The Insulin-like Growth Factor Axis in the Lactating Rat and in the Immune System of the Sheep

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SUMMARY

The insulin-like growth factors (IGFs) are important in several areas of animal production, for example, IGF-1 mediates the growth-promoting effects of growth hormone (GH). GH has effects on a variety of tissues including bone, mammary gland and adipose tissue; GH effects on adipose tissue are direct but its effects on other tissues are thought to be mediated by IGF production. The aim of this study was the investigation of the IGF axis in two areas important to animal performance: lactation and the immune system.

In many species the mother has an increased susceptibility to parasitic and fungal infections during the pre- and post-partum period with detrimental effects on the welfare of both the dam and the neonates dependent on the milk. Although a role for the IGFs and growth hormone (GH) have been implicated in the immune system, few studies have used farm species. Here I have shown that the immune system of the sheep has all the elements of the IGF axis: type 1 IGF receptors, IGF-1, insulin-like growth factor binding protein (IGFBP), and IGFBP protease. These preliminary findings warrant further evaluation of the IGF axis in the sheep immune response and in particular its assessment during the periparturient period when immunocompetence is compromised.

Although the galactopoietic effects of GH in dairy cows is well established the mechanism involved is uncertain. Functional GH receptors have not been shown on mammary epithelial cells and the effects of GH have therefore been considered to be indirect via either nutrient partitioning or IGF-1 since the mammary gland does possess IGF receptors. The IGFs are potent mitogens for mammary cells *in vitro*, however the *in vitro* culture of mammary tissue is difficult, particularly from

lactating glands. There is limited information on the IGFBPs produced by the mammary gland, their regulation and role in mammary gland biology.

The studies in this thesis have used two models of rat lactation in which mammary involution has been induced by either litter removal, or by treatment with anti-rGH serum to ablate GH and bromocriptine to ablate prolactin (PRL). When involution was induced by litter removal the mammary gland expressed IGFBP-2, -4 and -5, and high levels of IGFBP-5 were detected in milk. IGF-1 can act as a survival factor for several cell types and we hypothesised that IGFBP-5 production was increased to abrogate the effects of IGF-1 since serum IGF-1 concentrations increased after litter removal. Serum IGF-1 concentrations did not however correlate with increased IGFBP expression.

Hormone ablation and litter removal induce different physiological states: litter withdrawal induces milk accumulation, whereas after hormone ablation the gland is empty of milk because the young continue to suckle. PRL treatment of animals whose litters had been removed decreased the levels of IGFBP-5 in milk despite milk accumulation. Sealing of half the dams teats showed that IGFBP-5 levels increased in sealed mammary glands compared with their unsealed contralateral counterparts. Previous studies have shown that after litter removal there is a decrease in total mammary DNA and an increase in DNA laddering indicative of apoptosis; similarly teat sealing can also induce apoptosis in the sealed gland in the absence of endocrine changes. These findings support a role for IGFBP-5 in the induction of apoptosis. In contrast, PRL and GH ablation did not induce IGFBP-5 expression although previous studies showed increased DNA laddering (although to a lesser extent than litter removal).

GH- and PRL-deficiency decreased serum IGF-1 concentrations but did not affect mammary IGFBP-5 expression. GH-deficient lactating rats have decreased serum IGF-1 concentrations, but serum IGFBP-3 levels were reduced only when both PRL and GH were ablated suggesting dual control of IGFBP-3 during lactation. This may have important implications for IGF transport since IGFBP-3 is the main carrier of IGF-1 in blood. PRL and GH may thus maintain mammary epithelial cell integrity by modulating the IGF-1 survival signal: in this model, during lactation GH induces IGF-1 production (possibly produced locally within the mammary gland), and GH and PRL stimulate IGFBP-3 concentrations in blood whilst PRL suppresses the expression of IGFBP-5. In contrast, during involution the withdrawal of PRL permits the production of IGFBP-5 which serves to abrogate the anti-apoptotic effects of IGF-1.

We therefore postulate that IGFBP-5 is an initiator of cell death in the involuting mammary gland: the direct demonstration of the survival effects of IGF-1, and a causal role for IGFBP-5 in involution are required to support this hypothesis.

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ABBREVIATIONS

ALS	Acid labile subunit
APR	Acute phase response
BSA	Bovine serum albumin
CHO	Chinese hamster ovary
Con A	Concanavalin A
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme linked immunoadsorbent assay
FAB-MS	Fast atom bombardment mass-spectroscopy
FCS	Foetal calf serum
FSH	Follicle stimulating hormone
GAG	Glycosaminoglycan
GH	Growth hormone
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid])
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
MBS	m-Maleimidobenzoyl-N-hydroxysuccinimide ester
MMP	Matrix metalloproteinase
MOPS	3-(N-Morpholino)propanesulphonic acid
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
PL	Placental lactogen
PMSF	Phenylmethylsulphonyl fluoride
PRL	Prolactin
SSC	Standard saline citrate
TBS	Tris buffered saline
TIMP	Tissue inhibitor of matrix metalloproteinase
Tris	Tris(Hydroxymethyl)aminomethane
TLCK	N-α-ρ-tosyl-l-lysine chloromethyl ketone
WLB	Western ligand blot

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DECLARATION

The work contained in this thesis was carried out by myself in the Hannah Research Institute (HRI) with the following exceptions: *in vivo* hormonal manipulations of rats and serum radioimmunoassays in Chapter 5 were carried out by Margaret Gardner (HRI); preparation of template DNA for probe synthesis for use in Northern analyses and preparation of some RNA samples in Chapter 5 were by MandyVallance (HRI); *in situ* hybridisation in Chapter 5 was carried out at the Wolfson Research Laboratories, University of Birmingham under supervision of Dr Ann Logan; Dr Su Chen, University of Warwick carried out FAB-MS analyses of synthetic peptides; conditioned medium from mouse mammosphere cultures were obtained from Dave Blatchard and Lynda Quarrie (HRI).

PUBLICATIONS

Data presented in this thesis has been published in part in the following:

Flint DJ, Tonner E, Beattie J & Panton D 1992 Investigation of the mechanism of action of growth hormone in stimulating lactation in the rat. *J Endocrinol* **134** 377-383.

Flint DJ, Tonner E, Beattie J & Gardner M 1994 Several insulin-like growth factor-I analogues and complexes of insulin-like growth factors-I and -II with insulin-like growth factor-binding protein-3 fail to mimic the effect of growth hormone upon lactation in the rat. *J Endocrinol* **140** 211-216.

Tonner E, Beattie J & Flint DJ 1995 Production of an insulin-like growth factor binding protein by the involuting rat mammary gland. *Intercellular Signalling in the Mammary Gland*, pp 103-104. Wilde CJ, Peaker M & Knight CH. New York: Plenum Press.

Tonner E, Beattie J & Flint DJ 1995 Production of insulin-like growth factor binding protein (IGFBP), IGFBP-3 protease, and expression of IGF-I receptors by cells of the sheep immune system. *Eur J Endocrinol* **132** 118-22.

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Travers MT, Barber MC, Tonner E, Quarrie L, Wilde CJ & Flint DJ 1996 The role of prolactin and growth hormone in the regulation of casein gene expression and mammary cell survival: relationships to milk synthesis and secretion. *Endocrinology* **137** 1530-1539.

CHAPTER 1: INTRODUCTION

1.1 Overview

The insulin-like growth factors (IGFs) were discovered while searching for mediators of the growth promoting effects of growth hormone (GH). Although the suppression of matrix protein synthesis in hypophysectomised rats can be restored by *in vivo* GH administration, GH could not stimulate sulphate uptake into hypophysectomised rats cartilage *in vitro*. Serum from normal or GH treated hypophysectomised rats, but not from hypophysectomised rats had stimulatory activity establishing the presence of an intermediatory substance termed 'sulphation factor' or later 'somatomedin' (Salmon & Daughaday 1958). Two other types of plasma factors were independently discovered, an insulin-like activity which was not supressible by antiserum to insulin (NSILA), and a potent stimulator of cell growth in culture termed multiplication-stimulating activity (MSA). Purification and characterisation identified two 7.5 kDa peptides responsible for these biological activities, which were subsequently designated IGF-1 and IGF-2. The demonstration of IGF-1 production by several GH target tissues challenged the somatomedin hypothesis and established that in addition to its endocrine role, IGF-1 may also act as a paracrine growth factor. Local administration of GH into cartilage could stimulate growth and this effect could be blocked by antibodies to IGF-1 (Schlector *et al.* 1986), however the growth rate was a fraction of that induced by systemic GH administration showing the involvement of both endocrine and paracrine IGF-1 in growth. It is now clear that in addition to their metabolic role the IGFs are pleiotropic growth factors which affect the mitogenesis and differentiation of a wide variety of cell types.

The majority of IGF in physiological fluids are bound to one of a series of binding proteins (IGFBPs) which can modulate IGF action. The IGFBPs are synthesised in a wide range of tissues and although their actions are complex it is proposed that IGFBPs have several major functions: to prolong the half-lives of the IGFs; regulate metabolic clearance of IGFs; to transport IGFs in plasma and control their exchange with the tissues; and modulate IGF effects at the local level. The IGFBPs have a major impact on IGF action and they form the main subject of this thesis.

A large body of literature exists on the IGF-IGFBPs and the following review covers IGF-IGFBP biology and discusses in more detail the IGF axis in lactation and in the immune system.

1.2 Insulin-like growth factors: genes and proteins

The IGFs are 7.5 kDa proteins which contain four domain structures, called B,C,A, and D, based on their homology with insulin. The IGFs from several species have been cloned and sequenced and show a high degree of conservation between species (Ward & Elliss 1992). Northern blotting demonstrated that the IGF-1 gene gives rise to heterogeneous mRNA transcripts, the major mRNA species in the rat being about 1, 1.7, and 7.5 kb (Daughaday and Rotwein 1989). The rat IGF-1 gene consists of 6 exons, exons 3 and 4 coding for the mature protein. Exons 1 and 2 code for alternative 5'-untranslated regions and N-terminal sequences, and transcription is initiated at two different promotors upstream of exons 1 and 2.

IGF-1 is made as a propeptide containing a C-terminal E domain which requires proteolytic cleavage to form the mature peptide. There are two precursor

forms IGF-1 Ea and IGF-1 Eb, which differ in their C-terminal sequence but result in identical mature proteins. IGF-1 Ea is derived from exon 4 spliced to exon 6 in the rat or splicing of exon 4 to 5 in humans. IGF-1 Eb is derived from exon 4 spliced to exons 5 and 6 in the rat, or exon 4 spliced to exon 6 in humans. The use of alternative polyadenylation signals in exon 6 provide further mRNA heterogeneity (Hall *et al.* 1992). Both precursor forms are represented in all species of mRNA and the significance of the transcript heterogeneity remains unclear.

Natural variants of IGF-1 have been described, such as the N-terminal truncated des 1-3 IGF-1 which has a restricted distribution and is found only in brain, platelet lysates and bovine colostrum (reviewed in Humbel 1990). Site-directed mutagenesis studies demonstrated that the N-terminal part of IGF-1 is a recognition site for IGFBPs since deletion of residues 1-3 or substitution of residues in this region drastically decreases affinity for the IGFBPs (Humbel 1990). Several mutants with reduced affinity for IGFBPs but reasonable affinities for type 1 IGF receptors, have been useful in the study of receptor binding and the biological effects of IGF in the absence of modulation by IGFBPs (Francis *et al.* 1992).

The rat IGF-2 gene also has 6 exons and is under complex transcriptional control. An important feature of IGF-2 expression is parental imprinting such that only the paternally derived allele is active (DeChiara *et al.* 1991). Exons 1-3 represent alternative 5'-untranslated regions which are transcribed under the control of promotors P1-P3, giving rise to the major transcripts which in rats are about 3.8, 4.6 and 3.6 kb (Daughaday & Rotwein 1989). IGF-2 is secreted as a single precursor form and the natural variants of IGF-2 are apparently derived from the same gene and formed by differential RNA splicing. Large forms of IGF-2 are found in human brain, serum, spinal fluid and probably represent partially processed precursor forms (reviewed in Humbel 1990).

1.3 IGF receptors and cell signalling

The type 1 IGF receptor, structurally closely related to the insulin receptor, is a disulphide linked heterotetramer consisting of two 130 kDa alpha subunits which contain the IGF binding site, and two 95-105 kDa transmembrane beta subunits which have intrinsic tyrosine kinase activity (Massague & Czech 1982). The insulin receptor binds insulin with high affinity and IGF-1 with much weaker affinity, and does not bind IGF-2. In contrast the type 1 IGF receptor binds IGF-1 with high affinity, IGF-2 with a slightly lower affinity which sometimes approaches that of IGF-1, and binds insulin with much lower affinity. IGF-1 levels are low in foetal life and increase in the adult, but type 1 IGF receptors are most abundant in foetal and newborn tissues leading to the suggestion that the abundant IGF-2 in the foetus acts through the type 1 IGF receptor (Bondy *et al.* 1990)

A third member of the insulin receptor family is the insulin related receptor (IRR) which shares the common structure including the tyrosine kinase domain, but does not bind insulin, IGF-1 or IGF-2 (Zhang & Roth 1992) and the ligand is unknown.

Hybrid receptors consisting of one type 1 IGF receptor $\alpha\beta$-half-receptor and one insulin receptor $\alpha\beta$-half-receptor have been demonstrated in some tissues and cell lines. This receptor binds IGF-1 and insulin with affinities similar to those of the type 1 IGF receptor, and IGF-1 is more effective in eliciting its autophosphorylation (Soos *et al.* 1990), thus implying that it may behave more like a type 1 IGF receptor than an insulin receptor. Human placenta has a high percentage of hybrid receptors (Soos & Siddle 1989), however it is unknown whether hybrid receptors can mediate responses to IGF-1 or insulin *in vivo*.

There have been reports of type 1 IGF receptors with affinities for ligands differing from those of the cloned receptor and only some of the data can be explained by hybrid receptors or differential splicing of mRNA. IGF stimulation of adult and foetal muscle from rat and human demonstrated the presence of two species of type 1 IGF receptor with beta subunits of 105 kDa and 95 kDa (Alexandrides *et al.* 1993). The 105 kDa type is preferentially expressed on foetal cells and is stimulated *in vitro* by low concentrations of insulin as well as IGF-1, which led to the suggestion that it may have a role in mediating the effects of insulin in foetal tissues. Insulin is growth stimulatory in the foetus, whereas in the adult it is mainly a metabolic hormone. However insulin receptors are in similar numbers in foetal and adult life so receptor distribution cannot account for the developmental differences. Antibodies to the alpha subunit (anti-IR-3) are selective for the 105 kDa containing receptor suggesting there is a difference in both α and β subunits of foetal and adult receptors. Recently splice variants of the mRNA for the 105 kDa receptor have been described which differ at 3 nucleotides (CAG) in the extracellular portion of the beta chain. Although these variants have equal affinity for IGF-1, the CAG minus version shows an increase in IGF-1 stimulated signalling activity and a 50% decrease in receptor internalisation. Thus the receptor variants have distinct signalling properties suggesting that the beta subunit may play a role in both signalling and internalisation (Condorelli *et al.* 1994).

The intrinsic tyrosine kinase activity of the type 1 IGF and insulin receptor is believed to mediate their actions by the phosphorylation of cellular substrates. Several substrates for the type 1 IGF and insulin receptor kinase have been identified

including the insulin receptor substrate-1 (IRS-1), and the subsequent stimulation of PIP_3 or Ras activation are common elements (reviewed in Jones & Clemmons 1995). How insulin and IGF-1 signalling diverge remains unclear and is the subject of many studies. For example chimaeric receptor studies in which the C-terminal domain of one receptor was fused to the rest of the heterologous receptor have shown that replacement of the insulin receptor C-terminus with the type 1 IGF receptor C-terminus markedly affects the responses to insulin, but in contrast replacement of the type 1 IGF receptor C-terminus with that of the insulin receptor had little effect on IGF-1 responses suggesting the type 1 IGF receptor mitogenic activity is not affected by the insulin receptor C-domain (Faria *et al.* 1994). Receptors with the extracellular domain of the insulin receptor and the intracellular IGF-1 domain are more effective than the native insulin receptor in delivering a mitogenic signal (Andersen *et al.* 1990).

