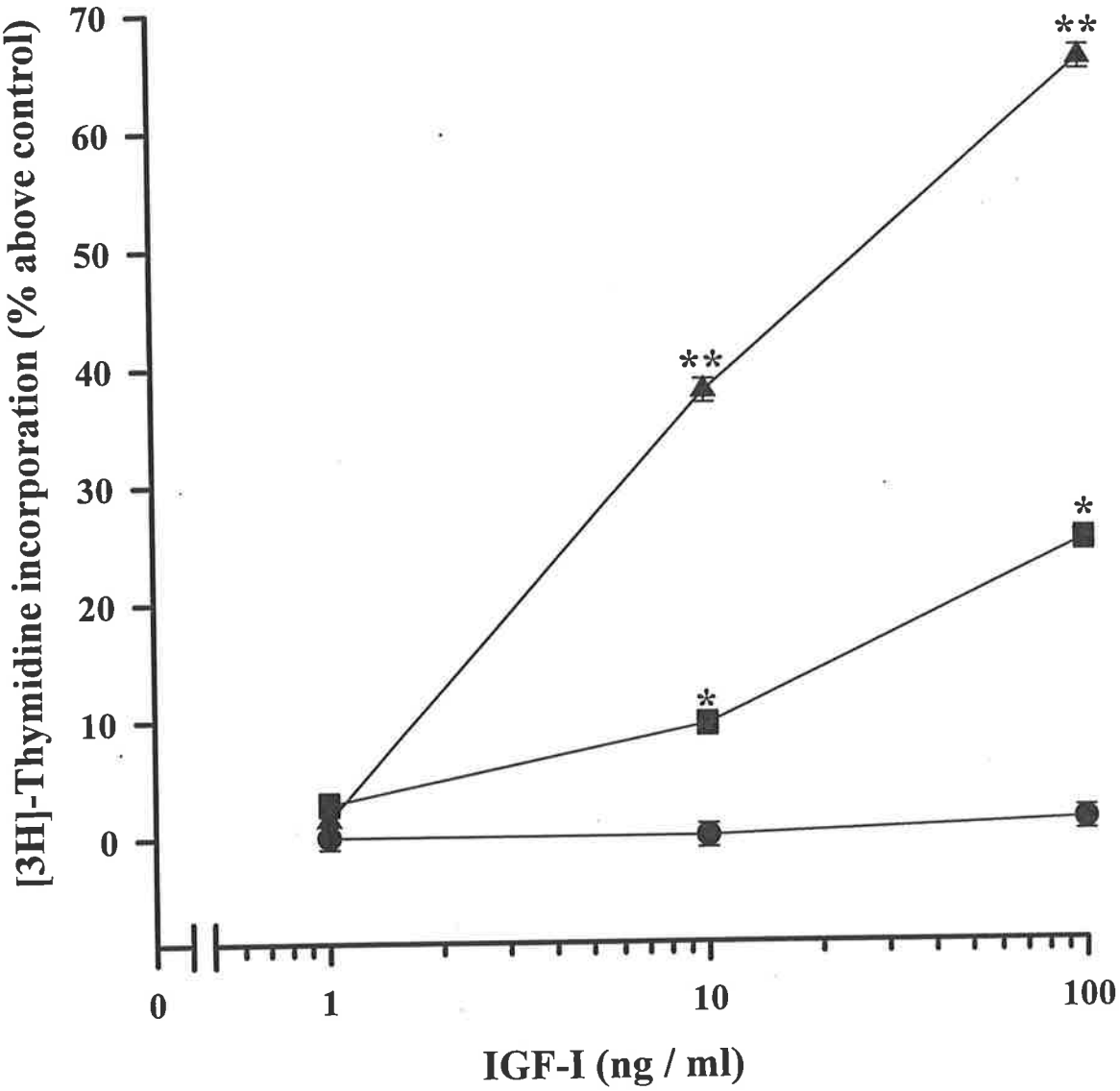
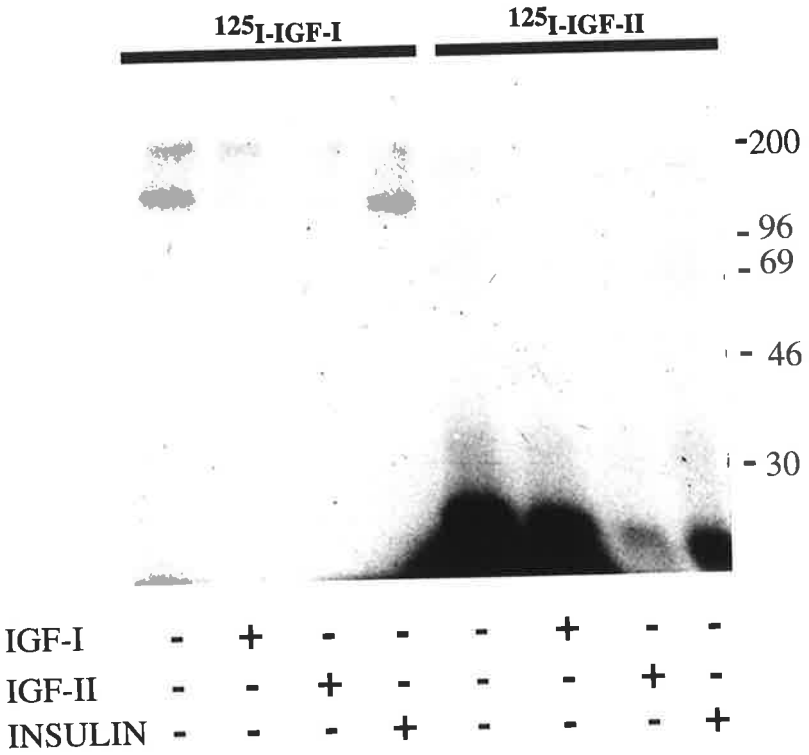

[3H]-Thymidine incorporation (% above control)
70
60
50
40
30
20
10
0
**
**
*
*
0
1
10
100
IGF-I (ng / ml)

Figure 4.3: Affinity label cross-linking of ^{125}I-IGF-I and ^{125}I-IGF-II to HUVE cell monolayers. Autoradiograph of ^{125}I-IGF-I and ^{125}I-IGF-II cross-linked to monolayers of HUVE cells. Cross-linking was carried out in the presence (+) or absence (-) of IGF-I (8 μg), IGF-II (8 μg), or insulin (80 μg). Cross-linked samples were subjected to SDS-PAGE on 12 % acrylamide gels under reducing conditions and bands visualized by exposure to X-ray film for 4 weeks. Positions of ^{14}C-labelled molecular weight markers are indicated (x 10^{-3}).


125I-IGF-I
125I-IGF-II
-200
- 96
- 69
- 46
- 30
IGF-I - + - - - + - -
IGF-II - - + - - - + -
INSULIN - - - + - - - +

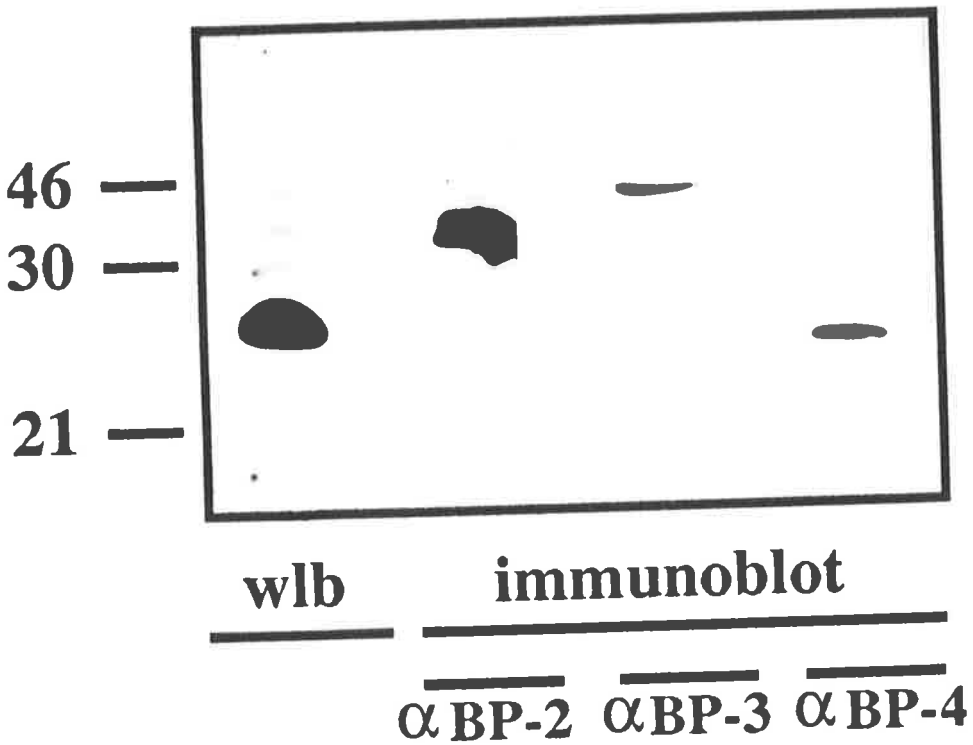
receptor α-subunit, and dimers of the cross-linked α-subunit, respectively. When cross-linking was performed in the presence of 8 μg/ml of unlabelled IGF-I or IGF-II, the intensity of these bands was reduced. In addition, the presence of 80 μg/ml of insulin only slightly diminished the intensity of these bands.

Cross-linking studies with ^{125}I-IGF-II produced a dominant band with a molecular weight of approximately 28 kDa and another faint band at 35 kDa. Binding of ^{125}I-IGF-II to these proteins was prevented by 8 μg/ml of unlabelled IGF-II, but not by 8 μg/ml IGF-I or 80 μg/ml of insulin. It is likely that these bands represent endothelial cell surface associated IGFBPs, which display a greater affinity for IGF-II than IGF-I. A faint band was also observed at 200 kDa, which was diminished in the presence of excess cold IGF-I and IGF-II.

4.3.3 Identification of IGFBPs in HUVE cell conditioned media

Serum-free media conditioned by HUVE cells maintained in the presence of bFGF (100 ng / ml) and heparin (25 μg / ml) for 24 h was examined for the presence of IGFBPs by both Western ligand and immunblotting techniques (Fig. 4.4). The most predominant species of IGFBP present on the western-ligand blot was a 24 kDa protein which was identified immunologically as IGFBP-4 using antiserum raised against ovine IGFBP-4. A less intense doublet was observed at approximately 38-43 kDa which cross reacted with anti-bodies specific for hIGFBP-3. The band at 32 kDa was similarly identified as IGFBP-2. The 28 kDa band visible on the ligand blot was not detected by immunoblotting. Antibodies to human IGFBP-1 and -5 did not bind any proteins present in the HUVE cell conditioned media samples (data not shown). Thus, under these culture conditions, HUVE cells secrete immunologically detectable IGFBP-2, IGFBP-3 and a 24 kDa form of IGFBP-4.

Figure 4.4: Western ligand blots and immunoblots of IGFBPs secreted by confluent monolayers of HUVE cells. Confluent cultures were exposed to serum-free HUVE media for 24 h. HUVE cell 24 h conditioned media (1 ml equivalence) was subjected to SDS-PAGE under nonreducing conditions, transferred to nitrocellulose sheets, probed with ^{125}I-IGF-II and exposed to X-ray film for 4 days (wlb = western ligand blot). For immunoblots of conditioned media of HUVE cells, nitrocellulose sheets were exposed to antibodies against human IGFBP-2 (αBP-2), human IGFBP-3 (αBP-3) and antiserum to ovine IGFBP-4 (αBP-4) as indicated. Positions of ^{14}C-labelled molecular weight markers are indicated (x 10^{-3}).

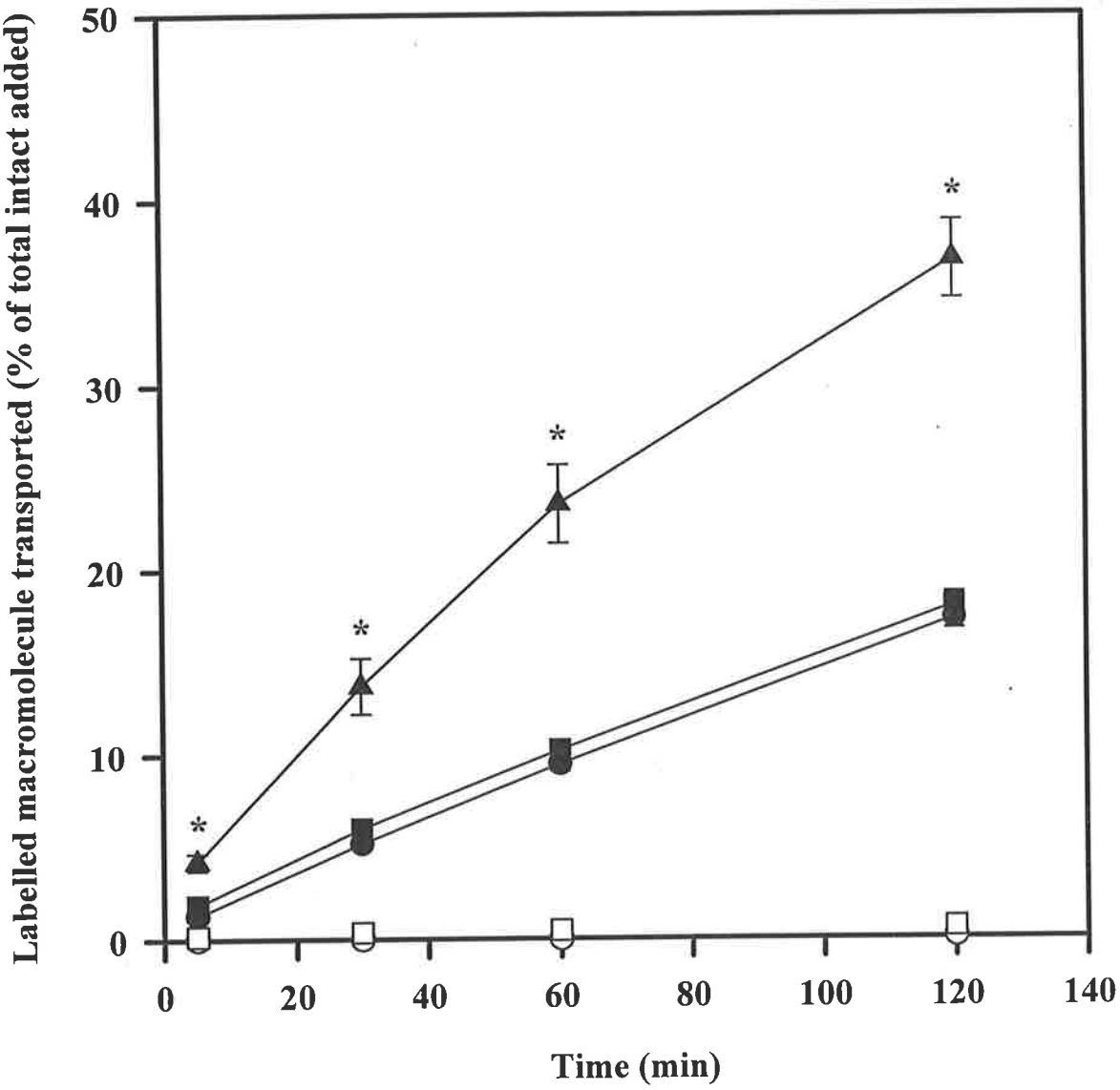

46
30
21
wlb
immunoblot
αBP-2
αBP-3
αBP-4

4.3.4 Transmigration Experiments

Several preliminary experiments were conducted to determine optimal growth conditions for the HUVE cells prior to, and during, IGF-I transport experiments. Daily media changes were required to support the culture of HUVE cells grown on transwell inserts, and after three days post-seeding the integrity of the monolayers began to regress (see sections 3.3.4 and 3.3.5). The integrity of the HUVE cell monolayers used in transmigration experiments was confirmed by light microscopy and both transmission and scanning electron microscopy (see section 3.3.4 and Fig. 3.6 and Fig. 3.7). Typically, epithelial cell monolayers are more restrictive to the passage of ions and solutes than endothelail cell monolayers (Schneeberger and Lynch, 1992). In these studies the MDCK cell line was used as a control since they produce highly occlusive tight junctions and generate high values of transepithelial electrical resistance (Cereijido *et al.*, 1978). The transepithelial electrical resistance of the control MDCK cell monolayers was approximately $70\Omega.cm^2$ three days after seeding onto transwells. As transendothelial electrical resistance could not be used as a gauge to determine the integrity of the HUVE cell monolayers, we routinely fixed, stained and viewed control HUVE cell monolayers immediately prior to each experiment, and each monolayer used in the experiments was viewed at the 2 h time point (see section 3.3.4).

When ^{125}I-IGF-I was added to the luminal side of the HUVE cell monolayer there was a time dependent increase in the amount of this peptide appearing intact in the abluminal chamber, with 17.3 ± 0.6 % (n = 51) of total ^{125}I-IGF-I added transported by 120 min (Fig. 4.5). The movement of ^{125}I-IGF-I across filters without cell monolayers was significantly greater (36.7 ± 2.1 %; n = 51, $P < 0.05$) than that across the HUVE cells, indicating that the endothelial cells had substantially excluded the passage of ^{125}I-IGF-I. However, movement of ^{125}I-IGF-I across the endothelial cell monolayer was not significantly different to [^{3}H]-inulin. [^{3}H]-inulin is impermeable to cell membranes and was used as a marker of intercellular

Figure 4.5: ^{125}I-IGF-I and [^{3}H]-inulin transport across HUVE cell and MDCK cell monolayers. ^{125}I-IGF-I and [^{3}H]-inulin transport is expressed as labelled macromolecule appearing in the abluminal chamber as a percentage of total intact ^{125}I-IGF-I and total [^{3}H]-inulin added to the luminal chamber at time zero, respectively. For HUVE cells, data are the pooled means ± sems of triplicate transwells from seventeen experiments, and for MDCK cells, data represents the pooled means ± sems of triplicate transwells from four experiments. ^{125}I-IGF-I and [^{3}H]-inulin transport across both cell monolayers is indicated by circles and squares, respectively. ^{125}I-IGF-I transport across filters without cells is indicated by triangles. Closed symbols represent HUVE cell data, open symbols MDCK cell data. Asterisks represent significant differences in transport with respect to ^{125}I-IGF-I across HUVE cell monolayers ($P<0.05$).

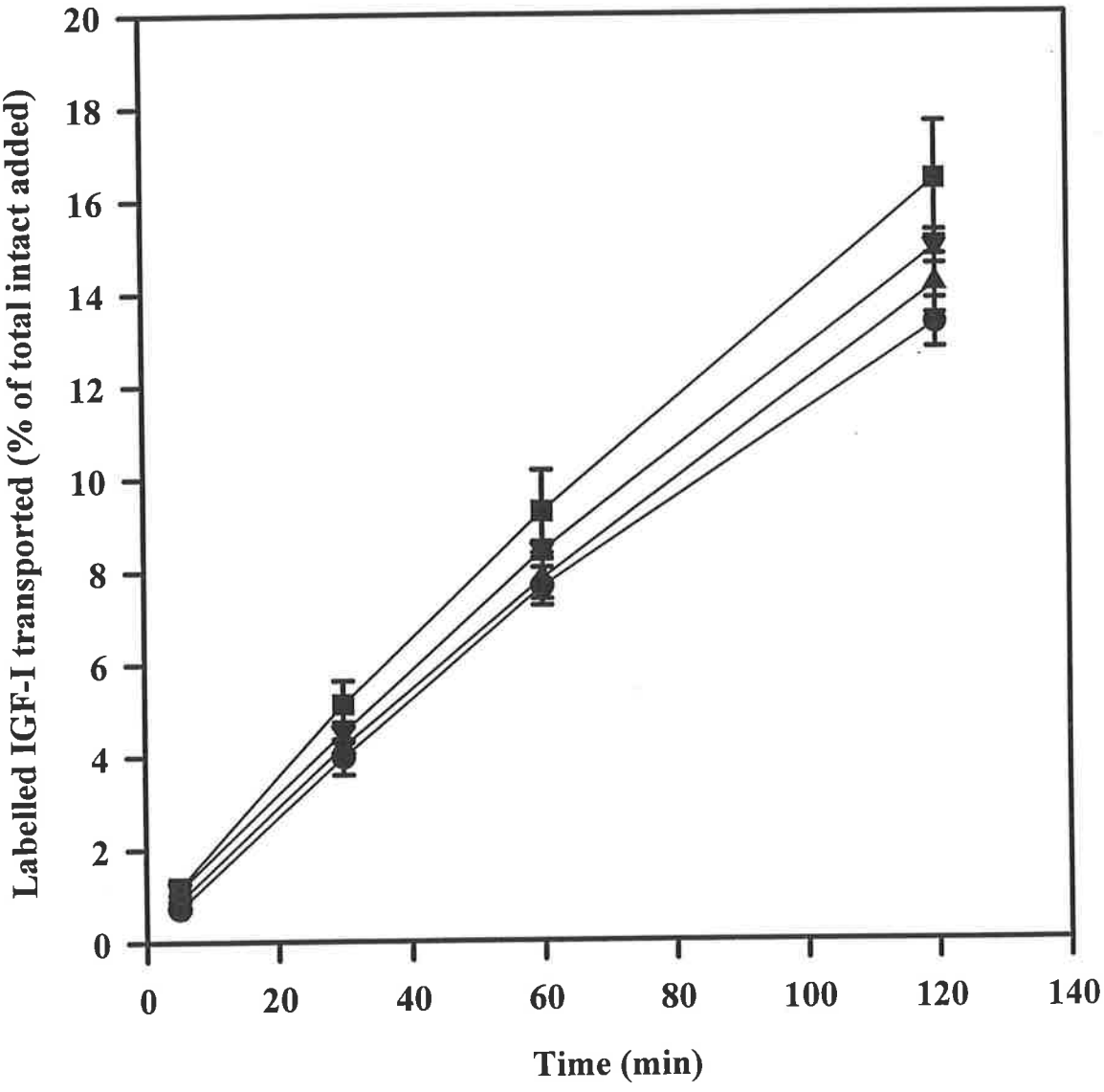

Labelled macromolecule transported (% of total intact added)
50
40
30
20
10
0
0
20
40
60
80
100
120
140
Time (min)
*
*
*
*

leakage. TCA precipitabilty of the ^{125}I-IGF-I tracer before addition to the luminal chamber was 95.7 ± 0.3 % (n = 51). TCA precipitability was also determined for ^{125}I-IGF-I in the abluminal chambers and gradually increased from 72.1 ± 1.2 % (n = 51) at 5 min up to 81.1 ± 0.9 % (n = 51) by 2 h, indicating a preferential movement of free ^{125}I at the early time points. In contrast, to the HUVE cell monolayers, MDCK cells essentially excluded the passage of both ^{125}I-IGF-I and [^{3}H]-inulin, with less than 1 % of total intact ^{125}I-IGF-I and [^{3}H]-inulin added to the luminal chamber appearing in the abluminal chamber at 2 hours (Fig. 4.5).

4.3.5 Effect of excess unlabelled IGF-I or antibody to the Type I IGF receptor on transport of ^{125}I-IGF-I

To determine whether transport of ^{125}I-IGF-I across HUVE cell monolayers follows a competitive and specific pathway, we investigated the effects of i) a 1000 fold excess of unlabelled IGF-I and of ii) αIR-3, an antibody to the type I IGF receptor on ^{125}I-IGF-I transport across the HUVE cell monolayer (Fig. 4.6). Both excess cold IGF-I and αIR-3 were added to the luminal chamber 30 min prior to the addition of IGF-I tracer and were present for the remainder of the experiment. Excess unlabelled IGF-I did not affect the transport of ^{125}I-IGF-I, indicating that it is a non-competitive process. To determine the appropriate concentration of αIR-3 to use in the transmigration study, αIR-3 inhibition of ^{125}I-IGF-I binding in HUVE cells was examined. The amount of ^{125}I-IGF-I bound in the presence of excess unlabelled IGF-I represented the non-specific binding and was approximately 16 % of total counts bound. Non-specific binding was subtracted from the total binding to calculate the specific binding of IGF-I. In the presence of 10 μg / ml of αIR-3, the specific binding of ^{125}I-IGF-I decreased significantly by 63 ± 1 % (n = 3; P<0.001). In contrast, the same concentration of the purified mouse myeloma protein IgG (a control antibody of the same

Figure 4.6: Effects of the presence of excess unlabelled IGF-I or antibody to the type I IGF receptor on ^{125}I-IGF-I transport across HUVE cell monolayers. ^{125}I-IGF-I transport is shown as a percentage of total intact ^{125}I-IGF-I added to the luminal chamber at time zero appearing in the abluminal chamber. ^{125}I-IGF-I transport across HUVE cell monolayers (circles) was measured in the presence of either a 1000 fold excess of cold IGF-I (8 μg) (squares), αIR-3 (10 μg / ml) (triangles) or mouse myeloma protein IgG-1 (10 μg / ml) (inverted triangles). Data represents the mean ± sem of three transwells from a representative experiment.


Labelled IGF-I transported (% of total intact added)
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
Time (min)

subclass), exerted no effect on ^{125}I-IGF-I binding. Furthermore, there was no inhibition in the transport of ^{125}I-IGF-I from the luminal to abluminal chamber, when either αIR-3 or the control antibody at 10 μg / ml was added to the upper chamber, confirming that the transport of free IGF-I across endothelial cell monolayers is a non-specific process, and is not mediated by the type I IGF receptor.

4.4 DISCUSSION

Macromolecules must first interact with the vascular endothelium prior to their egress from the blood to the underlying tissues. Transendothelial migration of macromolecules occurs via two separate pathways; (i) paracellular transport, involving diffusion at intercellular or transcellular junctions and (ii) vesicular transport, which is activated by a receptor-ligand interaction, or occurs by pinocytosis of macromolecules at the plasmalemmal surface. For example, insulin crosses endothelial cell monolayers via receptor-mediated transcytosis, a process that can be blocked by antibodies to the insulin receptor (Hachiya *et al.*, 1988; King and Johnson, 1985). Albumin transport is dependent on both a paracellular (Antohe *et al.*, 1993; Rippe and Haraldsson, 1987) and transcellular route (Antohe *et al.*, 1993; Ghitescu *et al.*, 1986) and albumin receptor glycoproteins exist on the plasma membranes of endothelial cells (Schnitzer and Oh, 1994; Schnitzer *et al.*, 1992).

Evidence exists suggesting that IGF-I does cross endothelial barriers. Recent studies by Taylor *et al.* (1993) have shown that IGF-I is able to transfer across monolayers of porcine aortic endothelial cells. Bar and colleagues have used a perfused cardiac model to demonstrate that IGF-I is able to cross capillary boundaries (Bar and Boes, 1988, Bar *et al.*, 1990a and Bar *et al.*, 1990b). Although the functional relevance of the 150 kDa complex is not well understood, it is thought that IGFs associated with the ternary complex remain in the vascular compartment as it is unable to cross the capillary barrier due to its size (Binoux and

Hossenlopp, 1988). Studies in specific cell systems show that IGFs associated with IGFBP-3 are still able to exert their mitogenic effect (Blum *et al.*, 1989). This being the case, a mechanism would be required to cause the dissociation of the 150 kDa complex, allowing IGFs or IGFs as a component of binary IGF-IGFBP complexes to be transported to the extravascular space. However, studies have reported the detection of the 150 kDa IGF-IGFBP complex in rat wound fluid (Robertson *et al.*, 1996), ovine mammary lymph and follicular fluid (Hodgkinson *et al.*, 1989b) and human peritoneal fluid (Giudice *et al.*, 1994; Bowsher *et al.*, 1991). Whether the complex as a whole crosses the endothelial barrier or whether the constituents cross individually and reassociate in these extracellular fluids is not known.

The aims of the current study were to (i) determine the IGF and IGFBP physiology of HUVE cells, and (ii) utilise the *in vitro* HUVE cell model of peptide tranport that was established and validated in chapter 3, to determine whether free IGF-I crosses endothelial cell monolayers via a paracellular route, possibly through endothelial intercellular junctions or large pore channels or by a transcellular process through the cells via receptor or cell surface binding proteins.

Preliminary experiments clearly demonstrated that HUVE cells were extremely intolerant to serum-free conditions. Even after 5 minutes in the absence of serum and growth supplements the characteristic, cobblestone morphology of the HUVE cells became spindle-like and the cells began to lift off the plate. HUVE cells were therefore maintained in serum-free culture media in the presence of heparin and recombinant human bFGF (100 ng/ml; cf Battista *et al.* 1995), and the presence of a continuous cell monolayer was checked by staining the cells and viewing each transwell at the end of each experiment. In addition, electron microscopy on selected wells confirmed the presence of an occlusive monolayer possessing tight junctions.

The presence of functional receptors for IGF-I and IGF-II on HUVE cells was demonstrated by radioreceptor, DNA synthesis and cross-linking studies. IGF receptors have

been demonstrated on endothelial cells isolated from other sources, including calf retinal capillaries, adipose capillaries, pulmonary arteries and aortas (Bar *et al.*, 1988; Bar and Boes, 1988; Bar *et al.*, 1986; Jialal *et al.*, 1985; King *et al.*, 1985; Bar and Boes, 1984). In addition, our affinity label cross-linking experiments consistently revealed lower molecular weight bands at approximately 28 and 35 kDa, possibly representing cell surface-associated IGFBPs. These bands were only observed when ^{125}I-IGF-II was the cross-linked ligand, suggesting these binding proteins possess a higher affinity for ^{125}I-IGF-II than ^{125}I-IGF-I. Other laboratories have reported the appearance of lower molecular weight bands on autoradiographs after cross linking ^{125}I-IGF to human and bovine endothelial cells (Feldman *et al.*, 1993; Ohashi *et al.*, 1993; Bar *et al.*, 1989).

When in combination with bFGF or low levels of FBS, IGF-I was able to stimulate DNA synthesis, confirming that HUVE cells possess functional IGF receptors and that in this assay IGF-I acts as a progression factor (Russell *et al.*, 1984), requiring additional competence factors to induce a mitogenic response in these cells. Similarly, King *et al.* (1985) found that endothelial cells originating from large vessels such as aorta were unresponsive to IGF-I alone. In contrast, IGF-I was mitogenic for small vessel endothelial cells.

As endothelial cell IGFBPs could affect the interactions of IGFs with endothelial cells, IGFBPs released into medium conditioned by HUVEs were characterised by Western ligand and immunoblotting. Under serum-free, bFGF-supplemented conditions, four IGF binding proteins were secreted into 24 h conditioned media of HUVE cells. The predominant 24 kDa band was identified as IGFBP-4 using antiserum raised against ovine IGFBP-4. The other two less prominent species at 32 kDa and the doublet between 38-43 kDa were immunologically identified as IGFBP-2 and IGFBP-3, respectively. Although these experiments were unable to immunologically confirm the identity of the fourth band occuring at approximately 28 kDa, it is possible it could represent glycosylated IGFBP-4. Large vessel endothelial cells established from bovine pulmonary artery and aorta also secrete

predominantly IGFBP-4, along with lesser amounts of IGFBP-3 (Boes *et al.*, 1992). In contrast, bovine microvessel endothelial cells derived from periaortic and omental fat secrete IGFBP-2 and IGFBP-3 (Moser *et al.*, 1992). Yang *et al.* (1993) observed that three bovine endothelial cell lines established from both micro- and macro- vessels secreted IGFBP-4. The differing IGFBP profiles observed in the conditioned media of the above cells could represent tissue, species and / or cell culture differences.

Both endothelial and epithelial cell monolayers generate tight junctions. In this series of experiments, MDCK cells were used as a control cell line because they are well characterised with respect to their barrier function. In general, endothelial cell intercellular junctions are less restrictive to the passage of ions and solutes, compared to their epithelial cell counterparts (Schneeberger and Lynch, 1992). Indeed, the electrical resistance across MDCK cell monolayers was much higher than the endothelial cells and they virtually excluded any passage of ^{125}I-IGF-I or [^{3}H]-inulin. In contrast, the HUVE cells did not produce values of electrical resistance above those observed with filters alone, although significantly excluded the passage of ^{125}I-IGF-I or [^{3}H]-inulin compared to filters without cells. These data are in agreement with the data of other investigators. Bovine aortic endothelial (BAE) cell monolayers produce electrical resistance values similar to those measured for cells which do not demonstrate tight junctions, namely 3T3-C2 fibroblasts. However, unlike fibroblasts, the endothelial cells restrict the passage of macromolecules (Milton and Knutson, 1990). Thus, HUVE cells restrict the passage of macromolecules, yet do not form a barrier to the passage of electrolytes. It is important to note that other properties of the endothelium, besides the presence of receptors or cell surface binding sites, contribute to the transport of macromolecules across endothelial cell monolayers. These include the charge of the endothelial cell barrier (Swanson and Kern, 1994) and the structure and characteristics of the endothelial extracellular matrix (Wheatley *et al.*, 1993a and b).