The type 2 IGF/mannose-6-phosphate receptor is a single polypeptide chain of 250 kDa comprising a large extracellular domain containing distinct binding sites for IGF-2 and mannose-6-phosphate, a single transmembrane domain, and a short intracellular domain (MacDonald *et al.* 1988). A soluble form of the receptor (210 kDa) has been described in serum which differs from the membrane bound form at the cytoplasmic C-terminal portion and it has been proposed that it arises by cleavage of the membrane bound form (Clairmont & Czech 1991). Although IGF-2 binds the type 2 IGF/mannose-6-phosphate receptor with greater affinity than IGF-1 (insulin does not bind) the function of the type 2 IGF/mannose-6-phosphate receptor is the targeting of lysosomal enzymes to lysosomes and the mediation of IGF-1 uptake and degradation (Neilsen 1992). Most biological responses to IGF-2 are thought to be

mediated by the type 1 IGF receptor, and the role of the type 2 IGF/mannose-6-phosphate receptor in mediating IGF-1 or IGF-2 signalling remains to be elucidated.

1.4 IGF actions *in vitro*

1.4.1 Metabolic and mitogenic effects

The IGFs exert acute metabolic effects on protein and carbohydrate metabolism on most cell types via the widely expressed Type 1 IGF receptors. IGF-1 stimulates amino acid transport, glucose utilisation, lipid formation and protein synthesis though its effects vary depending on cell type. In skeletal muscle the major *in vitro* effect of IGF-1 is in the stimulation of glucose uptake, glycogen synthesis and glycolysis (Dimitriadis *et al.* 1992).

The ability of IGFs to stimulate DNA synthesis has been widely studied. Competence factors, such as PDGF and FGF induce quiescent cells to enter G_1 and IGF-1 functions as a progression factor in the cell cycle late in G_1, allowing the cell cycle to continue resulting in DNA synthesis and proliferation (Pardee 1989). Since PDGF increases the numbers of type 1 IGF receptors in fibroblasts (Clemmons & Shaw 1983) and receptor over- expression bypasses the need for PGDF, the function of PDGF as a competence factor may be to increase type 1 IGF receptor levels (Baserga & Rubin 1993). Likewise haemopoietic IL-3 dependent cells overexpressing type 1 IGF receptors become IL-3 independent in the presence of excess IGF-1 and the high frequency of IL-3 independence suggested that no other mutation was necessary (McCubrey *et al.* 1991). As might be expected from its role in cell cycle progression IGF induces mitogenesis in a wide variety of cell types (see Lowe 1991 for review).

Analogous with IGF effects on cell proliferation is its capacity to act as a survival or anti-apoptotic factor (Raff 1992). It has been suggested that apoptosis has an important role in blood cell production (Williams *et al.* 1990). IGF-1 abrogated apoptosis of cytokine (CSF) dependent cells deprived of cytokine (Rodriguez-Tarduchy *et al.* 1992), supporting a role for IGF-1 in inhibiting apoptosis and in controlling the regulation of blood cell formation.

1.4.2 Effects on differentiation and cell function

IGF-1 and IGF-2 can induce differentiation of several cell types including osteoblasts, osteoclasts, chondrocytes, neural cells and adipocytes (Sara & Hall 1990 (review)). IGFs also affect a wide range of cell functions for example: regulation of hormone secretion in several cell types such as ovarian cells where they act synergistically with FSH and oestrogen (Adashi *et al.* 1994); the stimulation of extracellular matrix component synthesis by endothelial cells (Lowe 1991); increased chemotactic migration in several types of epithelial and endothelial cells (reviewed in Jones & Clemmons 1995).

Thus the IGFs regulate the growth, differentiation and function of a wide range of cell types. IGF-1 can elict a motility response, a mitogenic response, or a metabolic response (which may be part of a mitogenic response), but it is unclear whether the intracellular signalling pathways are the same for the different responses. Intracellular events including some of the cell signalling, are likely to be shared by different signalling molecules so that the overall "signal" and subsequent response depends on the context of the IGF-1 signal. The integration of all the signals from the microenvironment such as hormones, growth factors, extracellular matrix

components, and also the cells' receptivity e.g. the distribution and abundance of receptor subtypes and the differentiation state of the cell, determine the outcome.

1.5 *In vivo* effects of IGF-1

The anabolic effects of GH are well established and IGF-1 can mediate both the growth promoting and anabolic effects of GH. Anabolic effects of IGF-1 on protein synthesis and nitrogen balance have been demonstrated using a variety of stimulated catabolic states including rats treated with dexamethasone (Tomas *et al.* 1992) and in dietary protein restriction (Thissen *et al.* 1991). In protein restricted animals carcass weight was not increased but the weights of gut, kidneys and spleen increased (Tomas *et al.* 1992; Thissen *et al.* 1991). However IGF-1 infusion in well fed rats does stimulate carcass weight suggesting there is IGF-1 resistance in protein restricted rats (Thissen *et al.* 1991).

Infusion of IGF-1 into GH-deficient mature rats stimulated body weight gain and longitudinal bone growth, but not as potently as GH (Lowe 1991). Whereas GH administration causes proportional organ growth, IGF-1 treatment results in greater increases in kidney, spleen and thymus weights in neonatally hypophysectomised rats (Glasscock *et al.* 1992). There is also some evidence that GH and IGF-1 effects may differ depending upon the route of administration since a growth response was seen when IGF-1 was infused but not when it was injected (Skottner *et al.* 1987).

IGF-1 infusion in hypophysectomised and protein restricted rats restores the depressed IGFBP-3 level to normal (Clemmons *et al.* 1989). Therefore the difference in the effects of GH and IGF-1 may be related to tissue delivery of IGF-1 since although IGF-1 may modulate IGFBP-3, GH is required for ALS production

(Gargosky *et al.* 1994) thus affecting ternary complex formation; alternatively GH may have IGF-1-independent effects on tissues. In contrast to the wealth of evidence comfirming IGF-1 as a mediator of GH action, GH administration to GH deficient rats in the presence of antisera to IGF-1 failed to influence the response to GH in terms of growth rate (Spencer *et al.* 1991).

IGF-1 administration to humans has comparable effects to those seen in rats. In normal subjects GH and IGF-1 have comparable anabolic and growth effects but opposite effects on glucose and fatty acid metabolism. The ability of IGF-1 to restore the nitrogen balance in calorie restricted subjects was comparable to that of GH but there was a hypoglycaemia at doses well tolerated by fed subjects (Clemmons *et al.* 1992a). However in calorie restricted subjects combined IGF-1\GH treatment was more potent than individual treatment in restoring nitrogen balance and IGFBP-3 levels (Kupfer *et al.* 1993). This is probably due to an increased proportion of IGF-1 in ternary complexes (because GH regulates ALS) which will increase its half life thus increasing its anabolic effects and protecting from hypoglycaemia. Combined IGF/GH therapy may therefore have clinical uses in critically ill patients with hypercatabolism (Bentham *et al.* 1993).

1.6 IGFBP Structure

Six structurally distinct IGFBPs have been purified, cloned and sequenced and the strong sequence homology suggests the genes are closely related, probably arising from successive gene duplication (Shimasaki & Ling 1991). The human and rat genes have 4 exons and IGFBP-3 has a fifth exon which codes for 3'-untranslated sequences. The promotor regions of some IGFBPs have been reported; human

IGFBP-1 and -3 genes contain TATA boxes recognised by the RNA polymerase II initiation complex, rat IGFBP-4 and -5 also have a TATA box but the initiation site is not defined, and IGFBP-2 and -6 have no TATA box.

A single IGFBP mRNA species has been described for most tissues, with a few exceptions such as larger hepatic forms of IGFBP-1 mRNA. The IGFBPs are synthesised as prepeptides and form mature protein of approximately 200-300 amino acids. The amino and carboxyl termini of the six IGFBPs have 60-70% homology and contain 18 cysteines whose alignment is conserved, IGFBP-6 being the exception as it lacks 2 of the homologous cysteines in the rat and 4 homologous cysteines in the human. The central region of the molecule is most divergent with only 30% homology, this region lacks cysteines with the exception of IGFBP-4 which has 2 additional cysteines in this region.

There are several differences between IGFBPs in their structure and post-translational modifications which are summarised in Table 1.1. The C-terminal end of IGFBP-1 and -2 contain an RGD sequence which could bind to integrin receptors. IGFBPs contain putative glycosaminoglycan (GAG)-binding consenus sequences (XBBXBX and XBBBXXBX where B is any basic amino acid and X is undefined) which may interact with the extracellular matrix. All IGFBPs in rat and human contained at least one GAG-binding sequence, with the exception of rat and human IGFBP-4 and human IGFBP-6; both rat and human IGFBP-5 contain three such sequences (Hodgkinson *et al.* 1994).

	IGFBP					
	1	**2**	**3**	**4**	**5**	**6**
RGD sequence	+	+	−	−	−	−
GAG binding	+	+	+	−	+	+
phosphorylation	+	−	+	−	+	−
glycosylation	−	−	N	N	O	O
preference for IGF-1 or IGF-2	≈	IGF-2	≈	≈	IGF-2	IGF-2

Table 1.1 Structural features of the IGFBPs. N is N-linked glycosylation; O is O-linked glycosylation.

IGFBP-3 and to a lesser extent IGFBP-4, are N-glycosylated (Martin & Baxter 1986; Ceda *et al.* 1991). IGFBP-1, -3, -5 and -6 have potential O-linked glycosylation sites but this type of glycosylation has been demonstrated for IGFBP-5 and -6 only (Martin *et al.* 1990). There is little information regarding the role of carbohydrates in IGFBP function. Although recombinant glycosylated and non-glycosylated IGFBP-3 have the same affinity for IGF-1 (Keifer *et al.* 1992), the carbohydrate moiety could be important for other properties such as their half life, stability or targeting. Serine phosphorylation have been demonstrated for IGFBP-1, -3, and -5 (Jones *et al.* 1993a; Mukku & Chu 1990; Jones *et al.* 1992) although IGFBP-1 phosphorylation is best studied. IGFBP-1 from decidual cells and amniotic fluid is mainly phosphorylated, but as pregnancy progresses the ratio of phophorylated:non-phosphorylated forms in amniotic fluid increases (Koistenen *et al.* 1993); phosphorylation of IGFBP-1 increases its affinity for IGF-1. Human IGFBP-1 is phosphorylated solely on serines and although phosphorylation occurs at 3 sites the phosphorylation of only one of these sites is associated with alterations of the affinity for IGF-1 (Jones *et al.* 1993a). IGFBP-1 appears to be phosphorylated only at the intracellular level since functional ectokinases on the surface of tranfected CHO cells do not phosphorylate secreted or exogenous IGFBP-1 (Jones *et al.* 1991). Unlike IGFBP-1, dephosphorylation of IGFBP-3 does not alter its affinity for IGF-1, though it did increase the formation of the ternary complex (Mukku *et al.* 1991). Thus IGFBPs derived from different sources may vary in their post translational modifications, but the functional significance of these differences is unclear.

The affinities of IGFBPs for IGF-1 and IGF-2 have been determined using IGFBPs from a variety of sources. In general IGF-1 and IGF-2 bound with roughly

similar affinities to all IGFBPs with the exception of IGFBP-6 which consistently shows markedly greater affinity for IGF-2 (Martin *et al.* 1990); slightly higher affinity for IGF-2 has also been shown for IGFBP-2 and -5 (Clemmons *et al.* 1992b).

The homology of the cysteine rich regions between the different IGFBPs suggested that the IGF binding site resides here, furthermore reducing agents abolish IGF binding suggesting that the 3-D structure is important. The IGF-binding capacity of IGFBP fragments and mutants has been used to determine the binding site. Truncation of IGFBP-2 at the N-terminus (Wang *et al.* 1988) or C-terminus (Zapf *et al.* 1988) reduced but did not abolish IGF-binding suggesting both termini can bind IGF. Likewise an N-terminal truncated IGFBP-1 bound IGF (Huhtala *et al.* 1986). However mutation at the C-terminus of IGFBP-1 destroyed IGF binding and showed ^{226}Cys is essential for IGF binding (Brinkman *et al.* 1991). A study using several structural analogues of IGF-1 indicate that recombinant IGFBP-1 and -2 have very similar affinities for the analogues, but IGFBP-3, -4 and -5 have some similarities but significant differences. For example, mutations in the A or B chain of IGF-1 decreased its affinity for IGFBP-1 or -2. In contrast, although A chain mutations had little effect on IGFBP-3 binding, B chain mutations reduced affinity by 50% (Clemmons *et al.* 1992b). These studies provide further support for differences between IGFBPs in their structural requirements for IGF binding.

1.7 IGFBP action at the cellular level

1.7.1 Introduction

The IGFBPs were originally identified as serum proteins which inhibited the

potentially hypoglycaemic effects of serum IGF concentrations and it was therefore assumed that they had an inhibitory function. In simple binding assays IGFBPs appeared to inhibit IGF action by blocking IGF binding to receptors, however the use of whole cell systems challenged this view. It is now apparent that IGFBPs may also enhance IGF action, and in addition IGFBPs can have direct cellular effects which are IGF independent. The biological actions of IGFBPs are complex and may depend on their posttranslational modifications, ability to bind cell surfaces, or the presence of proteases which may alter their affinity for growth factors; these parameters vary between IGFBPs therefore the biological actions of each IGFBP will be considered separately.

1.7.2 IGFBP-1

IGFBP-1 in molar excess of IGF concentrations has a predominantly inhibitory action on [^{3}H]thymidine uptake in many cell types such as FRTL-5 thyroid cells (Frauman *et al.* 1989). In general, studies where inhibition has been seen have used serum free conditions, whereas there are several reports of enhancing effects in the presence of serum or plasma.

IGFBP-1 can enhance IGF-1 stimulated DNA synthesis in porcine aortic smooth muscle cells, but only in the presence of a low concentration of platelet poor plasma (Elgin *et al.* 1987). Furthermore, the potentiating capacity of IGFBP-1 was reduced when IGF-1 mutants with low binding to either IGFBP-1 or the type 1 IGF receptor-1 were used suggesting that binding to both IGF-1 and its receptor are necessary for potentiation (Clemmons *et al.* 1990a).

IGFBP-1 exists in differentially phosphorylated forms, which differ in their biological effects. Only the cell-adherent form of IGFBP-1 isolated from amniotic fluid could potentiate IGF-1 action on smooth muscle cells (Busby *et al.* 1988). The authors subsequently demonstrated that the adherent form was non-phosphorylated and this had a lower affinity for IGF-1 (Jones *et al.* 1991). However conflicting evidence comes from a separate study in which IGF-1 mediated [^{3}H]thymidine uptake into fibroblasts was potentiated by IGFBP-1 irrespective of the phosphorylation state of IGFBP-1 (Koistenen *et al.* 1993).

The presence of an RGD sequence in IGFBP-1 suggested that it may adhere to cells through integrin receptors, and it has been recently demonstrated that IGFBP-1 binds the $\alpha5\beta1$-integrin receptor (Jones *et al.* 1993b). The functional significance of this was shown using CHO cells expressing abundant $\alpha5\beta1$-integrin receptors. CHO cells expressing IGFBP-1 show enhanced migration which is abrogated by mutation of IGFBP-1 at the RGD sequence. This effect of IGFBP-1 was IGF-1 and IGF-2 independent but this does not preclude the involvement of IGFBP-1 cell adherance to integrin receptors in the potentiation of IGF action in other cell types.

1.7.3 IGFBP-2

Purified IGFBP-2 inhibited [^{3}H]thymidine uptake in rat astroglial cells (Knauer & Smith 1980) and foetal rat calvaria (Feyen *et al.* 1991). IGFBP-2 inhibits IGF binding to MDBK cells but des-(1-3)IGF stimulates DNA synthesis in these cells more potently than native IGF-2 suggesting that at least under the conditions used in the system, IGFBP is exerting its inhibitory action via IGF binding (Ross *et al.*

1989).

Although in most cases IGFBP-2 appears to inhibit IGF action, IGFBP-2 can be a weak potentiator of IGF action. In the presence of platelet poor plasma, IGFBP-2 potentiated the response of porcine smooth muscle cells to IGF-1 although less potently than IGFBP-1. In contrast, in serum free medium IGFBP-2 was a potent inhibitor of IGF-1 stimulated DNA synthesis (Bourner *et al.* 1992).

1.7.4 IGFBP-3

IGFBP-3 can inhibit IGF-1 action in several cell types. IGFBP-3 inhibits DNA synthesis in human skin fibroblasts when co-incubated with IGF-1 in a molar excess (DeMellow & Baxter 1988). Studies using transfected cell lines have suggested endogenously produced IGFBP-3 is inhibitory to cell growth and may have IGF-independent effects (Cohen *et al.* 1993). Hs578T breast cancer cell monolayer growth is inhibited by IGFBP-3 in the absence of IGF-1 administration (Oh *et al.* 1993). Although IGFs alone have no effect on monolayer growth, they can block IGFBP-3 growth inhibition. Subsequently membrane proteins of about 20, 26 and 50 kDa which specifically bind IGFBP-3 were demonstrated on these cells but the nature of these proteins is unknown (Oh *et al.* 1993).

Many studies have established the ability of IGFBP-3 to enhance IGF action. DeMellow & Baxter (1988) found that pre-incubation of IGFBP-3 with dermal fibroblasts followed by its removal, could potentiate IGF-1 action on DNA synthesis. Similarly IGFBP-3 only enhanced IGF action on bovine fibroblasts if previously incubated with cells (Conover *et al.* 1992). During incubation IGFBP-3 becomes associated with the cell surface and undergoes processing to lower molecular weight

forms; these surface bound forms had 10-fold lower affinity for IGF-1 compared with the intact IGFBP-3 in the solution phase. There is no evidence that treatment with IGFBP-3 increases the number of type 1 receptors or their affinity for IGF-1. However, the use of IGF analogues with low affinity for IGFBPs but which still bind type 1 IGF receptors, demonstrate that the ability of IGFBP-3 to enhance IGF-1 action may also depend upon altered reactivity of type 1 IGF-1 receptors.

These studies indicate that cell surface association is required for potentiation of IGF action but the mode of binding is unknown. The C-terminal end of IGFBP-3 has abundant basic amino acids which may interact with glycosaminoglycans on the cell surface or in the extracellular matrix. Heparin disrupts such non covalant interactions and increased the amount of IGFBP-3 in the medium of cultured fibroblasts (Martin & Baxter 1993).

1.7.5 IGFBP-4

In contrast with other IGFBPs, in most studies IGFBP-4 appears to inhibit rather than enhance IGF-1 action. For example IGFBP-4 isolated from osteosarcoma cells showed a dose dependent inhibition of IGF action on cartilage (Mohan *et al.* 1989). IGF-2 treatment of bone cell cultures increased IGFBP-4 proteolytic activity in the conditioned medium (Kanzaki *et al.* 1994). This effect did not require the presence of cells and because analogues with low affinity for IGFBPs were ineffective in enhancing IGFBP-4 proteolysis, IGF binding to IGFBP is important rather than binding to receptors. IGFBP-4 proteolysis is enhanced by IGF-1 in decidual cells, a process which is IGF-1 receptor independent (Myers *et al.* 1993). Similar effects are seen with fibroblasts, furthermore only intact IGFBP-4 could inhibit IGF-1 stimulated

[^{3}H]aminoisobutyric acid uptake in fibroblasts whereas proteolysed IGFBP-4 had no effect (Conover *et al* 1993). Since proteolytic cleavage of IGFBP-4 virtually abolishes its binding capacity for IGF, proteolysis may be a mechanism for releasing cells from this inhibition.

1.7.6 IGFBP-5

A molar excess of recombinant IGFBP-5 inhibited IGF-1-stimulated DNA and glycogen synthesis in human osteosarcoma cells (Keifer *et al.* 1992). An inhibitory function of IGFBP-5 is further suggested by its ability to inhibit steroidogenesis in granulosa cells stimulated with IGF-1 (Ling *et al.* 1993).

A characteristic of IGFBP-5 is its ability to avidly bind fibroblast ECM (Jones *et al.* 1993a). When incubated with the ECM IGFBP-5 potentiated IGF-1 action whereas IGFBP-5 added to the culture medium was proteolytically cleaved to a 21 kDa fragment which did not potentiate IGF-1 action. When attached to the ECM the affinity of IGFBP-5 for IGF-1 was lowered 7-fold suggesting that IGF-1 would be more available to receptors. This lowering of affinity and protection from proteolytic cleavage may be important in the potentiating capacity of ECM bound IGFBP-5.

A 23 kDa truncated form of IGFBP-5 derived from osteosarcoma cells, enhanced the mitogenic effect of IGF-1 on osteoblasts (Andress & Birmbaum 1992). This fragment bound the cell surface and had a greatly reduced affinity for IGF-1 compared with intact IGFBP-5, suggesting that proteolysis is associated with its enhancing action. In addition this fragment had some mitogenic capacity which was IGF-independent (Andress *et al.* 1993). Thus the lowering of IGF binding affinity, cell surface association, and proteolytic cleavage may be important in regulating the

potentiating action of IGFBP-5. IGF-2 treatment of bone cell cultures decreased IGFBP-5 proteolytic activity in the conditioned medium by a process requiring the presence of cells (Kanzaki *et al.* 1994).

1.7.7 IGFBP-6

There are very few reports of the *in vitro* effects of IGFBP-6 although a recent paper suggests it may have an antigonadotropin effect in the ovary (Rohan *et al.* 1993).

1.7.8 Summary

The functions of the IGFBPs at the local level are to enable site directed (tissue, cell type) localisation of IGF and modulation of IGF action. The molecular mechanisms of these IGFBP action are complex. Enhancing effects on IGF action have been demonstrated for IGFBP-1, -2, -3 and -5, although the mechanism involved differs between IGFBPs. IGFBPs possess GAG binding sequences which could interact with glycosaminoglycans on the cell surface or in the extracellular matrix (Hodgkinson *et al.* 1994). Thus the effects of IGFBPs involve complex interactions which are incompletely understood and caution is therefore required in interpreting changes in IGFBP levels in cell culture since these may result from alterations in synthesis rate, in post translational modifications of IGFBP (e.g. phosphorylation) altering functional status, or in the distribution between the cell surface and fluid phase. There are few *in vivo* studies using exogenously administered IGFBPs and it is unclear whether their overall effects *in vivo* are to inhibit or enhance IGF-1 action.

1.8 IGFBP regulation of IGF bioavailability *in vivo*

Although the relative role of endocrine and locally produced IGFs are unclear, circulating IGF provides a pool of IGF which can be exchanged with the tissues and IGFBPs can regulate this exchange. Most of the IGF in serum is bound to IGFBP-3 which along with an 88 kDa acid labile subunit (ALS) forms a ternary complex of 150 kDa (Baxter *et al.* 1989). Lesser amounts of IGF are bound in a 40 kDa complex with IGFBP-1, -2, -4, and -6 which contain most of the unsaturated IGF sites (Guler *et al.* 1989). IGFBP-5 and -6 are in very low amounts in serum and IGFBP-5 has not been detected in these complexes.

IGFBP increases the $t_{1/2}$ of IGF from 10 minutes for free IGF, to 30 minutes when in the 40 kDa complex, and about 15 hours when in a ternary complex; the IGF-1 analogue des(1-3)IGF-1, which has a low affinity for IGFBPs, is rapidly cleared from the circulation (Ballard *et al.* 1991). The acid labile subunit is in molar excess over the other components of the ternary complex and although in normal serum it does not alter the association constant of IGFBP-3 for IGF, it stabilises the IGF into a complex too large to cross the endothelial barrier (Martin & Baxter 1992). The ability of exogenous human IGFBP-3 to form ternary complexes in rats has been used to assess the amount of bioavailable IGF. Human IGFBP-3 rapidly formed a ternary complex which persisted in the circulation for hours, suggesting that there is a larger pool of free IGF than previously thought from the steady state free levels (Lewitt *et al.* 1993). In another study where human recombinant nonglycosylated IGFBP-3 was administered to rats, IGFBP-3 was initially cleared rapidly, but a portion which formed ternary complexes was stable for a few hours. IGFBP-3 which left the circulation was distributed to the tissues suggesting that

IGFBP-3 not associated with acid labile subunit rapidly leaves the vasculature (Arany *et al.* 1993). The initial high clearance rate contrasts with the studies of Lewitt *et al.* (1993) where complexes were formed rapidly, but the two studies used glycosylated or non-glycosylated IGFBP-3 respectively and, as suggested previously, glycosylated IGFBP-3 may equilbrate more rapidly in rat blood than non-glycosylated protein (Sommer *et al.* 1993).

Using a rat heart perfusion model IGFBPs -1 to -4 and IGF-1 were shown to cross the capillary boundary. IGFBP-1, -2, -3, and IGF-1 localised to muscle, but both glycosylated and non-glycosylated IGFBP-4 localised to connective tissue (Boes *et al.* 1992). When IGFBP-4 was administered with IGF-1 it localised to muscle like free IGF-1, suggesting that IGF-1 determines the distribution (Bar *et al.* 1990a). Administration of insulin along with the IGFBPs, did not change the pattern of localisation of the IGFBPs, but did have differential effects on transcapillary efflux of IGFBPs. Movement of IGFBP-4 was decreased, IGFBP-1 efflux increased and that of IGFBP-2 was unchanged (Bar *et al.* 1990b). The *in vivo* administration of [^{125}I]-IGFBP-1 and -2 to rats showed IGFBP $t_{1/2}$ was greater than that of free IGF-1 but less than that of ternary complex, suggesting these IGFBPs rapidly equilibrate with the extravascular compartment although a portion is stable with a $t_{1/2}$ of 2 hours (Young *et al.* 1992).

The mechanism of transport of IGFs and IGFBPs out of the vasculature is not clear. Bovine vascular endothelial cells possess IGF-1 receptors (Bar & Boes 1994) and there is evidence that endothelium itself may directly uptake and release intact IGF-1 (Bar *et al.* 1986). Circulating IGFBPs may attach to endothelial cells via their RGD sequences (IGFBP-1 and -2), or their GAG-binding regions (Hodgkinson *et al.*

1994), but the mode of translocation is unclear. Likewise the mechanistic basis for the selectivity of localisation or insulin effects on IGFBP translocation in the studies of Bar *et al.* 1990b has not been explained. This may involve the differential expression of extracellular matrix components between tissues (perhaps altered by insulin), and the differences in ECM binding between IGFBPs.

Thus the functions of circulating IGFBPs are: to prolong IGFs half-lives; regulate IGFs metabolic clearance; to transport IGFs in plasma and control their exchange with the tissues. During several pathophysiological conditions such as protein restriction (Clemmons *et al.* 1989) the amount of IGF bound in ternary:low molecular weight complexes decreases, however how this would alter IGF biological activity has not been determined.

1.9 Proteolysis of IGFBPs

IGFBP-3 was substantially reduced in the serum of pregnant women (Hossenlop *et al.* 1990, Giudice *et al.* 1990, rats (Davenport *et al.* 1990), and mice (Fielder *et al.* 1990) when analysed by Western ligand blotting (WLB). However since other studies (Baxter & Martin 1986) showed elevated levels of IGFBP-3 by radioimmunoassay it was postulated that IGFBP-3 protease activity in pregnancy serum altered IGFBP-3 affinity for IGFs rendering the IGFBP-3 undetectable by WLB (Hossenlopp *et al.* 1990; Giudice *et al.* 1990). This protease activity was demonstrated by the ability of serum from pregnant dams to degrade radiolabelled IGFBP-3 (Lamson *et al* 1991) or IGFBP-3 in normal serum. This phenomenon was subsequently shown in other catabolic states, such as after elective surgery (Davenport *et al.* 1992a), severe illness (Davies *et al.* 1991), and diabetes (Bang *et al.* 1994).

It has been postulated that the proteolytic cleavage of IGFBP-3 in pregnancy results in decreased affinity for IGF-1 and the increased dissociation kinetics (Lassarre & Binoux 1994) makes IGF more available. However the physiological significance of proteolysis is difficult to demonstrate although serum from pregnant women had an enhanced ability to stimulate DNA synthesis in chick fibroblasts cells suggesting increased IGF availability in pregnancy serum (Blat *et al* 1994).

IGFBP-3 from pregnant woman shows little impairment in ternary complex formation (Baxter *et al.* 1993) and IGFBP-3 still circulates in an 140 kDa complex (Suikkari & Baxter 1992) although there is an additional complex of around 110 kDa. IGFBP-3 from human pregnancy serum has a normal molecular weight of about 50 kDa after acid gel chromatography but several other alterations are apparent. Under the harsher *in vitro* conditions of SDS-PAGE, proteolysed IGFBP-3 has an apparent molecular weight of about 30 kDa which contrasts with 40-45 kDa shown for intact IGFBP-3 (Baxter *et al.* 1993). In addition reverse phase chromatography inactivated IGFBP-3 from pregnant women but not that of the non-pregnant state (Baxter *et al.* 1993). IGFBP-3 from pregnancy serum shows impaired solution phase binding to radioiodinated IGF (Hossenlopp *et al.* 1990; Suikkari & Baxter 1990). Proteolysed IGFBP-3 could not form ternary complexes in the presence of [Tyr^{60}]IGF-1 or and IGF-1 analogue altered in the A domain, though it could form complexes with native IGF-1 (Baxter *et al* 1993). How pregnancy IGFBP-3 can form 140 kDa complexes when IGF binding is apparently impaired is an anomaly since ALS only binds IGFBP-3 (at least *in vitro*) when the IGF binding site is occupied (Baxter *et al* 1989). The use of monoiodinated tracers has further clarified the nature of the IGFBP-3 alteration during pregnancy. Using recombinant

IGF-1 analogues it was shown that the amino acid substitution at Tyr^{60} and Tyr^{24}, but not Tyr^{31}, resulted in poor binding of binary complex to ALS (Baxter *et al.* 1993). Proteolysed IGFBP-3 has about a 10-fold lower affinity for Ser^{24} and Leu^{60} IGF-1 than for normal IGF-1 or Ala^{31}IGF-1. Thus the specificity of IGFBP-3 for IGF-1 is altered around the Tyr^{60} or Tyr^{24} of IGF-1, and the affinity of IGF/IGFBP-3 interaction is increased by acid labile subunit binding thus enabling the formation of ternary complexes with normal affinity (Baxter & Skriver 1993).

There is evidence for limited proteolysis of IGFBP-3 occurring in the normal state outside the bloodstream. Non-pregnant serum has low levels of proteolysis (Gargosky *et al* 1992) and Lalou & Binoux (1993) showed that proteolysis is higher in the lymph than serum suggesting that the site of proteolysis is the tissues, perhaps occurring at the cell surface or interstitium rather than in transit across capillaries. Normal rat serum contains IGFBP-3 proteolytic activity which is absent in the hypophysectomised rat. Proteolysis could be restored by the administration of GH but not IGF-1, suggesting that GH can modulate IGF action indirectly by altering IGFBP-3 (Rutishauser et al. 1993). Although proteolysis of IGFBP-3 is the best studied, early WLB studies of pregnancy serum detected a reduction in the intensity of IGFBP-4, and more recently IGFBP-4 and -5 protease activity has been demonstrated in human pregnancy serum (Claussen *et al.* 1994). IGFBP-2 can be proteolytically cleaved in the plasma of newborn animals (McCusker *et al.* 1991).