Several lines of evidence would suggest that free IGF-I crosses the endothelial barrier via a paracellular pathway. No significant difference between the amounts of [^{3}H]-inulin or ^{125}I-IGF-I transported across the HUVE cell monolayers was observed. As inulin (5600 Mr) is of similar molecular weight to IGF-I, is impermeable to cell membranes, and crosses endothelium through the intercellular junctions (Sonksen *et al.*, 1971), it is likely that ^{125}I-IGF-I transport could also be occurring via a paracellular pathway as opposed to a transcellular route. To examine this possibility further, the transport of ^{125}I-IGF-I in the presence of excess cold IGF-I or in the presence of antibody to the type I IGF receptor, was measured. ^{125}I-IGF-I transport across HUVE cell monolayers was non-competitive as determined by the inability of excess unlabelled IGF-I to compete with ^{125}I-IGF-I tracer transport. No inhibition in the transport of ^{125}I-IGF-I occurred in the presence of αIR-3 antibody at concentrations of antibody known to decrease binding of ^{125}I-IGF-I to HUVE cells by more than 60%.

The majority of ^{125}I-IGF-I appearing in the abluminal chamber following transport across HUVE cell monolayers was intact. Porcine aortic endothelial cells cultured on permeable membranes displayed polarised secretion of IGF-I plus IGF-I binding protein activity from the basal surface (Taylor *et al.*, 1993) suggesting the endothelium has the potential to regulate the exposure of subendothelial tissues to IGF-I. In addition to the results of the analysis of HUVE cell conditioned media in the present investigation, studies by independent researchers have shown that IGFBPs are secreted into the medium of cultured endothelial cells (Bar *et al.*, 1987, 1989, Moser *et al.*, 1992, Yang *et al.*, 1993, Taylor *et al.*, 1993). Along with IGFBPs circulating in the blood, these endothelial cell derived IGFBPs could affect the interactions of circulating IGFs with endothelial cells, acting as an additional regulatory mechanism that controls the transport of IGFs from blood to the interstitium harbouring the tissue cells.

In summary, studies described in this chapter have demonstrated that i) human umbilical vein endothelial cells possess receptors for both IGF-I and IGF-II, but are only responsive to IGF-I in the presence of other factors such as bFGF and serum, ii) HUVE cells secrete predominantly IGFBP-4, plus lesser amounts of IGFBP-2 and IGFBP-3, iii) HUVE cell monolayers do not form a restrictive barrier to electrolytes, but do limit the transendothelial passage of ^{125}I-IGF-I and iv) that transendothelial migration of ^{125}I-IGF-I across HUVE cell monolayers occurs via a paracellular route, with the majority of the ^{125}I-IGF-I appearing as intact peptide on the abluminal side of the monolayer. As IGF-I binding proteins are integral to the regulation of IGF-I, the effects of IGFBPs on the transfer of IGF-I across the endothelial cell barrier remains an important aspect of IGF physiology to be investigated in the following chapters.

CHAPTER: 5 EFFECTS OF IGFBPs ON TRANSPORT OF INSULIN-LIKE GROWTH FACTOR-I ACROSS ENDOTHELIAL CELL MONOLAYERS

5.1 INTRODUCTION

In the previous chapter, it was demonstrated that IGF-I transfers passively across a HUVE cell monolayer by a paracellular pathway. Similar to other systemic endothelia, the low electrical resistance values generated by HUVE cell monolayers is indicative that the paracellular pathway is the dominant conductance route for macromolecules (Shasby, 1988). Endothelial hyperpermeability in response to inflammatory agents including thrombin and histamine is accompanied by reversible cell rounding and interendothelial gap formation, further suggesting that the predominant transport pathway of endothelia is a paracellular one (Lum and Malik, 1996). Although endothelial cells, including HUVE cells, in monolayer culture contain intercellular junctions that contribute to the barrier function (Siflinger-Birnboim, 1987, Chapter 3, Fig. 3.7), current thought also acknowledges a number of other determinants of passive barrier properties of the endothelia. Studies have demonstrated that cell-cell contact between endothelial cells is an important determinant of endothelial macromolecule transfer. Manjo and Palade (1961) first proposed that the loss of the close approximation between venular endothelial cells following histamine exposure was a possible mechanism for histamine induced oedema formation. Killackey *et al.* (1986) have confirmed this concept *in vitro*, showing that following exposure to histamine, adjacent endothelial cells lost contact with each other, permitting dye to penetrate into a bead on which the cells were grown. α-Thrombin induces a loss of an HUVE cell selective barrier to albumin, which was demonstrated to be a result of cellular contraction and paracellular gap formation (Garcia *et al.*, 1992; Garcia and Natarajan, 1992; Garcia *et al.*, 1986). Tumor necrosis factor-alpha (TNF-α) treatment of bovine pulmonary microvessel endothelial (BPMVE) cell monolayers,

induces an increase in endothelial cell monolayer permeability (Partridge *et al.*, 1992). The increases in permeability paralleled changes in endothelial cell morphology from cobblestone to elongated cells and the formation of prominent intercellular gaps. In contrast, exposure of canine jugular vein endothelial cell monolayers to histamine or bradykinin for 15 min resulted in an increased permeability to albumin, yet no changes in the intercellular gaps were observed (De Fouw *et al.*, 1993). The authors speculated that the increased albumin flux may have arisen from transient intercellular gap formation or alternatively physical changes within the endothelial cell glycocalyx.

In addition to the barrier provided by the endothelial cells themselves, the cells secrete glycoproteins, glycolipids and proteoglycans that form a glycocalyx that coats the luminal plasmalemma (Lawrenson *et al.*, 1996; Haldenby *et al.*, 1994; Schneeberger and Hamelin, 1984). Plasma proteins, including albumin, are also incorporated into this glycocalyx (Schneeberger and Hamelin, 1984). The glycocalyx and incorporated proteins may represent the true active interface between blood and endothelial cells, and potentially contribute to the selectivity of the endothelial barrier to migration of macromolecules (Katz, 1992). Curry and Michel (1980) were the first to formulate the fiber matrix model, suggesting that the endothelial glycocalyx forms a fibrous matrix that confers macromolecular sieving properties on the vascular wall. Pore sizes within this matrix may change, regulating the macromolecular access to the paracellular and / or vesicular transendothelial pathways. More recent studies have indicated that glycocalyx-like material also exists in the intercellular tight junctions, which may also form part of the filter within the paracellular pathway (Leach and Firth, 1992). Incorporation of oligosaccharides may tighten the matrix since they have been shown to reduce the permeability of the endothelial fiber matrix of the chick chorioallantoic membrane (Henry *et al.*, 1997). In perfused rat hearts, hypoxia resulted in disruption and irregular clumping of the endothelial glycocalyx, which may explain the increased capillary permeability observed under this condition (Ward and Donnelly, 1993). The glycocalyx and

incorporated proteins present a negatively charged luminal surface, that occludes the passage of anionic macromolecules, but also resists water and neutral molecules (Ryan, 1986; Raviola and Butler, 1983; 1984; Simionescue *et al.*, 1981). Studies in the kidney suggest that glycosaminoglycans represent the main barrier to albumin transport across the glomerulus (Hunsicker *et al.*, 1981). Neutralisation of the negative charge of the anionic glycocalyx in nephrectomized rats by injection of polycations such as protamine sulfate, hexadimethrine or poly-*l*-lysine was shown to increase systemic vascular permeability (Vehaskari *et al.*, 1984). Molecules such as orosomucoid (alpha 1-acid glycoprotein), a serum glycoprotein, has been demonstrated to bind the endothelial glycocalyx increasing the overall negative charge and as a result decreases vascular permeability (Schnitzer and Pinney, 1992). More recent studies of the spinal cord of the rat demonstrated an association between loss of the charge of the endothelial glycocalyx due to spinal cord injury, and disruption of barrier function, suggesting that anionic sites may contribute to the maintenance of the blood-spinal cord barrier (Noble *et al.*, 1996). Although most endothelial beds have a luminal anionic glycocalyx, not all beds have a lower permeability to anions relative to cations (Shasby, 1988). This implies that other charge barriers could exist, and may reside in the extracellular matrix.

Appreciable literature exists relating to the importance of the extracellular matrix in endothelial permeability. Endothelial cells produce an underlying extracellular matrix consisting of collagens, fibronectin, laminin and glycosaminoglycans (Yurchenco and Schittny, 1990). The product is a complex meshwork of predominantly type IV collagen and laminin, associated with heparan sulfates with large hydration shells, that may form anionic channels, which in turn influence permeability of the ECM to molecules of different charge and sizes. As the ECM acts as a substratum for endothelial cell attachment, it also influences endothelial cell shape and cytoskeletal conformation (Madri, 1983), factors also considered important to endothelial cell permeability. A number of studies have highlighted the structure-function relationship between matrix composition and endothelial barrier

permeability. BPMVE cells seeded onto filters pre-coated with ECM produced by TNF-α treated BPMVE cells, developed large intercellular gaps and centralised actin filaments and displayed two- to three-fold higher ^{125}I-albumin permeability values than BPMVE monolayers grown on filters pre-coated with ECM of untreated BPMVE cell monolayers (Partridge *et al.*, 1992). Examination of ECM extracted from BPMVE cells treated with TNF-α showed a decreased fibronectin content, suggesting that TNF-α induced increases in endothelial permeability involves loss of fibronectin or remodelling of ECM. Therefore, increased permeability may be secondary to decreased endothelial cell-ECM contact, resulting in elongation of cells and formation of intercellular gaps. Furthermore, they found an increased level of a 96 kDa metalloproteinase in the conditioned medium of the TNF-α treated cells with the capability of degrading specific ECM components, including fibronectin (Partridge *et al.*, 1993). Wheatly *et al.*(1993a) have since shown that incorporation of soluble human fibronectin into the ECM reduced the TNF-α induced increase in lung endothelial monolayer permeability. Treatment of endothelial cells with heparin caused a loss of the ECM associated heparan sulfate proteoglycan and a concomitant increase in endothelial permeability to albumin (Guretzki *et al.*, 1994). Reduction in collagen content of ECM produced by bovine aortic, venous and human umbilical vein endothelial cell monolayers by treatment with medium conditioned by mouse melanoma cells led to hyperpermeability, a result which has implications for invasive cancer therapy (Utoguchi *et al.*, 1996). Exposure of the same types of endothelial cells with ascorbic acid increased collagen synthesis, resulting in a decreased permeability (Utoguchi *et al.*, 1995).

Although the above mentioned factors are involved in the passive barrier function of endothelia, determinants of the diffusive flux of IGF-I via the paracellular pathway are not known. One postulated role of the IGFBPs is to control rates of IGF transport from the vascular compartment. The predominant 150 kDa ternary complex, which consists of an IGFBP-3 molecule, an acid labile subunit (ALS) and either an IGF-I or -II molecule, carries

the majority of IGF-I in circulation (Baxter, 1988b). This complex is thought to act as a reservoir of readily releasable IGFs in plasma. Lower amounts of IGFs circulate in 50 kDa binary complexes, which contain IGF-I or -II and one of the six known IGFBPs. Sara and Hall (1990) hypothesised that the low molecular weight IGFBPs act as transporting proteins to 'shuttle' IGFs out of the vascular space to tissues. Evidence has been accumulating which indicates that IGFBPs cross the endothelial cell barrier. IGFBP-1, -2, -3 and -4 are all able to cross the capillary barrier in the perfused isolated beating rat heart (Bar *et al.*, 1990a, b, and c; Boes *et al.*, 1992). In addition, these researchers have shown that IGF-I bound to IGFBP-1 to -4 is able to cross capillary boundaries (Bar *et al.*, 1990a, b; Boes *et al.*, 1992). Furthermore, pharmacokinetic parameters for IGFBP-1 and -2 in rats suggest that these proteins equilibrate with the extravascular space, potentially providing a means for translocation of the IGFs out of the vasculature (Young *et al.*, 1992). Most recently, radiolabelled recombinant human IGFBP-3 unassociated with the ternary complex has been shown to rapidly cross capillary endothelia from blood to organs including kidney, liver and gut, suggesting a role for IGFBP-3 in delivering IGFs to peripheral tissues (Arany *et al.*, 1993). Taken together, these data infer that the lower molecular weight IGFBPs are involved in delivering IGFs to tissues. It has been demonstrated in the perfused rat heart model that insulin is capable of stimulating the transcapillary movement of IGFBP-1 and -4 (Bar *et al.*, 1990c; Boes *et al.*, 1992). Streptozotocin induced diabetes in rats has been shown to give rise to increased IGFBP-1 serum and hepatic mRNA levels, in association with a marked increase in gene transcription (Ooi *et al.*, 1990; Unterman *et al.*, 1990). Treatment of these animals with insulin resulted in a rapid decrease in serum and hepatic IGFBP-1 levels (Ooi *et al.*, 1990). Since IGFBP-1 levels are inversely related to insulin levels (Lee *et al.*, 1993), increases in insulin could result in IGFBP-1 crossing the vascular endothelium into tissues. These findings are indicative of a regulated, nutrient dependent mechanism for delivering circulating IGF from the vascular compartment to extracellular fluid and tissue sites.

The involvement of various IGFBPs in the transfer of IGFs from blood to extracellular fluids is not understood. It has been postulated that IGFs are transported through the capillary barrier by transference of the IGFs from the 150 kDa complex to smaller binary complexes. Alternatively, complete dissociation of the ternary complex may occur followed by unbound IGFs diffusing into the extracellular space. In culture, endothelial cells form a "cobblestone" monolayer with intercellular junctions that contribute to the barrier function of the monolayer (Siflinger-Birnboim *et al.*, 1987). However, ECM of endothelial cells also contributes to barrier function (Yurchenco and Schittny, 1990). Both IGFBP-3 and -5 bind to the endothelial cell surface and to ECM secreted by these cells (Bar *et al.*, 1994). Studies examining ECM of cultured human foetal fibroblasts identified IGFBP-5 and minimal amounts of IGFBP-3. In addition, when IGFBP-5 was bound to ECM it potentiated the growth stimulatory actions of IGF-I (Jones *et al.*, 1993a). The possible role of IGFBPs present in endothelial cell ECM in the transfer of IGF across the monolayer barrier is unknown.

As a first step in understanding how IGFBPs may affect the translocation of IGFs from plasma to interstitial fluid, I have used a HUVE cell monolayer *in vitro* model of an endothelial cell barrier, and examined the effects of the addition of exogenous IGFBPs on the transport of both IGF-I and LR^3 IGF-I across the monolayer. In addition, I examined HUVE cell derived extracellular matrix to identify IGFBPs and investigated the transport of IGF-I across HUVE cell derived ECM substratum.

5.2 MATERIALS AND METHODS

5.2.1 Materials

IGFBPs used in these studies were generously supplied by the following; IGFBP-1, DSL (Texas, USA), IGFBP-2, Dr. Eiji Kuto (Basel, Switzerland), IGFBP-3 and IGFBP-6, Dr Robert Baxter (Sydney, Australia), IGFBP-4, IGFBP-5 and carboxy truncated IGFBP-5 (tIGFBP-5), Dr Dennis Andress (Seattle, USA). Human ALS was kindly donated by Dr. Robert Baxter (Sydney, Australia). Triton X-100 was from Ajax Chemicals (Sydney, Australia). Ammonium acetate was from BDH Chemicals (Victoria, Australia).

5.2.2 Transmigration studies

HUVE cells were seeded on fibronectin coated transwells and maintained in HUVE medium under the same culture conditions as described in section 4.2.9. Prior to experiments, confluency of HUVE cell monolayers was confirmed by staining and light microscopy of control transwells (see section 4.2.9). Transmigration studies were performed as in section 4.2.9, with a few modifications. To examine whether factors in serum, possibly IGFBPs, have an affect on ^{125}I-IGF-I transport across HUVE cell monolayers, the transport of ^{125}I-IGF-I (8 ng) was examined in either serum-free HUVE medium plus 0.1 % BSA, bFGF (100 ng / ml) and heparin (25 μg / ml) as described in section 4.2.9, or in HUVE medium plus 0.1% BSA containing 20% FBS. In additional experiments, the transport of ^{125}I-LR^3 IGF-I (8 ng) was also determined in either serum-free or 20% FBS containing HUVE medium.

5.2.3 Transport of ^{125}I-IGF-I and ^{125}I-LR^3 IGF-I in the presence of IGFBP-1 to -6 or truncated IGFBP-5

Transmigration studies were performed as for section 4.2.9. However, prior to addition of ^{125}I-IGF-I or ^{125}I-LR^3 IGF-I to the luminal chamber at time zero, each labelled IGF was incubated for 2h at 37°C with a 1:1 molar amount of either hIGFBP-1, -2, -3,.-4, -5, -6 or truncated hIGFBP-5. Additional experiments were performed using a 1:2 and a 2:1 molar ratio of ^{125}I-IGF-I : IGFBP.

5.2.4 Transport of ^{125}I-IGF-I in the presence of hIGFBP-3 and human acid labile subunit (ALS)

Transmigration studies were performed as described in section 4.2.9. In these experiments, ^{125}I-IGF-I (8 ng), a 1:1 molar equivalence of hIGFBP-3 (45 ng) and hALS (450 ng) at a 5-fold molar excess over the hIGFBP-3 amount were incubated for 2h at 37°C, prior to addition to the luminal chamber at time zero.

5.2.5 Preparation of HUVE cell derived extracellular matrix (ECM)

The procedure for the preparation of ECM has been described previously (Knudsen *et al.*, 1988). HUVE cells were subcultured onto fibronectin coated tissue culture flasks (T80) or transwells. Cells were maintained in HUVE medium at 37° C, containing 20% FBS and 25 μg / ml of both ECGS and heparin, with daily media changes until confluence. Cells were then washed twice in 0.5% Triton X-100 in PBS pH 7.4 for 10 min to remove cell membranes. The remaining cytoskeleton and any adherent nuclei were removed by a 5 min incubation in 25 mM ammonium acetate, pH 9.0. The remaining ECM was rinsed twice with PBS. All solutions, flasks and transwells were kept on ice throughout the procedure. ECM remaining on transwells was used as a substratum for transmigration experiments. To obtain

ECM samples for Western ligand blot and immunoblot analysis, SDS-loading buffer (500 µl, see section 4.2.6) was added to each tissue culture flask, the bottom of the flask scraped with a rubber policeman and the samples stored at -20°C.

5.2.6 Western ligand blot and immunoblot analyses of HUVE cell derived ECM

ECM samples (40 µl, see section 5.2.5), human plasma (2 µl equivalence) and hIGFBP-1, -2, -3, -5 and oIGFBP-4 (100 ng of each) were incubated at 65°C for 15 min in SDS-loading buffer (see section 4.2.6). All samples were subjected to discontinuous SDS-PAGE, transferred to nitrocellulose sheets, incubated with ^{125}I-IGF-II and exposed to X-ray film as outlined in section 4.2.6. The nitrocellulose sheets were then cut into strips for immunoblotting, as described in section 4.2.6.

5.2.7 Transport of ^{125}I-IGF-I through transwells coated with HUVE cell derived ECM substratum

Transmigration studies were performed essentially as described in section 4.2.9, with the exception that ^{125}I-IGF-I transport across HUVE cell monolayers and transwell filters without cells was compared to transport across transwell filters with HUVE cell derived ECM substratum as prepared in section 5.2.5.

5.2.8 Statistical analyses

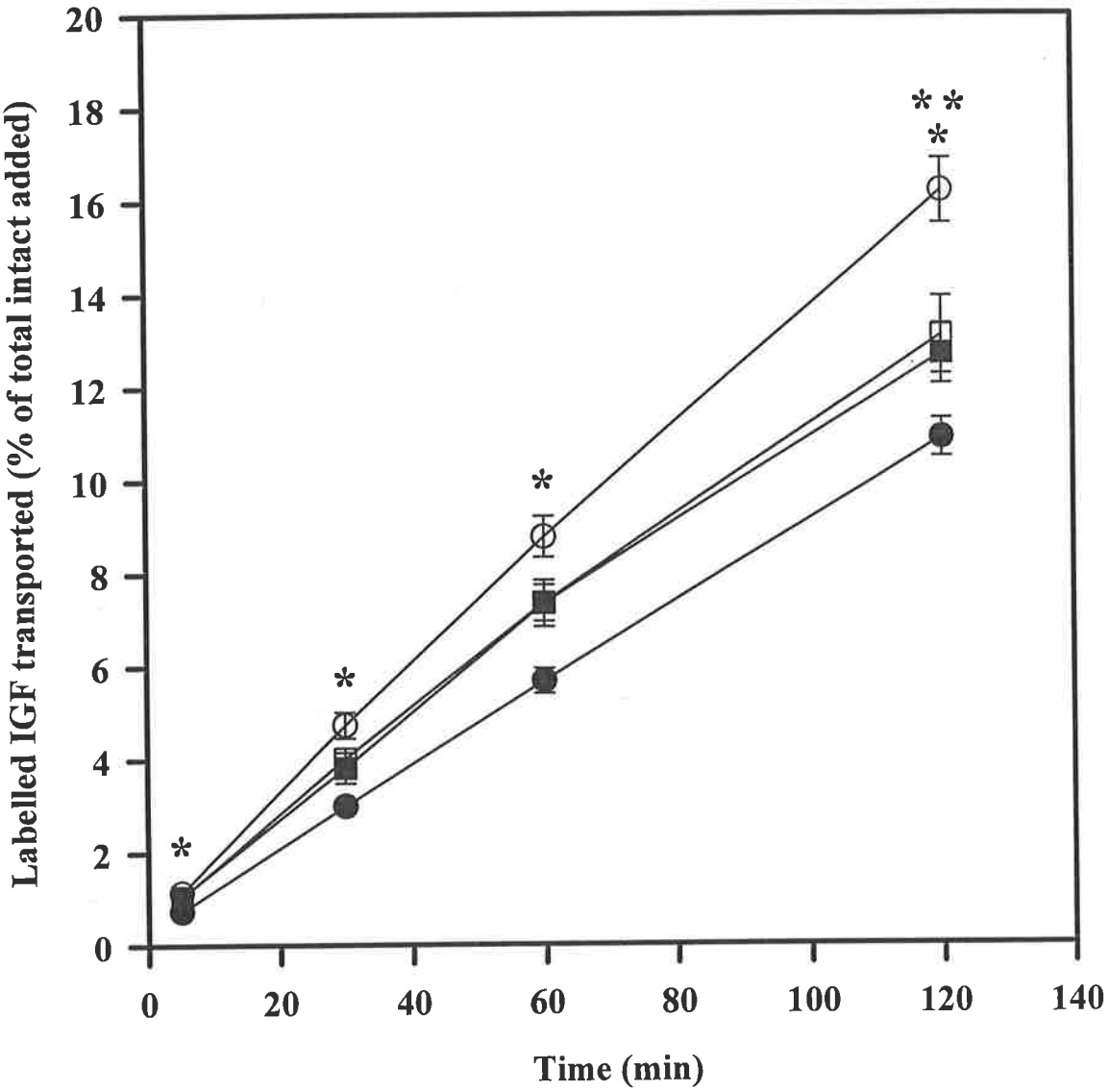
All data were analysed by one-way analysis of variance with all pairwise multiple comparisons using Student Newman-Keuls method. $P<0.05$ was considered significant.

5.3 RESULTS

5.3.1 Transport of ^{125}I-IGF-I or ^{125}I-LR^3 IGF-I across HUVE cell monolayers in the presence of 20% FBS or serum-free experimental medium

To determine whether transport of ^{125}I-IGF-I across HUVE cell monolayers is affected by components present in serum, including the IGFBPs, the effect of the presence of 20% FBS in the experimental medium was examined (Fig 5.1). In addition, the transport of ^{125}I-IGF-I and ^{125}I-LR^3 IGF-I (an IGF-I analogue with a reduced affinity for IGFBPs) in both FBS containing medium and serum-free conditions was compared. The presence of 20% FBS in the experimental medium significantly inhibited the transport of ^{125}I-IGF-I across HUVE cell monolayers from 16.2 ± 0.7 % at 120 minutes under serum-free conditions, to 10.9 ± 0.4 % (Fig 5.1). At the 2 h time point, the transport of ^{125}I-LR^3 IGF-I across HUVE cell monolayers in 20% FBS containing or serum-free medium, was significantly lower than the transport of ^{125}I-IGF-I in serum-free conditions. Furthermore, transport of ^{125}I-LR^3 IGF-I in the presence of 20% FBS was not inhibited compared with transport of ^{125}I-LR^3 IGF-I in serum-free conditions. These data show that the transport of free IGF-I across endothelial cell monolayers is inhibited by the presence of serum components, possibly IGFBPs.

Figure 5.1: ^{125}I-IGF-I and ^{125}I-LR^{3} IGF-I transport across HUVE cell monolayers in either serum-free medium (open figures) or 20% FBS containing medium (closed figures). ^{125}I-IGF-I (circles) and ^{125}I-LR^{3} IGF-I (squares) transport is expressed as a percentage of total intact ^{125}I-IGF-I or ^{125}I-LR^{3} IGF-I added to the luminal chamber at time zero. Data are the pooled means ± sems of triplicate Transwells from 8, 3, 4 and 3 experiments for transport of ^{125}I-IGF-I in serum-free medium, ^{125}I-IGF-I in 20% FBS containing medium, ^{125}I-LR^{3} IGF-I in serum-free medium and ^{125}I-LR^{3} IGF-I in 20% FBS containing medium, respectively. * represent significant differences in transport between ^{125}I-IGF-I in serum-free medium compared with 20% FBS containing medium ($P<0.05$). ** represent significant differences in transport between ^{125}I-IGF-I in serum-free medium compared with ^{125}I-LR^{3}IGF-I in both 20 % FBS containing media and under serum-free conditions ($P<0.05$).


Labelled IGF transported (% of total intact added)
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
Time (min)
*
*
*
* *

5.3.2 Effect of IGFBPs on transport of ^{125}I-IGF-I and ^{125}I-LR^3 IGF-I across HUVE cell monolayers

To determine whether transport of IGF-I across HUVE cell monolayers is affected by the presence of each of the known IGFBPs, the effects of pre-incubating labelled IGF-I with each individual IGFBP for 2h at 37°C before addition to the luminal chamber was investigated. These experiments were carried out at a 1:1 molar ratio of ^{125}I-IGF-1:IGFBP. With the exception of truncated hIGFBP-5, which has a greatly reduced affinity for IGF-I (Andress and Birnbaum, 1992), each of the IGFBPs significantly inhibited the transport of ^{125}I-IGF-I from the luminal to abluminal chamber (Fig 5.2A). In contrast, when ^{125}I-LR^3 IGF-I was the labelled ligand under examination (Fig 5.2B), there was no inhibition of transport of this peptide, confirming that the IGFBPs inhibit transport by binding to free IGF-I. No difference in transport between inulin and IGF-I was seen as determined in chapter 4. However, there was a significant difference between inulin and LR^3 IGF-I transport.

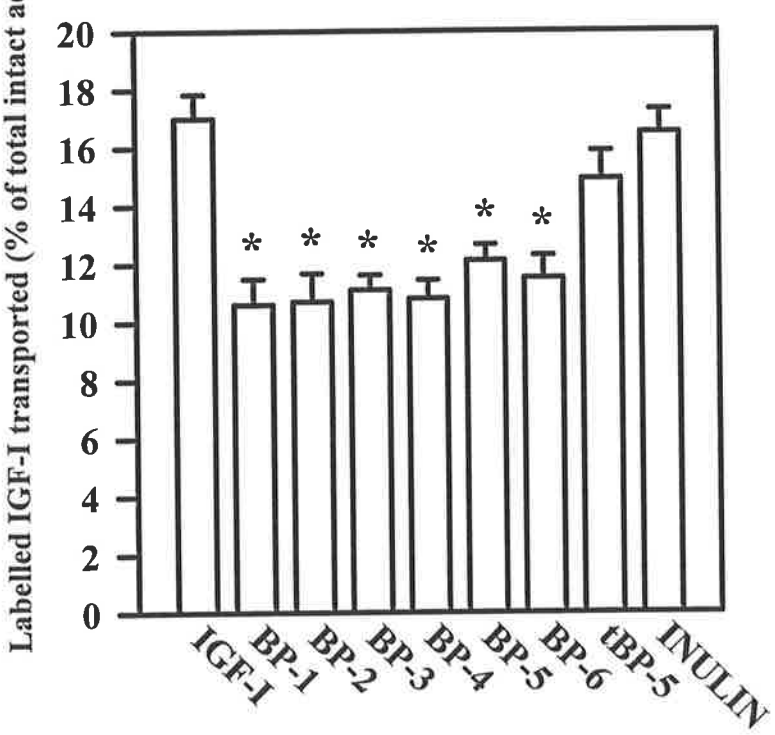
When the same experiments were carried out at a 1:2 IGF:IGFBP molar ratio (Fig 5.3A), similar transport of ^{125}I-IGF-I was observed. However, the effect was less dramatic when a 2:1 molar ratio of IGF-I to IGFBP was used (Fig 5.3B), indicating that this molar ratio may be less favourable for the binding of ^{125}I-IGF-I and the human IGFBPs under these experimental conditions, allowing increased flux of ^{125}I-IGF-I.

5.3.3 Effect of the presence of hIGFBP-3 and hALS on transport of ^{125}I-IGF-I across HUVE cell monolayers

Since the 150 kDa ternary complex is thought to act as a store of IGFs within the vascular compartment and studies showed that IGFBPs inhibited IGF-I transport in the HUVE cell monolayer model, the transport of ^{125}I-IGF-I across HUVE cell monolayers was compared to that of ^{125}I-IGF-I in the presence of hIGFBP-3 and ALS (Fig 5.4). Due to the low binding

Figure 5.2: Effects of IGFBPs on the transport of ^{125}I-IGF-I and ^{125}I-LR^3 IGF-I across HUVE cell monolayers. ^{125}I-IGF-I (*A*) and ^{125}I-LR^3 IGF-I (*B*) transport is shown as a percentage of total intact labelled IGF added to the luminal chamber at time zero appearing in the abluminal chamber at 2h. Labelled IGF transport across HUVE cell monolayers was measured in the presence of a 1:1 molar ratio of either hIGFBP-1, -2, -3, -4, -5, -6 or truncated hIGFBP-5. Data represents the pooled means ± sems of triplicate Transwells from three experiments. * represent significant differences in transport with respect to either ^{125}I-IGF-I or ^{125}I-LR^3 IGF-I across HUVE cell monolayers ($P<0.05$).

A


Labelled IGF-I transported (% of total intact added)
20
18
16
14
12
10
8
6
4
2
0
*
*
*
*
*
*
IGF-I
BP-1
BP-2
BP-3
BP-4
BP-5
BP-6
tBP-5
INULIN

B

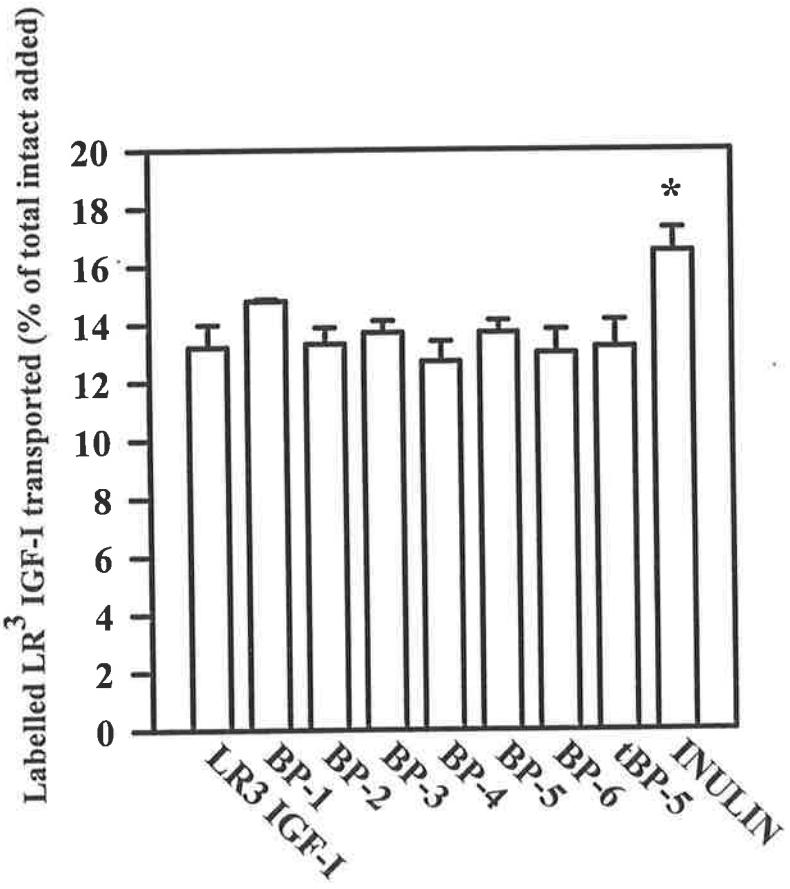

Labelled LR3 IGF-I transported (% of total intact added)
20
18
16
14
12
10
8
6
4
2
0
*
LR3 IGF-I
BP-1
BP-2
BP-3
BP-4
BP-5
BP-6
tBP-5
INULIN

Figure 5.3: Effects of different IGF to IGFBP molar ratios on the transport of ^{125}I-IGF-I across HUVE cell monolayers. ^{125}I-IGF-I transport is shown as a percentage of total intact labelled IGF added to the luminal chamber at time zero appearing in the abluminal chamber at 2h. Labelled IGF-I transport across HUVE cell monolayers was measured in the presence of a either a 1:2 (*A*) or 2:1 (*B*) IGF:IGFBP molar ratio. Data represents the pooled means ± sems of triplicate Transwells. * represent significant differences in transport with respect to ^{125}I-IGF-I across HUVE cell monolayers ($P<0.05$).