Proteolysis of IGFBPs has also been demonstrated in cell culture medium. Conditioned medium from bone cultures contains a protease of between 67 and 169 kDa by gel filtration, which degrades IGFBP-4 and -5 (Kanzaki *et al.* 1994). A calcium-dependent protease which degraded IGFBP-5 but did not affect IGFBP-1 to

-4, has been purified from human fibroblast conditioned medium (Nam *et al.* 1993). Fibroblast conditioned medium may contain proteases for IGFBP-4 and -5 (Conover *et al* 1993; Fowlkes & Freemark 1992). Proteolytic cleavage of IGFBP-2 and -4 usually results in the abolition of its IGF binding capacity; this contrasts with proteolysis of IGFBP-3 and -5 where proteolytic fragments tend to retain some IGF binding capacity (reviewed in Jones & Clemmons 1995). The physiological importance of the proteolytic activity in conditioned medium is unclear but their presence may alter the biological activity of the IGFBP in the assay system used.

Proteolysis plays an important role in regulating many physiological processes such as the activation of growth factors and zymogens. Physiological fluids therefore contain many extracellular proteases, many of which can cleave IGFBPs *in vitro*. Most proteolytic modifications of IGFBPs are due to metal dependent serine proteases, however comparison of the proteolytic activity of proteases from different sources established that serum IGFBP-3 proteolytic activity is similar during pregnancy and critical illness but differs from that of common proteases like plasmin (Frost *et al* 1993).

It is difficult to identify the protease responsible for the effects seen since many extracellular proteases like plasmin can proteolyse IGFBP-3 *in vitro* but *in vivo* they would be in an inactive form or blocked by the presence of inhibitors. Little is known about the regulation of the IGFBP-protease activities and the physiological role of proteolytic cleavage of IGFBPs *in vivo* remains to be demonstrated.

1.10 Regulation of IGF and IGFBP synthesis

1.10.1 Developmental regulation

IGF-1 and IGF-2 are present in almost all foetal tissue although their abundance varies between tissues (Han *et al.* 1988). In the rat, IGF-1 mRNA levels are lower than those of IGF-2 in foetal liver, kidney and heart, and these rise postnatally, whereas expression of IGF-1 in foetal lung, heart and stomach is higher than those found postnatally (Adamo *et al.* 1989). The abundant IGF-2 levels in the foetal rat decrease postnatally (except in the brain), however in other species such as man and ruminants there are significant IGF-2 levels in the adult. The high IGF-2 levels in the foetal and embryonic tissues suggest a role for IGF-2 in foetal growth. Studies of IGF-1 and -2 gene knockout mice show that while deletion of either gene reduced birth weight to 60%, postnatal growth of IGF-2 knockout mice was normal but IGF-1 knockouts had a reduced growth rate, extreme muscle hypoplasia and most died before adulthood. The growth retardation effect of IGF-2 deletion is associated only with failure of early embryonic growth, whereas IGF-1 deletion causes progressive growth failure, suggesting that growth effects of IGF-2 are dominant in the embryo, but IGF-1 is dominant in late gestation and in the perinatal period (Baker *et al.* 1993).

IGF-1 levels in the foetus are largely independent of GH but are under nutritional control. Maternal starvation leads to a rapid fall in foetal plasma IGF-1 in sheep (Bassett *et al.* 1990) and in rats (Bernstein *et al.* 1991). In contrast only extreme placental failure or undernutrition alters IGF-2 levels in foetal sheep, suggesting that IGF-2 may have a constitutive role in foetal growth but IGF-1 is acutely modulated by nutrition (Bassett *et al.* 1990).

The IGFBP profile also differs with developmental stage. Foetal serum IGFBP-3 levels are comparatively low and IGFBP-1 and-2 levels comparatively high. However species differences exist as there is little IGFBP-3 in foetal rat serum but it is easily detectable in foetal sheep serum (Osborn *et al.* 1992) although less abundantly than postnatally. IGFBP-1 and -2 are both expressed in a variety of foetal tissues; the expression of both is high in the liver but IGFBP-2 mRNA is abundant in foetal brain, kidney and stomach (Ooi *et al.* 1990). Serum and hepatic mRNA levels of IGFBP-1 and -2 decrease postnatally in rat, although IGFBP-2 is the major IGFBP in neonatal rat serum. IGFBP-2 levels progressively decrease and IGFBP-3 levels gradually increase, becoming detectable at about day 10 (Glasscock *et al.* 1990).

1.10.2 Hormonal regulation

It is well established that GH has major effects on IGF-1 levels (Daughaday & Rotwein 1989). In neonatal rats growth is under partial pituitary control and becomes pituitary dependent after a few weeks when IGF-1 and IGFBP-3 levels rise and IGFBP-2 decreases (Cooke & Nicoll 1983; Yang *et al.* 1989). In neonatally hypophysectomised rats IGF-1 and IGF-2 serum levels decrease, IGFBP-2 increased after an initial drop, and IGFBP-3 became detectable at day 20 at only 10% of normal levels (Glasscock *et al.* 1990). This reciprocal relationship of IGFBP-2 and -3 was thought to be coordinately regulated by GH, however when neonatal rats were treated with anti-GH serum in the first few weeks of life, their subsequent IGFBP-3 levels were much reduced compared with controls but immunoreactive IGFBP-2 levels still declined as usual (Pallmer *et al.* 1994).

GH is the major hormonal influence on serum IGF-1, IGFBP-3 and ALS in adults. Administration of GH or IGF-1 to GH-deficient rats demonstrated that while GH directly regulates serum ALS and IGF-1, IGFBP-3 is primarily regulated by IGF-1 (Gargosky *et al.* 1994). Although IGF-1 and IGFBP-3 mRNA are coexpressed in a large number of tissues, mRNA levels are not always coordinately regulated (Albiston & Herington 1992).

Serum IGFBP-1 levels are inversely proportional to insulin levels (Brismar & Hall 1993), and insulin is thought to be a major regulator of serum IGFBP-1, though there may also be a role for intracellular glucose (Lewitt & Baxter 1991a). Insulin treatment of streptozotocin diabetic rats could restore normal hepatic IGF-1 and IGFBP-1 gene transcription (Pao *et al.* 1992). Insulin also increases IGF-1 mRNA in hepatocytes in culture suggesting its effects on transcription are direct (Johnson *et al.* 1989).

In contrast IGFBP-1 is increased in insulin-induced hypoglycaemia and it is now apparent that the glucose counter regulatory hormones regulate IGFBP-1 (Lewitt & Baxter 1992). Glucocorticoids and glucagon increase IGFBP-1 mRNA in cultured rat hepatocytes (Kachra *et al.* 1994). Although insulin suppresses IGFBP-1 expression in rat H4IIE hepatoma cells and glucocorticoids increase it, insulin effects are dominant (Unterman *et al.* 1991). Dexamethasone administration to rats does not affect serum IGF-1 levels but reduces weight gain while increasing IGFBP-1 in serum and mRNA in the liver (Luo *et al.* 1990). Glucocorticoids have however a suppressive effect on the expression of other IGFBPs. For example *in vitro* glucocorticoid treatment of human osteoblast like cells increased IGFBP-1 mRNA but decreased mRNA for IGFBP-3, -4, -5 and -6 (Okazaki *et al.* 1994). Likewise

dexamethasone decreased IGFBP-3 and -5 in foetal fibroblasts (Camacho-Hubner *et al.* 1992).

Serum IGFBP-2 levels are inversely related to GH status, decreasing in diabetes and acromegaly, and increasing in GH deficiency (Clemmons *et al.* 1991). However serum IGFBP-2 also increases in states of IGF excess, for example IGF-1 infusion (Zapf *et al.* 1990); thus serum IGFBP-2 levels increase when IGFBP-3 levels are insufficient to bind the available IGF (Baxter 1993). Insulin does not seem to be a major regulator of IGFBP-2, and it has been suggested that IGF-2 could be the major regulator of circulating IGFBP-2 *in vivo* (Blum *et al.* 1993).

Less is known about hormonal regulation of IGFBP-4 and -5. FSH decreases IGFBP-4 and -5 mRNA in rat granulosa cell culture (Liu *et al.* 1993). In human bone cells IGFBP-4 levels are increased by calcium mobilising hormones PTH and vitamin D but decreased by bone morphogenic protein-7, IGF-1 and progesterone. In contrast, IGFBP-5 levels in human bone cells are increased by IGF-1 and progesterone (reviewed by Mohan (1993)). IGFBP-4 in conditioned medium was increased by IGF-1 in muscle cells (McCusker *et al.* 1989). In contrast IGF-1 treatment decreased IGFBP-4 and -5 in foetal fibroblasts culture, although there was no decrease in mRNA (Camacho-Hubner *et al.* 1992).

1.10.3 Nutritional regulation

The IGFs are important regulators of tissue growth, and nutrition is a major regulator of the IGFs. In most species GH is elevated by fasting, but IGF-1 levels fall in undernourished children and animals, showing the dominance of nutritional control of IGF-1 over that of GH. Fasting decreases GH receptors which may partly account

for this apparent GH resistance (Straus & Takemoto 1990a). In contrast there is little change in GH receptor expression in protein restriction (Straus & Takemoto 1990b) suggesting a post receptor mechanism of GH resistance. IGF-1 and GH receptor mRNA are decreased in the liver and in other tissues of fasted rats though they are not coordinately regulated in all tissues (Lowe *et al.* 1989) but the physiological relevance of this is not determined. IGF-1 infusion does not normalise growth in protein restricted rats but spleen and kidney growth is enhanced (Thissen *et al.* 1991). Dietary energy or protein restriction decreased IGF-1 message but only protein restriction affects serum albumin production demonstrating that serum IGF decreases are not merely reflecting a general decrease in serum proteins.

The effects of nutrition on IGF-2 levels have been less well studied than those of IGF-1. IGF-2 appears less affected by acute fasting than IGF-1 but is significantly decreased by chronic dietary restriction (see Straus 1994 for review).

Changes in nutritional status markedly alter the expression of both IGFBP-1 and -2; in general the modulation of IGFBPs-1 and -2 by nutrition follows a pattern inversely related to IGF-1 expression. IGFBP-1 mRNA is increased in fasting animals in both protein and energy restriction due to increased transcription (Straus *et al.* 1993). Hepatic IGFBP-2 mRNA increases in rats by fasting (Tseng *et al.* 1992), protein restriction (Straus & Takemoto 1990b), or energy restriction (Straus & Takemoto 1991). In contrast IGFBP-3 levels are decreased by fasting or protein deprivation in growing rats (Clemmons *et al.* 1989).

Normally IGFBP-2 and -3 levels are constant throughout the day, however IGFBP-1 levels show marked diurnal variation. Insulin and carbohydrate which are major inhibitory regulators of IGFBP-1 (Cotterill *et al.* 1988) and a role for IGFBP-

1 in glucose homeostasis has been suggested (Lewitt & Baxter 1991b). Possible alternative roles for IGFBP-1 have also been suggested such as an IGF shuttle or regulator of mitogens (Holly 1991).

Nutrition is not only a major regulator of IGF-1 but also alters the expression of IGFBPs although the physiological consequences of changes in IGFBPs are unclear. IGF-1 clearance from the serum into tissues is increased in protein deprived rats, and this increased clearance may be due to an increase in the amount of IGF-1 complexed to IGFBP-1 and -2 (Thissen *et al.* 1992).

1.11 The GH/IGF axis and the immune response

1.11.1 Introduction

It is now established that the endocrine and immune systems interact and there is growing evidence that polypeptide hormones play a supporting role in immunoregulation. The growth stimulatory effects of GH on lymphoid tissue was first suggested in 1930 when Smith reported thymic atrophy in hypophysectomised rats (Smith 1930). In subsequent studies GH or PRL restored thymus and spleen growth in hypophysectomised rats (Berczi & Nagy 1987). Similarly, in immunodeficient dwarf dogs GH treatment improved clinical condition and increased thymic size and cellularity (Roth *et al.* 1984). These studies described the role of GH only in gross anatomical terms but many subsequent studies demonstrated a specific role for GH in the development and function of immune tissues. For example, administration of a specific antiserum to GH resulted in defects in lymphatic tissue and humoral immunity in neonatal rats (Crilly *et al.* 1994).

Many studies have suggested that GH actions are mediated via the endocrine or paracrine synthesis of insulin-like growth factor-I (IGF-1) and since cells of the immune system can produce and respond to GH and IGF-1, some of GH's effect may thus be mediated by IGF-1. For example, recombinant human IGF-1 treatment restored thymus and spleen weights to normal in hypophysectomised rats (Guler *et al.* 1988). Similarly, in diabetic rats, IGF-1 treatment restored thymus weight and increased thymocyte proliferation (Binz *et al.* 1990). In some studies administration of IGF-1 but not GH increased the weight of spleen and thymus disproportionally to overall body size (Guler *et al.* 1988, Skottner *et al.* 1989, Binz *et al.* 1990). These *in vivo* studies described the effects of IGF-1 in gross anatomical terms, but more recent *in vivo* studies have investigated the effects of IGF-1 administration on the immune functionality as well as cell numbers and changes in cell size (Clark *et al.* 1993). There are an increasing number of studies of IGF effects on immune cells *in vitro*, initally these concentrated on T-lymphocytes but recently the effect on immunoglobulin synthesis and B-cell lymphopoiesis have received more attention.

1.11.2 Production of IGFs by cells of the immune system

GH stimulates IGF-1 production from rat spleen cells (Baxter *et al.* 1991) in culture and stimulates the growth of T-lymphoblasts lines via local IGF-1 production (Geffner *et al.* 1992). IGF-1 production also has been demonstrated by transformed, but not freshly isolated, human B-lymphocytes (Merimee *et al.* 1989) however the amount of IGF-1 released was low though IGFBPs did not seem to be interfering with IGF-1 measurement.

An IGF-like molecule is released from cultured alveolar macrophages (Rom *et al.* 1988) and IGF-1 mRNA is expressed by wound macrophages (Rappolee *et al.* 1988). Arkins *et al.* (1993) detected IGF-1 expression in differentiated mouse macrophages (but not pre-myeloid cells) using Northern blotting or PCR amplification; since IGF-1 mRNA was negligible in lymphoid cell lines and lymphoid tissues this suggests that in haemopoietic cells it is the myeloid rather than lymphoid cells which express IGF-1.

IGF-2 appears to be the major IGF within the thymus of the young rat and human (Geenan *et al.* 1993). IGF-2 was localised by immunohistochemistry to stromal cells in the subcapsular cortex and medulla of human thymus, and to stromal cells dispersed throughout the parenchyma of rat thymus. In contrast IGF-1 was localised to non epithelial-like cells in the interlobular septa; this confirms Han's earlier study of IGF-1 mRNA distribution in the human foetus, which showed IGF-1 mRNA exclusively in the capsule and interlobular septa (Han *et al.* 1987).

1.11.3 Production of IGFBPs by cells of the immune system

Few studies have investigated the production of IGFBPs in the immune system. Although there are satisfactory serum-free culture media for lymphoid cells, the use of serum is routine and since serum contains IGFBPs this interferes with the detection of endogenously produced IGFBPs. IGFBP production has been demonstrated from human leukaemic blasts (Neely *et al.* 1991) and human peripheral blood cells express mRNA for several IGFBPs only some of which are demonstrable by WLB (Nyman & Pekonen 1993). Spleen tissue from adult rats expresses mRNA for IGF-1, IGFBP-2, and IGFBP-4 (Domene *et al.* 1994). Hypophysectomy reduced

splenic mRNA for IGF-1 and IGFBP-2 but increased IGFBP-4 mRNA, and GH treatment can partially prevent these effects. Hypophysectomy of juvenile rats has similar effects (Yakar *et al.* 1994).