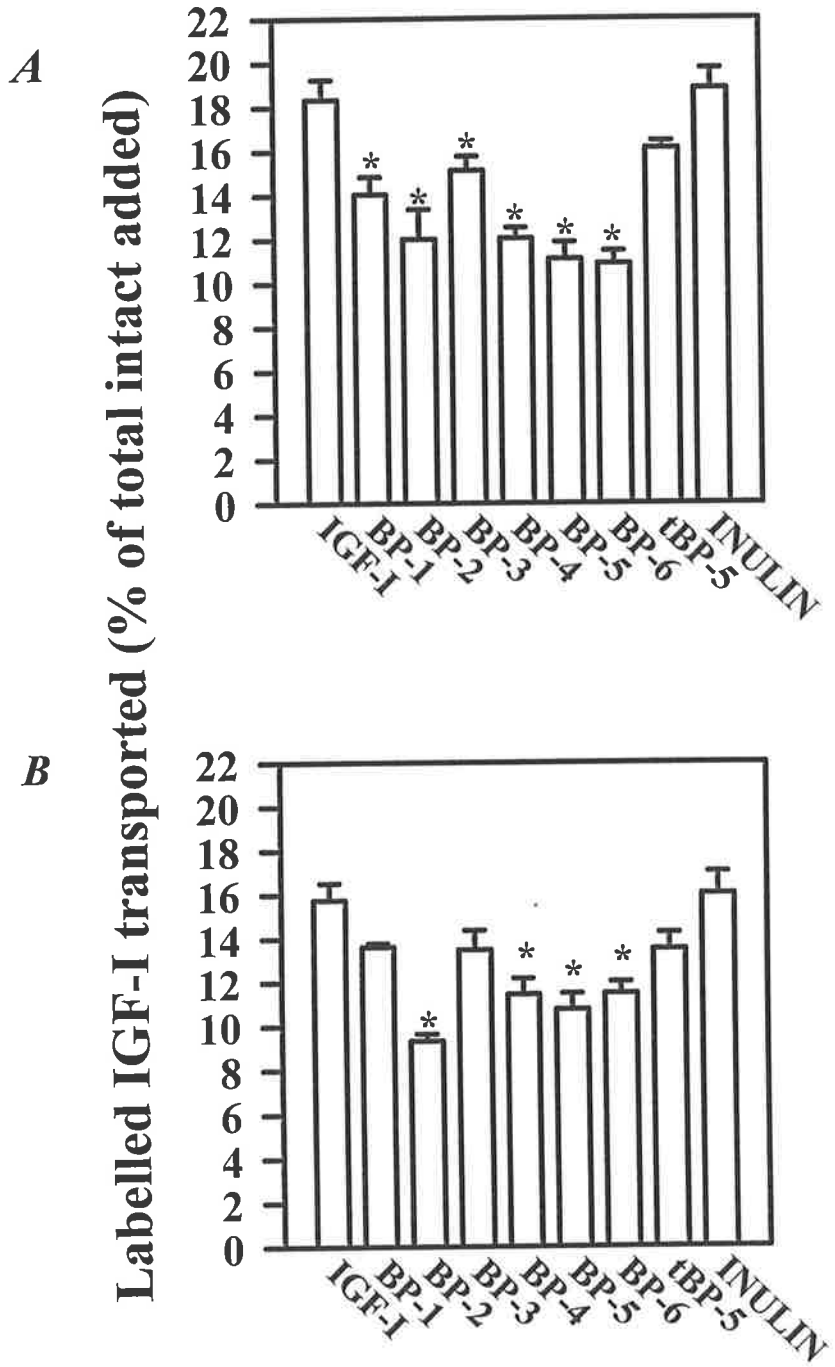
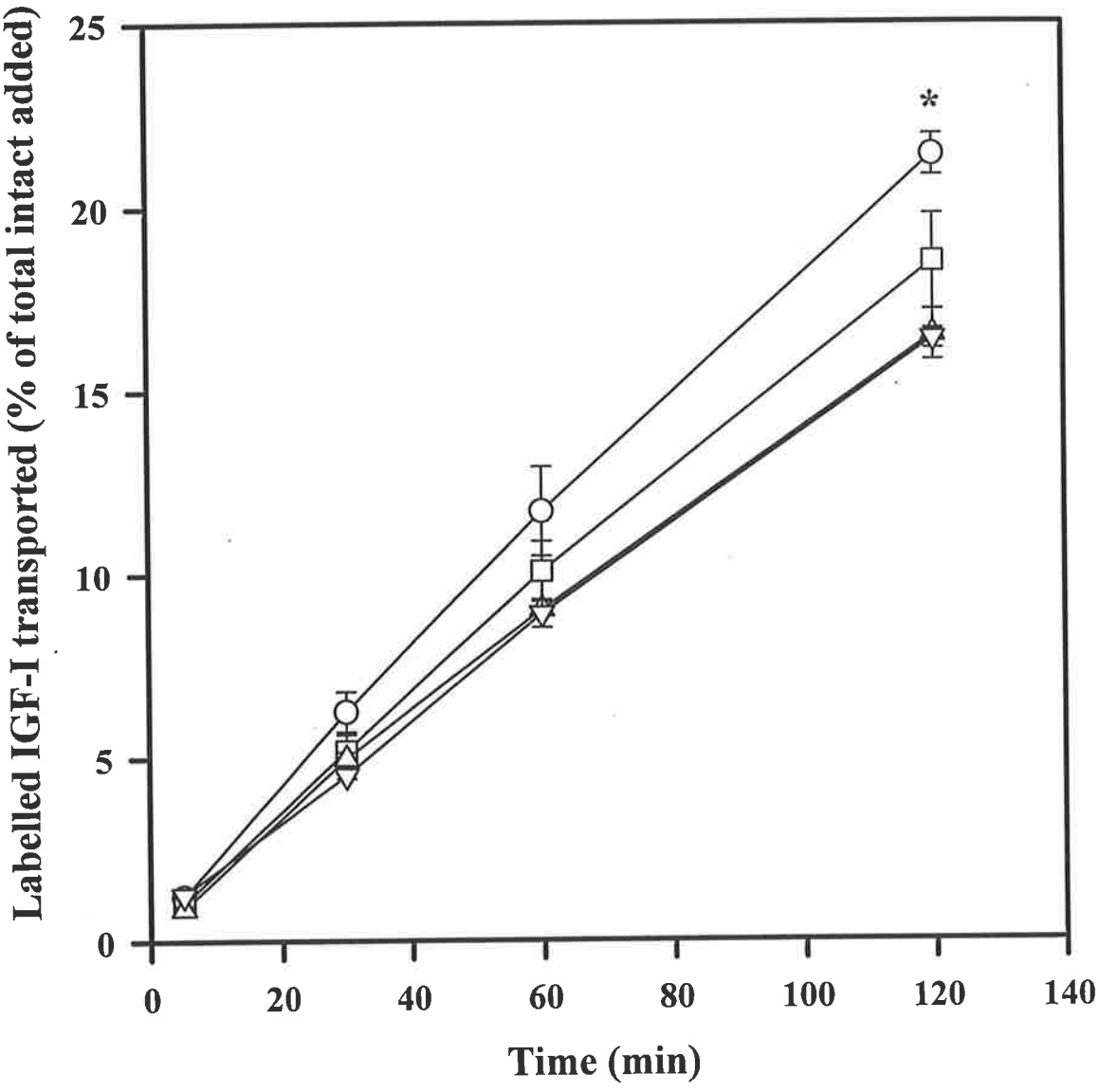

A
B
Labelled IGF-I transported (% of total intact added)
0
2
4
6
8
10
12
14
16
18
20
22
IGF-1
BP-1
BP-2
BP-3
BP-4
BP-5
BP-6
tBP-5
INULIN

Figure 5.4: Effects of the presence of hIGFBP-3, hIGFBP-3 plus hALS or hALS alone on ^{125}I-IGF-I transport across HUVE cell monolayers. ^{125}I-IGF-I is expressed as a percentage of total intact ^{125}I-IGF-I added to the luminal chamber at time zero appearing in the abluminal chamber. ^{125}I-IGF-I transport across HUVE cell monolayers (circles) was measured in the presence of either hIGFBP-3 (squares), hIGFBP-3 and hALS (triangles) or hALS (inverted triangles). Data represents the pooled means ± sems of three Transwells from two individual experiments. * represents a significant difference in transport of each data point at 2h with respect to ^{125}I-IGF-I across HUVE cell monolayers ($P<0.05$).

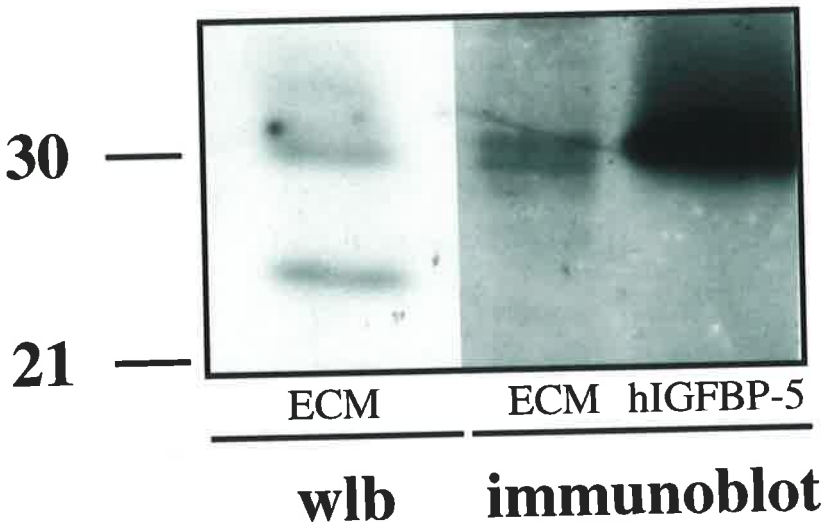

Labelled IGF-I transported (% of total intact added)
25
20
15
10
5
0
*
0
20
40
60
80
100
120
140
Time (min)

affinity of ALS for IGF-I-IGFBP-3 complexes at 37° C / neutral pH 7.0, ALS was used at a 5-fold molar excess (450 ng) over the IGFBP-3 concentration (45 ng) to ensure ternary complex formation (Dr Robert Baxter, personal communication). ^{125}I-IGF-I (8 ng) and hIGFBP-3 were used at a 1:1 molar ratio. At the 2 h time point, the presence of hIGFBP-3 alone significantly inhibited transport of ^{125}I-IGF-I from 21.4 ± 0.6 % to 18.5 ± 0.3, confirming earlier studies. Similarly, by 2 h the transport of ^{125}I-IGF-I across HUVE cell monolayers was significantly reduced from 21.4 ± 0.6 % to 16.5 ± 0.6 % by the presence of hIGFBP-3 / hALS. Furthermore, the presence of hALS alone significantly inhibited transport of ^{125}I-IGF-I to 16.4 ± 0.2 %, indicating that hALS at such high concentrations may have been able to form a ternary complex with ^{125}I-IGF-I bound to low levels of secreted endogenous HUVE cell IGFBP-3.

5.3.4 Identification of IGFBPs in HUVE cell derived extracellular matrix (ECM)

Extracellular matrix (ECM) derived from HUVE cells was examined for the presence of IGFBPs by both Western ligand blotting and immunoblotting techniques (Fig 5.5). Two bands were present on the Western ligand blot. The 31 kDa protein was identified immunologically as IGFBP-5 using an antibody specific for hIGFBP-5. The 24 kDa band visible on the ligand blot was not detected by immunoblotting. Antibodies to human IGFBP-1, -2 and -3 and antiserum raised against oIGFBP-4, failed to bind any proteins present in HUVE cell ECM samples. Thus, ECM derived from HUVE cells contains immunologically detectable IGFBP-5 and a 24 kDa protein whose identity was unable to be determined by immunoblotting techniques.

Figure 5.5: Western ligand blot and immunoblot of IGFBPs contained in HUVE cell derived ECM samples. HUVE cell derived ECM was obtained as described in materials and methods. HUVE cell derived ECM (40 μl) was subjected to SDS-PAGE under non-reducing conditions, transferred to nitrocellulose sheets, probed with ^{125}I-IGF-I and exposed to X-ray film for 2 weeks (wlb = Western ligand blot). For immunoblots, the same nitrocellulose sheets were exposed to an antibody specific for hIGFBP-5 (α-BP-5) as indicated. Positions of ^{14}C-labelled molecular weight markers are indicated (x10^{-3}).


30
21
ECM
ECM hIGFBP-5
wlb
immunoblot

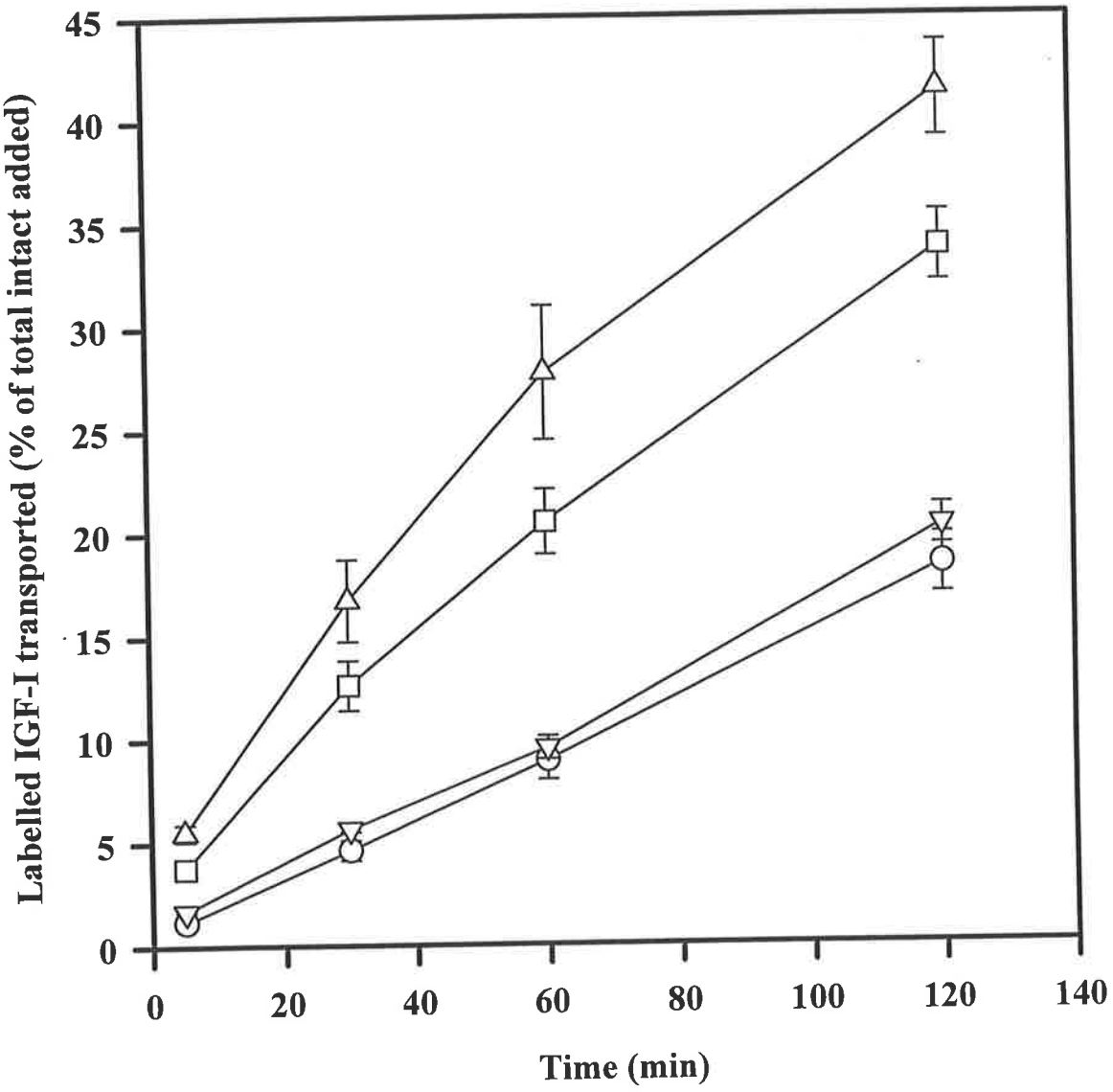
5.3.5 Transmigration of ^{125}I-IGF-I across HUVE cell derived ECM

As Western ligand blotting and immunoblotting revealed that HUVE cell derived ECM contained IGFBPs, the transport of ^{125}I-IGF-I across HUVE cell monolayers compared to that across HUVE cell derived ECM was examined. At each time point, the transport of ^{125}I-IGF-I across HUVE cell derived ECM was significantly greater than that across HUVE cell monolayers, but was significantly less than that across filters without cells (Fig 5.6). As observed earlier in chapter 4, there was no significant difference in the transport of [^{3}H]-inulin and ^{125}I-IGF-I across HUVE cell monolayers. Additionally, the transport of ^{125}I-IGF-I across HUVE cell monolayers was significantly restricted compared to transport across filters without cells. These data indicate that the ECM may play a regulatory role in the transport of free IGF-I across an endothelial cell monolayer.

5.4 DISCUSSION

The endothelial cell barrier is thought to determine whether IGF-I is retained within the plasma associated with the 150 kDa ternary complex, or transferred to the extracellular space either in association with an IGFBP or as a free peptide (Rechler, 1993; Bar *et al.*, 1990b; Guler *et al.*, 1989a; Binoux and Hossenlopp, 1988). How IGF:IGFBP complexes are transported or by what mechanism IGF dissociates from these complexes to transport as free peptide is unknown. Due to the likely involvement of IGFBPs in transport of IGFs across the endothelial cell barrier, this chapter examined their effect on IGF-I transport in the HUVE cell monolayer model. LR^{3} IGF-I is a useful tool for comparison against IGF-I in determining the effect of reduced IGFBP interaction on endothelial transfer of plasma IGF-I. Preliminary studies examining the transport of ^{125}I-IGF-I across HUVE cell monolayers in media containing 20% FBS showed a significant decrease in the flux of ^{125}I-IGF-I, compared to ^{125}I-IGF-I transport in serum-free media. Unlike ^{125}I-IGF-I, there was no difference in transport of

Figure 5.6: ^{125}I-IGF-I transport across HUVE cell monolayers and HUVE cell derived ECM. ^{125}I-IGF-I and [^{3}H]-inulin transport is expressed as a percentage of total intact ^{125}I-IGF-I and total [^{3}H]-inulin added to the luminal chamber at time zero, respectively. Data are the pooled means ± sems of triplicate Transwells from three experiments. ^{125}I-IGF-I and [^{3}H]-inulin transport across HUVE cell monolayers is indicated by circles and inverted triangles, respectively. ^{125}I-IGF-I transport across HUVE cell derived ECM or filters without cells is indicated by squares and triangles, respectively.


Labelled IGF-I transported (% of total intact added)
45
40
35
30
25
20
15
10
5
0
0
20
40
60
80
100
120
140
Time (min)

^{125}I-LR^3 IGF-I across HUVE cell monolayers in serum-free media to that of ^{125}I-LR^3 IGF-I transport in the presence of 20% FBS. These data indicate that the transport across HUVE cell monolayers of IGFs with the ability to bind IGFBPs is inhibited in the presence of FBS containing factors, which are likely to be IGFBPs. The significant difference between the transport of ^{125}I-IGF-I and ^{125}I-LR^{3}IGF-I could be because LR^{3}IGF-I is a larger molecule than IGF-I, or it could arise because of the differences in charge between the two peptides. The difference in the transport of ^{125}I-IGF-I and ^{125}I-LR^3 IGF-I observed in the presence of 20% FBS, supports the *in vivo* findings that radiolabelled des(1-3) IGF-I and LR^3 IGF-I are cleared more rapidly than IGF-I from the circulation of rats following bolus intravenous injections (Bastian *et al.*, 1993; Ballard *et al.*, 1991). In pregnancy, circulating IGFBP-3 has a reduced affinity for IGF-I which is thought to be a result of proteolytic enzyme activity within pregnancy serum (Binoux *et al.*, 1991b; Guidice *et al.*, 1990). In pregnant rats LR^3 IGF-I was cleared at the same rate as in non-pregnant animals, whereas IGF-I clearance was increased compared to non-pregnant values, approximating a rate closer to that of LR^3 IGF-I (Bastian *et al.*, 1993). The lower transendothelial transport values for ^{125}I-IGF-I in the presence of 20% FBS was also expected from evidence indicating that association with IGFBPs increases the plasma half-life of IGF peptides (Davis *et al.*, 1989; Hodgkinson *et al.*, 1989a; Francis *et al.*, 1988b).

Since the preceding results suggested that the presence of IGFBPs inhibited the transport of ^{125}I-IGF-I across HUVE cell monolayers, the effects of each of the known individual IGFBPs on ^{125}I-IGF-I transport in this model were examined to determine whether i) the inhibition observed with FBS could be reproduced by IGFBPs, and ii) if differences existed between the different classes of IGFBPs. Due to insufficient quantities of IGFBPs, rigorous examination of the most appropriate conditions under which to perform these experiment was not undertaken, however, on the basis of discussions with Dr Robert Baxter, ^{125}I-IGF-I and each of the IGFBPs were incubated for 2h at 37 °C prior to addition to the

luminal chamber in an attempt to reflect physiological conditions. Importantly, in each instance the presence of an IGFBP significantly decreased the transport of ^{125}I-IGF-I across the HUVE cell monolayer.

Andress *et al.* (1993) have recently produced a recombinant form of carboxy-truncated IGFBP-5 (tIGFBP-5) which has a reduced affinity for IGF-I but is able to bind osteoblast monolayers and stimulate osteoblast mitogenesis in a similar fashion to the native truncated 23 kDa IGFBP-5 isolated from osteoblasts. Specific ^{125}I-IGF-I binding to intact IGFBP-5 reached a maximum of 28 % at a binding protein concentration of 25 ng / ml while specific ^{125}I-IGF-I binding to tIGFBP-5 only reached a maximum of 7.7 % at a concentration of 2500 ng / ml (Andress *et al.*, 1993). Under the present experimental conditions, tIGFBP-5 was used as a specific control to determine the effect of IGFBPs with a reduced affinity for IGF-I. Carboxy-truncated IGFBP-5 was the only IGFBP tested that did not inhibit transmigration, consistent with the hypothesis that binding of IGFBPs to IGF-I inhibits the transmigration of IGF-I. Further evidence is provided by the results obtained with LR^3 IGF-I. The present study was also designed to take advantage of the reduced binding protein affinity of LR^3 IGF-I compared with IGF-I. Conditions were established in which labelled IGFs used were of similar specific activity, and held at a constant IGF:IGFBP ratio on a molecular weight basis. None of the IGFBPs inhibited the transport of ^{125}I-LR^3 IGF-I across the HUVE cell monolayer, further supporting the notion that binding of IGF-I to IGFBPs inhibits its transport across the endothelial barrier. A 1:2 IGF:IGFBP binding ratio also resulted in a significant reduction in ^{125}I-IGF-I transport, although a molecular weight binding ratio of 2:1 was less effective at reducing ^{125}I-IGF-I transport, with IGFBP-1 and -3 along with tIGFBP-5 failing to inhibit transport. Although a rigorous examination of binding conditions was not possible due to insufficient quantities of IGFBPs, these data indicate that a 1:1 molar ratio may be the most favourable for the binding of ^{125}I-IGF-I to the human IGFBPs under these experimental conditions, and that slight variations exist between the IGFBPs in the ability to inhibit ^{125}I-

IGF-I transport across HUVE cell monolayers. The difference in IGF-I and inulin transport observed between these two experiments may have arisen because they were performed with different cell isolates, which could possibly differ slightly in the permeability of their tight junctions.

Preincubation of ^{125}I-IGF-I with a 1:1 molar ratio of IGFBP-3 and a five-fold molar excess of ALS relative to IGFBP-3 resulted in a significant reduction in the flux of ^{125}I-IGF-I across the HUVE cell monolayer. Incubation with both IGFBP-3 and ALS also significantly reduced the flux of ^{125}I-IGF-I compared to that observed in the presence of IGFBP-3 alone. This is in agreement with the notion that the 150 kDa ternary complex retards IGFs in the vascular compartment, serving as a reservoir of IGFs that may be mobilised to target sites outside of the vasculature. The fact that ^{125}I-IGF-I transport is not totally occluded in the HUVE cell model could be a result of not all the ^{125}I-IGF-I being totally complexed. This may arise from the binding conditions *in vitro*, or the fact that the binding kinetics are dynamic, resulting in transient dissociation which would permit flux of free ^{125}I-IGF-I. In cultured endothelial cells, IGFBP-3 is able to specifically associate with both the endothelial cell surface and the endothelial extracellular matrix (Booth *et al.*, 1995). This is thought to occur via binding to glycosaminoglycans, since IGFBP-3 is known to have two heparin-binding concensus sequences (Booth *et al.*, 1996; Fowlkes and Serra, 1996; Hodgkinson *et al.*, 1994). Oh *et al.* (1993) have also reported evidence that IGFBP-3 may interact with a specific cell-surface receptor. Lee and Rechler (1996) have demonstrated that proteolysis of IGFBP-3 within the 150 kDa complex occurs *in vitro*, which releases IGF-I from the complex. Thrombin and plasmin are known IGFBP-3 proteases that are present at the endothelial cell surface (Booth *et al.*, 1996). IGFBP-3 is also proteolysed by activity in serum including cation-dependent protease activity in adult rat serum (Lee and Rechler, 1996), gestation serum protease activity (Giudice *et al.*, 1990) and post-operatively increased serum protease activity (Davenport *et al.*, 1992a and b). In the HUVE cell model used in the current experiments, the

150 kDa complex could localise at the apical surface of the cell monolayer via the affinity of IGF and /or the IGFBP-3 for the endothelial cell surface. Proteolysis of IGFBP-3 within the complex due to endothelial cell associated IGFBP-3 proteases, may result in the release of free ^{125}I-IGF-I that can then translocate across the endothelial monolayer. A similar scenario can easily be envisaged *in vivo*. Booth *et al.* (1996) have recently shown that IGFBP-3 exposed to pregnancy serum, plasmin or thrombin results in the production of numerous fragments which display altered affinities for both IGF and heparin. They propose that *in vivo* some of these fragments with reduced IGF affinity that incorporate into ECM could act as intermediary transporters for IGF out of the vascular compartment. It would be of interest to examine whether the addition of specific protease inhibitors to the current HUVE cell model would decrease the flux of ^{125}I-IGF-I which had been pre-incubated with IGFBP-3 and ALS. An unexpected finding was that ^{125}I-IGF-I diffusive transport across the HUVE cell monolayer was reduced in the presence of ALS alone, approximating values of that seen with ALS and IGFBP-3. One explanation for this phenomenon is that the large molar excess of ALS may have driven the formation of a ternary complex between ^{125}I-IGF-I, ALS and endogenous HUVE cell IGFBP-3.

Experiments within this chapter demonstrate that IGFBP-5 and a 24 kDa IGFBP which was unable to be immunologically identified, are incorporated within HUVE cell ECM. The present study also indicates that the ECM contributes to the endothelial permeability of IGF-I. Other investigators have found that IGFBP-3 and IGFBP-5 specifically bind to endothelial cell monolayers at both the cell surface and extracellular matrix (Booth *et al.*, 1995). IGFBP-5 and minimal amounts of IGFBP-3 have also been found to be present in ECM of cultured human fibroblasts (Jones *et al.*, 1993a). It has been shown that IGFBP-5 has 3 glycosaminoglycan-binding concensus sequences, which may explain why it is detected readily in the ECM of different cell types (Hodgkinson *et al.*, 1994). Association of IGFBP-5 with the ECM reduces its affinity for IGF-I eight-fold and ECM associated IGFBP-5

potentiates the effects of IGF-I on cell growth (Jones *et al.*, 1993a). It has been further suggested that heparin-like molecules may induce a conformational change in IGFBP-5 resulting in the decreased affinity (Arai *et al.*, 1994). Although it is possible that IGFBPs in HUVE cell derived ECM may also have a reduced affinity for IGF-I, the reduction in diffusive flux of ^{125}I-IGF-I across HUVE cell ECM compared to filters alone may still be due to binding to ECM associated IGFBPs. Additionally the ECM selectivity for ^{125}I-IGF-I passage could be on the basis of charge or size of the IGF molecule.

The possible mechanisms by which IGFBPs retard the transport of IGF-I across the HUVE cell monolayer are multiple. Firstly, intercellular junctions have been identified in human umbilical vein endothelial cells in monolayer culture (Larson and Sheridan, 1982, Chapter 3, Fig 3.7). The junctional complexes which consist of tight junctions, which are the most apical intercellular junctions, and the adherens or gap junctions which are more basolateral relative to the tight junctions, represent the site of diffusional transport of macromolecules (Schneeberger and Lynch, 1992; Simionescu and Simionescu, 1984; Simionescu *et al.*, 1978). Tracer studies indicate that junctional pores of approximately 7.5 nm in radius are present in pulmonary vascular endothelium, which are sufficiently large to transport molecules < 7 nm in radius (Taylor and Granger, 1984). Diffusive nonvesicular flux of proteins through these hypothesised pores is size selective, since molecules > 7.5 nm in radius were not permeable (Taylor and Granger, 1984). Siflinger-Birnboim *et al.*(1987) demonstrated size selectivity of bovine pulmonary artery endothelial monolayers *in vitro* to a number of molecules of varying sizes. Their data was best fitted to a two-pore model, which is also consistent with evidence of size selectivity with studies in intact lung, where a two-pore model was also proposed to account for diffusional transport of solutes across endothelial cells (Taylor and Garr, 1970). It is likely that the small pores represent the intercellular junctional pathway. The possibility has been raised that the large pore system is represented by transcellular channels formed by transient fusion of two or more plasmalemmal vesicles

(Ogawa *et al.*, 1993; Bundgaard *et al.*, 1983). In the bovine pulmonary artery endothelial monolayers, molecules of molecular weight between 0.182 and 12 kDa diffused in an unrestricted manner, molecules between 36 and 82 kDa demonstrated restricted diffusion proportional to their molecular weight, whereas diffusion of fibrinogen (340 kDa) was restricted (Siflinger-Birnboim *et al.*, 1987). In the HUVE cell monolayer model it is possible that IGF-I diffuses unrestricted on the basis of its size. However, the decreased transport of ^{125}I-IGF-I observed when in the presence of IGFBPs or ALS may be due to the formation of binary IGF-I:IGFBP and ternary complexes. These larger molecular weight ^{125}I-IGF-I complexes would have proportionally increased interactions with the pore's walls, which in turn would slow down the egress of ^{125}I-IGF-I transport across the HUVE cell monolayer. The two-pore model of transport of macromolecules has also been shown to be dependent on charge interactions (Malik *et al.*, 1989; Perry *et al.*, 1983), although whether this has relevance to transendothelial transport of IGF-I and IGF-I:IGFBP complexes is not known. A limitation to this model is that it fails to indicate via which route the molecules cross the endothelium, that is via interendothelial junctions or transvesicular channels.

Secondly, IGFBP-3 and -5 but not -1, -2, -4 or -6 can specifically bind to endothelial cell monolayers and extracellular matrix (Booth *et al.*, 1995). Potentially, IGFBP-3 and -5 could localise at the endothelial cell surface or ECM and concentrate free IGF-I at these sites, retarding its egress across the endothelium.

Thirdly, the initial barrier to the transvascular movement of proteins is believed to be the endothelial glycocalyx. It has been suggested that this fibrous meshwork acts as a macromolecular sieve. Thus, when IGF-I is bound to IGFBPs or is associated with the ternary complex, this sieve may regulate its access to the paracellular and or vesicular pathways. In addition, the glycocalyx represents a negative charge barrier to the passage of anionic macromolecules (Raviola and Butler, 1983). Whether this electrostatic charge at the luminal surface represents a barrier to IGF-I is unknown.

Fourthly, transport of macromolecules across the endothelium is also dependent on components of the subendothelial ECM, such as collagen, fibronectin and integrins (Utoguchi *et al.*, 1996; 1995; Qiao *et al.*, 1995; Partridge *et al.*, 1992). The ECM is thought to form an anionic channel, which may influence the permeability of various solutes on the basis of charge (Yurchenco and Schittny, 1990). The dense negative charge of glycosaminoglycans is already known to be an important factor in restricting albumin transport (Taylor and Granger, 1984). It is possible that components of the ECM may act as determinants for the transvascular passage of IGF-I. If so, binding of IGF-I to the IGFBPs may add another level to this regulation.

Finally, the actual binding or incorporation of IGFBPs into the glycosaminoglycan rich glycocalyx or ECM may alter the permeability properties of these structures to IGF-I. IGFBP-5 has been demonstrated to bind other fibroblast ECM components including types III and IV collagen, laminin and fibronectin (Jones *et al.*, 1995). In addition, all human IGFBPs, with the exception of IGFBP-4, have glycosaminoglycan-binding concensus sequences (Hodgkinson *et al.*, 1994). Arai *et al.*(1994) have demonstrated that recombinant human IGFBP-3, -4 and -5, but not IGFBP-1 or -2, bind to the glycosaminoglycan heparin. However, IGFBP-2 will bind heparin if IGF-I or IGF-II is added to the incubation buffer (Arai *et al.*, 1996). In all, this would permit the IGFBPs to bind to both the glycocalyx and the ECM, which may alter the selectivity of the endothelial cell barrier for IGF-I. Future experiments examining the effects on permeability of IGFBP binding to ECM or glycocalyx of HUVE cell monolayers may elucidate their role on HUVE cell IGF-I permeability.

So far the studies contained within this thesis have shown that IGF-I is transported via a paracellular route across the HUVE cell monolayer model, and that IGFBPs are involved in the regulation of this process. Since this only provides information at the cellular interface, I wanted to examine the transport of IGF-I from the vascular compartment to extracellular sites and the effects of IGFBPs on this transport *in vivo*. To accomplish this, I utilised a model of

extracellular fluid accumulation in the rat, and investigated the transport of intravenously administered bolus doses of ^{125}I-IGF-I or ^{125}I-LR^{3} IGF-I to these extracellular fluid sites. These studies are described in chapter 7.

CHAPTER: 6 TRANSPORT OF INSULIN-LIKE GROWTH FACTOR-I ACROSS EPITHELIAL CELL MONOLAYERS

6.1 INTRODUCTION

The previous three chapters described the validation of an *in vitro* endothelial cell barrier model, the transport of IGF-I across an endothelial cell monolayer and the effects of IGFBPs on this transport. Since transporting epithelial cells share similarities with endothelial cells, including growing as monolayers and containing lateral membrane specialisations such as tight junctions, I utilised the Transwell bi-chamber system to examine the transport of IGF-I across epithelial cells. Specific emphasis was placed on gut epithelial cells, and IGF-I transport across these cells was compared to that observed with kidney and lung epithelial cells.

Transport of macromolecules across epithelial surfaces occurs either via active transcellular or passive paracellular pathways. For example, proteins such as horseradish peroxidase, insulin, immunoglobulin-G and beta-lactoglobulin have been shown to be actively transported across the epithelium lining small intestine in membrane-bound vesicles after binding to cell surface receptors or binding sites (Kiliaan *et al.* 1996; Bendayan *et al.* 1994; Jochims *et al.* 1994; Wheeler *et al.* 1993; Walker *et al.* 1972). In contrast, a component of the transport of horse-radish peroxidase and bovine serum across epithelial monolayers occurs by passive intercellular diffusion (Kiliaan *et al.* 1996; Warshaw *et al.* 1974; Rhodes & Karnovsky, 1971).

Several growth factors including insulin, epidermal growth factor, nerve growth factor and insulin-like growth factor-I (IGF-I) are taken up by the gut in active form (Phillips *et al.* 1990; Thornburg *et al.* 1984; Aloe *et al.* 1982; Kelly, 1960). Significant attention has been

focussed on the transport of IGF-I. IGFs have been detected in the milk of many species (Olanrewaju *et al.* 1996; Donovan *et al.* 1991; Vega *et al.* 1991; Baxter *et al.* 1984a) and orally administered ^{125}I-labelled IGF-I is absorbed by the gut and appears in the circulation intact (Xu & Wang, 1995; Odle *et al.* 1996; Vacher *et al.* 1995; Baumrucker *et al.* 1992). IGF-I is a potent promoter of growth, metabolism and differentiation of numerous cell types including intestinal epithelial cells (Corps & Brown, 1987). IGF-I administered systemically promotes gut growth and maturation (Read *et al.* 1992) and orally administered IGF-I acts locally to stimulate gut epithelial cell proliferation (Burrin *et al.* 1996; Baumrucker *et al.* 1994).

To exert its mitogenic actions, IGF-I binds to the type I IGF receptor on target cell membranes (LeRoith *et al.* 1995). Type I IGF receptors have been identified on porcine small intestinal mucosal membrane preparations (Schober *et al.* 1990), on the basolateral membranes of enterocytes in piglets (Morgan *et al.* 1996) and the villus and crypt epithelium of the rat (Laburthe *et al.* 1996; Ryan & Costigan, 1993; Heinz Erian *et al.* 1991). Intestinal cell lines derived from rat jejunal crypts (IEC-6), as well as the rat, rabbit and human colon (Caco-2) also express IGF type I receptors (Hoeflich *et al.* 1994; Rouyer Fessard *et al.* 1990; Park *et al.* 1990; Pillion *et al.* 1989). Whether these receptors mediate the transport of IGF across epithelial monolayers is currently unknown.

In the present studies I have examined the transport of ^{125}I-labelled IGF-I across monolayers of a rat small intestinal epithelial cell line and compared this transport to that observed across kidney and lung derived epithelial cell lines. These studies present evidence for the restricted transport of intact IGF-I across these epithelial cell monolayers via a paracellular route.

6.2 MATERIALS AND METHODS

6.2.1 Materials

All the materials used for studies within this chapter have previously been described in sections 3.2.1 and 4.2.1.

6.2.2 Cell culture

Madin Darby bovine kidney (MDBK; CRL 6071), mink lung epithelial cells (Mv1Lu; CCL 64) and rat small intestinal crypt (IEC-6; CRL 1592) epithelial cell lines were all purchased from American Type Culture Collection, (Rockville, USA). All cells were grown in DMEM containing 10% FBS, together with 100 mg of streptomycin, 60 mg penicillin and 1 mg fungizone per litre of growth medium. The cells were grown and used for experiments as monolayers at 37^{0}C in 5% CO_2.

6.2.3 Radioreceptor assays

The procedure for this assay has been described previously in section 4.2.3 with the following exceptions; i) the 24 place multiwell plates were not fibronectin-coated and ii) the competing ligand was IGF-I only.

6.2.4 Affinity label cross-linking experiments

Cross-linking was performed essentially as described in section 4.2.4, with the exception that confluent epithelial cell monolayers in 6-place multiwell plates were washed

twice and incubated at 4°C in DMEM plus BSA (0.1% w/v) for 2 h prior to incubation of the cells with radiolabelled IGFs and unlabelled competing ligands.

6.2.5 Measurement of DNA synthesis

Confluent monolayers of epithelial cells grown in 24-place multiwell plates were washed twice for 30 min in serum-free DMEM plus BSA (0.1% w/v). Each monolayer was subsequently incubated for 24 h in serum-free medium containing a dilution series of IGF-I. A 10% FBS and serum-free control was also incorporated on each plate. During the last 6 h of this period, 1 μCi (5 nmol) of [^{3}H]-thymidine was added to each well. Each monolayer was washed with Hanks' balanced salt solution, twice with 5% TCA and once with water. Cells were solubilised by trituration in NaOH (0.5 mol/l) containing Triton X-100 (0.1% v/v), and incorporated [^{3}H]-thymidine quantified using a beta counter. Results are expressed as the percentage of DNA synthesis above that observed in serum-free conditions.

6.2.6 Transmission electron microscopy and measurement of transepithelial electrical resistance

TEM of confluent epithelial cell monolayers was performed to examine the presence of tight junctions. Transwells were seeded with 20 x 10^3 epithelial cells per well in DMEM plus 10% FBS, placed in 24 place multiwell plates containing the same medium and cultured for 6 days with media changes every 2 days. Transwell filters were excised and the cell monolayers fixed and processed for TEM as described in section 3.2.6.2.

Measurement of electrical resistance was undertaken to monitor the integrity of each cell monolayer used in the transport experiments. Transwells were seeded with 20 x 10^3 epithelial cells per well in DMEM plus 10% FBS, placed in 24 place multiwell plates

containing the same medium and cultured for 16 days with media changes every 2 days. Transepithelial resistance measurements were made every 24 h using a Millicell-Electrical Resistance System (Millipore, Bedford, MA, USA). To calculate the resistance measurement of each cell monolayer, the mean resistance measurements of transwells without cells were subtracted from the monolayer measurements and corrected for the area of the transwell (0.33 cm^2).

6.2.7 Transmigration experiments

These experiments were performed as described in section 4.2.9 with the following changes; i) epithelial cells were grown for 6 days in transwells as described above for measurement of transepithelial resistance, ii) the integrity of every monolayer was confirmed prior to and after each experiment by measuring the transepithelial resistance, and iii) experiments were performed in serum free DMEM plus BSA (0.1% w/v).

6.2.8 Statistical analyses

Transmigration data were analysed by one-way analysis of variance with all pair wise multiple comparisons using Student-Newman-Keuls method. DNA synthesis data and data arising from the effects of excess unlabelled IGF-I on ^{125}I-labelled IGF-I transport were analysed by Student's t tests.

6.3 RESULTS

6.3.1 Competitive binding and ligand-receptor cross-linking experiments

To confirm the presence of IGF binding sites on epithelial cell lines, competitive binding studies were performed on confluent cell monolayers. IEC-6 and Mv1Lu epithelial cell lines bound 3.7% and 4.1% of the total ^{125}I-labelled IGF-I added to each well respectively, while the MDBK cells bound 23% of total ^{125}I-labelled IGF-I. The IGF-I receptor displacement curve is shown in Fig. 6.1. Fifty percent of bound ^{125}I-labelled IGF-I was displaced by 3.6, 3.8 and 6.0 ng/ml of unlabelled IGF-I from Mv1Lu, IEC6 and MDBK cells, respectively. To further characterise IGF binding sites on each of the epithelial cell lines, bound ^{125}I-labelled IGF-I was cross-linked to cell surface receptors in the absence or presence of saturating concentrations of unlabelled IGF-I, IGF-II or insulin. Receptor-ligand complexes identified by SDS-PAGE and autoradiography were visualised at approximately 140 and 270 kD for both the IEC-6 and Mv1Lu cell lines (Figs. 6.2 A and B). Both bands were sensitive to displacement when cross-linking was performed in the presence of unlabelled IGF-I, IGF-II or insulin. The molecular weights of these two bands are consistent with the cross-linked type 1 IGF receptor α-subunit and dimers of the cross-linked α-subunit, respectively. No cross-linking of ^{125}I-labelled IGF-I to MDBK cell membranes was observed (Fig. 6.2C). However, a band at 21 kDa was apparent when lysates of MDBK cells incubated with ^{125}I-labelled IGF-I were run on a 12% polyacrylamide gel (Fig. 6.2D).

Figure 6.1: IGF-I radioreceptor assays. Inhibition of ^{125}I-labelled IGF-I binding to IEC-6 (circles), Mv1Lu (triangles) and MDBK (squares) cells by unlabelled IGF-I. Values are expressed as the fraction of binding in the absence of competing ligand. Each point is the mean ± sem of triplicate determinations from three experiments.

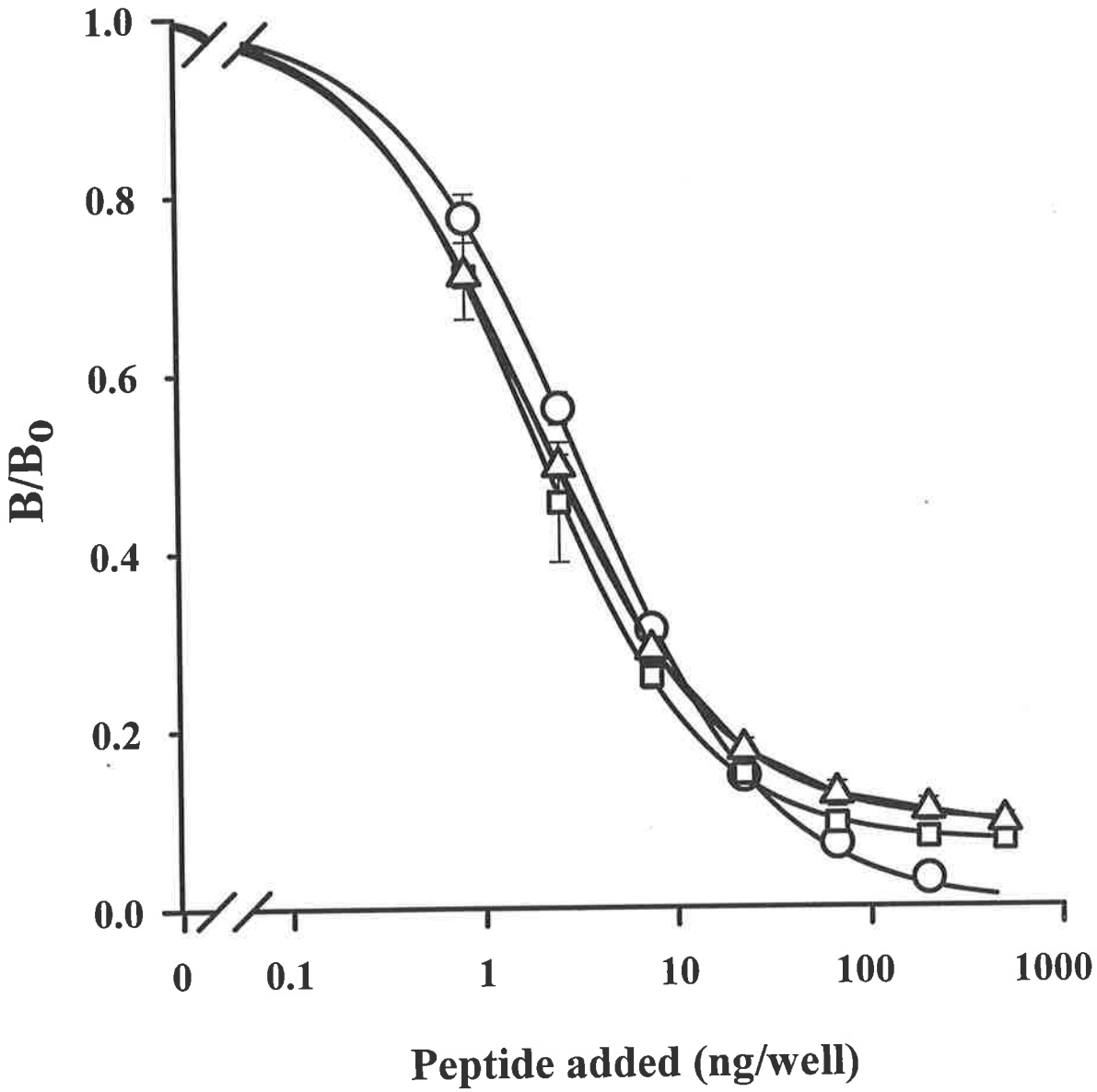
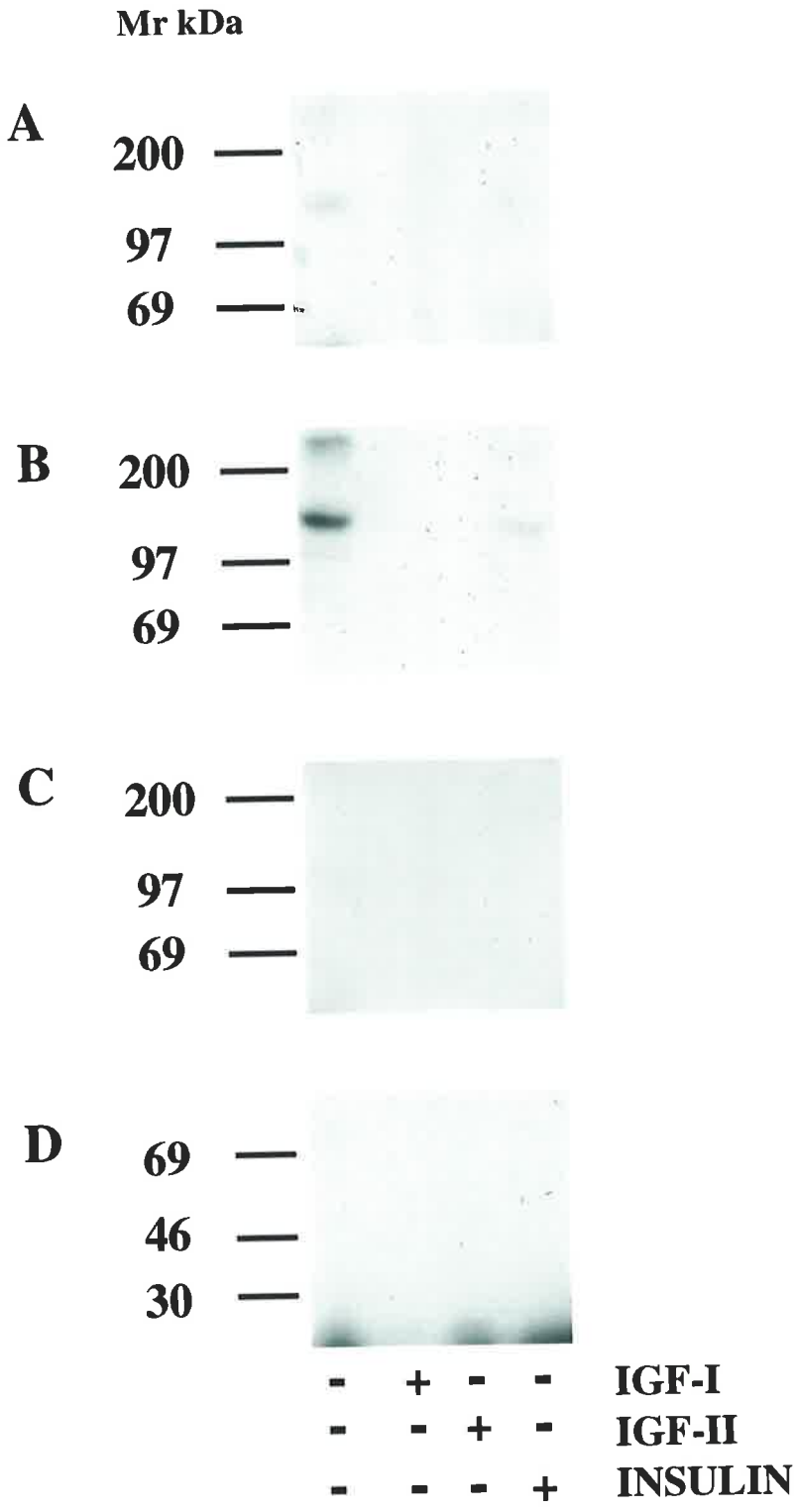

1.0
0.8
0.6
0.4
0.2
0.0
B/B0
0
0.1
1
10
100
1000
Peptide added (ng/well)

Figure 6.2: Autoradiograph of ^{125}I-labelled IGF-I cross-linked to monolayers of IEC-6 (A), Mv1Lu (B) and MDBK (C and D) cells. Cross-linking was carried out in the presence (+) or absence (-) of IGF-I (8 μg), IGF-II (8 μg) and insulin (80 μg). Cross-linked samples were subjected to SDS-PAGE on 6% (A, B and C) or 12% (D) acrylamide gels under reducing conditions and bands were visualised by exposure to X-ray film for 28 days. Positions of ^{14}C-labelled molecular weight markers are indicated (x 10^{-3}).


Mr kDa
A
200
97
69
B
200
97
69
C
200
97
69
D
69
46
30
- + - - IGF-I
- - + - IGF-II
- - - + INSULIN

6.3.2 DNA synthesis assays

The presence of functional type I IGF receptors was further assessed using [^{3}H]-thymidine incorporation as a measure of DNA synthesis. All concentrations of IGF-I tested significantly increased [^{3}H]-thymidine incorporation into the DNA of Mv1Lu cells (Fig. 6.3). At the highest concentration tested (100 ng/ml), the Mv1Lu cells were stimulated to increase [^{3}H]-thymidine incorporation by 74 ± 2% (mean ± sem; n=3). The incorporation of [^{3}H]-thymidine into IEC-6 and MDBK cells was increased only after addition of 100 ng/ml of IGF-I, resulting in significant increases of 20 ± 4% and 80 ± 3% over serum-free conditions, respectively (mean ± sem; n=3).

6.3.3 Transmigration experiments

The ability to grow confluent epithelial cell monolayers on transwell membranes was confirmed by transmission electron microscopy. Each cell line formed a polarised monolayer of cells with a clearly defined apical membrane and produced distinct tight junctions (Fig. 6.4 A, B and C, junctions indicated by arrows). Transepithelial resistance values for all cell lines on the first 16 days after seeding onto the transwells at a standard concentration 20 x 10^3 are shown in Fig. 6.5. As all monolayers were confluent and independent experiments produced consistent transepithelial resistance values at day 6, all transepithelial transport studies were undertaken at this time point. The mean transepithelial electrical resistance values on day 6 post-seeding were 61.5 ± 7.9, 12.6 ± 1.7 and 7.9 ± 1.0 $\Omega.cm^2$ for MDBK, IEC-6 and Mv1Lu cells respectively (mean ± sem; n = 5-7 cultures). Integrity of the monolayers was routinely monitored by measuring transepithelial electrical resistance values on all cultures prior to and at the end of every transmigration experiment. These two values did not significantly differ.

Figure 6.3: [^{3}H]-thymidine incorporation into IEC-6 (open bars), Mv1Lu (solid bars) and MDBK (hatched bars) cells treated with the indicated concentrations of IGF-I. Results are expressed as a percentage above incorporation observed under serum-free conditions (means ± sem, n=3, *P<0.05, †P<0.01).

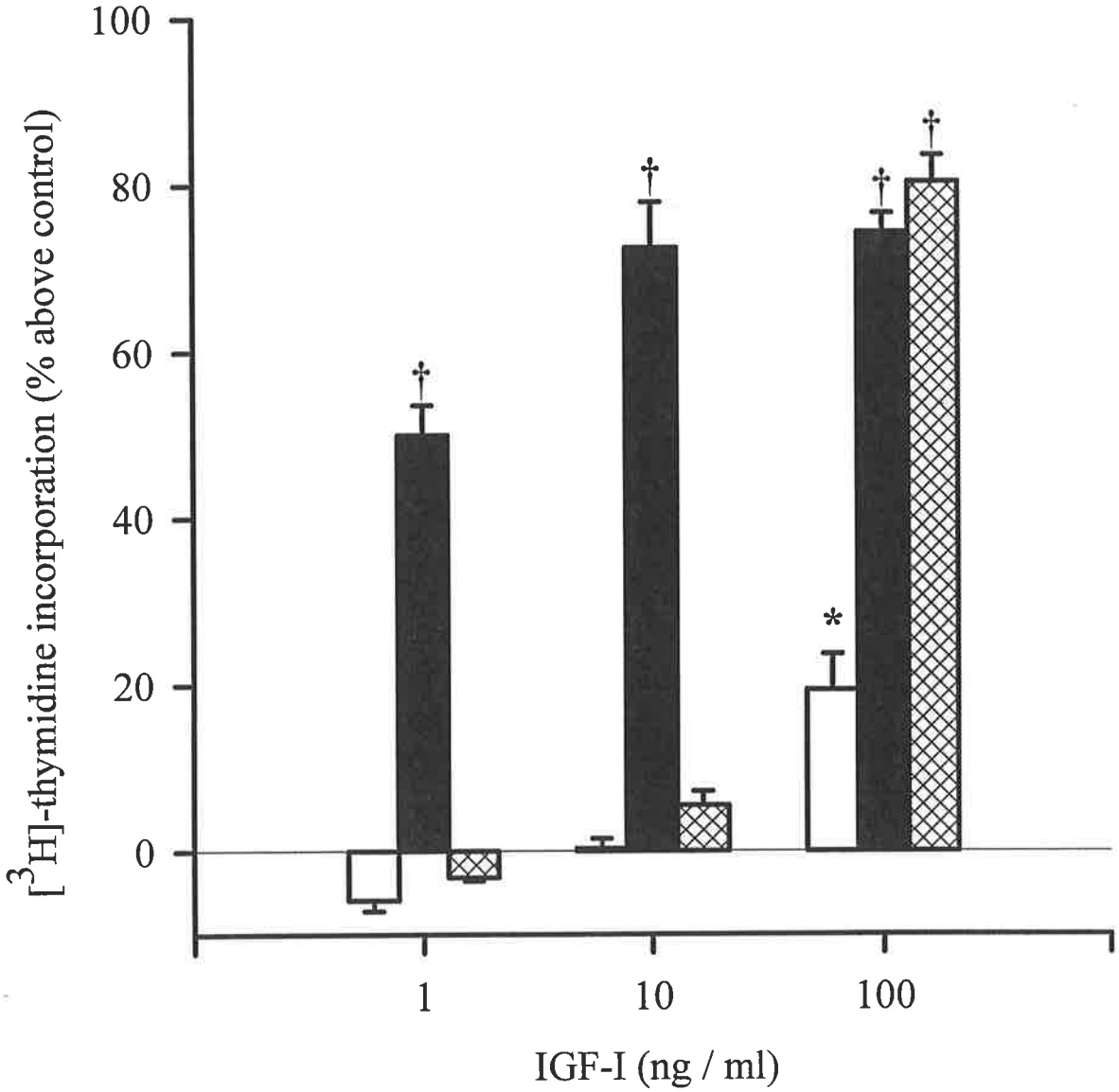

100
80
60
40
20
0
[3H]-thymidine incorporation (% above control)
†
†
†
†
*
1
10
100
IGF-I (ng / ml)

Figure 6.4: Transmission electron microscopy of epithelial cell monolayers grown on Transwells. IEC-6 (A), Mv1Lu (B) and MDBK (C) cells were seeded on transwells in DMEM plus 10% FBS and the media changed every 2 days. On day 6 post-seeding, the cells were fixed and stained for transmission electron microscopy as described in Materials and Methods. Arrows indicate the presence of tight junctions between adjacent cells (x 30 000).

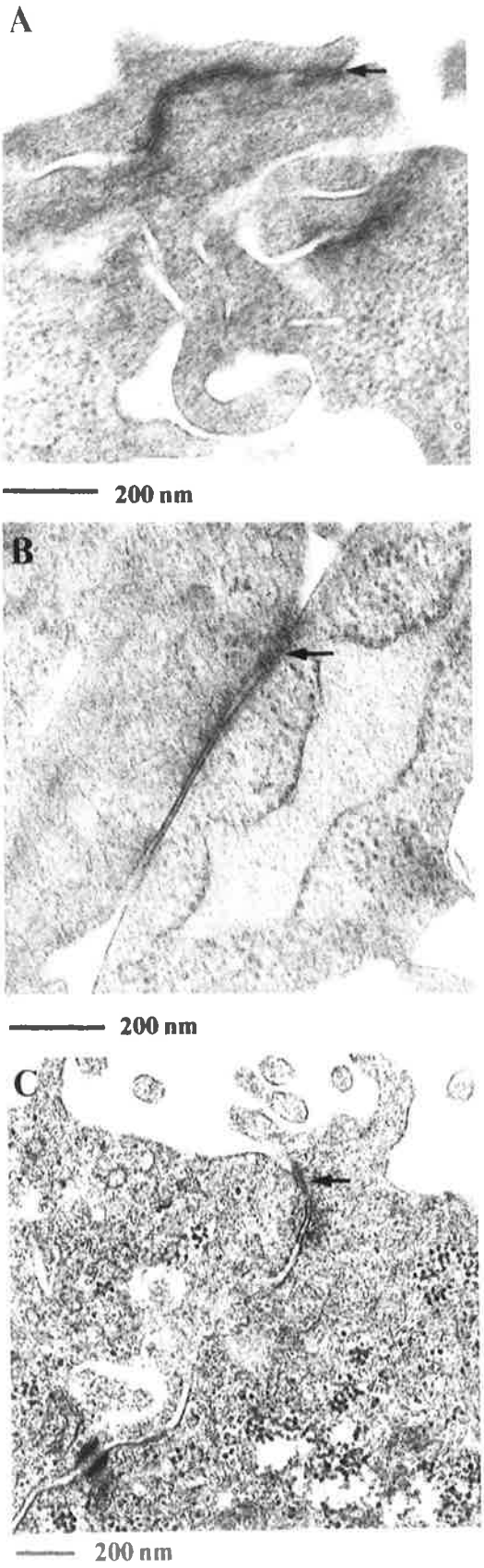
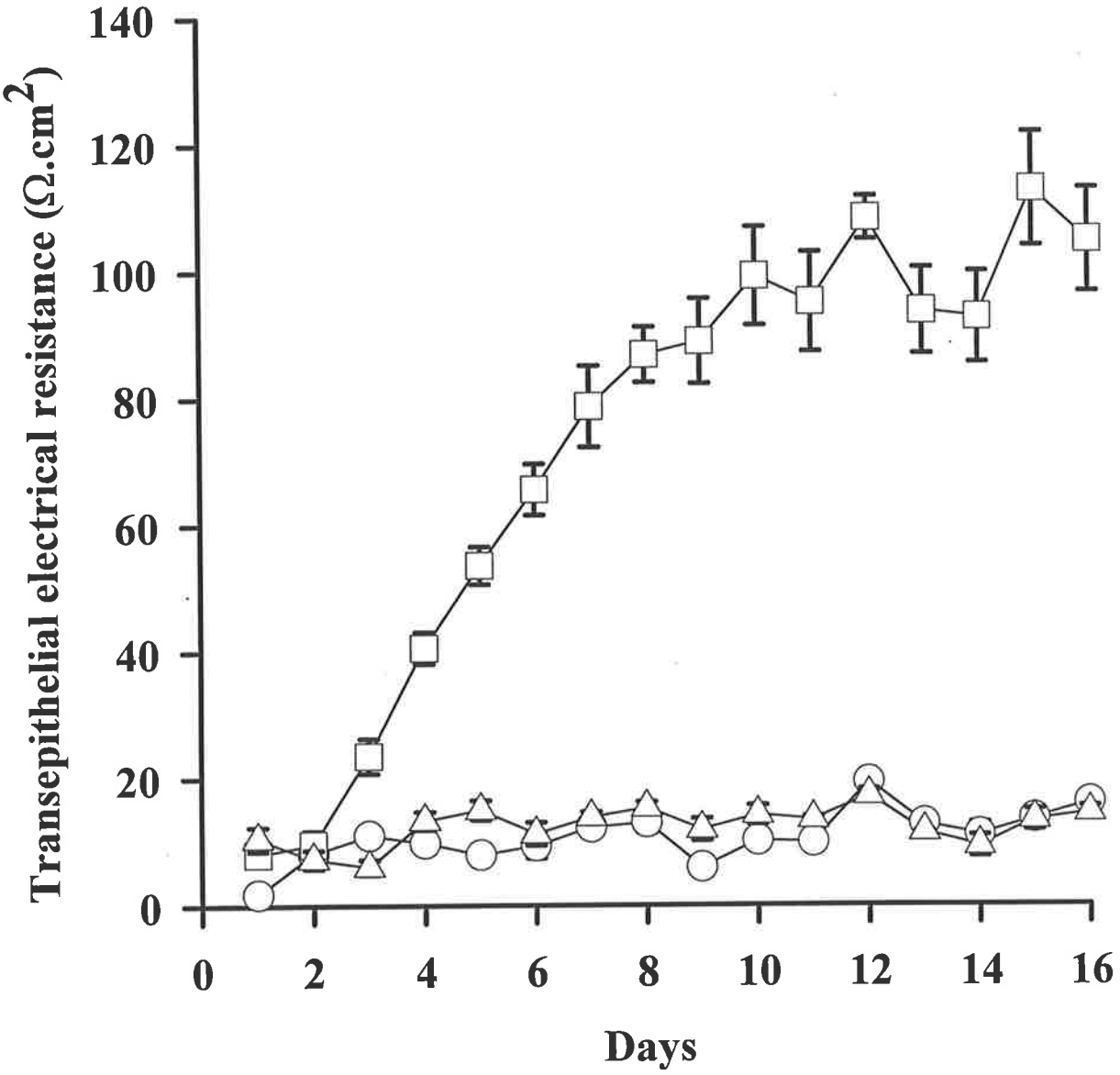

A
200 nm
B
200 nm
C
200 nm

Figure 6.5: Transepithelial electrical resistance measurements of cell monolayers. IEC-6 (circles), Mv1Lu (triangles), and MDBK (squares) cells were seeded on transwells in DMEM plus 10% FBS (20 x 10^3 cells per well). Electrical resistance values were measured every 24 h, each value representing the mean ± sem of 3 transwell cultures.