1.11.4 Expression of IGF receptors

Type 1 IGF receptors have been demonstrated on a variety of myeloid and lymphoid cells: human peripheral blood T-lymphocytes (Tapson *et al.* 1988, Johnson *et al.* 1992), B-lymphocytes, monocytes and Natural killer cells (Kooijman *et al.* 1992a), platelets (Hartmann *et al.* 1989); human malignant myeloid (Sinclair *et al.* 1988), B-cell (Freund *et al.* 1994) and T cell lines (Lee *et al.* 1986); rat thymus and thymoma cells (Verland & Gammeltoft 1989); bovine peripheral blood mononuclear cells, neutrophils and to a lesser extent, erythrocytes (Zhao *et al.* 1992).

1.11.5 Effects of IGFs on immune cell function

IGF-1 enhances H_2O_2 production by bovine neutrophils (Zhao *et al.* 1993) and enhances human neutrophil phagocytosis (Jin *et al.* 1993). IGF-1 and GH can prime human and porcine neutrophils for O_2^- production (Fu *et al.* 1991) although GH effects are not mediated by IGF-1. In contrast GH or IGF-1 stimulate human thymic epithelial cell proliferation and enhance thymic hormone production; both the IGF-1 and growth hormone effects could be abrogated by an antibody to IGF-1 suggesting that the GH effect may be mediated by IGF-1 secretion (Timsit *et al.* 1992).

The use of serum in culture medium has complicated the interpretation of some studies of IGF effects since serum contains IGF and insulin, and IGFBPs which could modify IGF responses. IGF-1 augments [^{3}H]thymidine incorporation

into human peripheral blood mononuclear cells (PBMC) (Kooijman *et al.* 1992b), lymphoblastoid cell lines and human tonsillar B-lymphocytes (Kimata & Yoshida 1994), bovine PBMC (Zhao *et al.* 1993), rat thymocytes (Yamada *et al.* 1994), and stimulates normal and transformed T-lymphocyte growth (Geffner *et al.* 1992). However other studies failed to show stimulatory effects of IGFs e.g. on [^{3}H]thymidine incorporation into rat thymocytes (Verland & Gammeltoft 1989). Some studies using human peripheral blood cells showed little enhancement in the presence of serum but significant enhancement in the presence of serum treated with reducing agent to inactivate growth factors (Kooijmann *et al.* 1992b). The enhancement was greater when purified T-cells depleted of monocytes were used, though supplementation with IL-1 showed the IGF effects were independent of monocytes or IL-1 production. The authors suggest the absence of monocytes enhanced IGF-1 effects because monocytes secrete IGFs or IGFBPs which may inhibit IGF-1 action on mitogen stimulation. This is plausible since Arkins *et al.* (1993) showed IGF-1 expression by myeloid rather than lymphoid cells, and although IGFBP production has not been demonstrated by macrophages, exogenous IGFBP-1 could block the polyclonal activation of human peripheral blood cells (Kooijman *et al.* 1992b). The direct effect of IGF-1 on lymphoid cells was demonstrated when antisense oligonucleotide strategy showed that IGF-1 receptor expression was necessary for mitogenic stimulation of mononuclear cells (Reiss *et al.* 1992). Recently IGF-1 has been shown to enhance PHA stimulated [^{3}H]thymidine uptake in human lymphocytes by reducing G_1 phase length thus enhancing clone expansion rather than altering cell numbers (Schillaci *et al.* 1994). IGF-2 alters surface determinants on neutrophils and lymphocytes and may thus affect their reactivity (Vetvicka & Fusek 1994).

Human platelets show enhanced aggregation in response to stimulants in the presence of IGF-1; since the platelet is anucleate this demonstrates a metabolic response to IGF-1 in the absence of its mitogenic effect (Motani *et al.* 1992).

IGF-1 has effects on haematopoiesis of myeloid and lymphoid cells. IGF-1 augments human granulopoiesis *in vitro* (Merchav *et al.* 1988) and *in vivo* administration of IGF-1 increases bone marrow B-lineage cells (Jardieu *et al.* 1994). IGF-1 increases proliferation of intrathymic pre-T lymphocytes *in vitro* (Gjerset *et al.* 1990) and stimulates the differentiation of pro-B cells in response to IL-7 (Landreth *et al.* 1992). Mature B-lymphocyte function can also be modulated by IGF-1 and GH. GH and IGF-1 can enhance immunoglobulin synthesis by tonsillar B-lymphocytes as well as human B-lymphocyte lines *in vitro* (Kimata & Yoshida 1994). There is evidence that in human peripheral B-cells, IGF-1 can induce IgE and IgG_4 production by class switching through an IL4- and IL3-independent mechanism. GH had a similar effect which was direct i.e. not mediated by IGF-1 (Kimata & Fujimoto 1994). Infusion of IGF-1 into mice increased lymphocyte numbers in peripheral lymphoid tissues, and enhanced Ig production in response to *in vivo* challenge. In addition IGF-1 alone could stimulate Ig production by antigen primed splenocytes *in vitro* (Robbins *et al.* 1994).

1.11.6 Role of IGFs in the immune response

There is clearly evidence for a role of IGFs in the immune response, in ontogeny and in the mature differentiated function of both specific and non-specific arms of the immune response. IGF-1 may mediate some of the effects of GH but not all, for

example GH has direct effects on mature neutrophils (Fu *et al.* 1991) but its effect on granulocyte maturation is mediated by IGF-1 (Merchav *et al.* 1988).

Immune responses require complex interactions between different cell types and the ECM within the micro-environment. Although IGF-1 production by lymphoid cells is low, IGF-1 produced by stromal cells including macrophages, may act by a paracrine mechanism. Murine bone marrow stromal cells produce IGF-1 and IGFBPs (Abboud *et al.* 1991) and thymic epithelial cells produce IGF-1, in response to GH (Timsit *et al.* 1992). In addition to its mitogenic effect IGF-1 is chemotactic for human T-lymphocytes (Tapson *et al.* 1988). Some cytokines bind ECM components, where they not only form a reservoir of cytokine, but are often better motility agents than free cytokine. IGFBPs attached to the ECM via their GAG binding regions could likewise sequester IGFs in the microenvironment thus enhancing the chemotactic properties of IGFs.

The endocrine role of the IGFs is not fully elucidated, but they are thought to play a role in carbohydrate, lipid, and whole body protein metabolism. Evidence suggests that changes in IGF-1 and GH are permissive to protein catabolism. GH and IGFBP-1 levels are raised but IGF-1 and IGFBP-3 are low (IGFBP-3 protease activity is increased) in some catabolic states such as critical illness, cancer, and septicaemia (Bentham *et al.* 1993). Hypercatabolic states such as injury and severe illness, are associated with immunosuppression and since IGFs appear to be important in the maintenance of the immune response, the alterations in IGFs may be contributing to the observed immunosuppression.

The acute phase response (APR) consisting of fever, reduced food intake, weight loss, hypercatabolism etc. is the first phase in the inflammatory response to

injury or infection. The APR normally lasts for a few days and may be detrimental if it continues e.g. in rheumatoid arthritis. Cortisol initiates the hepatic response but is also inhibitory to the production of secondary cytokines, thus forming a loop limiting the response. Insulin can modulate the APR by attenuating the response of the liver to IL-1 and IL-6 (Campos & Bauman 1991). Recent evidence suggests IGF-1 may be a component of the APR. Endotoxin administration to rats immediately reduced plasma GH and IGF-1 but IGFBP-3 was unaltered, showing the dissociation of serum IGF/GH and IGFBP-3 (Fan *et al.* 1994). Because nutrition is a major regulator of IGF-1, the endotoxin-related changes in the IGF axis are confounded by the effect of reduced food intake induced by endotoxaemia. In ruminants short term reduction in food intake has little effect on circulating IGF-1 levels making them a good model in which to study the effects of endotoxin. Similar to its effects in rats, endotoxin administration to cattle reduced serum IGF-1 but IGFBP-3 was unaffected (Elsasser *et al.* 1995). In contrast yeast administration to lambs increased circulating IGF-1 concentration (Moore *et al.* 1994). Although circulating IGF-1 and IGFBP-3 levels decrease in many catabolic states, serum IGF-1 is unaltered in sepsis (Dahn *et al.* 1988) and serum IGFBP-3 was unaltered in infected pigs (Prickett *et al.* 1992). IGF-1 levels tend to rise after moderate injury and fall in severe trauma; the catabolic response may vary with the severity of the insult.

IGF-1 and GH have been used in preliminary clinical trials to restore the nitrogen balance in catabolic states, but the effect of treatment on the immune responses was rarely determined in spite of the clinical problem of post-trauma immunosuppression. Aggressive nutrition eliminated the depressed $CD4^+/CD8^+$ T-cell ratio seen in patients with head injuries; IGF-1 administration in addition to

nutrition increased the $CD4^+/CD8^+$ ratio while IGF-1 levels were elevated (Kudsk *et al.* 1994).

IGF-1 and IGF-2 are found high amounts in wounds and poor wound healing is associated with IGF-1 deficiency states. Wound IGF-1 is likely to be derived not only from the circulation, but by local production by wound macrophages and fibroblasts, and from the lysis of platelets which contain IGF-1 and IGFBP-3. IGF-1:IGFBP-3 complexes are effective in promoting wound healing in animal models (Sommer *et al.* 1991). IGF-1 and GH have potential clinical uses in restoring immune function in immunosuppressed states such as the regeneration of bone marrow after irradiation. Alternatively, ablation of IGF-1 or GH may be of use in leukaemias (Hooghe *et al.* 1993). IGF-1 and GH clearly modulate the immune response and the clinical and agricultural importance of the interactions between the immune and endocrine systems is beginning to be recognised.

1.12 The IGF axis during pregnancy

During pregnancy the maternal metabolism adapts under hormonal control to meet the increased energy requirements imposed by the foetus and placenta, and maternal glucose utilisation is reduced to spare glucose for the foetus. In rats maternal muscle is degraded to help meet this energy demand and because IGF-1 inhibits the breakdown of protein it is possible that the decrease in IGF-1 in late pregnancy is involved in the regulation of glucose and protein metabolism during pregnancy (Chiang & Nicoll 1990).

Serum IGF-1 levels abruptly decrease in the second half of pregnancy in rats (Travers *et al.* 1993, Davenport *et al.* 1990), pigs (Lee *et al.* 1992), and the baboon

(Putney *et al.* 1990). The decreased serum IGF-1 levels in rats during late pregnancy are believed to be caused by decreased transcription in the liver as IGF-1 mRNA decreases per unit of RNA. However since total hepatic IGF-1 mRNA is higher if the total RNA content (because of increased liver size during pregnancy) is taken into account, decreased transcription alone cannot explain the decrease in serum IGF-1 and other mechanisms are likely such as increased clearance from the circulation (Travers *et al.* 1993). In contrast, in human pregnancy there is a steady rise in serum IGF-1, although IGF-2 was either unchanged (Hall *et al.* 1984) or raised (Gargosky *et al.* 1990). Serum IGF-1 and IGF-2 are unchanged in pregnancy in sheep (Gluckman *et al.* 1979) and in mice (Fielder *et al.* 1990). Rats support a comparatively greater weight of foetal tissue than humans in late pregnancy. It has been suggested that this higher metabolic burden induces a maternal catabolic state which accounts for the decrease in serum IGF-1 in the rat but not in humans (Gargosky *et al.* 1990).

In man, mice and rats IGFBP-3 protease activity is greatly increased in late pregnancy rendering IGFBP-3 undetectable by WLB. The appearance of protease activity in rat serum coincides with the switch of maternal metabolism to a catabolic state and since IGFBP-3 protease activity occurs in other catabolic states it has been suggested that the function of the protease is to reduce IGFBP-3 affinity for IGF and therefore increase its availablity. However ewes are often in a negative energy balance in late pregnancy but both serum IGF and IGFBP-3 concentrations are normal and there is no evidence of increased IGFBP-3 protease activity by WLB. Neither pigs nor humans are in a negative energy balance in late pregnancy, but whilst pig serum IGF-1 decreases in the absence of marked IGFBP-3 protease

activity (Lee *et al.* 1992), IGF-1 levels remain high in human pregnancy serum in spite of increased protease activity. Therefore increased IGFBP-3 protease and low serum IGF-1 do not necessarily correlate with catabolism and the physiological function of this IGFBP-3 protease in pregnancy remains unclear.

The Baboon, Pig and Rhesus monkey, which exhibit little or no IGFBP-3 protease activity in late pregnancy, have superficial placentas, however species like rats and humans which do exhibit increased IGFBP-3 protease activity have an invasive trophoblast. Decidualisation is associated with increased production of proteases which enable trophoblast invasion. The source of IGFBP-3 protease may be the decidua since human decidual cell explants (Deal & Lamson 1991) and rat uterine decidua (Davenport *et al.* 1992b) produce IGFBP-3 protease in culture. Therefore the sparse endometrial decidualisation in some species may explain the absence of IGFBP-3 protease expression in late pregnancy (Giudice *et al.* 1993).

IGFBP-3 from human term placental trophoblasts (Deal *et al.* 1991) and from pregnant baboon serum (Giudice *et al.* 1993) is about 2 kDa higher than that in normal serum, and deglycosylation studies suggest they may be differentially glycosylated or differ in primary structure, though the functional significance of this alteration is unknown.

IGFBP-3 is not the only serum IGFBP which shows alterations during pregnancy. Human pregnancy serum shows decreased IGFBP-2 and -4 by WLB after about week 10 (Giudice *et al.* 1990). IGFBP-1 serum levels measured by RIA increase in human(Rutanen *et al.* 1982). IGFBP-1 is also present in human amniotic fluid (Rutanen *et al.* 1982) and is abundantly produced by decidual cells in culture (Clemmons *et al.* 1990b). Amniotic levels of IGFBP-1 increase throughout human

pregnancy and the proportion of phosphorylated IGFBP-1 increases (Koistenen *et al.* 1993).

In contrast, serum IGFBP-4 in pregnant rats appears resistant to proteolysis (Davenport *et al.* 1990) and hepatic mRNA for IGFBP-1 and -4 is increased although hepatic IGFBP-2 mRNA was barely detectable (Donovan *et al.* 1991). IGFBP-2 was the major binding protein in rat decidua, placenta, and uterus by day 15 (Davenport *et al.* 1992b). IGFBP-1 protein is expressed by rat uterus but only in the non-decidualised endometrium (Sadek *et al.* 1994).

Although IGFBP-3 is apparently reduced in pregnancy when detected by WLB, human serum IGFBP-3 levels are actually raised and rat hepatic IGFBP-3 mRNA is normal (Donovan *et al.* 1991a). Since rats are GH resistant in late pregnancy this shows the dissociation of IGFBP-3 and GH levels, and implies that IGFBP-3 expression is not under such strict GH control during pregnancy. The effect of GH administration on foetal growth has been investigated in several studies using *ad lib.* fed rats, but conflicting results were obtained; foetal growth was unaffected, increased, or the effect varied with maternal size. The marked GH resistance during late pregnancy in the rat can only be overcome with high doses of GH (Chiang *et al.* 1990), but lower GH doses administered to pregnant rats on a low food intake could reduce foetal and placental growth, and high doses led to advanced resorption of the foetuses (Chiang & Nicoll 1991). Ablation of GH in pregnant rats by administration of anti-rGH serum decreased maternal muscle mass and increased foetal weights (Palmer *et al.* 1996). These studies suggest that maternal GH resistance is an important adaptation in diverting nutrients from dam to foetus.

There is increasing evidence that maternal IGF-1 is a key determinant of maternal-foetal nutrient partitioning. For example Gluckman *et al.* (1992) showed that the administration of IGF-1 (but not GH) overcomes maternal constraint, abolishing the negative correlation between foetal weight and litter size, but does not affect foetal and placental weights. Similarly Gargosky *et al.* (1991) showed administration of IGF-1, but not GH, to *ad lib.* fed rats increased serum IGF-1 levels and maternal weight but did not affect the foetal placental unit. IGF-1 does not cross the placenta and probably exerts its effects by altering placental function in favour of nutrient transfer to the foetus.