Transepithelial electrical resistance (Ω.cm²)
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
Days

In addition, light microscopy on all cultures confirmed the presence of confluent monolayers at the end of each experiment.

The transport of ^{125}I-labelled IGF-I across transwell filters alone and both ^{125}I-labelled IGF-I and [^{3}H]-inulin across confluent monolayers of IEC-6, MDBK and Mv1Lu cells is shown in Fig. 6.6. Addition of ^{125}I-labelled IGF-I to the apical side of each cell monolayer resulted in a time dependent increase in the amount of this peptide appearing intact in the basolateral chamber. At 2 h, 7.9 ± 0.8%, 10.8 ± 0.7% and 1.2 ± 0.2% of total intact ^{125}I-labelled IGF-I added to the apical chamber was transported by IEC-6, Mv1Lu and MDBK cell monolayers, respectively (mean ± sem., n = 18 cultures). The flux of ^{125}I-labelled IGF-I across filters without cell monolayers was significantly greater (34.1 ± 0.9%, mean ±.sem, n = 18) than that across the epithelial cells, indicating that the IEC-6 and Mv1Lu cells had greatly restricted and the MDBK cells virtually excluded the passage of ^{125}I-labelled IGF-I. In addition, the flux of ^{125}I-labelled IGF-I across the epithelial cell monolayers was significantly less than that observed for [^{3}H]-inulin.

TCA-precipitability of ^{125}I-labelled IGF-I immediately prior to its addition to the apical chamber was 95.2 ± 0.56% (n = 72 cultures). For IEC-6 and Mv1Lu cell monolayers, the majority of radioactivity appearing in the basolateral chamber was TCA-precipitable. TCA-precipitability gradually increased from 52 ± 2% at 5 min to 63 ± 2% by 2 h and from 64 ± 4% at 5 min up to 76 ± 3% by 2 h for the IEC-6 and Mv1Lu cells, respectively (mean ± sem, n = 18 cultures), indicating the preferential movement of free ^{125}I at the earlier time points. In contrast, less than 30% of radioactivity appeared as TCA-precipitable counts in the basolateral chamber of the MDBK cell cultures by the end of the 2 h experiment.

In order to examine whether the transport of ^{125}I-labelled IGF-I is concentration dependent, a 1000-fold excess of unlabelled IGF-I was added to the apical chamber 30 min prior to the addition of ^{125}I-labelled IGF-I. As can be seen from Fig. 6.7, excess unlabelled

Figure 6.6: Transport of ^{125}I-labelled IGF-I (circles) and [^{3}H]-inulin (squares) across IEC-6 (A), Mv1Lu (B) and MDBK (C) cell monolayers. Transport of ^{125}I-labelled IGF-I across control transwell filters without cells is shown in each case (triangles). Transport of ^{125}I-labelled IGF-I and [^{3}H]-inulin transport is expressed as a percentage of total intact ^{125}I-labelled IGF-I and total [^{3}H]-inulin added to the apical chamber at time zero, respectively. Data are the pooled means ± sem of triplicate transwells from 6 experiments. *P<0.05, †P<0.01 vs ^{125}I-labelled IGF-I across epithelial cell monolayers.

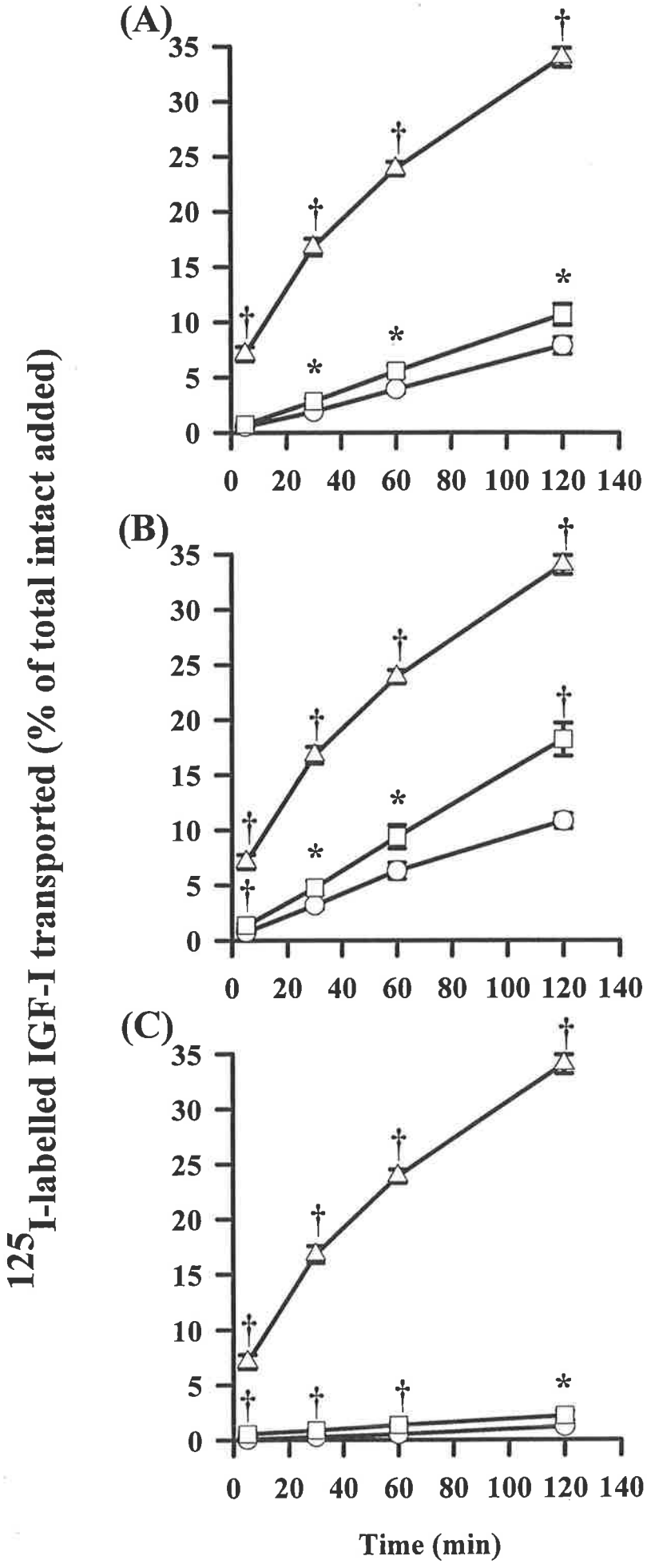
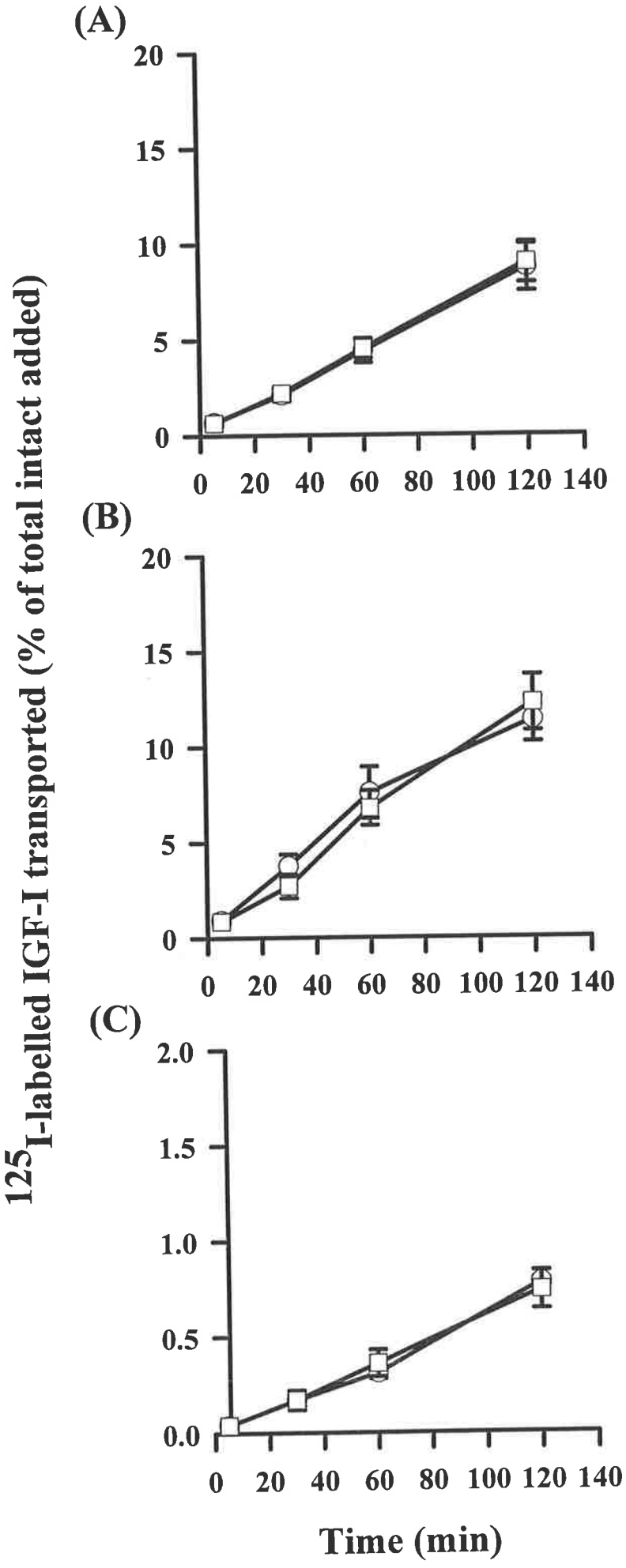

(A)
(B)
(C)
125I-labelled IGF-I transported (% of total intact added)
Time (min)
0
5
10
15
20
25
30
35
0 20 40 60 80 100 120 140
†
*

Figure 6.7: Effect of excess IGF-I on transport of ^{125}I-labelled IGF-I across epithelial cell monolayers. Transport is shown as a percentage of total intact ^{125}I-labelled IGF-I added to the apical chamber at time zero appearing in the basolateral chamber of IEC-6 (A), Mv1Lu (B) and MDBK (C) cell cultures in the presence (squares) and absence (circles) of a 1000-fold excess of IGF-I. Data represents the means ± sem of triplicate transwells from 3 experiments.


(A)
20
15
10
5
0
0 20 40 60 80 100 120 140
(B)
20
15
10
5
0
0 20 40 60 80 100 120 140
(C)
2.0
1.5
1.0
0.5
0.0
0 20 40 60 80 100 120 140
125I-labelled IGF-I transported (% of total intact added)
Time (min)

IGF-I did not alter the transport of ^{125}I-labelled IGF-I across epithelial cell monolayers, suggesting the non-saturable flux of intact ^{125}I-labelled IGF-I across epithelial cell barriers.

6.4 DISCUSSION

The major function of any epithelium is to form a selective barrier to the movement of solutes including peptides and proteins. Solute flux across an epithelial barrier can occur by either a transcellular route, involving transport systems located in the apical and basolateral membranes or a paracellular route via intercellular spaces. The absorption of biologically active intact protein species, including growth factors, from the intestine is well described. For example, absorption of ^{3}H-labelled bovine serum albumin has been demonstrated in adult rats (Warshaw *et al,*. 1974). IgG is transported from the gut lumen to the circulation by a receptor-mediated pathway in newborn calves (Jochims *et al.*, 1994). In addition, hypoglycaemia induced in newborn pigs (Asplund *et al,*. 1962) and calves (Pierce *et al.*, 1964) following orogastric administration of insulin originally suggested that the intestine absorbs an intact and metabolically active form of this peptide. In contrast, more recent studies in which insulin was added to milk replacer given to minature pigs or administered orally to calves just prior to feeding colostrum did not effect serum insulin or glucose levels (Grütter and Blum, 1991; Shulman, 1990). This result is in contrast to that observed in the rat, where biologically active insulin is absorbed by the intestinal mucosa and transferred to the circulation.

Insulin administered to the lumen of the duodenum resulted in rapid increases in circulating levels of insulin followed by significant decreases in blood glucose (Bendayan *et al.*, 1994). Using closed-loop *in situ* experiments, Schilling and Mitra (1992) showed that insulin delivered to the distal small intestine gave rise to severe and prolonged

hypoglycaemia. Absorption of insulin by the intestinal mucosa has been since demonstrated to occur by a transcytotic pathway in both normal and diabetic rats (Bendayan *et al.* 1994).

Of direct relevance to the current study are several reports demonstrating intestinal absorption of intact IGF-I. Plasma IGF-I levels increase in calves orally administered IGF-I at a concentration of 750 ng / ml (Baumrucker *et al.*, 1992). In 1-day old calves, supraphysiological amounts of IGF-I or ^{125}I-labelled IGF-I administered into a clamped segment of the jejunum results in the appearance of trace amounts of immunoreactive IGF-I or ^{125}I-labelled IGF-I in the mesenteric vein draining the segment (Vacher *et al.*, 1995). Orally administered ^{125}I-labelled IGF-I is absorbed after administration to piglets and calves with some 20% of total plasma radioactivity appearing as intact ^{125}I-labelled IGF-I, the majority of which is associated with IGFBPs (Odle *et al.*, 1996; Xu & Wang, 1995). Similarly, Donovan *et al.* (1997) found that in piglets orally administered ^{125}I-rhIGF-I, 18-20% of total radioactivity in both portal and arterial blood was intact, with absorbed ^{125}I-rhIGF-I representing 0.205% of the total plasma IGF-I pool. Although studies report the ability of certain animals species to absorb orally administered IGF-I, the physiological relevance of this absorption is a contentious issue since studies usually use pharmacological doses and the absorption plus the side effects on metabolism and growth are negligible. However, the rat seems to differ in this respect. Philipps *et al.* (1997) showed that rat pups fed rat milk substitute containing recombinant human IGF-I within a physiological range gained more weight, had increased brain and liver wet weight, demonstrated increased small intestine and liver protein contents, plus had serum IGF-I levels twofold above that of control animals fed rat milk substitute devoid of IGF-I. In addition to IGF-I, the amount of low molecular weight IGFBPs was greater in the IGF-I supplemented pups than in pups fed rat milk substitute alone. Whether these effects are related to direct absorption of exogenous peptide or enhanced gut differentiation with subsequent stimulation in nutrient uptake is unclear. IGF-I concentrations in serum of rat milk substitute fed rats were 30 % of IGF-I serum values measured in dam-fed

pups (Philipps *et al.*, 1989), yet in rats fed rat milk substitute supplemented with IGF-I, the serum IGF-I concentration was normalised, suggesting that milk-borne IGF-I may be an important factor for maintaining circulating IGF-I levels in infant rats. Interestingly, Baumrucker and Blum (1994) showed transient alterations in serum prolactin and IGF-I in new born calves given milk replacer containing physiological levels of IGF-I. Although orally ingested IGF-I exerts local effects on the gut, the functional relevance of IGF-I absorption by the intestine is not understood. However, it is plausible that IGF-I absorption by the gut into the circulation may permit it to exert growth actions on various tissues, alter its own regulation or govern the secretion of other hormones, possibly in an endocrine manner.

The presence of functional IGF receptors on IEC-6 and Mv1Lu cells were determined by radioreceptor, affinity label cross-linking and [^{3}H]-thymidine incorporation studies. The increased sensitivity of Mv1Lu cells to IGF-I in the latter study may reflect the production or secretion of lower levels of IGFBPs compared to the other cell lines. My results confirm earlier data indicating that IEC-6 cells possess type I IGF receptors (Simmons *et al.* 1995; Martin & Baxter, 1992a). Interestingly, the finding that MDBK cells possess type I IGF receptors using radioligand binding and DNA synthesis assays could not be extended to cross-linking experiments, possibly reflecting the radioligand cell binding kinetics over the time course of the study. In this regard receptors for IGF-I and IGF-II have been identified on cultured canine and opossum proximal tubule cells (Fawcett & Rabkin, 1995; Hammerman & Rogers, 1987). While the type II IGF receptors were shown to be equally distributed on the basolateral and brush border membranes, the distribution of the type I IGF receptor was asymmetrical with the basolateral membrane exhibiting a several fold higher specific binding capacity (Hammerman & Rogers, 1987). As MDBK cells display properties of proximal tubule cells, it may be that the radioligand is unable to access basolateral surface receptors over the time course of the cross-linking experiments. The binding of ^{125}I-labelled IGF-I to the surface of these cells by a competitive mechanism may reflect binding to a cell surface

binding protein. Indeed a band at 21 kDa was observed when cross-linked ^{125}I-labelled IGF-I was run out on a 12% polyacrylamide gel.

In the present study, epithelial cell monolayers were maintained in serum-free conditions to minimise potential interference of serum-derived IGFs or IGFBPs. The presence of confluent cell monolayers was checked using light and electron microscopy as well as transepithelial resistance measurements. Under these conditions the epithelial cell monolayers produce tight junctions. The electrical resistance measured across MDBK cells in the present studies was much higher than the Mv1Lu and IEC-6 cell monolayers, but comparable to the value of 83.4 ± 15.8 $\Omega.cm^2$ for MDBK cells cited in Cereijido *et al.*, (1978). In addition, the MDBK cells which originate from the proximal tubule virtually excluded any passage of ^{125}I-labelled IGF-I or [^{3}H]-inulin. This may be a reflection of the role of these cells *in vivo*. Proximal tubule cells are not involved in protein transport, but are the site of reabsorption of amino acids originating from the small amount of protein present in the glomerular filtrate. Proteins are hydrolysed to amino acids by brush border enzymes and are either utilized by the tubule cells or returned to the blood for use by other cells (Junqueira *et al.*, 1986). The IEC-6 and Mv1Lu cell lines produced low transepithelial electrical resistance values, yet still greatly restricted the passage of ^{125}I-labelled IGF-I or [^{3}H]-inulin compared to filters without cells. In contrast, previous studies have shown T_{84} colonic carcinoma cells generate high electrical resistance values, while greatly impeding the movement of electrolytes as well as macromolecules such as insulin, inulin and albumin (Milton & Knutson, 1990), and thus display characteristics more comparable to the kidney-derived MDBK cell line than the gut-derived line in the current experiments. This observation possibly reflects the role of colonic epithelium as the site of water rather than protein absorption. IEC-6 and Mv1Lu cells exhibit similar characteristics to endothelial cells which produce tight junctions and low transcellular electrical resistance, although significantly impede the transport of macromolecules (Chapter 4).

The majority of ^{125}I-labelled IGF-I that bound to each of the three epithelial cell monolayers was almost totally displaced by concentrations of unlabelled IGF-I (Fig. 6.1) which were 10 fold less than the concentration of excess unlabelled IGF-I used in the transmigration experiments (Fig. 6.7). The transport of ^{125}I-labelled IGF-I across epithelial cell monolayers was unaltered in the presence of this excess concentration of IGF-I, suggesting a non-saturable transport mechanism. I interpret these results as demonstrating that ^{125}I-labelled IGF-I crosses IEC-6, Mv1Lu and MDBK epithelial cell barriers via a paracelluar route, and not by a receptor mediated pathway. Indeed, as ^{125}I-labelled IGF-I transport across each epithelial cell monolayer was significantly less than inulin, ^{125}I-labelled IGF-I transport may have been retarded by binding to cell surface IGF receptors or IGFBPs.

CHAPTER: 7 TRANSFER OF LABELLED IGF-I AND LR^{3}IGF-I FROM BLOOD TO EXTRACELLULAR WOUND FLUID SITES

7.1 INTRODUCTION

In chapter 2 it was shown that IGF-I and LR^{3}IGF-I are able to leave the systemic circulation and distribute to various tissues in rats. Further studies in this thesis have described the development of an *in vitro* model of an endothelial cell barrier and have determined the pathway of IGF-I movement across monolayer cultures of HUVE cells. In addition, this research has shown that IGFBPs along with the ECM that HUVE cells secrete, regulate the transfer of IGF-I across these endothelial cell monolayers. These studies have therefore generated new data regarding the mechanism and regulation of the transfer of IGF-I across endothelial barriers using an *in vitro* model. However, in the whole animal the process of IGF-I transfer becomes more complex since it is potentially influenced by circulating IGFBPs plus the presence of multiple tissues which possess IGF receptors and have the ability to produce local IGFs and IGFBPs. Therefore, to gain further insight into this complex situation at the tissue level, *in vivo* studies are necessary.

In vivo, IGFs circulate in the blood complexed to multiple specific binding proteins. The majority of IGF-I is present in the circulation associated with IGFBP-3, which together with an acid-labile subunit, forms a ternary complex of approximately 150 kDa (Baxter *et al.*, 1988a). The role of the IGF binding proteins in the transfer of IGF-I from blood to tissues is not well understood (see sections 1.9, 1.13 and 5.1). However, it is apparent that association with binding proteins, particularly in the 150 kDa complex, increases the circulating half-life of IGF-I, (Ballard *et al.*, 1991; Davis *et al.*, 1989; Cascieri *et al.*, 1988; Francis *et al.*, 1988b and Zapf *et al.*, 1986). Association of IGF-I with lower molecular weight IGFBPs is thought to permit targeting of IGF-I to specific tissues (Sara and Hall, 1990). It has been hypothesized

that the 150 kDa complex does not leave the vascular space, yet this is a contentious issue (see section 1.9). Although the specific function of the 150 kDa complex is unknown, the discovery of increased protease activity for circulating IGFBP-3 occurring in pregnancy, post-surgery, cancer, critical illness, renal failure and HIV infection, has led to the proposal that it may serve as a functional pool of IGFs available during stress (see section 1.11; Frost *et al.*, 1996; Timmins *et al.*, 1996; Lee *et al.*, 1994). Limited proteolysis of IGFBP-3 now appears to represent a normal process to increase IGF bioavailability. Cleavage of IGFBP-3 results in the generation of a 31 kDa fragment with a reduced affinity for IGF (Clemmons, 1993). This fragment, which although may form the ternary complex, will permit bound IGF to more readily equilibrate with the lower molecular weight IGFBPs, which possibly transport bound IGFs to the extravascular compartment.

Schilling *et al.* (1959) designed a stainless steel, wire mesh chamber to study wound healing in animals. This model was later popularized by Hunt *et al.* (1967), who investigated the wound microenvironment created by these chambers. It is now often referred to as the 'Hunt-Schilling' chamber. This device is placed beneath the panniculus carnosus of loose skinned animals such as rats (Schilling *et al.*, 1959). In-growth of granulation tissue consisting of fibroblasts, capillaries and abundant collagen fibrils occurs, producing an artificial space that contains wound fluid and which eventually becomes filled with connective tissue matrix (Wakui, 1992; Lynch, 1991; Schilling *et al.*, 1959).

Numerous studies have demonstrated that growth factors can regulate all aspects of tissue repair in normal and impaired wound healing models. Concentrations of IGF-I in wound fluid approximate levels found in serum (Hunt, 1991). It is likely that plasma contributes IGF-I to wounds, however, lysis of platelet α-granules (Karey *et al.*, 1989) and proliferating fibroblasts (Sumi *et al.*, 1984) contribute to the surge in IGF levels in wound fluid just after injury. IGF mRNAs have also been shown to be expressed in granulation tissue (Gartner *et al.*, 1992; Steenfos, 1994). Evidence also exists suggesting that

administration of exogenous IGF-I can stimulate wound healing. IGF-I infused into wounds restores the healing impairment due to treatment with corticosteroids (Suh *et al.*, 1992). Although treatment with IGF-I alone had no effect on the healing of skin wounds in pigs, it potentiated the effect of PDGF on regeneration of dermal connective tissue and on epithelial proliferation (Lynch *et al.*, 1989; 1987). Sommer *et al.*(1991) have reported that locally administered IGF-I / IGFBP-3 complex enhances collagen deposition above that seen after administration of IGF-I alone. Similarly, coadministration of IGFBP-1 enhances the stimulation of wound healing by IGF-I (Jyung *et al.*, 1994), suggesting that IGF-I and its binding proteins interact to affect the process of wound healing.

The Hunt-Schilling wound chamber model offers a site of readily accessible extracellular fluid free of blood contamination that enables investigation of the transfer of radiolabelled, tracer amounts of IGFs out of the blood to extracellular sites. The use of LR^3IGF-I permits examination of the effects of IGFBPs on IGF-I transfer from the blood to extracellular fluid. Additionally, studies examining transfer of IGF-I and LR^3IGF-I from blood to extravascular wound fluid sites will increase our understanding of the mechanism which may be responsible for regulating the bioavailability of IGF-I to tissues.

This chapter presents the results of two *in vivo* experiments. Experiment 1, a preliminary trial, examined the feasability of utilizing the Hunt-Schilling wire mesh chamber model to examine the transport of IGF-I from blood to extracellular fluid sites. Experiment 2, the main trial, examined the effects of chamber implantation on plasma IGFBP profiles and clearance of IGF-I peptides in rats. Using rats implanted with wound chambers, transfer of IGF-I and LR^3IGF-I from blood to extravascular wound fluid sites was analysed to examine the effect of IGFBPs on IGF-I transport.

7.2 MATERIALS AND METHODS

7.2.1 Materials

Stainless steel Hunt-Schilling wound chambers 3 cm long by 1 cm in diameter (Schilling *et al.*, 1959; Hunt *et al.*, 1967) were constructed from no. 40 mesh (Crestware Industries, Adelaide, Australia) and the ends plugged with silicon polymer (see Fig 7.1B). Male Sprague-Dawley rats were obtained from the Central Animal House, University of Adelaide. Isoflurane was from Abbott Australasia (Sydney, Australia). Povidone-iodine came from Delta-West Pty. Ltd. (Bentley, Australia). Vetafil nonabsorbable suture was purchased from Clements Stansen Medical (Sydney, Australia).

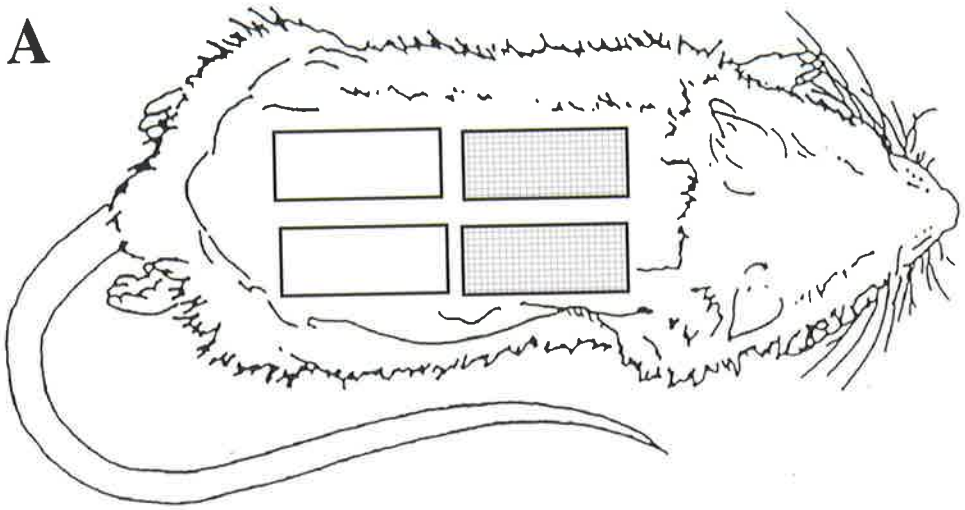
7.2.2 EXPERIMENT 1 :Transfer of ^{125}I-IGF-I from blood to wound fluid

7.2.2.1 Implantation of Hunt-Schilling subcutaneous wound chambers

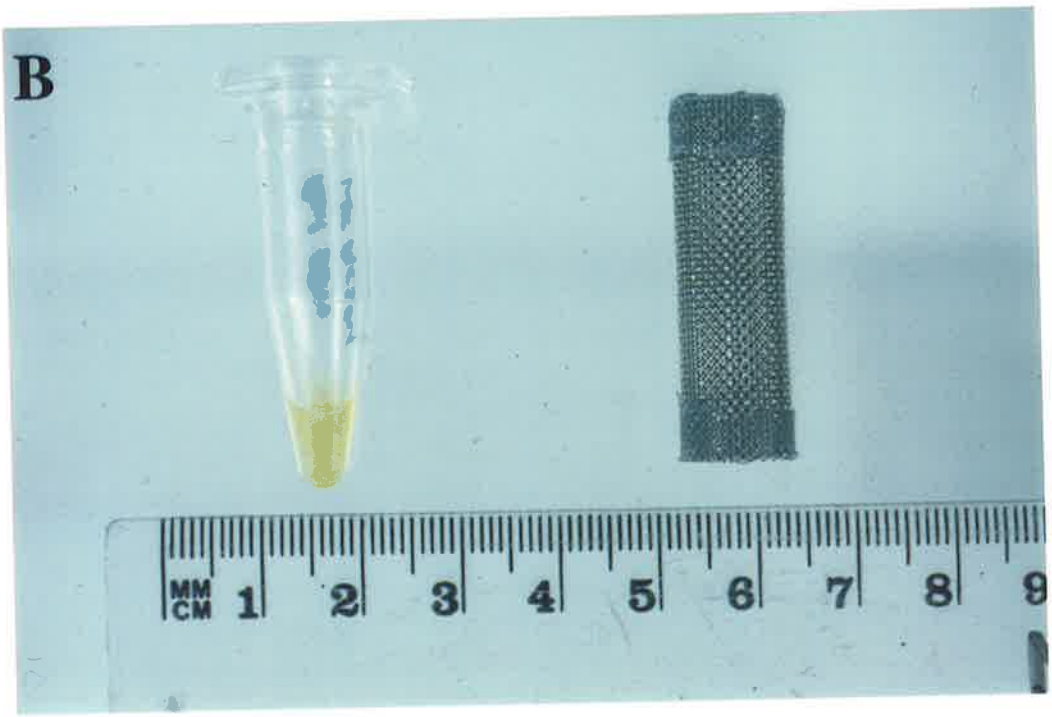
Wound chambers were washed in ethanol and rinsed several times with distilled water prior to autoclaving. Four male Sprague-Dawley rats with a mean weight ± sem of 290.61 ± 5.06g were anaesthetized with isoflurane and a oxygen-nitrous oxide mix. The back of each animal was shaved of hair and the skin aseptically prepared by swabbing with povidone-iodine and 70 % (v/v) ethanol. Two 1-1½ cm abaxial incisions were made, one either side of the midline at a point just craniad to the sacrum, permitting creation of two subcutaneous pockets by blunt dissection for implantation of chambers. Each chamber was introduced using a modified 5 ml syringe craniad to each incision and finally located in a dorso-lateral orientation above each scapula (Fig 7.1A, hatched rectangles). The incisions were sutured with nonabsorbable material. The animals were kept warm until conscious and allowed to

Figure 7.1: Location of either two (hatched rectangles only) or four Hunt-Schilling wound chambers implanted in rats (A) and a photograph of a Hunt-Schilling wound chamber and a sample of wound fluid (B).

A



B



recover with free access to food and water for 14 days prior to experimentation. The experimental protocols were approved by the University of Adelaide Animal Ethics Committee and The Women's and Children's Hospital Ethics Committee.

7.2.2.2 Infusion of ^{125}I-IGF-I into rats implanted with Hunt-Schilling subcutaneous wound chambers and sample collection

On day 14 following insertion of wound chambers, the rats had a mean weight ± sem of 372.4 ± 5.1g. Each rat received 100 µl of ^{125}I-IGF-I (10 x 10^6 cpm) via injection into the tail vein. The specific activity of the radiolabelled IGF-I was 84 µCi / µg. At 15, 30, 60 or 120 min after injection of the bolus dose of ^{125}I-IGF-I, one rat was anaesthetized as described above and three minutes later killed by cardiac puncture. Blood was collected into tubes heparinized to a final concentration of 10 U / ml, aliquotted into 1 ml subsamples and immediately placed into tubes and centrifuged for 2 min at 10 000g at room temperature. Plasma was removed, frozen in liquid nitrogen and stored at -20°C. Immediately following cardiac puncture, the fluid contents of each wound chamber was aspirated by percutaneous insertion of a 19 gauge needle through the silicon plug. Caution was taken to avoid damaging the granulation tissue on the inner surface of the chamber to prevent contamination of wound fluid with blood, giving rise to straw coloured wound fluid samples (Fig. 7.1B). Wound fluid from each chamber was collected into separate tubes, heparinized to a final concentration of 10 U / ml and centrifuged at 10 000g for 2 min at room temperature. Supernatant was aliquotted into tubes, frozen in liquid nitrogen and stored at -20°C.

7.2.2.3 Trichloroacetic acid precipitation of ^{125}I-IGF-I in plasma and wound fluid samples

TCA precipitation was used to determine the percentage of TCA-precipitable (intact) ^{125}I-IGF-I in plasma and wound fluid. Triplicate portions (100 µl) of plasma samples and wound fluid from both chambers in each rat were counted in a γ-counter for 10 min to determine total counts. Plasma and wound fluid samples (25 µl) were then mixed with 475 µl of ice-cold 10 % (w / v) TCA in PBS, and following the addition of 0.1% (w / v) BSA, incubated for 2h on ice and centrifuged at 4°C for 20 min at 10 000g. The supernatant was removed and the radioactivity in both the TCA-soluble and TCA-insoluble portions measured in a γ-counter for 10 min.

7.2.2.4 Neutral gel permeation chromatography of plasma and wound fluid samples

A separate sample of plasma and wound fluid (200 µl) were subjected to FPLC. Wound fluid was first centrifuged at 4°C at 10 000g for 5 min to remove solids. Lipids were removed from both the plasma and wound fluid samples by mixing them with an equivalent volume of Freon (1,1,2-trichloro-1,2,2-trifluroethane), centrifuged at 10 000g for 5 min at 4°C, and the aqueous layer removed. The lipid extracted plasma and wound fluid samples were then chromatographed on a Superose-12 column (HR 10/30), which had been previously equilibrated with a solution of 50mM sodium phosphate, 150 mM NaCl, 0.02 % (w / v) sodium azide and 10 IU / ml heparin, pH 7.2. The flow rate was 0.5 ml / min and 0.5 ml fractions were collected. Radioactivity of fractions was counted in a γ-counter for 10 min. The column was calibrated using ^{125}I-IGF-I, and the molecular mass standards carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (69 kDa) and human immunoglobulin G (150 kDa).

In addition, samples of day 14 wound fluid and plasma pooled from 6 different rats were incubated with 100,000 cpm of ^{125}I-IGF-I at 4°C for 18 h and were chromatographed on the same Superose-12 column.

7.2.3 EXPERIMENT 2: Study of the transfer of ^{125}I-IGF-I and ^{125}I-LR^3 IGF-I from blood to wound fluid

7.2.3.1 Implantation of four Hunt-Schilling subcutaneous wound chambers

Twenty male Sprague-Dawley rats with a mean weight ± sem of 472.6 ± 11.1g were anaesthetized as described above. All animals were treated in the same manner as in the preliminary trial, with the exception that in this study two extra incisions were made at the sacrum, to allow the implantation of four wound chambers per rat (see Fig. 7.1A, hatched and open rectangles). Eight of the twenty rats acted as sham controls and were not implanted with wound chambers.

7.2.3.2 Infusion of ^{125}I-IGF-I and ^{125}I-LR^3 IGF-I and collection of blood and wound fluid samples

Two days prior to experimentation, all animals were anaesthetized by an i.p. injection of a 9:1 solution of methohexitone sodium and pentobarbitone sodium (3.75 ml / kg of body weight) and a catheter inserted into the jugular vein, which was filled with saline containing 10 IU heparin to maintain patency. The animals were kept warm until conscious, and allowed free access to feed and water and left to recover for two days. On the experimental day, rats had a mean weight ± sem of 481.0 ± 9.5 g. Prior to infusion of radiolabelled IGFs, a 200 µl blood sample was withdrawn from the catheter of each rat. Following each blood sampling, the equivalent volume of saline / heparin was injected back into the catheter line. Blood

samples were processed as described in section 7.2.2.2 and stored for Western ligand blot analysis. Rats were randomly chosen for infusion with either ^{125}I-IGF-I or ^{125}I-LR^{3}IGF-I. A description of the treatment groups and an outline of the sampling regimen is given in Table 7.1. Volumes of the blood samples taken at the different time points varied to allow FPLC and pharmacokinetic analyses without seriously reducing blood volume of the animals. At time zero, each rat received a bolus dose (100 µl) of 10 x 10^6 cpm of ^{125}I-IGF-I or ^{125}I-LR^{3}IGF-I in physiological saline plus 0.01 % BSA via the jugular catheter, and the catheter line was flushed with 300 µl of saline. Blood samples of 150 µl were withdrawn at 1, 15, 60 and 180 min for TCA precipitation analyses, while 300 µl blood samples were collected at 5, 30, 120 and 240 min as described above which would be subjected to TCA precipitation analyses and FPLC. All samples were processed as before and stored for later analysis. At 5, 30 120 and 240 min, one of the four wound chambers was aspirated of its contents which was treated as described in section 7.2.2.2.

7.2.3.3 Appearance of ^{125}I-IGF-I and ^{125}I-LR^{3}IGF-I in wound fluid and clearance from plasma

Duplicate portions of plasma (25 µl) and wound fluid (50 µl) from each rat and wound chamber were counted in a γ-counter for 10 min. Plasma and wound fluid samples were then mixed with 475 µl and 450 µl of ice-cold 10 % (v/v) TCA in PBS plus 0.1 % (w/v) BSA, respectively. TCA precipitations were performed as in section 7.2.2.3.

Pharmacokinetic parameters for each rat were calculated by fitting the data to a bi-exponential equation that describes a two-compartment model (Roland and Tozer, 1989), with utilization of a non-linear curve fitting programme (Tablecurve Windows V1.0; Jandel Scientific, Corte Madera, CA, USA) in which $C = Ae^{-\alpha t} + Be^{-\beta t}$, where C equals the plasma concentration of TCA-precipitable radioactivity per ml; A and B correspond to the concentrations of TCA-precipitable radioactivity in each compartment at time 0; α and β are

Table 7.1 Experiment 2 treatment groups and sample regimen

Peptide Infused	0 min	1min	5 min	15 min	30 min	60 min	120 min	180 min	240 min
IGF-I sham control (n=4)	200 µl	150 µl	300 µl + WF	150 µl	300 µl + WF	150 µl	300 µl + WF	150 µl	300 µl + WF
LR³IGF-I sham control (n=4)	200 µl	150 µl	300 µl + WF	150 µl	300 µl + WF	150 µl	300 µl + WF	150 µl	300 µl + WF
IGF-I chamber (n=6)	200 µl	150 µl	300 µl + WF	150 µl	300 µl + WF	150 µl	300 µl + WF	150 µl	300 µl + WF
LR³IGF-I chamber (n=6)	200 µl	150 µl	300 µl + WF	150 µl	300 µl + WF	150 µl	300 µl + WF	150 µl	300 µl + WF

the decay rate constants for each compartment and *t* is the time. The area under the curve (AUC) method was used to determine total plasma clearance (CL) using the relationship CL = dose/AUC, where the dose equals IGF radioactivity administered. Initial volume of distribution (Vc) was calculated as Vc = dose/(A+B). The volume of distribution at steady state (Vss) was calculated as $Vss = (A/\alpha^2 + B/\beta^2)/AUC$.

7.2.3.4 Neutral gel permeation chromatography

Pools of time 5, 30, 120 and 240 min plasma (200 µl) and wound fluid (150 µl) samples from chamber implanted rats infused with ^{125}I-IGF-I or ^{125}I-LR^{3}IGF-I, and plasma samples (200 µl) from sham controls infused with ^{125}I-IGF-I or ^{125}I-LR^{3}IGF-I, were lipid extracted and applied to a Superose-12 column as in section 7.2.2.4.

7.2.3.5 Western ligand blot analysis

To examine the plasma IGFBP profiles of rats implanted with chambers compared to those of the sham control animals, 10 µl samples of blood collected from each rat before radiolabelled IGF infusion were mixed with 190 µl of SDS-loading buffer (see section 4.2.6) and incubated for 15 min at 65°C. Rat plasma samples (1 µl equivalence) were subjected to SDS-PAGE, proteins were transferred to nitrocellulose sheets and IGFBPs detected as described in section 4.2.6. Nitrocellulose sheets were probed with either 5 x 10^5 cpm of ^{125}I-IGF-I or ^{125}I-LR^{3}IGF-I.

To compare the IGFBP profiles of wound fluid and plasma, pools (10 µl) of 14 day wound fluid or plasma samples from rats were mixed with 190 µl of SDS-loading buffer, incubated for 15 min at 65°C and subsamples (1 µl equivalence) subjected to SDS-PAGE and protein transferred to nitrocellulose as in section 4.2.6. Nitrocellulose sheets were probed with 5x10^5 cpm of ^{125}I-IGF-II

7.2.3.6 Statistical analysis

Data were analysed by one-way analysis of variance with all pairwise multiple comparisons using Student-Newman-Keuls method. Pharmacokinetic data was analysed by two-way analysis of variance with replications. $P < 0.05$ was considered significant.

7.3 RESULTS

7.3.1 EXPERIMENT 1: Preliminary trial

7.3.1.1 Appearance of systemically infused ^{125}I-IGF-I in wound fluid

The preliminary study was performed to determine whether one can measure the appearance of ^{125}I-IGF-I in wound fluid aspirated from Hunt-Schilling chambers following systemic infusion of the radiolabelled tracer. Four rats implanted with two Hunt-Schilling wound chambers each, were infused with ^{125}I-IGF-I (10×10^6 cpm) via the tail vein. One rat was killed at 15, 30, 60 or 120 min following infusion, and the wound fluid contents of the two chambers collected. Wound fluid samples were straw coloured with no visible evidence of blood cells (Fig 7.1B). Wound fluid samples were counted in a gamma counter, and then subjected to TCA-precipitation. The mean amount of intact ^{125}I-IGF-I appearing per ml of wound fluid in the two chambers as a percentage of total intact ^{125}I-IGF-I infused, increased from 0.01% at 15 min up to 0.11% by 120 min (Table 7.2). Amounts of ^{125}I-IGF-I appearing in each chamber at one time point were in good agreement. The TCA-precipitability of ^{125}I-IGF-I infused at time zero was 97.4%. At 15 min, the mean percentage of total radioactivity in wound fluid samples that represented intact ^{125}I-IGF-I was 35.1%. At 30 min, the mean percentage of intact radioactivity in wound fluid had decreased to 19.2% and this value persisted for the remaining hour and a half. In contrast, the amount of intact ^{125}I-IGF-I

Table 7.2: Appearance of TCA precipitable (intact) ^{125}I-IGF-I in day 14 wound fluid and plasma as a percentage of total intact ^{125}I-IGF-I infused.

Time after infusion	Intact ^{125}I-IGF-I per ml of wound fluid (% of total infused)		% TCA precipitability of ^{125}I-IGF-I in wound fluid		Intact ^{125}I-IGF-I per ml of plasma (% of total infused)	% TCA precipitability of ^{125}I-IGF-I in plasma
	Chamber 1	Chamber 2	Chamber 1	Chamber 2		
15 min	0.01%	0.01%	35.4%	34.7%	3.6%	94.3%
30 min	0.01%	0.02%	17.4%	20.7%	2.3%	86.7%
60 min	0.07%	0.05%	22.3%	16.6%	2.9%	81.6%
120 min	0.12%	0.10%	20.4%	18.7%	2.3%	77.2%

Two Hunt-Schilling wound chambers were implanted per rat. Fourteen days later ^{125}I-IGF-I was infused via the tail vein. Plasma and wound fluid from both chambers from one rat was collected at either 15, 30, 60 or 120 min following infusion of ^{125}I-IGF-I. Plasma values are means of triplicate samples per rat.

appearing per ml of plasma as a percentage of total intact ^{125}I-IGF-I infused, decreased from 3.6% at 15 min to 2.3% at 120 min. With time, intact ^{125}I-IGF-I TCA-precipitable radioactivity in plasma decreased from 94.3% at 15 min to 77.2% by 120 min.

7.3.1.2 Neutral gel chromatography of day 14 wound fluid and plasma samples following infusion of ^{125}I-IGF-I

Equivalent volumes of wound fluid and plasma samples taken from the rats at each time point were chromatographed on a size-exclusion column at neutral pH. It is important to note that much less IGF-I specific radioactivity appeared in wound fluid relative to plasma as reflected in the scale of the Y-axis in Fig 7.2. At 30 min the majority of the radioactivity in wound fluid corresponded to a molecular mass of 7.5 kDa, representing free ^{125}I-IGF-I. The remainder of the radioactivity occurred in a region corresponding to a molecular mass of 50 - 30 kDa, which likely represents ^{125}I-IGF-I in association with wound fluid IGFBPs. In contrast to wound fluid, most of the radioactivity in plasma eluted at a position corresponding to a molecular mass of the ternary complex (150 kDa), with less radioactivity recovered in fractions corresponding to 50 - 30 and 7.5 kDa.

In a separate experiment aimed at elucidating the molecular weight of the IGF:IGFBP complexes in both wound fluid and plasma, pools of day 14 wound fluid and plasma from 6 different rats were incubated *in vitro* with ^{125}I-IGF-I at 4 °C for 18 h before size-exclusion chromatography. Three peaks of radioactivity were eluted from both wound fluid and plasma samples, at molecular masses corresponding to 150, 30-50 and 7.5 kDa (Fig 7.3). In contrast to plasma, less radioactivity was recovered in wound fluid in the 150 kDa region relative to the 40-50 and 7.5 kDa regions, and recovery of wound fluid radioactivity in the 7.5kDa region was greater than that in plasma.

Figure 7.2: Neutral gel permeation chromatography of day 14 plasma or wound fluid collected at 30 min following infusion of ^{125}I-IGF-I into rats. Samples (200 μl) were applied to a Superose-12 column previously equilibrated in PBS (pH 7.2), and eluted at a flow rate of 0.5 ml / min with the same buffer. The column was calibrated with γ-globulin (G, 150 kDa), BSA (B, 66 kDa), ovalbumin (O, 49kDa), carbonic anhydrase (C, 30 kDa) and ^{125}I-IGF-I (I, 7.5 kDa). Fractions (0.5 ml) were collected and radioactivity counted on a γ-counter.

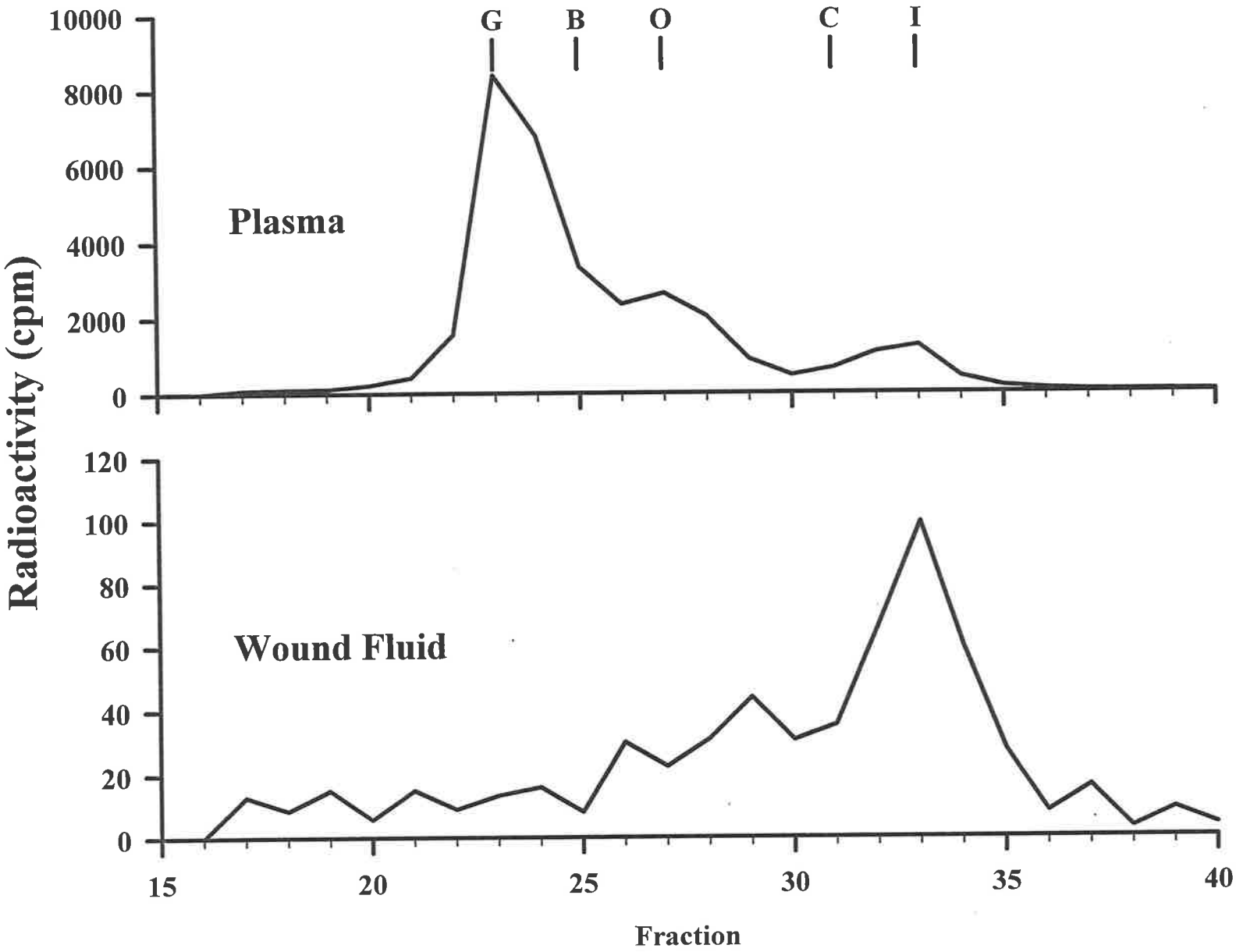
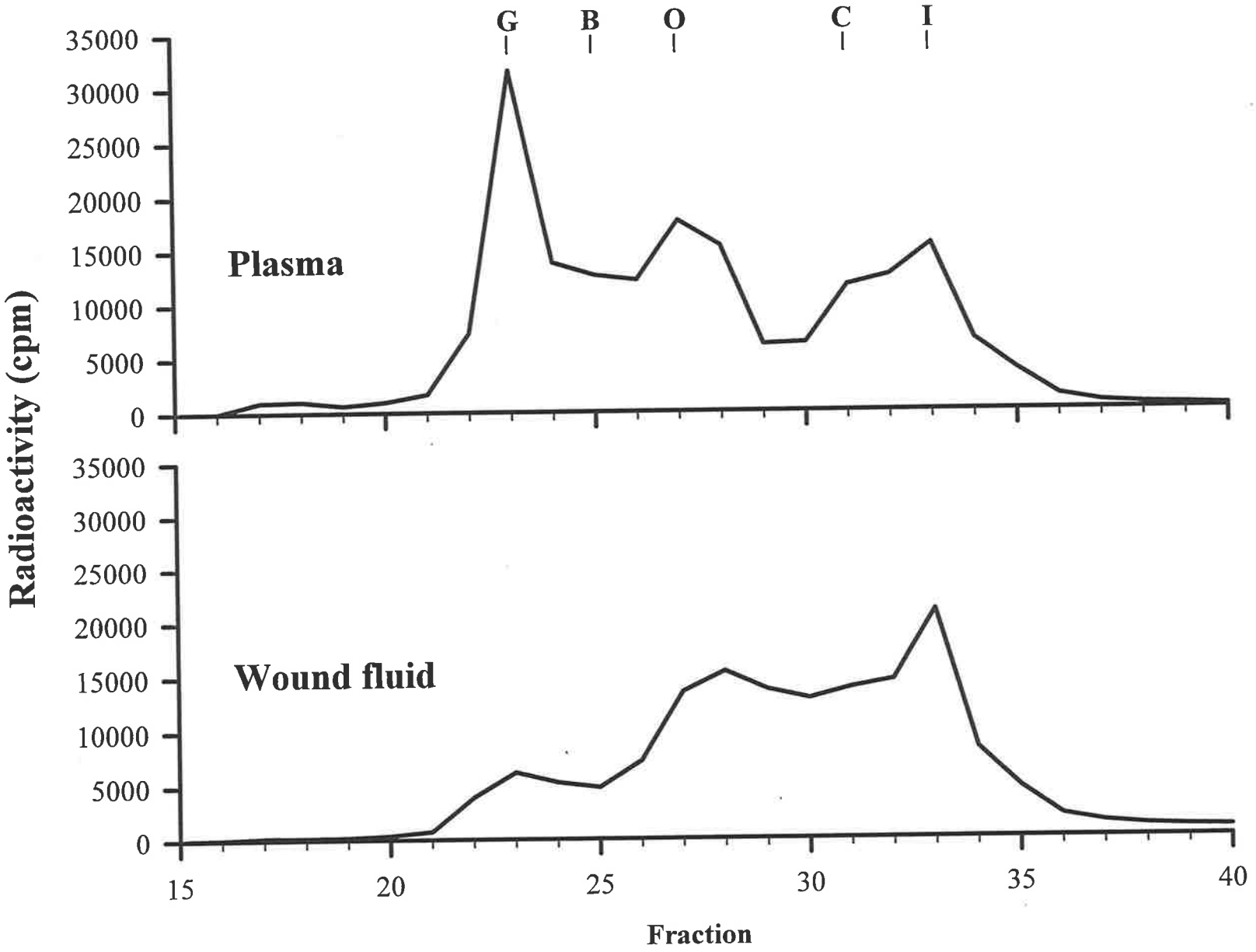

G
B
O
C
I
10000
8000
6000
4000
2000
0
Plasma
Radioactivity (cpm)
120
100
80
60
40
20
0
Wound Fluid
15
20
25
30
35
40
Fraction

Figure 7.3: *In vitro* characterization of IGFBPs in day 14 plasma and wound fluid by neutral gel permeation chromatography. Samples (200 μl) were pooled and mixed with ^{125}I-IGF-I (1x10^5 cpm) for incubation at 4°C for 18 h and applied to a Superose-12 column equilibrated in PBS (pH 7.2). Samples were eluted at 0.5 ml / min with the same buffer. The column was calibrated with γ-globulin (G, 150 kDa), BSA (B, 66 kDa), ovalbumin (O, 49kDa), carbonic anhydrase (C, 30 kDa) and ^{125}I-IGF-I (I, 7.5 kDa). Fractions (0.5 ml) were collected and radioactivity counted on a γ-counter.


G
B
O
C
I
35000
30000
25000
20000
15000
10000
5000
0
Plasma
Radioactivity (cpm)
Wound fluid
15
20
25
30
35
40
Fraction

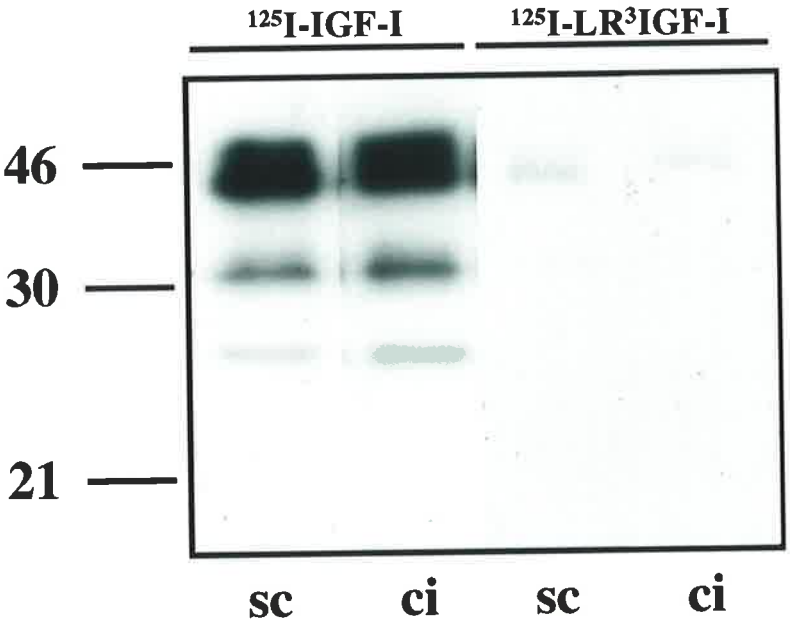
7.3.2 EXPERIMENT 2: Main study

The preliminary trial indicated that systemically infused ^{125}I-IGF-I transfered intact into extravascular wound fluid sites, where it appeared in measurable amounts. It also indicated that the amounts appearing in either chamber were concordant. As a consequence of this preliminary experiment, a main trial was designed to examine the transfer of ^{125}I-IGF-I from blood to extracellular wound fluid sites. However, this main trial differed from the preliminary experiment in the following ways. To examine the rate of transfer of ^{125}I-IGF-I from blood to wound fluid, six rats were implanted with four Hunt-Schilling chambers to permit aspiration of wound fluid at four different time points following infusion of radiolabelled tracer. This was done to decrease the overall number of animals used, but provide enough animals to achieve statistical significance. In addition, ^{125}I-LR^{3}IGF-I was infused in a separate group of animals to determine the effects of IGFBPs on transfer of IGF-I from blood to extracellular fluid. Two groups of four rats acted as ^{125}I-IGF-I and ^{125}I-LR^{3}IGF-I infused sham operated controls. Finally, rats were inserted with jugular catheters to permit regular blood sampling for concurrent pharmacokinetic experiments.

7.3.2.1 Comparison of IGFBP profiles in plasma of wound chamber implanted and sham control rats

To examine whether the implantation of chambers in rats caused a perturbation of the plasma IGFBP profile which could potentially alter the transport of IGFs from blood to extracellular fluid sites, Western ligand blot analysis of implanted and sham control rat plasma was performed. In both implanted and sham control rats, a doublet of bands at approximately 46 to 55kDa, along with two fainter lower molecular weight bands at 31 and 24 kDa were observed when ^{125}I-IGF-I was the radiolabelled ligand (Fig 7.4). These studies indicated that implantation of chambers did not alter the plasma IGFBP profile. When ^{125}I-

Figure 7.4: Western ligand blots of day 14 plasma from sham control and chamber implanted rats. Plasma (1 μl equivalence) from representative sham control (sc) and chamber implanted (ci) rats prior to infusion of labelled IGF-I or LR^3IGF-I were added to SDS-sample buffer and electrophoresed on 12.5 % SDS-PAGE gels under non-reducing conditions. The proteins were transferred to nitrocellulose sheets and incubated with 5 x 10^5 cpm of either ^{125}I-IGF-I or ^{125}I-LR^3 IGF-I, and exposed to X-ray film for 4 days. The positions of molecular mass markers in kDa are indicated.


125I-IGF-I
125I-LR3IGF-I
46
30
21
sc
ci
sc
ci

LR^3 IGF-I was the radiolabelled probe, a faint band at 46 kDa was seen in both wound chamber implanted and sham control rat plasma, confirming the weak affinity of the IGF-I analogue for rat plasma IGFBPs.

7.3.2.2 Pharmacokinetic parameters of ^{125}I-IGF-I and ^{125}I-LR^3 IGF-I in wound chamber implanted and sham control rats

Clearance studies were performed to determine whether the implantation of wound chambers affected the clearance values of IGFs in rats. The mean TCA-precipitable radioactivity in plasma over time is shown in Fig 7.5. In all cases an early rapid and subsequent slower decline in plasma concentration of the labelled growth factors was observed. The areas under the curves definine the rapid (α) and slower (β) components and are shown in Fig 7.5. The clearance, half-lives plus initial and steady state volumes of distribution of the rapid and slower components are presented in Table 7.3. Half-lives for the fast component were significantly greater for chamber implanted animals compared to controls. The area under the curve for the fast component of IGF-I infused chamber implanted animals was significantly decreased relative to control IGF-I infused and chamber implanted LR^3IGF-I infused rats. For the slower phase, the AUCβ was significantly greater for ^{125}I-IGF-I than ^{125}I-LR^3IGF-I in both sham control and chamber implanted rats. This indicates that LR^3IGF-I radioactivity is cleared more rapidly and that the amount of LR^3IGF-I radioactivity in the slow pool is reduced. This is reflected in the metabolic clearance rate of ^{125}I-IGF-I, which was significantly reduced, being 2.3-fold and 2.5-fold lower than ^{125}I-LR^3IGF-I in sham control and chamber implanted rats, respectively. Additionally, the half-lives of IGF-I were significantly greater than LR^3IGF-I for the slow component. For LR^3IGF-I in chamber implanted rats, AUCβ and the half-life of the slow component were significantly higher than that obtained in sham controls, however, the steady state volume of distribution for LR^3IGF-I was significantly higher in chamber implanted rats which is reflected in a higher

Figure 7.5: Clearance of trichloroacetic acid-insoluble radioactivity from plasma of (A) sham control and (B) wound chamber implanted rats following injection of either ^{125}I-IGF-I (circles and solid lines) or ^{125}I-LR^{3}IGF-I (squares and dashed lines) at time zero. Values are means ± sems for six animals in chamber implanted groups and four animals in sham control groups. Decay curves were calculated as described in Materials and Methods. Solid lines represent ^{125}I-IGF-I and dashed lines ^{125}I-LR^{3}IGF-I.

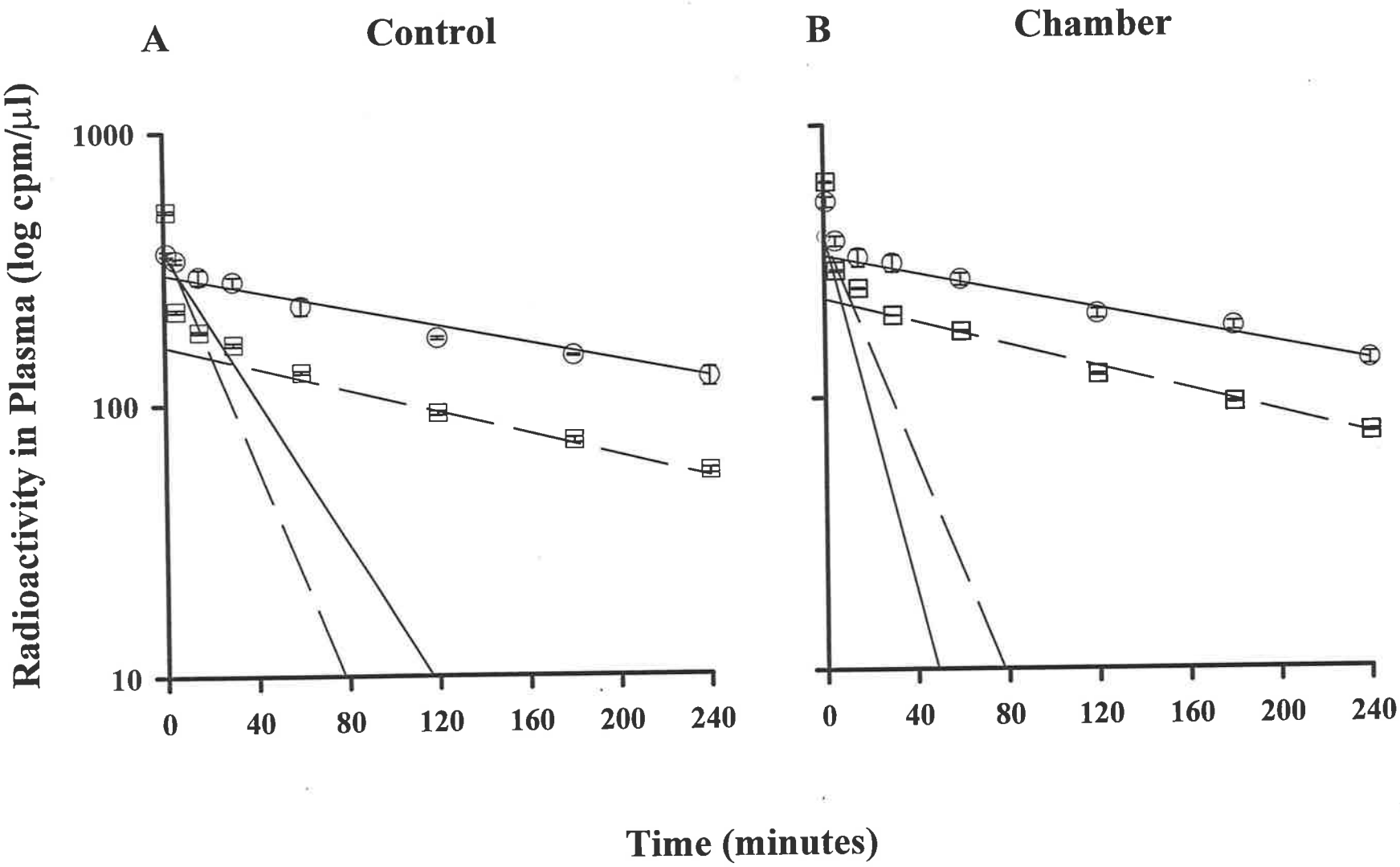

A
Control
B
Chamber
Radioactivity in Plasma (log cpm/μl)
1000
100
10
0
40
80
120
160
200
240
Time (minutes)

Table 7.3 Pharmacokinetic parameters for labelled IGF-I and LR³ IGF-I administered to wound chamber implanted and sham control rats. Values are means ± sems for 4 animals in each sham control group and 6 animals in each wound chamber implanted group.