The type of placentation and the consequent relationship of maternal-foetal circulations differs between species and their different IGF-IGFBP profiles may reflect this. However the role of the IGFBPs and their regulation in mother and foetus during pregnancy are poorly understood.

1.13 The IGF axis and the mammary gland

1.13.1 Introduction

The mammary gland cycle of growth, development and involution comprises distinct physiological states: mammogenesis, the development of mammary tissue during puberty and pregnancy; lactogenesis, the initiation of milk secretion at parturition; galactopoiesis, the maintenance of established lactation; and involution, when milk secretion ceases and the gland regresses to a resting state. The regulation of these events involves the complex coordination of a range of hormonal stimuli, growth factors and cell-cell interactions. The hormonal control of each stage is largely independent and marked species differences exist.

1.13.2 Mammary differentiation and development

1.13.2.1 *In vivo*

Mammary development is dependent on a variety of lactogenic and mammogenic factors. The presence of a lactogen, PRL or Placental lactogen (PL) has been considered essential, but there is increasing evidence of the importance of GH although the relative role of the different hormones and the mechanisms involved are unclear. The effect of GH was originally attributed to its similarities with PRL but Kleinberg and coworkers have in a series of studies provided evidence for a role of GH in mammary development using the castrated, estradiol-treated male rat as a model. rGH which is non-lactogenic, was more potent than rPRL in directly stimulating mammary gland growth and differentiation when implanted in rat mammary gland (Kleinberg *et al.* 1990). Feldman *et al.* (1993) subsequently compared the ability of implanted GH, PRL and PL to induce mammary development; only hormones which could bind to GH receptors induced mammary development, regardless of their lactogenic activity. This supports a role for GH by a mechanism independent of lactogenic receptors.

A direct mode of action for GH within the mammary gland seemed unlikely because GH receptors have not been convincingly demonstrated on mammary cells. For example GH receptors could not be found on mammary glands of the pregnant non-lactating ewe using conventional binding assays (Akers 1985). GH receptor mRNA has been detected during late gestation and early lactation in mammary acini tissue of rabbit (Jammes *et al.* 1991) and cow (Hauser *et al.* 1990) though adipocyte GH receptors may account for some data. However in pregnant and early lactating rats, immunocytochemistry showed GH receptor protein localised to ductal epithelial

cells, alveolar cells and myoepithelial cells as well as adipocytes (Lincoln *et al.* 1990).

Several lines of evidence support a role for IGF-1 in mammary development. IGF-1 can substitute for the pituitary in mammary development (Ruan *et al.* 1992). The mammary gland expresses IGF-1 mRNA (Murphy *et al.* 1987) and expression was stimulated by systemic GH administration (Kleinberg *et al.* 1990) suggesting that GH may exert its effects on mammary development through IGF-1. IGF-1 expression in mammary glands of pregnant rats decreases during pregnancy; IGF-1 was immunolocalised to myoepithelial cells (Marcotty *et al.* 1994) suggesting a paracrine role for IGF-1 in mammary development. Consistent with this hypothesis, mammary type 1 IGF receptors have been demonstrated in several species. In rats type 1 IGF receptors increase in pregnancy compared with virgin animals (Collier *et al.* 1989). In the ewe also, type 1 IGF receptors are higher in gestation than lactation, and IGF-2 receptors are higher prepartum than during lactation (Disenhaus *et al.* 1988). In contrast, type 1 IGF receptors on lactating cow mammary gland declined during the prepartum period, although IGF-2 receptors, which are more abundant than type 1 IGF receptors, were unchanged (Hadsell *et al.* 1990; Dehoff *et al.* 1988). In the pig also, type 1- and -2 IGF receptor mRNA expression in pregnant mammary tissue were greater during early growth than later stages (Lee *et al.* 1993) suggesting that IGF-1 is more important in early mammary development.

1.13.2.2 *In vitro*

IGF-1 is mitogenic for mammary cells in culture, for example IGF-1 stimulated [^{3}H]thymidine uptake in undifferentiated bovine mammary epithelial cells (Gertler

et al. 1983). Mammary tissue in culture produces IGF-1 and IGFBPs, the production of which can be modulated by IGF-1. Mammary explants from pregnant cows synthesise IGF-1 and IGFBPs but acini synthesise only IGFBPs; GH, PRL, insulin or cortisol had no effect on IGFBP production (Campbell *et al.* 1991). However IGF-1, des-IGF-1, and to a lesser extent IGF-2, enhanced the production of both IGFBP-2 and -3 in conditioned medium of mammary cells from pregnant heifers (McGrath *et al.* 1991).

In vitro culture of mammary tissue has generally used tissue from pregnant animals induced into lactogenesis by combination treatment with insulin, cortisol and PRL. The effects of GH, PRL and IGF-1 on mammary growth have been studied *in vitro* using mammary glands from young mice implanted with oestogen and progesterone (Plaut *et al.* 1993). Explants cultured in insulin, hydrocortisone, aldosterone and EGF with either PRL or GH showed lobular-alveolar development, but higher doses of GH than PRL are required. Similarly PRL is more potent than GH in stimulating β-casein expression. These findings contrast with those of Kleinberg's *in vivo* studies in which GH was more potent. Plaut *et al.* suggest the conflicting results may reflect differences in the models used or the use of homologous GH in Kleinberg's study, which is not available for the mouse studies. IGF-1 did not substitute for GH, PRL or insulin in tissue maintenance of mouse explants, perhaps due to the absence of appropriate IGFBPs in culture or alternatively, GH action may not be mediated by IGF-1 but may work through a GH receptor-independent mechanism (Plaut *et al.* 1993). IGF-1 can however stimulate the proliferation of mammary cells from mice (Imagawa *et al.* 1986), rats (Deeks *et al.* 1988), non-pregnant (Peri *et al.* 1992) and pregnant cows (McGrath *et al.* 1991).

In addition IGF-1 stimulated β-casein expression and the transport of glucose and alpha-lactalbumin in mammary explants (Prosser *et al.* 1987).

The effects of IGFs, GH and placental lactogens have been investigated *in vitro* using floating collagen gel culture of mouse mammary epithelial cells from pregnant mice. Cells were cultured in floating collagen gels, treated with mouse PRL, PL-1, PL-2, GH, IGF-1 and IGF-2. Cells in basal conditions produced IGFBPs at 40-45 kDa and at 29 kDa though at a lower level. Treatment with PRL and PLs increased the 29 kDa IGFBP, but the 40-45 kDa IGFBP was not as lactogen dependent. GH was a less potent stimulator of 29 kDa IGFBP but IGFs increased this IGFBP as potently as lactogens; IGF effects were additive with one of the lactogens. These IGF-1 effects did not seem to be type 1 IGF receptor mediated. IGFs however had no effect on alpha-lactalbumin production nor did they enhance the lactogenic effects of PL-1. The 40-45 kDa IGFBP was thought to be IGFBP-3, the 29 kDa IGFBP however did not react with antibody to IGFBP-1 or -2 and could not be identified (Fielder *et al.* 1992).

A mouse mammary epithelial line (Comma-D/MME) produced predominantly IGFBP-2 and -3 when cultured in serum free medium and their production was regulated by several hormones and growth factors. The mammogenic growth factors IGF-1 and EGF both stimulated DNA synthesis, but IGFBP-2 and -3 secretion was stimulated by IGF-1 and inhibited by EGF. Combinations of lactogenic hormones were also tested: insulin stimulated DNA synthesis and IGFBP-3 secretion but not IGFBP-2 secretion; cortisol inhibited IGFBP-3 secretion and DNA synthesis but increased IGFBP-2; whereas PRL had no effect on production of either IGF or IGFBPs (Skaar & Baumrucker 1993).

Thus mammary tissue produces and responds to IGFs and there is strong evidence for a role of IGFs in the development of the mammary gland, probably acting in a paracrine manner mediating mesenchymal-parenchymal interactions.

1.13.3 Galactopoiesis

1.13.3.1 *In vivo*

GH is well established as the major galactopoietic hormone in ruminants although the mechanism of action is not clear (Breier *et al.* 1991). GH also increases milk volume in lactating women (Milsom *et al.* 1992). In contrast, PRL is the major galactopoietic hormone in rodents although GH plays a lesser but significant role, especially when PRL is low (Madon *et al.* 1986).

There are several possible mechanisms of action of GH: nutrient partitioning, direct action on the mammary gland, or indirect effects via IGF-1. GH does not seem to increase milk yield by acting directly on the ruminant mammary gland because local infusion of GH could not show a unilateral stimulation of milk yield in sheep (McDowell *et al.* 1987). Consistent with this was failure to detect GH receptors on mammary epithelial cells (Akers 1985). However GH receptor mRNA has been detected in lactating mammary gland of the cow (Hauser *et al.* 1990) and rabbit (Jammes *et al.* 1991). Some of this mRNA could be derived from other cell types such as adipocytes, but in the rabbit during the phase of epithelial cell proliferation in late gestation the proportion of adipocytes and connective tissue is decreasing as the GH-receptor mRNA increases. Furthermore, during lactation mRNA is still detectable even though milk protein expression is high suggesting GH-receptor mRNA expression is by epithelial cells. Systemic administration of GH

increased immunoreactive IGF-1 in the cytoplasm of bovine secretory cells which may reflect internalistion of IGF or local production (Glimm *et al.* 1988). Glimm *et al.* (1990) subsequently used *in situ* hybridisation to show that GH receptor mRNA was expressed in alveolar epithelial cells and GH treatment decreased mRNA levels. One study did demonstrate GH receptor protein in lactating rat mammary epithelial cells (Lincoln *et al.* 1990). Receptor protein decreased at the onset of lactation and was only weakly detectable by day 18, suggesting that while GH may act directly in mammary development in the rat, this may not be the case in the maintenance of lactation. Another implication of this finding is that the contribution of GH, as a stimulant of locally produced IGF-1, is a minor contribution in galactopoiesis in the rat.

Consistent with a role for IGF-1, IGF receptors have been detected on lactating mammary tissue in several species. In the lactating cow both type 1- and -2 IGF receptors are expressed on mammary microsomes with type 2 receptor in 10-fold abundance. Binding to type 1 IGF receptors increased by 75% at the onset of lactation but declined throughout the postpartum period (Hadsell *et al.* 1990). Bovine mammary gland membranes expressed both 135 kDa and 127 kDa species of type 1 IGF receptor, but non-lactating have only the 135 kDa type suggesting that lactation is associated with structural changes in receptors as well as the level of expression (Dehoff *et al.* 1988). In contrast, rat type 1 IGF receptors levels decrease in lactation compared with pregnancy, although IGF-2 receptors were unchanged (Collier *et al.* 1989). In the ewe type 2 IGF receptors are lower during lactation than during the prepartum period (Disenhaus *et al.* 1988).

A role for circulating IGF-1 in galactopoiesis is supported by the ability of GH administration to increase serum IGF-1 and mammary blood flow thus increasing the supply of IGF-1 to the gland (Prosser *et al.* 1989). However systemic IGF-1 administration elevated circulating IGF-1 levels two-fold but milk yield was unaffected (Davis *et al.* 1989). Although GH consistently increased serum IGF-1, the galactopoietic response correlated with the amount of IGF-1 in milk and mammary tissue suggesting that the availability of circulating IGF-1 to the mammary gland is important in the response to GH (Prosser *et al.* 1991a). Local infusion of IGF-1 into mammary gland of goats apparently stimulated milk production (Prosser *et al.* 1990) but subsequently Prosser *et al.* (1992) showed that although intra-mammary infusion of IGF-1 increased mammary blood flow and milk yield, more frequent milk removal on the day before intra-mammary infusion attenuated both milk yield and mammary blood flow effects whilst not attenuating the response to GH. The role of IGF-1 in mediating GH effects within the gland was therefore questionable. The effects of GH and IGF-1 administration differ in several ways including their nutrient partitioning effects (Prosser & Mepham 1989) and depression of insulin and IGF-2 by IGF-1 infusion (Prosser & Davis 1992). IGF-1 and GH administration elicit different IGFBP profiles (Davis *et al.* 1989) with consequent affects on IGF availability; it is feasible that IGF-1 does mediate GH action within the gland but the delivery to the gland or local availability of exogenously administered IGF does not mimic that obtained with GH administration.

Although IGF-1 concentrations in milk and mammary tissue correlated with galactopoiesis in goats a role of IGF-1 has not been demonstrated. Mammary tissue

can express IGFs and IGFBPs but whether those found in milk are derived from the mammary tissue or enter by the paracellular route is unclear. The milk of all species studied contains IGFs and several of the IGFBPs are present in milk depending on the species. The presence of IGFs in the milk follows the pattern of other growth factors and immunoglobulin: higher in colostrum than in mature milk. Because type 1 IGF receptors are found in the rat gastrointestinal tract (Laburthe *et al.* 1988) and colostrum stimulates intestinal growth in suckling rats, a role for IGF-1 in the development of the gastrointestinal tract has been postulated (Berseth *et al.* 1983). A truncated IGF-1 which has reduced affinity for IGFBPs accounts for 50% of the IGF-1 in bovine colostrum but 3% of milk IGF-1 (Francis *et al.* 1986). It is unknown if this exists in the milk of other species, however this variant IGF-1 is particularly potent in promoting growth of the rat gastrointestinal tract (Read *et al.* 1992).

Serum IGF-2 concentrations in the adult rat are low and IGF-1 is the major IGF in both serum and rat milk. The concentration of IGF-1 decreased in both milk and serum during lactation although serum IGF-1 concentrations were still higher than those of milk. In contrast serum and milk IGF-2 concentrations were low and did not change during lactation (Donovan *et al.* 1991b). IGF-1 and -2 mRNA was low or absent in day 11 lactating rat mammary gland (Murphy *et al.* 1987) although IGF-2 mRNA was detectable in lactating rat mammary gland in another study (Manni *et al.* 1992). Marcotty *et al.* 1994 confirmed that IGF-1 mRNA is low at day 5 and 10 of lactation although IGF-1 could still be immunolocalised to myoepithelial cells. The low levels of expression suggested that serum may be the source of milk IGF-1 in the rat. Rat milk contains IGFBP-3, IGFBP-2, and a 24 kDa IGFBP identified by

WLB. IGFBP-2 was undetectable by WLB in maternal serum but lactating mammary gland expressed IGFBP-2 mRNA suggesting that IGFBP-2 is synthesised in the gland (Donovan *et al.* 1991b). However mammary gland IGFBP-3 mRNA is decreased at parturition and remains low in lactation suggesting that in rats, IGFBP-3 in milk is serum derived (Marcotty et al. 1994).

IGF-2 is usually the major milk IGF in species where it is the major serum IGF such as ruminants and man. Bovine colostrum has high IGF-1 levels although serum IGF-1 is low (Vega *et al.* 1991). Both IGF-1 and IGF-2 are transported into milk in goats, however IGF-1 is specifically transported and IGF-2 is non-specifically transported (Prosser *et al.* 1991b; Prosser & Fleet 1992). Bovine colostrum contains IGFBP-3 and -2, and IGFBPs at 30 kDa and 25 kDa (Skaar *et al.* 1991). Bovine serum IGFBP-2 is high in early lactation and lower in the dry period (Vicini *et al.* 1991) and may be the source of the IGFBP-2 in bovine milk. GH treatment of lactating cows decreased serum IGFBP-2 concentrations whilst increasing milk yield but whether IGFBP-2 is involved in galactopoiesis is unknown (Vicini *et al.* 1991). Similarly, GH administration lowered hepatic IGFBP-2 mRNA and plasma IGFBP-2 in lactating ewes (Klempt *et al.* 1993). Hepatic IGFBP-2 mRNA expression was much higher than that of mammary gland suggesting that in sheep, milk IGFBP-2 is serum derived.