	Area under the curve (cpm / min per nl)		Clearance (ml / min per kg)	Volume of Distribution (ml per kg)		Half-lives (min)	
	AUCα	AUCβ		Vc	Vss	$t_{½\alpha}$	$t_{½\beta}$
IGF-I (sham control)	3.1 ± 0.4	81.1 ± 0.5	0.3 ± 0.02	69.7 ± 4.5	84.9 ± 2.9	3.2 ± 0.1	203.1 ± 10.2
IGF-I (chamber implanted)	1.51 ± 0.3*	88.6 ± 0.2	0.4 ± 0.01	62.4 ± 3.2	101.4 ± 5.6	4.9 ± 0.02*	185.4 ± 4.7
LR³IGF-I (sham control)	2.1 ± 0.4	26.5 ± 0.1*	0.7 ± 0.02*	70.8 ± 0.6	109.1 ± 4.6*	2.8 ± 0.1	111.1 ± 3.9*
LR³IGF-I (chamber implanted)	2.7 ± 0.6**	46.8 ± 0.2‡**	1.0 ± 0.03‡**	78.7 ± 7.1	197.2 ± 6.9‡**	4.6 ± 0.5‡	140.2 ± 2.5‡**

P<0.05 compared to IGF-I sham control

****P<0.05 compared to IGF-I chamber implanted rats***

‡P<0.05 compared to LR³IGF-I sham control

AUCα and AUCβ are the area under the curve for the rapid and slow component, respectively.

Vc and Vss are the initial and steady state volumes of distribution, respectively.

$t_{½}\alpha$ and $t_{½}\beta$ are the half-lives for the rapid and slow component, respectively.

metabolic clearance rate. Steady state volumes of distribution were larger for LR^{3}IGF-I than IGF-I, which indicates that more IGF-I is confined to the circulation, as expected on the basis of the differences between these two peptides in binding to plasma IGFBPs. These results showed that ^{125}I-LR^{3}IGF-I was removed from the circulation more rapidly than ^{125}I-IGF-I in both control and chamber implanted animals. ^{125}I-IGF-I in sham control and chamber implanted rats is cleared with similar kinetics. Furthermore, ^{125}I-LR^{3}IGF-I is cleared more rapidly in chamber implanted rats, reflecting the larger volume of distribution for this peptide in these animals.

7.3.2.3 Appearance and TCA-precipitability of ^{125}I-IGF-I and ^{125}I-LR^3 IGF-I in wound fluid following infusion via a jugular catheter

To determine whether IGFBPs affect the transfer of IGF-I from blood to extracellular fluid sites, the appearance of both ^{125}I-IGF-I and ^{125}I-LR^3 IGF-I in wound fluid was examined following systemic infusion of these peptides into rats implanted with wound chambers. Five min following infusion of ^{125}I-IGF-I into rats, 0.003 ± 0.001% (mean ± sem: n=6) of total infused, intact ^{125}I-IGF-I had appeared per ml of wound fluid. By 240 min, this figure had increased up to 0.05 ± 0.004% (Table 7.4). This intact radioactivity represented 35.7 ± 1.7% (mean ± sem: n=6), 32.8 ± 0.46%, 21.3 ± 0.63% and 20.0 ± 0.87% of total radioactivity in wound fluid at 5, 30, 120 and 240 min, respectively (Table 7.4). In contrast, significantly more ^{125}I-LR^3 IGF-I than ^{125}I-IGF-I was recovered per ml of wound fluid at each time point (Table 7.4). At 5 min 0.01 ± 0.001% (mean ± sem: n=6, $P<0.001$) of total infused intact ^{125}I-LR^3 IGF-I had appeared per ml of wound fluid. By 240 min the amount had increased to 0.08 ± 0.01% (n=6, $P<0.01$). These data indicate that the egress of ^{125}I-IGF-I from blood to extracellular wound fluid sites is impeded relative to ^{125}I-LR^3 IGF-I. The total amount of radioactivity in wound fluid of rats infused with ^{125}I-LR^3 IGF-I was 40.28 ± 2.0%, 31.80 ±

Table 7.4: Appearance of TCA precipitable (intact) ^{125}I-IGF-I and ^{125}I-LR^3 IGF-I in day 14 wound fluid as a percentage of total intact ^{125}I-IGF-I or ^{125}I-LR^3 IGF-I infused.

Time after infusion	Intact ^{125}I-IGF-I per ml of wound fluid (% of total infused)	% TCA precipitability of ^{125}I-IGF-I in wound fluid	Intact ^{125}I-LR^3 IGF-I per ml of wound fluid (% of total infused)	% TCA precipitability of ^{125}I-LR^{3}IGF-I in wound fluid
5 min	0.003 ± 0.001%	35.7 ± 1.7%	0.01 ± 0.001%***	40.3 ± 2.0%
30 min	0.02 ± 0.003%	32.8 ± 0.5%	0.04 ± 0.01%*	31.8 ± 2.1%
120 min	0.04 ± 0.002%	21.3 ± 0.6%	0.08 ± 0.01%***	21.7 ± 1.3%
240 min	0.05 ± 0.004%	20.0 ± 0.9%	0.08 ± 0.01%**	18.4 ± 1.3%

Four Hunt-Schilling wound chambers were implanted per rat. Fourteen days later ^{125}I-IGF-I or ^{125}I-LR^3 IGF-I was infused via a jugular cannula. Plasma and wound fluid from one chamber from each rat was collected at either 5, 30, 120 or 240 min following infusion of ^{125}I-IGF-I or ^{125}I-LR^3 IGF-I. Values represent the mean ± sem of samples from one chamber per rat from six rats at each time point. P<0.05*, P<0.01, P<0.001*** compared to IGF-I.**

2.1%, 21.65 ± 1.3% and 18.43 ± 1.3% TCA precipitable at 5, 30, 120 and 240 min, respectively (Table 7.4).

7.3.2.4 Comparison of wound fluid and plasma IGFBP profiles

To examine any differences between wound fluid and plasma IGFBP profiles, pools of day 14 wound fluid and rat plasma were subjected to Western ligand blot analysis with ^{125}I-IGF-II as radiolabelled probe. Representative profiles of wound fluid and plasma are presented in Fig 7.6. Western ligand blotting of wound fluid identified an IGFBP profile of three bands, including a doublet at approximately 46 kDa and two single bands at 31 and 24 kDa. Similar banding patterns were revealed on comparison of equivalent loading volumes (1 µl) of wound fluid and plasma. However, the intensities of the 46 kDa doublet and the 31 kDa band in wound fluid were dramatically reduced compared to their plasma counterparts. In contrast, the 24 kDa bands seen in wound fluid and plasma were of similar intensities.

7.3.2.5 Profiles of IGF radioactivity in plasma and wound fluid

Pooled plasma from six animals sampled 5 min after infusion with ^{125}I-IGF-I chromatographed as a broad region of radioactivity at 150 kDa, a second at approximately 50 kDa and a third minor peak at the position corresponding to the molecular mass of free IGF-I (Fig 7.7). By 30 min the radioactivity of each region had slightly decreased. At 120 min the radioactivity of the 150, 50 and 7.5 kDa regions has decreased by approximately 50 %, with a further reduction in the high molecular mass region evident by 240 min. At each time point a preferential decrease of IGF radioactivity associated with low molecular weight IGFBPs was observed.

The chromatograms of pooled plasma samples after administration of ^{125}I-LR^{3}IGF-I are shown in Fig 7.8. Unlike IGF-I radioactivity in plasma, less total counts were observed and approximately 40% of the radioactivity in these samples was detected as free ^{125}I-LR^{3}IGF-

Figure 7.6: Comparison of IGFBP profiles of wound fluid and plasma. Day 14 wound fluid (1 μl equivalence) and a rat plasma control (1 μl equivalence) were incubated with SDS-loading buffer and electrophoresed on 12.5 % SDS-PAGE gels under non-reducing conditions. Proteins were transferred to nitrocellulose sheets, incubated with ^{125}I-IGF-II (5 x 10^5 cpm) and exposed to X-ray film for 4 days. The position of molecular mass markers are indicated in kDa.

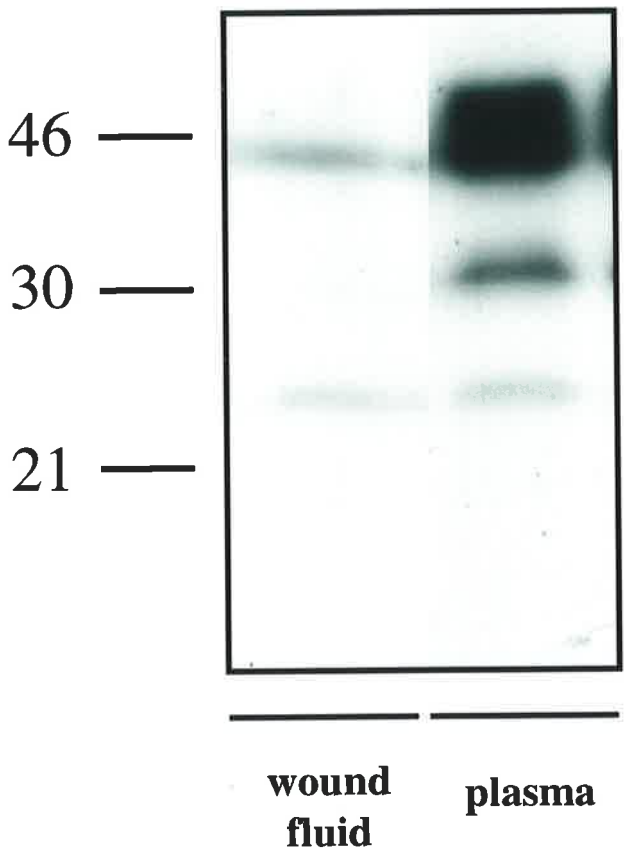

46
30
21
wound fluid
plasma

Figure 7.7: Neutral gel permeation chromatography of plasma from rats 5 min, 30 min, 120 min and 240 min after an intravenous bolus of ^{125}I-IGF-I. Pools of plasma from 6 rats (200 μl) were mixed and applied to a Superose-12 column equilibrated in PBS (pH 7.2). Samples were eluted at 0.5 ml / min with the same buffer. The column was calibrated with γ-globulin (G, 150 kDa), BSA (B, 66 kDa), ovalbumin (O, 43 kDa), carbonic anhydrase (C, 30 kDa) and ^{125}I-IGF-I (I, 7.5 kDa). Fractions (0.5 ml) were collected and radioactivity counted on a γ-counter. Values per μl of plasma are shown.

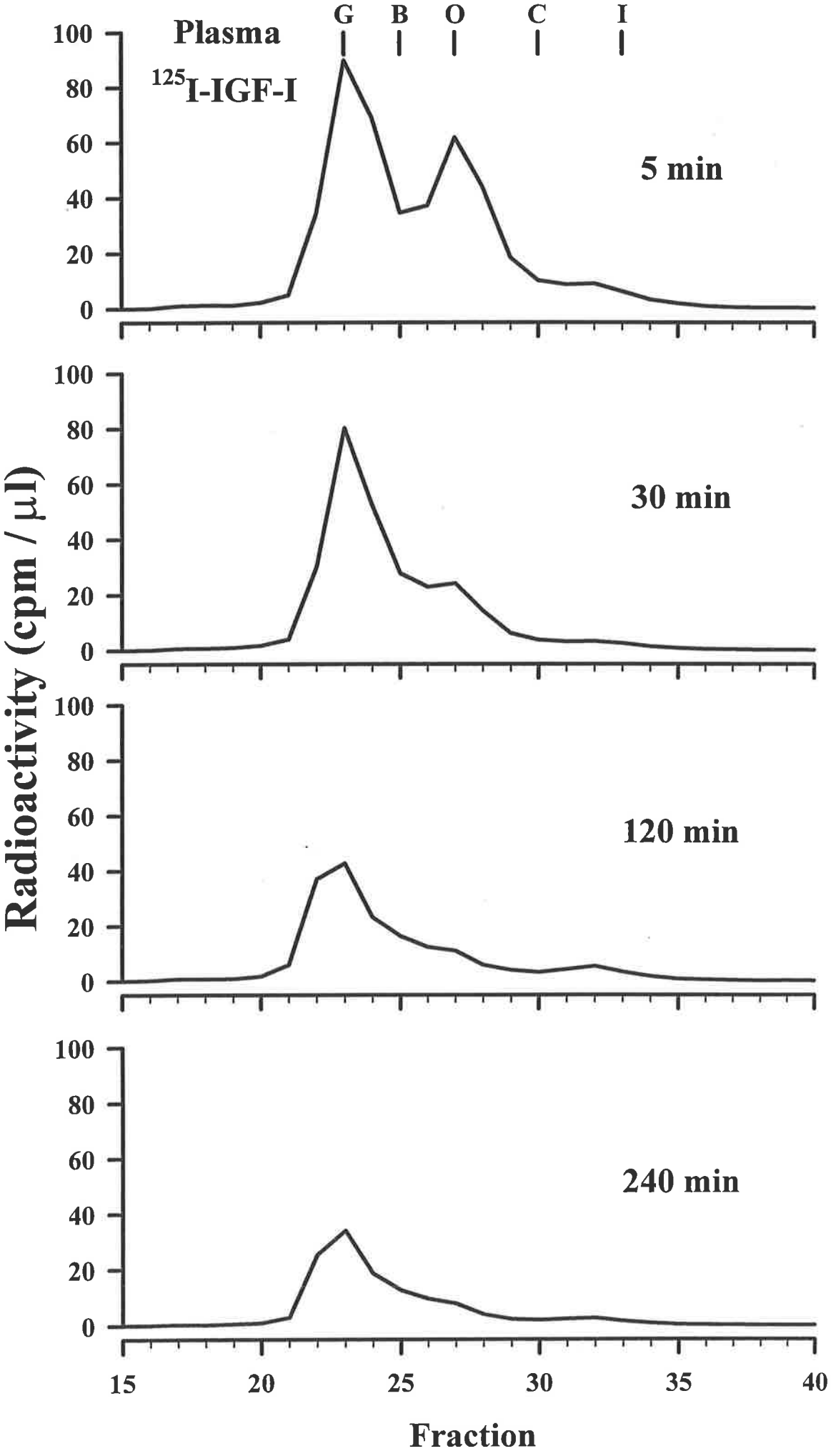
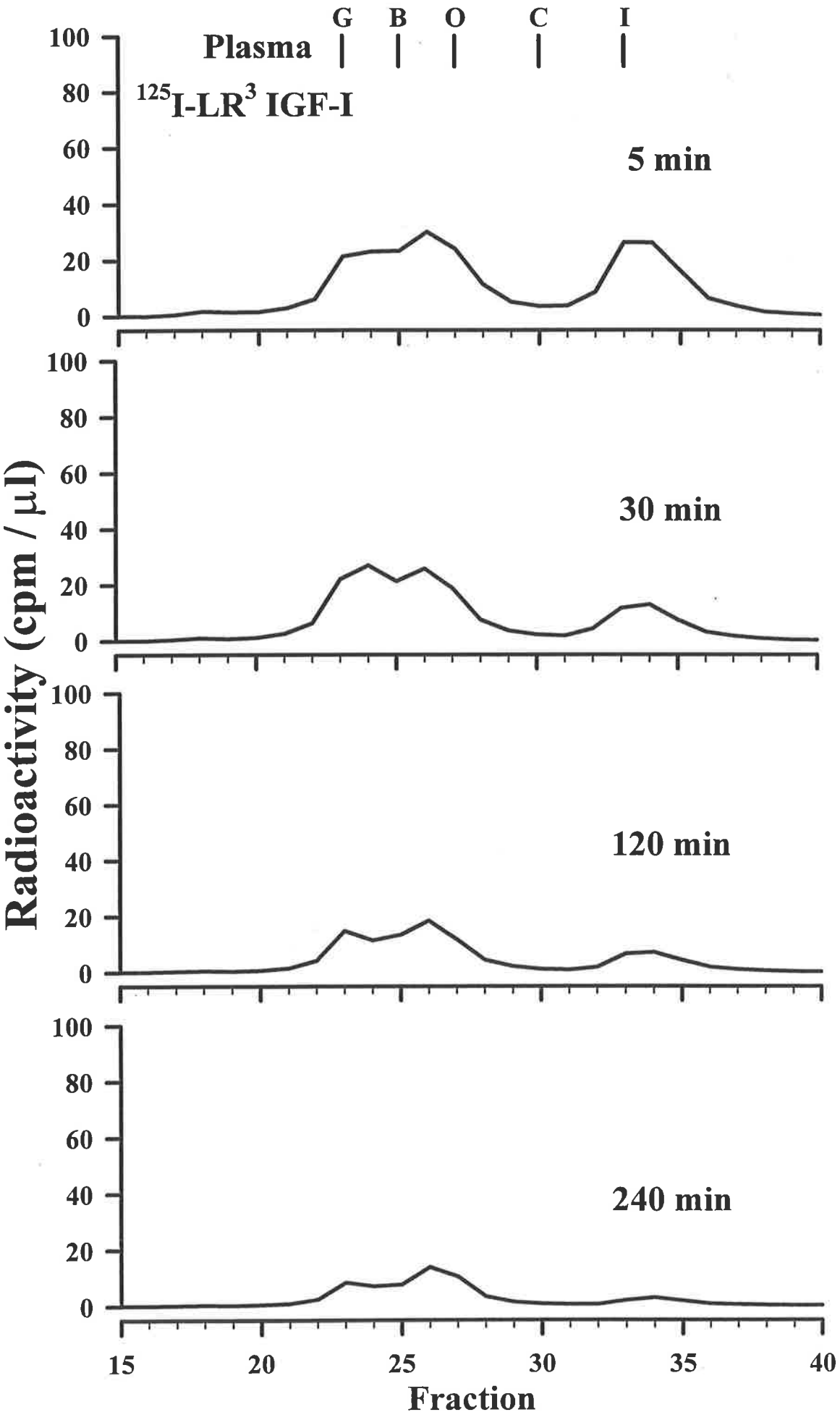

Plasma
125I-IGF-I
G
B
O
C
I
5 min
30 min
120 min
240 min
Radioactivity (cpm / µl)
Fraction
0
20
40
60
80
100
15
20
25
30
35
40

Figure 7.8: Neutral gel permeation chromatography of plasma from rats 5 min, 30 min, 120 min and 240 min after an intravenous bolus of ^{125}I-LR^{3}IGF-I. Pools of plasma from 6 rats (200 μl) were mixed and applied to a Superose-12 column equilibrated in PBS (pH 7.2). Samples were eluted at 0.5 ml / min with the same buffer. The column was calibrated with γ-globulin (G, 150 kDa), BSA (B, 66 kDa), ovalbumin (O, 43 kDa), carbonic anhydrase (C, 30 kDa) and ^{125}I-IGF-I (I, 7.5 kDa). Fractions (0.5 ml) were collected and radioactivity counted on a γ-counter. Values per μl of plasma are shown.


G
B
O
C
I
Plasma
125I-LR3 IGF-I
5 min
30 min
120 min
240 min
Radioactivity (cpm / µl)
100
80
60
40
20
0
15
20
25
30
35
40
Fraction

I with much lower amounts appearing in the 150 kDa and 50 kDa regions. Radioactivity collected in fractions corresponding to free IGF peptide was cleared more quickly than radioactivity in higher molecular weight peaks. By 30 min the radioactivity in the 7.5 kDa region had decreased by 50 %, while the radioactivity in the 150 kDa and 50 kDa regions evident at 5 min remained approximately the same. At 120 min and 240 min the radioactivity in each of the three regions continued to decrease at a relatively similar rate.

In contrast to plasma, less radioactivity was contained within wound fluid following infusion of labelled IGFs. It is important to note the difference in scale of the y-axis for the plasma chromatography profiles (Fig 7.7 and Fig 7.8), compared with the scale of the y-axis for the wound fluid chromatography profiles (Fig 7.9 and Fig 7.10). Pooled wound fluid from six animals sampled at 5 min following infusion of ^{125}I-IGF-I chromatographed as a broad region at approximately 50 kDa and a peak at the position corresponding to free IGF-I (Fig 7.9). By 30 min the radioactivity in the 7.5 kDa region had decreased slightly, however a third peak in the 150 kDa region had become apparent. By 120 and 240 min there were no peaks of radioactivity in the 150 kDa region and IGF-I specific radioactivity had become mainly associated with lower molecular weight IGFBPs.

When wound fluid samples taken from rats 5 min after infusion of ^{125}I-LR^{3}IGF-I were chromatographed, a similar 5 min profile was obtained to that of ^{125}I-IGF-I containing wound fluid with a broad region at 50 kDa and a peak at 7.5 kDa (Fig 7.10). However, by 30 min the profile differed from that of ^{125}I-IGF-I containing wound fluid, in that two large distinct peaks were observed at 50 kDa and 7.5 kDa and no activity occurred in the 150 kDa region. By 120 min the peaks of radioactivity in the 7.5 kDa region had decreased, but the radioactivity in the 50 kDa region had increased by about 25 %. A similar profile was observed for the 240 min samples, but a 25 % decrease in radioactivity in the 50 kDa region had occurred. Unlike plasma, ^{125}I-LR3IGF-I in wound fluid was not associated with the 150 kDa complex. In

Figure 7.9: Neutral gel permeation chromatography of wound fluid from rats 5 min, 30 min, 120 min and 240 min after an intravenous bolus of ^{125}I-IGF-I. Pools of wound fluid from 6 rats (150 μl) were mixed and applied to a Superose-12 column equilibrated in PBS (pH 7.2). Samples were eluted at 0.5 ml / min with the same buffer. The column was calibrated with γ-globulin (G, 150 kDa), BSA (B, 66 kDa), ovalbumin (O, 43 kDa), carbonic anhydrase (C, 30 kDa) and ^{125}I-IGF-I (I, 7.5 kDa). Fractions (0.5 ml) were collected and radioactivity counted on a γ-counter.

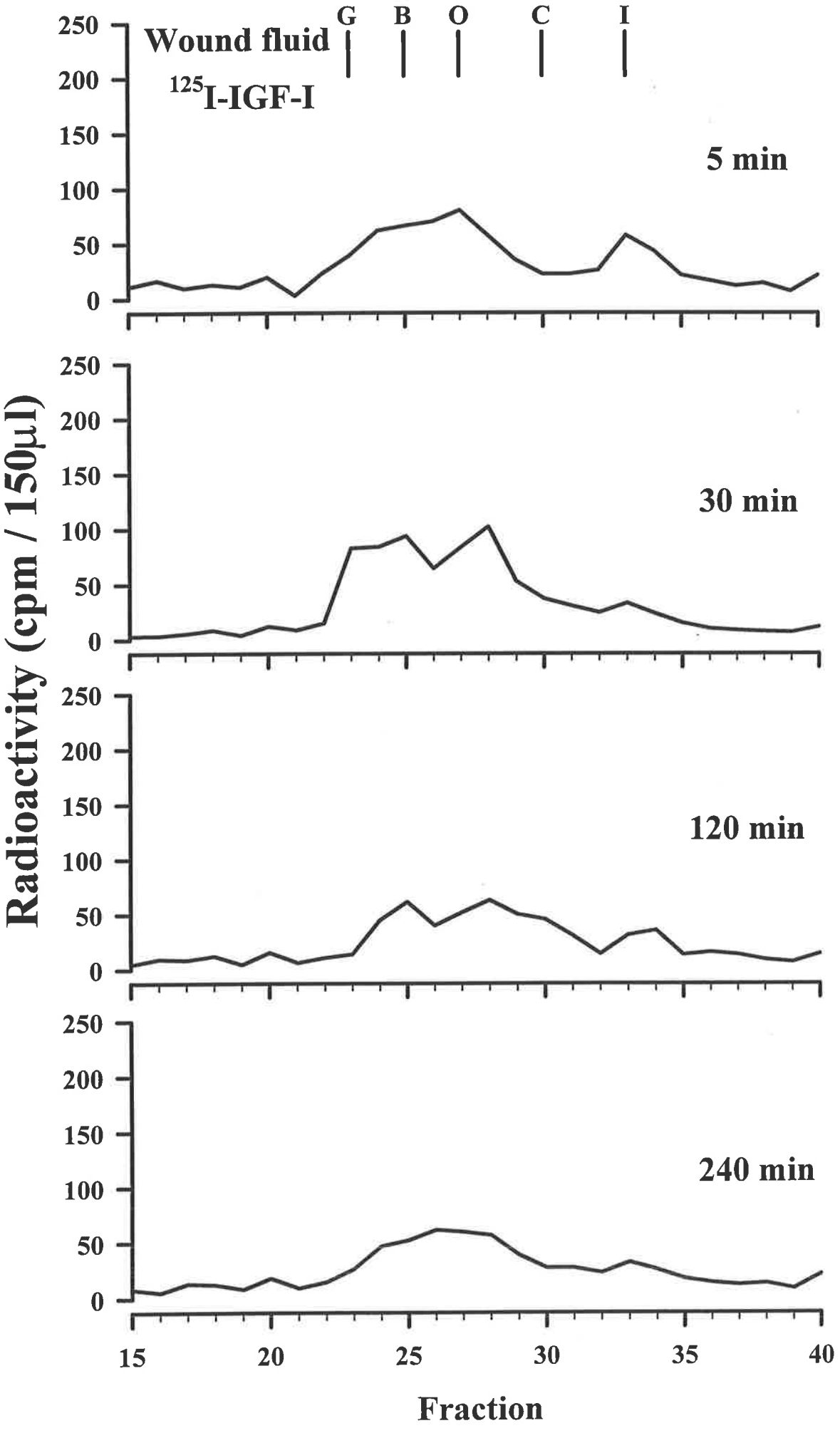
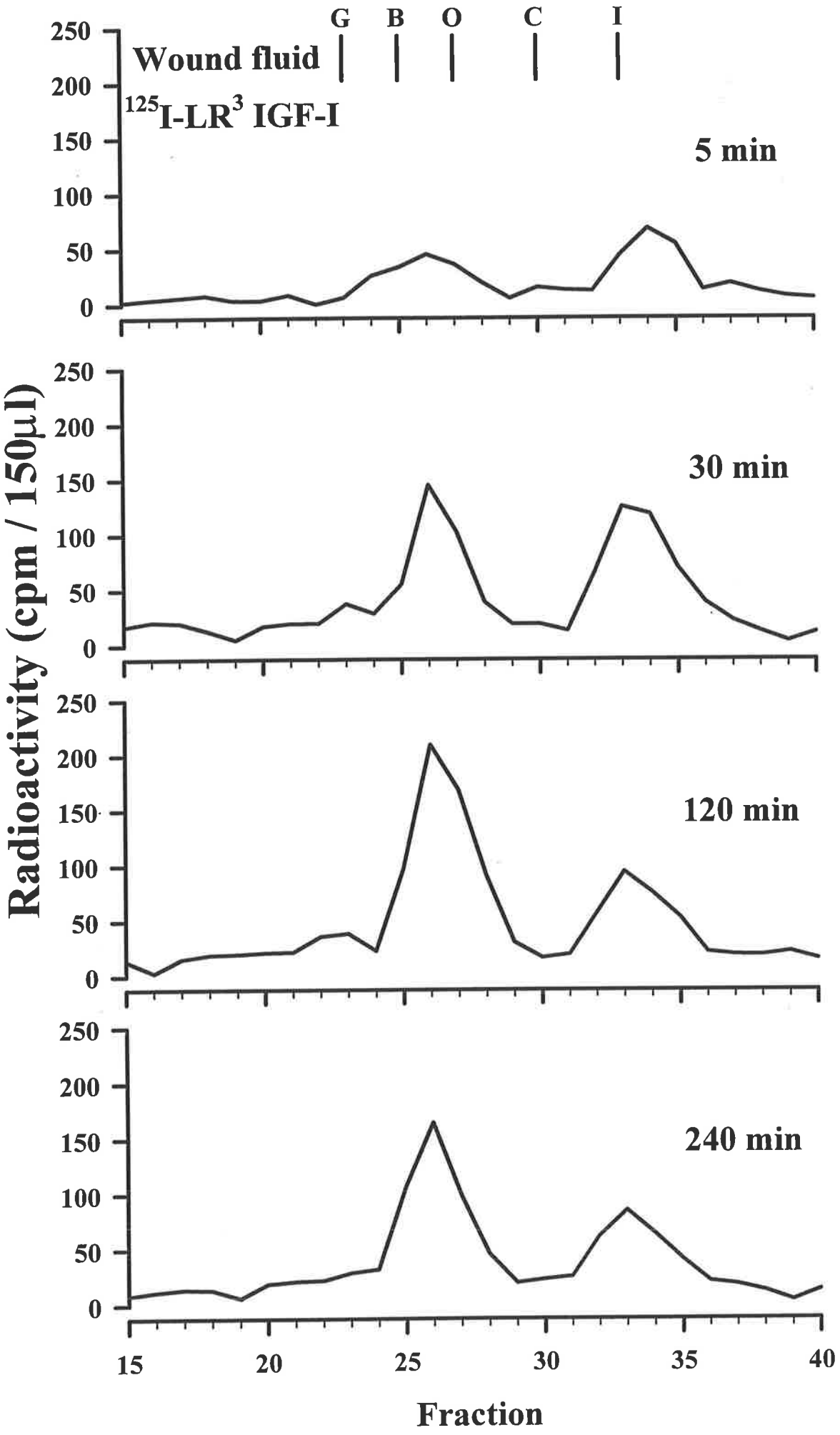

Wound fluid
125I-IGF-I
G
B
O
C
I
5 min
30 min
120 min
240 min
Radioactivity (cpm / 150µl)
250
200
150
100
50
0
15
20
25
30
35
40
Fraction

Figure 7.10: Neutral gel permeation chromatography of wound fluid from rats 5 min, 30 min, 120 min and 240 min after an intravenous bolus of ^{125}I-LR^{3}IGF-I. Pools of wound fluid from 6 rats (150 μl) were mixed and applied to a Superose-12 column equilibrated in PBS (pH 7.2). Samples were eluted at 0.5 ml / min with the same buffer. The column was calibrated with γ-globulin (G, 150 kDa), BSA (B, 66 kDa), ovalbumin (O, 43 kDa), carbonic anhydrase (C, 30 kDa) and ^{125}I-IGF-I (I, 7.5 kDa). Fractions (0.5 ml) were collected and radioactivity counted on a γ-counter.


G
B
O
C
I
Wound fluid
125I-LR3 IGF-I
5 min
30 min
120 min
240 min
Radioactivity (cpm / 150µl)
Fraction
250
200
150
100
50
0
15
20
25
30
35
40

addition, in the 120 and 240 min samples more ^{125}I-LR3IGF-I total radioactivity was found relative to ^{125}I-IGF-I.

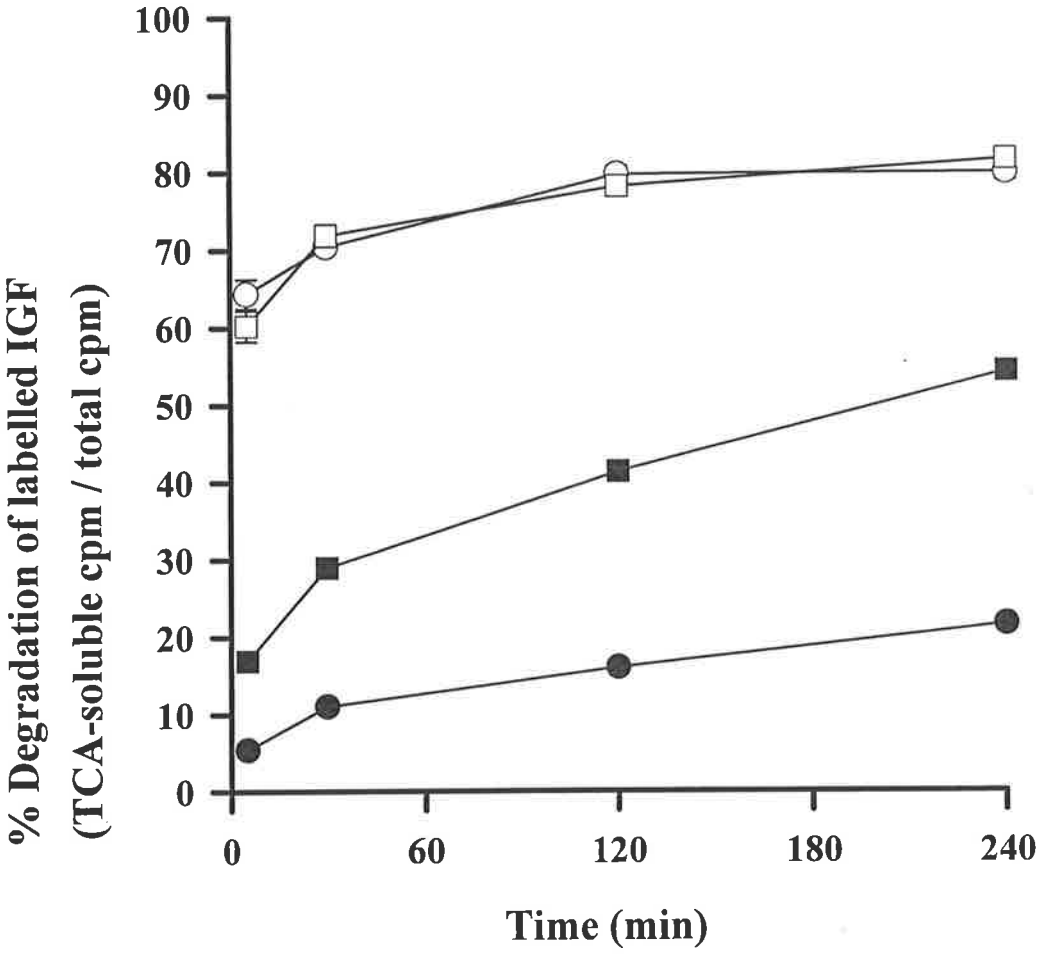
7.3.2.6 Degradation of ^{125}I-IGF-I and ^{125}I-LR^3 IGF-I in wound fluid and plasma

The degradation of growth factors as a function of time after infusion was calculated as a percentage of total plasma or wound fluid radioactivity accounted for by the TCA-soluble fraction (Fig 7.11). In plasma, ^{125}I-LR^{3}IGF-I was degraded more rapidly and to a greater extent than ^{125}I-IGF-I. In contrast, in wound fluid ^{125}I-LR^{3}IGF-I and ^{125}I-IGF-I were degraded to the same extent. These data suggest that degradation of IGF-I in plasma, or after uptake and release by cells is inversely related to the binding of IGF-I to plasma IGFBPs. However, the ability of IGF-I to bind IGFBPs does not afford it protection from degradation in wound fluid.

7.4 DISCUSSION

Several observations would suggest that IGF-I is able to leave the vascular compartment and distribute to various tissues (Hill *et al.*, 1997; Ballard *et al.*, 1991). It has also been demonstrated that IGFBPs have the ability to increase the circulating half-lives of IGFs (Thissen *et al.*, 1992; Cascieri *et al.*, 1988; Francis *et al.*, 1988b; Zapf *et al.*, 1986; Cohen and Nissley, 1976). Results such as these have led to the proposal that a potential role of the IGFBPs is to control rates of IGF transport from the circulation to extravascular sites. The preliminary trial in this chapter showed that systemically administered tracer amounts of radiolabelled IGF-I peptide is able to transfer from blood to wound fluid contained within wound chambers implanted in rats. At these sites, IGF-I appears in amounts that are readily measurable and comparable to other organs (see chapter 2), permitting meaningful analysis of data. To examine the effects of IGFBPs on this transfer, a major study was conducted which

Figure 7.11: Mean percent degradation of ^{125}I-IGF-I and ^{125}I-LR^{3}IGF-I in plasma and wound fluid. TCA-soluble radioactivity is expressed as a per centage of total radioactivity at each time point. Data points are means ± sems for 6 animals in each group. IGF-I (circles) and LR^{3}IGF-I (squares) from plasma is represented by closed symbols and from wound fluid by open symbols.


% Degradation of labelled IGF
(TCA-soluble cpm / total cpm)
100
90
80
70
60
50
40
30
20
10
0
0
60
120
180
240
Time (min)

investigated the effects of chamber implantation on plasma IGFBP profiles and IGF-I peptide pharmacokinetics in rats plus the transfer of both ^{125}I-IGF-I and ^{125}I-LR^{3}IGF-I from blood to wound fluid. Little variation occurred in the amount of peptide appearing in either chamber in the preliminary trial, so to reduce the variability arising from use of individual animals and decrease the overall number of animals used, it was decided that four chambers would be implanted per rat.

Other techniques for sampling sites of extracellular fluid were considered, but these methods have inherent problems. For example, microdialysis is likely to cause blood and cellular contamination of the extracellular fluid following insertion of the microdialysis probe, plus insertion gives rise to an inflammatory response which may cause extravasation of proteins (Wiig *et al.*, 1991). IGFs and IGFBPs are also known to be particularly adherent to the commercially available probes (Dr Anne Martin, Personal communication). Sampling of ascites fluid was attempted, but only very small quantities of fluid were recovered in normal rats. Use of the wound healing model of wound fluid accumulation provided the best opportunity to study the transfer of IGF-I peptides from blood to extracellular fluid, free of contamination of cells and blood.

A more rapid clearance of IGF-I peptides has been reported in rats under conditions where circulating levels of IGFBPs are reduced, including hypophysectomy (Cohen and Nissley, 1976) and protein restriction (Thissen *et al.*, 1992) or where the affinity of IGFBP-3 for IGFs is decreased such as in pregnancy (Davenport *et al.*, 1990). Surgery is also known to give rise to IGFBP protease activity, which can cause alterations in IGFBP levels (Cwyfan Hughes *et al.*, 1992). With this knowledge, I examined the IGFBP profiles in day 14 plasma of rats implanted with chambers compared to sham control animals, and determined that they were the same. This allowed me to exclude the possibility that implantation of chambers would affect the transfer of IGF peptides from blood to wound fluid, as no alterations in IGFBP plasma profiles were evident.

Pharmacokinetic studies of IGF-I variant molecules which display reduced association with IGFBPs other than LR^{3}IGF-I, have been shown to have increased clearance rates, including des (1-3) IGF-I (Ballard *et al.*, 1991) and (Glyn^3, Ala^4, Tyr-^{15}, Leu^{12})-IGF-I (Cascieri *et al.*, 1988). Previously I have also found that LR^{3}IGF-I is cleared more rapidly than IGF-I from plasma in rats (Bastian *et al.*, 1993). However, it was not known whether implantation of wound chambers in rats would alter the clearance of the plasma IGF-I peptides. The comparison between IGF-I and LR^{3}IGF-I clearance in both sham control and wound chamber implanted rats, qualitatively confirmed earlier studies that LR^{3}IGF-I was cleared more rapidly than IGF-I. However, the pharmacokinetic parameters differed, with the clearance rates for both peptides in both groups being somewhat lower than in the earlier investigation, a difference maybe associated with the use of larger animals in the present study. More importantly the clearance rates of IGF-I between the sham control and wound chamber implanted animals did not significantly differ. In contrast, the clearance rate of LR^{3}IGF-I in wound chamber implanted animals was significantly greater than clearance rates in sham control animals. Why this was not the case for IGF-I in wound chamber implanted rats could be attributed to the weaker affinity of LR^{3}IGF-I for IGFBPs, rendering it less confined to plasma.

A relatively limited number of studies have examined the transfer of plasma IGF-I into other extravascular fluids. Infusion of ^{125}I-labelled IGF-I into the arterial supply of a mammary gland in lactating goats resulted in the appearance of this peptide in milk (Prosser *et al.*, 1991). IGF-I injected intravenously into goats and sheep has also been recovered in lymph (Prosser *et al.*, 1992; Hodgkinson *et al.*, 1991). However, no study has examined the influence of IGFBPs on the transfer of IGF-I from blood to extracellular wound fluid sites in the whole animal. A key part of the present investigation involved a comparison between IGF-I and LR^{3}IGF-I with respect to translocation from plasma to extracellular fluid sites. Since LR^{3}IGF-I was known to bind weakly to rat plasma IGFBPs and that the clearance rate

of LR^{3}IGF-I is greater than IGF-I, it was hypothesized that more of the IGF-I analogue would be detected in extracellular fluid relative to IGF-I following intravenous infusion. It was found that radiolabelled IGF-I administered to rats implanted with wound chambers for 14 days before infusion of peptide, transferred from the blood to these sites of extracellular wound fluid accumulation. In parallel experiments with LR^{3}IGF-I as radiolabelled tracer, it was determined that when expressed as a percentage of intact radiolabelled IGF peptide infused, significantly more LR^{3}IGF-I specific radioactivity was recovered in wound fluid. These results confirm the prediction and support the conclusion that transfer of IGF-I peptides from blood to extracellular fluid is inversely related to the ability of the peptides to associate with binding proteins. The results of this comparison are also supported by the *in vitro* studies of chapter 5 which showed that the presence of IGFBPs in experimental medium inhibited transport of IGF-I across an endothelial cell monolayer model of the blood vessel wall, but did not affect transport of LR^{3}IGF-I. Lord *et al.*(1994) examined the uptake of IGF-I, LR^{3}IGF-I and des (1-3) IGF-I from blood by a segment of the small intestine in a non-recirculating, vascular perfused intestine model in the lamb. They found that there was no difference in the net transfer between the three IGF-I peptides from blood to intestine, and concluded that IGFBPs are not major determinants in this process. However, unlike the current study, the study by Lord *et al.* (1994) employed a one-pass perfusion through one specific organ. In contrast, the study in this chapter involved a complex whole animal model, which potentially is influenced by other tissues, plus the recirculation of intact and degraded forms of the infused labelled IGF-I peptides. Goats constantly infused with IGFBP-2 were shown to have increased IGFBP-2 plasma levels, and labelled IGF-I injected into these same animals distributed to lower molecular weight IGFBPs. Additionally, appearance of systemically administered ^{125}I-IGF-I in the mammary lymph was reported to be decreased up to four hours after ^{125}I-IGF-I injection (Prosser and Schwander, 1996). This observed decrease in the appearance of ^{125}I-IGF-I in lymph, while supporting the finding in the current study that

IGFBPs inhibit transfer of IGF-I from blood to extracellular wound fluid sites, is contrary to the "shuttle" hypothesis for IGFBP-2 suggested by Sara and Hall (1990). However, it may be possible that IGFBP-2 targets IGF-I to some specific extracellular sites, while inhibiting its transfer to others.

Western ligand blot analysis of the IGFBP profile in day 14 wound fluid confirm those of Robertson *et al.* (1996). The IGFBP band pattern of wound fluid and plasma were similar, except that the intesities of the band at approimately 31 kDa and the band at a molecular mass corresponding to IGFBP-3 were reduced in wound fluid compared to plasma. Similarly, studies investigating other extracellular fluid IGFBPs, including human skin interstitial fluid (Xu *et al.*, 1995) and human peritoneal dialysate (Kale *et al.*, 1996), have demonstrated reduced levels of IGFBP-3 relative to plasma levels.

Neutral gel chromatography profiles support the results of the Western ligand blot analyses. Administration of ^{125}I-IGF-I to rats implanted with wound chambers and *in vitro* incubations of ^{125}I-IGF-I with day 14 wound fluid followed by neutral gel filtration chromatography of wound fluid samples, revealed that ^{125}I-IGF-I was distributed to the lower molecular weight IGFBPs, however, a small amount was associated with the 150 kDa complex and some radioactivity was recovered in the 7.5 kDa region. Chromatograms of plasma from rats infused with ^{125}I-IGF-I also revealed three peaks of radioactivity, with the majority of ^{125}I-IGF-I being recovered in the 150 kDa complex. These results are supported by the studies of Robertson *et al.* (1996) who observed that *in vitro* incubations of ^{125}I-IGF-I with day 14 wound fluid and plasma resulted in three peaks of radioactivity at 150, 40-50 and 7.5 kDa. They reported that unlike plasma, less radioactivity in wound fluid was recovered in the 150 kDa region, while recovery was increased in the 40-50 kDa region. Similarly, in the current study LR^{3}IGF-I radioactivity in wound fluid was found associated with low molecular weight IGFBPs, however, it was not detected in the 150 kDa complex. Considerably more LR^{3}IGF-I than IGF-I existed in wound fluid as free peptide. LR^{3}IGF-I radioactivity in plasma

was distributed mainly to the low molecular weight IGFBPs. The source of IGFBPs in wound fluid is not clear. IGFBPs in wound fluid could be derived from plasma. However, with the formation of granulation tissue surrounding the wound chambers, fibroblasts or endothelial cells could possibly represent significant sources (Taylor and Alexander, 1993; Gartner *et al.*, 1992). Robertson *et al.* (1996) describe the presence of IGFBP-3 protease activity in wound fluid, which could possibly explain the observed decrease in IGFBP-3 levels in wound fluid relative to plasma. Existence of IGFBP-3 protease activity has also been reported for other extracellular fluids, including human skin interstitial fluid and peritoneal dialysate (Kale *et al.*, 1996; Xu *et al.*, 1995). Overall these results imply that IGF-I in extracellular fluids, including wound fluid, appears to occur in a form that is more readily available for interacting with specific IGF tissue receptors. The fact that more LR^{3}IGF-I exists as a free form in wound fluid relative to IGF-I may mean that it would have even greater accessibility to interact with tissue IGF receptors than IGF-I.

The accelerated clearance of LR^{3}IGF-I in rat plasma is in agreement with previous studies, and may be associated with increased rates of degradation, either in the blood or by tissues where it is degraded and returned to the circulation (Bastian *et al.*, 1993). No difference in the rate of degradation between IGF-I and LR^{3}IGF-I in wound fluid was observed. Thus, the accelerated clearance of LR^{3}IGF-I resulted in considerably more LR^{3}IGF-I accumulating in wound fluid.

In summary, I have shown that systemically administered IGF-I peptides are transferred from blood to extravascular wound fluid sites, where they exist in forms that may be more readily available to interact with tissue IGF receptors. These results show that the rate of transfer of LR^{3}IGF-I from blood to extracellular fluid is dramatically increased over that of IGF-I. This observation suggests that the ability to bind plasma IGFBPs inhibits translocation of IGF-I from blood to extracellular fluid. The decreased association of LR^{3}IGF-I with the 150 kDa complex, and the increased rate of delivery of this peptide from

the systemic circulation to wound sites relative to IGF-I suggests that this IGF-I analogue may be more effective in wound repair.

CHAPTER: 8 FINAL DISCUSSION

In recent years, research directed towards identification of drugs appropriate for the treatment of disease or malfunction of specific organs has extended to naturally occurring growth factors that enhance tissue growth, function and repair. IGF-I was first recognised to be the endocrine mediator of GH action. It was postulated that GH stimulated the liver to produce IGF-I, which in turn promoted growth of peripheral tissues (Salmon and Daughaday, 1957). More recently numerous observations have suggested that IGF-I produced by specific tissues acts locally by paracrine / autocrine mechanisms, giving rise to a review of the validity of the original somatomedin hypothesis (Holly and Wass, 1989). An alternative theory, known as the dual effector theory, has been proposed in which IGF-I has both paracrine / autocrine and endocrine functions (Green *et al.*, 1985). An important implication of the dual effector theory is that circulating IGF-I may merely represent an excess pool of paracrine / autocrine peptide which is simply being cleared from the circulation. However, evidence has been accumulating that suggests that IGF-I of endocrine origin may reach and act on many tissues.

Numerous data also suggest that the IGFBPs are able to prolong the half-life of IGF-I in the circulation, resulting in large stores of potentially releasable IGF-I activity. *In vitro*, IGFBPs modulate the actions of IGF-I, where they have both inhibitory and stimulatory effects. *In vivo*, analogues of IGF-I with weak affinities for IGFBPs, have been demonstrated to be more potent than IGF-I (see section 1.11).

For circulating IGF-I to exert its action on tissue growth, function and repair in an endocrine manner, it is necessary for it to leave the circulation by crossing the capillary barrier to reach extravascular target tissues. IGF-I alone or complexed to IGFBPs has been shown to cross endothelial cell monolayers and the capillary boundary to localise in specific tissues (Taylor *et al.*, 1993; Boes *et al.*, 1992; Bar *et al.*, 1990a; 1990c). However, how IGF-I crosses the endothelial barrier and a general role for IGFBPs in regulating this process is unknown.

The current investigation has examined the hypothesis that transfer of IGF-I from blood to extravascular sites is impeded by association with IGFBPs and as a result the aims of this thesis were to:

- determine whether and to what extent blood-borne IGF-I and LR^{3}IGF-I are distributed into peripheral tissues under normal and perturbed IGFBP conditions in rats
- develop and validate an *in vitro* model of an endothelial barrier
- utilising this model elucidate the pathway of IGF-I transfer across endothelial cells and compare this transport with that of IGF-I transport across epithelial cells
- examine the regulation by IGFBPs of IGF-I transport across endothelial cells
- compare the transfer of IGF-I and LR^{3}IGF-I from blood to extracellular fluid sites using an *in vivo* wound fluid accumulation model.

8.1 Determination of the distribution of IGF-I and LR3IGF-I outside the vascular space in rats under both IGFBP normal and IGFBP perturbed conditions

Earlier studies had shown that non-IGFBP associated IGF-I, whether due to reduced circulating IGFBP concentrations, an inability of IGFs to bind IGFBPs, or limited proteolysis of IGFBP-3 results in increased IGF-I clearance rates. It is reasonable to assume that non-IGFBP associated IGF-I peptides are less confined to the plasma compartment and as a result more readily available for transfer to tissues. However, the findings of chapter 2 indicated that this was not generally the case. In both pregnant and non-pregnant animals, IGF-I radioactivity was greater than LR^{3}IGF-I in caecum, brain, liver and heart, while LR^{3}IGF-I radioactivity was greater only in adrenals and ovaries. Since the levels of circulating intact IGFBP-3 and the differing abilities of the IGF-I peptides to bind IGFBPs appears to have no consistent bearing on the pattern of IGF-I peptide uptake by these specific tissues, it is possible that the distribution of IGF-I and LR^{3}IGF-I to these tissues is the same, but the

degradation rates of these two peptides and consequent return of the degradation products back to the circulation may differ in these organs. Other apparent reasons as to why the observed levels of blood-derived radiolabelled IGF-I and LR^{3}IGF-I peptides varied between different tissues exist. For example, the degree of vascularization of the organ, local production of IGFBPs and the IGF-I peptide translocation process may vary on a tissue specific basis.

The observation that less LR^{3}IGF-I radioactivity was observed in placenta, foetus and foetal plasma compared with IGF-I in pregnant rats raises the possibility that binding of IGF-I to IGFBPs enhances its uptake by the placenta and conceptus. Additionally, the fact that both IGF-I and LR^{3}IGF-I radioactivity was lower in small intestine, caecum, spleen and muscle of pregnant rats compared to non-pregnant controls, may imply that these peptides are diverted to other tissues such as placenta and mammary glands which display remarkable growth in pregnant animals.

The present study provides further evidence that circulating IGF-I peptides are distributed outside the vascular space into peripheral tissues which occurs on a tissue-specific basis. The data indicate that IGF-I and LR^{3}IGF-I are able to target different tissues for reasons which are unclear, but apparently unrelated to circulating IGFBP status, since the pattern was similar in both pregnant and non-pregnant rats. Determining these reasons will be difficult to demonstrate in the whole animal model. Although one study has examined transfer of IGF-I and LR^{3}IGF-I from blood to intestine with no apparent differences in this transport between the two peptides (Lord *et al.*, 1994), it may be necessary to isolate each specific tissue in question.

8.2 Development and validation of an *in vitro* model of an endothelial cell barrier

Numerous investigators have reported evidence of IGF-I receptors on endothelium (Bar *et al.*, 1988; King *et al.*, 1985; Jialal *et al.*, 1985; Bar and Boes, 1984), and more recently Bar and colleagues have demonstrated transcapillary permeability and subendothelial distribution of IGF-I (Boes *et al.*, 1992; Bar *et al.*, 1990a; 1990c). Yet no studies had defined the transport mechanism of IGF-I transfer across the vascular endothelial cell barrier.

In chapter 3 development and validation of an *in vitro* model of the blood vessel wall was described. This involved growing endothelial cells in a bi-chamber system which would allow examination of the transfer of IGF-I across an endothelial cell barrier. Although other researchers had previously utilised this model to study the transport of other proteins, cells and drugs across an endothelial barrier and to gain further insight into the barrier function of endothelial cells, this model had not been used in the laboratories in which research for this thesis had been undertaken.

HUVE cells were chosen because they represent a readily accessible source of primary human endothelial cells. Once the isolation technique and routine culture conditions, including the type of media, concentrations of FBS, growth factor supplement requirements, and appropriate substrata were established, these cells were positively identified as endothelial cells.

At the time of these studies, serum-free media for growth of human endothelial cells were appearing on the market. However, they did not support the growth of HUVE cells. Thus, a serum free medium of M199 containing bFGF and heparin was formulated and the model validated. TEM confirmed the presence of tight junctions and electrical resistance measurements produced by HUVE cells were within the range of other large vessel endothelial cells. In addition, these cells were able to transport albumin, which is known to be transported by other endothelial cells.

Having defined the HUVE cell monolayer model of a blood vessel wall in chapter 3, the experiments described in chapter 4 addressed two main issues. Firstly, they determined the IGF and IGFBP biology of HUVE cells and secondly, the pathway by which IGF-I crosses an endothelial cell barrier.

8.3 Elucidation of the transport pathway of IGF-I across vascular endothelium

In accord with data relating to other endothelial cells (Bar *et al.*, 1988; King *et al.*, 1985; Jialal *et al.*, 1985; Bar and Boes, 1984), it was demonstrated that HUVE cells posses both type I and type II IGF receptors. However, the studies presented in chapter 4 presented several lines of evidence suggesting that IGF-I crosses HUVE cell monolayers via a paracellular pathway. Inulin, a paracellular marker, is a molecule similar in size to IGF-I and is impermeable to cell membranes and crosses endothelial cells via intercellular junctions (Sonksen *et al.* 1971). Since no significant difference between the amount of [^{3}H]-inulin and ^{125}I-IGF-I transport was observed, it is likely that ^{125}I-IGF-I transport also occurs by a paracellular pathway. This was examined further by comparing the transport of ^{125}I-IGF-I across HUVE cells with that of ^{125}I-IGF-I in the presence of either excess unlabelled IGF-I or an antibody to the type I IGF receptor. Excess cold IGF-I was unable to compete with ^{125}I-IGF-I tracer transport. In addition, concentrations of αIR-3 antibody which were demonstrated to decrease binding of ^{125}I-IGF-I to HUVE cells, did not inhibit ^{125}I-IGF-I transport. These data suggest that IGF-I transport across HUVE cell monolayers is non-competitive and not mediated by the type I IGF receptor. Although these studies did not examine the possibility of IGF-I transport being mediated by either the type II IGF or insulin receptors, the low affinity of IGF-I for these receptors relative to the type I IGF receptor would make this an unlikely scenario.

In addition to the examination of IGF-I transport across endothelial cells, chapter 6 investigated IGF-I transport across epithelial cells since they too form physiological barriers to the passage of solutes, contain intercellular junctional complexes and in the case of kidney, lung and gut derived cell lines, form monolayers *in vitro*. Like endothelial cells, IGF-I transmigrated across Mv1Lu, MDBK and IEC6 epithelial cell lines in a paracellular manner. In the studies of chapter 4, the MDCK cell line was utilised as an alternative cell monolayer model, since they consistently occluded the passage of IGF-I. This in part could be due to the nature of these cells which produce much "tighter" intercellular junctions than HUVE cells, an observation supported by the fact that they produced significantly higher TER values. MDCK cells originate from the proximal tubule, which is not involved in protein transport, and occlusion of the passage of IGF-I by the MDCK cell line may be a reflection of these cells' *in vivo* role.

8.4 Effects of IGFBPs on IGF-I transport across endothelial cell monolayers

In vivo, endothelial cells are continually bathed by IGFBPs. HUVE cells were shown to secrete IGFBP-4 plus lesser amounts of IGFBP-2 and IGFBP-3 (Chapter 4). Studies by independent researchers have also reported that IGFBPs are secreted into the medium of other cultured endothelial cells (Taylor *et al.*, 1993; Yang *et al.*, 1993; Moser *et al.*, 1992 and Bar *et al.*, 1989 and 1987). Six known IGFBPs have been identified and cloned, although recent evidence points to the existence of other family members based on structural homology (Oh *et al.*, 1996). IGFBPs have been demonstrated to have both inhibitory and stimulatory effects on IGF-I action in various *in vitro* model systems (Baxter and Martin, 1989b). They are known to affect the half-life of IGF-I and the subendothelial distribution of IGF-I (Thissen *et al.*, 1992; Boes *et al.*, 1992; Bar *et al.*, 1990a, b, c; Davenport *et al.*, 1990; Zapf *et al.*, 1986; Cohen and Nissley, 1976,). A potential role for the low molecular weight IGFBPs has been

proposed in which they 'shuttle" the IGFs out of the vascular compartment (Sara and Hall, 1990). Binoux and Hossenlopp (1988) compared the IGF / IGFBP size distribution in serum and human lymph, and found that in contrast to serum, the smaller IGFBP species were more abundant than IGFBP-3 as a proportion of total IGFBPs. These data suggest that the 150 kDa complex is retained in plasma and does not cross the endothelial barrier, while the 50 kDa binary complexes are capable of leaving the circulation. However, other investigators have since shown the presence of the 150 kDa complex in wound fluid, peritoneal fluid, lymph and follicular fluid (Robertson *et al.*, 1996; Giudice *et al.*, 1994; Hodgkinson *et al.*, 1989b). It might be possible that free ALS crosses the capillary barrier and binds to IGFBP-3-IGF complexes in extracellular fluid to form the 150 kDa complex. Alternatively, the possibility exists that ALS is produced locally. In support of this, Dai and Baxter (1992) have reported the expression of mRNA for ALS in rat brain, kidney, heart, lung, spleen, muscle and liver. With respect to the two-pore model, it is likely that the small pores represent the intercellular junctional pathway and the large pores, transcellular channels formed by transient fusion of two or more plasmalemmal vesicles (Ogawa *et al.*, 1993; Bundgaard *et al.*, 1983). The present study did not resolve via which of these routes IGF-I crosses the endothelium, although the molecular weight would allow it to pass through either pathway. Regulation of the paracellular transport of macromolecules is currently thought to involve the intercellular junctions, the glycocalyx secreted by the endothelial cells, the size and charge of the macromolecule, and the endothelial extracellular matrix However, it is possible that the IGFBPs may also regulate the transport of IGF-I across the endothelial cell barrier. This issue was examined in chapter 5.

The effect of the presence of IGFBPs on IGF-I transport was examined in the HUVE cell model. In each case, the presence of an IGFBP significantly decreased the transport of ^{125}I-IGF-I across the HUVE cell monolayer. However, no single IGFBP was uniquely more or less effective than another. To determine the effect on IGF-I transport by IGFBPs with

reduced affinities for IGF-I, tIGFBP-5 was used as a specific control. This was the only IGFBP tested unable to inhibit transmigration. However, the presence of IGFBPs did not significantly effect the transport of LR^3IGF-I. These data are consistent with the proposed hypothesis that IGFBPs are able to inhibit transendothelial migration of IGF-I, unlike the data in chapter 2. The presence of a combination of IGFBP-3 and ALS resulted in a significant reduction in flux across the HUVE cell monolayer of IGF-I alone and IGF-I in the presence of IGFBP-3. These data support the notion that the 150 kDa complex retards IGF-I in the vascular compartment, where it may act as a reservoir of IGF-I which may be mobilised to target tissues.

Studies demonstrated that IGFBP-5 and an unidentified 24 kDa IGFBP are present in HUVE cell ECM (chapter 5). Importantly, they also indicated that HUVE cell ECM contributed to the endothelial permeability of IGF-I. How IGFBPs retard the transport of IGF-I across HUVE cell monolayers is not known, but possible mechanisms are multiple and were discussed in chapter 5. The glycocalyx represents a negative charge barrier to the passage of anionic molecules (Raviola and Butler, 1983). Binding of molecules such as polycations to neutralise this barrier may shed some light on the regulation of IGF-I passage across endothelial cell monolayers. IGFBPs have the capacity to bind to the glycocalyx and the ECM, although whether such binding alters their permeability properties is not known. Incubation of endothelial cell monolayers or endothelial cell derived ECM with IGFBPs, followed by examination of the clearance of permeability markers such as albumin and dextrans may elucidate the IGFBPs potential role in affecting glycocalyx and ECM permeability and as a result HUVE cell IGF-I permeability.

8.5 Transfer of IGF-I and LR^{3}IGF-I from blood to extracellular wound fluid sites.

Implantation of subcutaneous, stainless steel wound chambers in rats provided a source of readily accessible extracellular fluid free of blood contamination which permitted the examination of the transfer of IGF-I from the vascular compartment to extracellular wound fluid sites. A key part of this current investigation also involved a comparison between IGF-I and LR^{3}IGF-I in an attempt to examine the influence of IGFBPs on this translocation process. LR^{3}IGF-I is known to bind very weakly to rat IGFBPs and the clearance rate of LR^{3}IGF-I is greater than IGF-I in rats (Bastian *et al.*, 1993; Francis *et al.*, 1992). These findings led to the formulation of the hypothesis that more LR^{3}IGF-I would be detected in extracellular wound fluid relative to IGF-I. The studies in chapter 7 revealed that intravenously administered intact IGF-I and LR^{3}IGF-I is transferred from blood to sites of extracellular wound fluid accumulation, and that significantly more LR^{3}IGF-I specific radioactivity is recovered there than IGF-I specific radioactivity. These results support the idea that transfer of IGF-I peptides from blood to extracellular fluid is inversely related to the ability of the peptides to associate with IGFBPs. They are also supported by the *in vitro* data of chapter 5, which demonstrated that the presence of IGFBPs inhibited transport of IGF-I but not that of LR^{3}IGF-I across endothelial cell monolayers.

The finding that levels of IGFBP-3 in day 14 wound fluid are reduced relative to those levels in plasma confirmed earlier studies by Robertson *et al.*(1996), who also suggest that this reduction may be due to the presence of IGFBP-3 protease activity. In other forms of extracellular fluid, including skin interstitial fluid and peritoneal dialysate, a similar reduction in IGFBP-3 levels relative to plasma has been found along with the presence of IGFBP-3 protease (Kale *et al.*, 1996; Xu *et al.*, 1995). Examination of the distribution of IGF-I and LR^{3}IGF-I among IGFBPs in wound fluid indicated that compared to plasma, less IGF-I was associated with the 150 kDa complex, while more IGF-I radioactivity was recovered in the 40

-50 kDa region. The majority of LR^{3}IGF-I radioactivity in both plasma and wound fluid was found in the 40 -50 kDa peak, however, considerably more LR^{3}IGF-I than IGF-I in day 14 wound fluid existed as free peptide.

IGF-I is transported across the HUVE cell monolayer model via a paracellular pathway. The model presented here also suggests that IGFBPs inhibit this transport. In addition, in rats IGF-I peptides with weak affinities for rat IGFBPs transfer from blood to extracellular fluid to a greater extent than the parent IGF-I molecule. However, a number of other aspects require further validation. For example,

1) does IGF-I transport across HUVE cells occur via intercellular spaces or transcellular channels;

2) does IGF-I and LR^{3}IGF-I transport from blood to other extracellular fluid sites in specific tissues differ;

3) do alterations in plasma IGFBP levels or affinities of plasma IGFBPs for IGF-I affect transport of circulating IGF-I to extravascular fluid compartments and

4) what are the role of locally produced IGFBPs in determining the bioavailability of IGF-I peptides at extracellular sites.

If data from proposed questions such as these could be included into the current model, a more definitive model of IGF-I transport from blood to tissues would exist.

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