IGF-1 is the predominant IGF in human colostrum but concentrations decreased in the first 2 days of lactation whereas IGF-2 concentrations increased at day 6 (Eriksson *et al.* 1993). During this period IGF-1 and -2 in serum decreased although IGF levels were still much higher than those of milk. Whereas several IGFBPs are detectable in bovine or rat milk, IGFBP-2 is the major IGFBP in human

milk. However on day 1 postpartum IGFBP-3 was detectable in colostrum although maternal serum IGFBP-3 was poorly detectable. IGFBP-2 concentrations were 10-fold higher in milk than maternal serum suggesting that milk IGFBP-2 is made in the mammary gland (Eriksson *et al.* 1993). GH administration to lactating women increased IGF-1 in both serum and milk but IGF-2 or IGFBP-1 levels were unaffected (Breier *et al.* 1993). Similar to its effect in ruminants GH treatment reduced IGFBP-2 in serum of lactating women, although milk levels were unaffected. Although GH did not alter milk IGFBP-3 levels, plasma levels (100 fold higher than milk levels) showed a delayed rise compared with IGF-1 and correlated with milk yield (Breier *et al.* 1993). Thus GH may cause a coordinated rise in plasma IGFBP-3 and mammary gland IGFBP-2, and decrease in plasma IGFBP-2 to facilitate the partitioning of serum IGF-1 to the gland.

1.13.3.2 *In vitro*

In vitro studies of mammary gland tissue generally utilise tissue from pregnant animals induced into lactogenesis *in vitro* by lactogenic hormones (insulin, PRL and cortisol); few studies use fully lactating tissue because milk stasis rapidly leads to cell death. Although GH is the major galactopoietic hormone in the ruminant, administration of GH *in vitro* had no galactopoietic effect on cultured lactating bovine mammary gland (Gertler *et al.* 1983). Nor could IGF-1 affect fatty acid synthesis and alpha-lactalbumin secretion by cultured lactating bovine mammary tissue (Shamay *et al.* 1988). Similarly des-IGF-1 or IGF-2 were not galactopoietic for lactating bovine mammary gland in culture using lactogen responsive fat synthesis as a test, nor did they affect the galactopoietic effects of PRL (Peri *et al.*

1992). However IGF-1 has been shown to increase [^{3}H]thymidine uptake in bovine lactating mammary explants (Baumrucker 1986). Lactating bovine mammary tissue explants synthesise IGFs and a variety of IGFBPs (28-46 kDa), but cultured acini synthesise IGFBP although they release IGF into the medium (Campbell *et al.* 1991); this is similar to findings with tissue from non-lactating pregnant cows.

1.13.4 Breast cancer

1.13.4.1 *In vivo*

Human and animal breast cancer cells have receptors for both steroid and peptide hormones and tumour growth has been shown to be hormone dependent. Clinical studies indicate that patients with tumours expressing high levels of oestogen receptors have a better prognosis than those with receptor negative tumours. However not all receptor positive tumours respond to endocrine treatment suggesting a role for other hormones and growth factors; therefore the interactions of the steroid hormones and growth factors is obviously an area of importance.

The IGFs are among the variety of growth factors involved in the growth of breast cancer cells. IGF-1 is a potent mitogen for these cells and may also elicit a motile response in some breast cancer cell lines (Kohn *et al.* 1990). The majority of studies of the IGF axis in breast cancer tissue have utilised cell lines; primary tissue culture or *in vivo* studies are less well characterised. In clinical studies IGF-2 mRNA is commonly expressed in both tumour and stromal tissue (Yee *et al.* 1988), but IGF-1 mRNA is only expressed by stromal cells (Yee *et al.* 1989). Furthermore fibroblasts from mammary carcinomas preferentially express IGF-2 mRNA rather

than IGF-1 whereas fibroblasts from benign tumours preferentially express IGF-1, suggesting IGF-2 was assoociated with neoplasia (Cullen *et al.* 1991).

Type -1 and -2 IGF receptor mRNA is ubiquitous in tumour homogenates (Cullen *et al.* 1990), although type 1 IGF receptors were localised to the neoplastic tissue rather than the stroma (Pollack & Tremblay 1989). These studies suggest the IGF-1 and IGF-2 from the stroma, and IGF-2 from the tumour cells themselves may act by an autocrine or paracrine mechanism, and since human breast tumour tissue expresses mRNA for IGFBP-2, -3, -4 and -5 (none expressed IGFBP-1), local IGFBP production may modulate IGF action (Yee *et al.* 1991).

IGF and IGFBP expression has been studied in animal models of hormone responsive mammary tumours. In the well characterised nitrosomethylurea-induced rat mammary tumour, IGFs have been identified as having a major role in mediating hormonally stimulated growth *in vitro*. Tumour tissue *in vivo* synthesised mRNA for IGF-2 and IGFBP-2, -3 and -4, whereas normal lactating rat mammary tissue synthesised IGF-2 and IGFBP-2; neither tissue expressed IGFBP-1 (Manni *et al.* 1992). In agreement with the findings in human breast cancer cells, IGF-1 mRNA was not detected in either tumour or normal tissue in this rat model. *In situ* hybridisation studies localised mRNA for IGF-2, IGFBP-5 and -6 to the stromal cells but IGFBP-2 mRNA was expressed by epithelial cells (Manni *et al.* 1994). Ovariectomy caused regression of the tumour and was associated with a marked increase in IGFBP-6 and a smaller increase in IGFBP-2, and these changes were reversible with hormone repletion.

Similar compartmentalised IGFBP expression was demonstrated in the rat insulin-responsive R3230AC adenocarcinoma model. Type 1 and 2 IGF receptors are

expressed by these cells although neither IGF-1 or -2 was mitogenic *in vitro*, however IGF-1 enhanced cell adherance to plastic suggesting a role for IGF-1 in tumour growth. Tumours from normoglycaemic animals expressed IGFBP-2, -5 and lesser amounts of IGFBP-3, -4 and -6. *In situ* hybridisation analysis identified IGFBP-3 mRNA mainly on vascular endothelial cells, IGFBP-4 mRNA mainly on tumour stromal cells, and IGFBP-5 mainly on epithelial cells. This pattern of expression contrasts with that of the nitrosomomethyl-induced tumour described above in which IGFBP-5 mRNA was localised to the stromal cells. R3230AC tumours from diabetic rats showed increased IGFBP-2 mRNA which could be normalised by insulin treatment, although the other IGFBPs were unaffected. Insulin treatment however increased tumour IGFBP-4 and -5 mRNA to higher levels than those of normoglycaemic or diabetic animal. IGFBP-5 expression was decreased in long-term cultured cells but insulin addition to cultures or implantation *in vivo* could increase its expression suggesting a role for insulin as a modulator of IGFBP-5 expression in this mammary tumour (Korc-Grodzicki *et al.* 1993). These data show the differential regulation of IGFBPs by hormones and stresses the importance of stromal-epithelial interactions in controlling IGF action on tumour cells.

1.13.4.2 *In vitro*

A range of human breast cancer cell lines (HBCC) are widely used as model systems in breast cancer research and many studies have investigated the role of IGFs. In general the findings of these studies have agreed with those in human tissues. IGF-2 mRNA is expressed by some HBCC although using an RNAse protection assay recent studies found that none expressed IGF-1 mRNA (Yee *et al.* 1989).

Type 1 and 2 IGF receptors are expressed on both ER-positive and ER-negative lines (De Leon *et al.* 1988) and the potent mitogenic effect of IGF-1 on HBCC probably acts through the type 1 receptor as this effect of can be blocked by anti receptor antibody (Arteaga & Osborne 1989). Anti-IGF-1 antibody treatment *in vivo* inhibited MDA231 cell growth in athymic mice, though MCF-7 cell growth was not inhibited (Arteaga 1992). Interestingly the type 1 IGF receptors in several breast cancer cell lines abundantly express the 105 kDa β-subunit found on foetal cells but not the 95 kDa β-subunit found on adult cells (Alexandrides *et al.* 1993).

Several recent studies have investigated the production of IGFBPs by HBCC, and in general IGFBP production has correlated with oestrogen receptor (ER) status. ER positive lines express IGFBP-2, -4 and -5. ER negative lines express IGFBP-3 and -4, with IGFBP-1 as a minor component, and at least one line expresses IGFBP-5. Expression of IGFBP-6, although less well studied, has been demonstrated in three ER negative lines and also on the ER positive line MCF-7 (Figueroa & Yee 1992; Clemmons *et al.* 1990c; Sheikh *et al.* 1992). IGFBP expression is modulated by IGF-1 treatment of both ER-positive and ER-negative cells. IGF-1 increased IGFBP-2 levels and stimulated cell proliferation in MCF-7 cells (Adamo *et al.* 1992), but in MDA-MB231 cells (ER negative) IGF-1 decreased IGFBP-1 and-2 levels, and increased IGFBP-3 (Camacho-Hubner *et al.* 1991).

Oestrogens modulate IGFs and IGFBPs although its effects depend on ER status. Oestrogen stimulates IGF-2 mRNA expression in ER-positive T47D cells (Yee *et al.* 1988) but not in ER-negative MDA-MB-231 cells (Brunner *et al.* 1989). Anti-oestrogens are believed to antagonise oestrogen effects and to modulate the actions of growth factors such as IGF-1 (Winston *et al.* 1994). Oestradiol is

synergistic with IGF-1 in stimulating growth of MCF-7 cells and combined treatment increases IGFBP-4 and -5 expression (Sheikh *et al.* 1992), whereas anti-oestrogen treatment reduced IGFBP-4 and -5 in ER-positive T47D cells (Coutts *et al.* 1993). Anti-oestrogen treatment reduced type 1 IGF receptors in both ER-positive lines (MCF-7, T47D), but not ER-negative BT20 cells (Freiss *et al.* 1990), suggesting that although anti-oestrogens reduce circulating IGF-1 levels this would mainly affect ER-positive cells.

The retinoids have a wide range of effects which include antagonising the oestrogen stimulation of breast cancer cells. Retinoic acid inhibits IGF-1 stimulation of MCF-7 cell proliferation and greatly increased the levels of IGFBP-3 in conditioned medium. Treatment with retinoic acid alone increased IGFBP-3 mRNA and this was not further increased by combination treatment with IGF-1 suggesting that retinoic acid increases IGFBP-3 transcription whilst the effects of combination treatment on IGFBP-3 are postranscriptional (Fontana *et al.* 1991; Adamo *et al.* 1992).

Several studies have investigated the effects of IGFBPs on cell growth. Chen *et al.* (1994) showed that recombinant IGFBP-3 and -2, but not -4 and -5, enhanced IGF-1 stimulation of MCF-7 cells. In contrast IGFBP-3 treatment had an inhibitory effect on Hs578T cell growth, and the specific binding of IGFBP-3 to cell surface proteins suggest the presence of IGFBP-3 receptors which mediate this inhibitory effect on monolayer growth (Oh *et al.* 1993a; Oh *et al.* 1993b). IGFs can regulate IGFBP-3 in conditioned medium of this cell line (Hs578T) by non-receptor mediated dissociation of cell surface binding and protection from proteolytic activity (Oh *et al.* 1993c). IGFBP-3 proteolytic activity has been detected in the sera of women with

breast cancer (Frost *et al.* 1993) and this may result in IGFBP-3-mediated enhancement of IGF-1 action.

The mitogenic potency of the IGFs make them a potential target for clinical intervention. Cancer cells which are IGF-responsive may respond to endocrine or paracrine IGF expression, and the modulation of IGFBPs descibed above in *in vitro* systems may contribute to the modulation of IGFs effects. However these studies *in vitro* are an over-simplication of the *in vivo* situation which is undoubtedly more complex, therefore a greater understanding of the IGF axis in breast cancer is required before therapeutic agents can be designed.

1.14 Aims of study

The IGFs are of importance to several areas of animal production such as the mediation of the growth-promoting effects of GH. GH exerts effects on a variety of tissues including bone, mammary gland and adipose tissue; GH effects on adipose tissue are direct but its effects on other tissues are thought to be mediated by IGF production.

The galactopoietic effects of GH in dairy cows is well established but the mechanisms involved remain uncertain. It has been suggested that GH may exert its effects through the the IGFs and indeed IGFs are potent mitogens for mammary cells. The mammary gland is responsive to IGFs but their importance may vary with the stage in the mammary gland cycle. There is strong evidence of IGF-1 involvement in mammary gland development during pregnancy; type 1 and type 2 IGF receptors are high, and IGF-1 is mitogenic *in vitro*.

In contrast, although mammary epithelial cells lack functional GH receptors but possess type 1 and 2 IGF receptors, no clear galactopoietic effect of IGF-1 can be shown *in vitro* or *in vivo*. However the increase in milk and mammary gland IGF-1 in goats is correlated with increased milk yield thus supporting a role for IGF-1. IGFBPs have a major role in modulating IGF action, therefore it is plausible that the absence of appropriate IGFBPs (*in vivo* and *in vitro*) accounts for the failure of IGF-1 to mimic GH action. Mammary tissue synthesises IGFBPs and hormonal control has been demonstrated in *in vitro* studies. However the *in vitro* culture of mammary tissue is difficult (particularly from lactating glands) and many studies have used transformed cells. Therefore there is limited information on the IGFBPs produced in the mammary gland, their regulation, and their role in mammary gland biology.

The role of GH during lactation has previously been investigated using hormonally manipulated rats given an antiserum to GH to ablate GH, and bromocriptine to ablate prolactin (PRL) (Madon *et al.* 1986). Although PRL is the major galactopoietic hormone in rats, GH is important, especially when PRL is low and furthermore the importance of GH increases as lactation progresses. The use of a rodent model was necessary to enable *in vivo* hormone replacement studies which would be impossible in larger animals. This model has subsequently been used to investigate if the galactopoietic effect of GH are mediated by IGFs, and to study the hormonal control of IGFBPs during lactation; work contained in this thesis forms part of these studies (Flint *et al.* 1992; Flint *et al.* 1994). The major aim of this thesis was to investigate the IGF axis in the rat during lactation and involution with emphasis on the role of the IGFBPs.

In many species the mother has an increased susceptibility to parasitic and fungal infections during the pre- and post-partum period with detrimental effects on the welfare of both the dam and the neonates dependent on the milk. A role for GH and the IGFs has been implicated in the immune response, however few studies have used farm species although altered immunity would be of importance to animal welfare and hence production. It is feasible that modulation of the IGFs and their binding proteins may be involved in the immunosuppression of pregnancy and lactation. Therefore this study aimed to establish a role for IGFs and their binding proteins in the immune system of sheep which would then provide a basis for investigating the IGF axis in the immune system during pregnancy and lactation.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 General laboratory chemicals and reagents

General reagents were obtained from BDH (Thornliebank, Glasgow, UK) or Fisons (Loughborough, UK), and most other chemicals were from Sigma (Poole, Dorset, UK) unless stated otherwise. Molecular biology reagents were from Promega (Southhampton, UK) or Boehringer (Lewes, UK). Tissue culture media and supplements were from Gibco (Paisley, UK) or ICN Biomedicals (High Wycombe UK). Plasticware was from Greiner Labortechnik Ltd (Glos, UK) Water was double distilled tap water.

2.1.2 Animals

Wistars rats were allowed free access to food (Labsure irradiated CRM diet, Labsure, Poole, Dorset, UK) and water. Sheep were Finn-Dorset cross given free access to hay plus 400g/day cereal mix for at least four weeks prior to slaughter. Dutch breed rabbits were housed in groups and allowed free access to food (RG1 diet, BS & S, Edinburgh, UK) and water.

2.1.3 Radionuclides

Iodinations were carried out using the iodogen method (Fraker *et al.* 1986). IGF-1 or IGFBP-3 (2.5 μg) was added to 0.5 mCi ^{125}I in an 20 μl volume to an Iodogen (Pierce) coated tube (1 mg/30 μl chloroform) for 20 min before adding 100 μl KI (200 mg/ml) and 100 μl of phosphate buffer containing 0.5% BSA. The mixture was then removed and passed down a Sephadex G10 column to separate labelled protein

from free iodine. Specific activities were 100-280 μCi/μg for IGFs, and about 100 μCi/μg for IGFBP-3. Gamma counting used a Cobra auto-gamma counter (Canberra Packard, Pangbourne, UK)

2.1.4 Cell lines

Clone 9 hepatocyte cell line derived from normal rat liver (ATCC code CRL 1439) was obtained frozen from the European Collection of Animal Cell Cultures (ECACC, PHLS, Porton Down, UK).

2.1.5 IGF and IGFBP peptides

Recombinant human IGF-1 was purchased from Bachem (UK), Saffron Walden, Essex, UK and Peninsula Laboratories Europe, St Helens, Merseyside, UK. Recombinant human IGF-2 was a gift from Monsanto Europe, Brussels, Belgium. Recombinant human IGFBP-3 (*Escherichia coli* expressed) was a gift from Celltrix Pharmaceuticals, Santa Clara, CA, USA. IGF-1 analogues, Long IGF-1, R_3IGF-1, and Long R_3-IGF-1, were a gift from Dr J Ballard, Cooperative Centre for Tissue Growth and Repair, Adelaide, Australia.

2.1.6 Anti-IGF-1 and IGFBP antisera

Polyclonal rabbit antisera raised against purified bovine IGFBP-2 was purchased from TCS Biologicals (Bucks, UK). Antisera to a synthetic peptide sequence of rat IGFBP-2 was a gift from Dr N. Ling (The Whittier Institute, Dept of Molecular Endocrinology, La Jolla, CA). Polyclonal guinea-pig antisera raised against human IGFBP-5 was a gift from Dr D.R. Clemmons, Dept Medicine, University of North

Carolina, North Carolina). Polyclonal rabbit anti-rhIGF-1 was a gift from NIDDK (Bethesda Maryland, USA).

2.1.7 IGF-1 and IGFBP nucleic acid probes

IGF-1 mRNA was detected using either a ^{32}P-labelled DNA probe derived from a rat cDNA cloned into pBluescript (Davenport *et al.* 1990) or a ^{32}P-labelled RNA probe generated from this using T7 RNA polymerase (Zinn *et al.* 1983). Dr S. Shimasaki (The Whittier Institute, Dept of Molecular Endocrinology, La Jolla, CA) kindly provided constructs of IGFBP fragments subcloned into pBluescript: IGFBP-1, 407 bp; IGFBP-2, 397 bp; IGFBP-3, 699 bp; IGFBP-4, 444 bp; IGFBP-5, 300 bp; IGFBP-6, 245 bp. Sense and antisense IGFBP riboprobes were generated from these using T7 or T3 polymerase depending on the orientation of the clone.

2.2 Methods

2.2.1 Cell line culture

Conditioned medium containing IGFBP-2 was prepared from Clone 9 cells. Cells at passage 21 or 22, were grown in Hams-F12 supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C in 5% CO_2/95% air. When cells were confluent conditioned medium was prepared by washing the monolayer in Hanks balanced salt solution (HBBS), and incubating in basal Hams F-12 supplemented with antibiotics (in some cultures with 0.5% BSA) for 48 h. Medium was then harvested and centrifuged at 13,000 x *g* for 10 min, then frozen in liquid nitrogen and stored at -20 °C.

2.2.2 DNA measurement

DNA was measured using the method of Labarca & Paigen (1980). Tissue was homogenised on ice in 4 volumes of extraction buffer (2 M NaCl, 50 mM NaH_2PO_4, pH 7.4) using an ultraturax homogeniser. The homogenate was then diluted 1/100 in buffer, and a 500 μl sample of this was mixed with Bisbenzamidine (Fluka H33258) at a final concentration of 1 μg/ml for 1 h before reading in a fluorimeter set at 355 nm exitation, 445 nm emission.

2.2.3 Protein assay

Protein estimates were made using Biorad Protein Assay reagent, a commassie brilliant blue G-250 dye-binding assay. Samples were incubated for 10 min in a 50 μl volume with 240 μl Biorad reagent diluted 1:4 in distilled water, and absorbances at 600 nm were read in a Titertek Twinreader (Flow labs). Protein content was determined using a standard curve of 1-5 μg BSA or casein for milk samples.

2.2.4 IGF-1 Radioimmunoassay (RIA)

IGF-1 concentrations were determined by a non-equilibrium RIA using a modified double antibody technique. IGFBPs were extracted by adding 200 μl of extraction medium consisting of 2 N HCl:95% ethanol (1:7 v/v), to 50 μl of sample, mixing and incubating at room temperature for 30 min. After centrifuging at 1700g for 5 min, 100 μl of neutralisation buffer (330 mM Tris) was added. When serum samples were used 20 μl neutralised sample was diluted 10-fold in dilution buffer (4 ml extraction buffer:5 ml neutralisation buffer in 100 ml RIA buffer). RIA buffer consisted of 60 mM $NaPo_4$ pH 7.4, 0.9% w/v NaCl, 0.5% w/v BSA, 0.1% w/v

NaN_3 or 0.1% w/v thiomersal. Diluted sample (100 μl) was mixed with 100 μl of a 1/2000 dilution of polyclonal rabbit anti-rhIGF-1 and pre-incubated for 24 h at room temperature before adding 100 μl [^{125}I]IGF-1 (20,000 cpm/tube in RIA buffer) for a further 24 h. Complexed IGF-1-antibody was precipitated by adding 300 μl of donkey anti-rabbit IgG precipitating serum (Scottish Antibody Production Unit, Carluke, Strathclyde, UK) diluted in RIA buffer:16% polyethylene glycol:antibody (15:15:1), containing 10 mM EDTA. After incubating at room temperature for 4 h, tubes were centrifuged at 1700 x *g* for 30 min, the supernatant was decanted and the radioactivity in the pellets counted. The assay was validated for use in rat serum by demonstrating parallelism of displacement by sample and standard after acid-ethanol extraction.

2.2.5 Solution phase assay for IGFBP activity

IGFBP activity was measured as previously described by Conover *et al.* (1989). Samples in a 200 μl volume were mixed with 100 μl of [^{125}I]IGF-1 (20-30,000 cpm) in 50 mM Tris HCl pH 7.4 containing 0.5% BSA and incubated overnight at 4°C. Bound and unbound radiolabel were separated by adding 300 μl 1% activated charcoal containing 0.2 mg/ml protamine sulphate, incubating at 4°C for 10 min, then centrifuging at 1700 x *g* for 10 min at 4°C. The supernatants were then decanted and the pellets counted. Non-specific binding was defined as the amount bound to buffer or unconditioned medium, and this was subtracted from the total bound radioactivity to the samples to determine specific binding activity.

2.2.6 IGFBP-3 proteolytic activity

Proteolysis was assayed as previously described by Lamson *et al.* (1991). Samples were incubated with 30,000 cpm [^{125}I]IGFBP-3 at 37 ° C for 5 h in a water bath,

then electrophoresed on a 12.5 % SDS-PAGE, the gel was then dried and exposed to film for 6-18 h.

2.2.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method of Laemmli (1970). Samples were prepared by boiling for 3 min in sample buffer composed of 62.5 mM Tris-HCl (pH 6.8), 0.1% SDS, 10% glycerol, and 0.05% bromophenyl blue. Where reducing conditions were required 5% 2-mercaptoethanol was included in the sample buffer.

Separating gels consisted of 10% or 12.5% total acrylamide (2.7% Bis-acrylamide crosslinker), 375 mM Tris-HCl (pH 8.8), 0.1% SDS (w/v). Stacking gels consisted of 4% acrylamide, 125 mM Tris-HCl (6.8), 0.1% SDS. Polymerisation was initiated by adding ammonium persulphate (Biorad) and TEMED. Samples were electrophoresed at 100-150 V on a Biorad mini protean II, using running buffer consisting of 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 0.1% SDS.

2.2.8 Western blotting

Western blotting was carried out as in Towbin *et al.* (1979). Following electrophoresis gels were equilibrated for 15 min in transfer buffer consisting of 25 mM Tris, 192 mM glycine, 20% methanol at 4 °C. Blotting medium was nitrocellulose, either 0.2 μm pore size (Biorad transblot transfer membrane) or 0.45 μm (Biotrace, Gelman). The nitrocellulose filter was pre-wetted in transfer buffer, the gel sandwich assembled and transfer was carried out using a Biorad mini transblot cell at 100 V for 1 h. After transfer the molecular weight markers (Sigma SDS-7) were visualised by staining with 0.02% Ponceau S (0.3% TCA/0.3%

sulphosalicylic acid), and their position recorded in pencil before eluting the Ponceau S by incubating in Tris buffered saline (TBS). Blots were then air dried and stored at 4 °C until used. The efficiency of transfer was assessed by staining the gels in Coomassie Brilliant Blue R in 40% methanol, 10% acetic acid, and destained in 40% methanol, 10% acetic acid.

2.2.9 Western ligand blotting

Western ligand blotting was carried out essentially as in Hossenlopp *et al.* (1986). After SDS-PAGE and Western blotting, blots were wetted in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl (TBS), blocked in 3% Nonidet P-40 in TBS for 30 min, 1% BSA in TBS for 2 h, then 0.1% Tween 20 in TBS for 10 min, all at 4 °C. Blots were then incubated overnight at 4 °C in a sealed plastic bag with [^{125}I]IGF-1 (2 x 10^6 cpm/ml in TBS with 1% BSA, 0.1% Tween 20. After incubation the blot was washed twice in TBS/0.1% Tween and three times in TBS (15 min each wash) then air dried and exposed to film at -80 °C or a PhosphoImager screen at room temperature.

2.2.10 Western immunoblotting

Western blots for IGFBP-2 detection were blocked for 30 min in 2% dried milk powder (Marvel) in TBS pH 7.4, then incubated with 1/1000 dilution anti-IGFBP-2 serum in TBS/0.2% Marvel at room temperature for 1-2 h. The blots were then washed for 10 min using 4 changes of TBS and incubated at room temperature for 45 min with affinity purified alkaline phosphatase conjugated anti-rabbit IgG antiserum diluted in TBS/0.2% Marvel. The blot was washed as above and rinsed

in alkaline phosphatase substrate buffer consisting of 100 mM Tris/HCl pH 9.5, 5 mM $MgCl_2$, 5 mM $ZnCl_2$. Substrate consisting of 0.4 mM nitro blue tetrazolium/0.38 mM BCIP (5 bromo-4-chloro-3-indolyl phosphate) in substrate buffer was added and incubated at room temperature for 20 min or until colour developed.

Western blots for IGFBP-5 detection were blocked in 3% Nonidet P-40 in TBS for 30 min, 1% BSA in TBS for 2 h, 0.1% Tween 20 in TBS for 10 min, all at 4 °C. Blots were then incubated overnight at 4 °C in a sealed plastic bag with 1/1000 dilution of anti-IGFBP-5 serum in TBS with 1% BSA, 0.1% Tween 20. The blots were then washed for 10 min using 4 changes of TBS/0.1% Tween and incubated at room temperature for 45 min with affinity purified peroxidase conjugated anti-guinea-pig IgG antiserum diluted in TBS/1% BSA/0.1% Tween 20. The blot was washed as above and incubated for 1 min with Enhanced Chemiluminesence (Amersham) reagent, then exposed to Reflection film (Du Pont) at room temperature.

2.2.11 RNA isolation

RNA was isolated from tissues using guanidinium isothiocyanate homogenisation and caesium chloride centrifugation (Chirwin *et al.* 1979). Tissues were collected and immediately frozen in liquid nitrogen. Tissue was ground to a fine powder in liquid nitrogen and added to an appropriate amount of denaturing solution (1 M guanidinium isothiocyanate, 50 mM Tris-HCl, 10 mM EDTA, 2% sodium lauryl sarkosine, 0.1% 2-mercaptoethanol) while frozen, the mixture was then passed through an 19g and then a 23g needle to disperse the tissue and shear the DNA.

Solid caesium chloride was dissolved in the homogenate (to a final 40% w/v) which was then overlaid on a cushion of 5.7 M caesium chloride/100 mM EDTA pH 7.5 and centrifuged at 35,000 rpm (150,000 x *g*) for 18 h. The RNA pellet was resuspended in water and an equal volume of chloroform/butanol (4:1) added, then mixed and centrifuged at 4000 x rpm for 5 min at 4° C. This extraction was repeated, the aqueous layers were collected and added to 0.1 volume of 3 M sodium acetate pH 5.3 and 2.5 volumes of absolute alcohol and precipitated at -20 °C overnight. RNA was recovered by centrifugation at 12,000 x *g* for 20 min at 4 °C, freeze dried and resuspended in water. RNA concentrations were determined by measuring absorbance at 260 nm.

2.2.12 Northern blotting

RNA (usually 20-40 μg) was resolved on a 1.2% agarose gel (20 mM MOPS pH 7.0, 5 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde, 0.5 μg/ml ethidium bromide), for 1-2 h. Samples were heated at 65 °C for 5 min in a 10 μl volume with denaturing buffer (6.6 M formaldehyde, 50% formamide, 20 mM MOPS pH 7.0, 5 mM NaCl, 1 mM EDTA), then cooled and loading buffer added (final 0.08% bromophenol blue, 5% Ficoll 400). The gel was run in 20 mM MOPS pH 7.0, 5 mM sodium acetate, 1 mM EDTA at 70 V for 1-2 h. Gels were photographed and rinsed in 10 x standard saline citrate (SSC) for 20 min (1 times SCC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) before diffusion blotting overnight onto Hybond N nylon filters (Amersham). RNA was bound to the filter using a Spectrolinker XL-1000 UV crosslinker (Spectronics Corporation, New York, USA).

2.2.13 Probe preparation for Northern analysis

A labelled IGF-1 cDNA probe was prepared by incubating 25 ng denatured insert DNA with 1 U of Klenow fragment *E. coli* DNA polymerase (BRL 8012), 25 μCi ^{32}P dCPT, 2.7 OD units/ml random oligonucleotide primers, 10 μM dATP/dTTP/dGTP, 200 μg/ml BSA, 0.04% 2-mercaptoethanol, 25 mM Tris HCl pH 8.0, 2.5 mM $MgCl_2$, 100 mM HEPES pH 6.0 in a final volume of 50 μl at room temperature for 2.5 h. The reaction mixture was then diluted to 100 μl with 50 mM NaCl, 1 mM EDTA pH 7.5 and the labelled probe was separated from incorporated label using a Sephadex G-50 column.

Labelled IGF-1 and IGFBP riboprobes were prepared by incubating 1 ng linearised plasmid DNA template with 10 U RNA polymerase, 20 μCi ^{32}P CTP, 12 μM CTP, 0.5 mM GTP/ATP/UTP, 20 U ribonuclease inhibitor, 10 mM dithiothreitol (DTT), 2 mM spermidine, 20 μg/ml BSA, 10 mM NaCl, 6 mM $MgCl_2$ and 40 mM Tris HCl pH 7.5 in a final reaction volume of 10 μl for 90 min at 37 °C. The reaction was stopped by adding 90 μl 50 mM NaCl, 1 mM EDTA pH 7.5 and the labelled probe was separated from incorporated label using a Sephadex G-50 column.

2.2.14 Hybridisation to Northern blots

Northern blots were prehybridised for 4 h at 55 °C in a solution containing 50% formamide, 900 mM NaCl, $NaPO_4$ 50 mM pH 7.0, 5 mM EDTA, 2 mg/ml Ficoll 400, 2 mg/ml BSA, 2 mg/ml polyvinylpyrrolidone, 0.1% SDS, 200 μg/ml denatured salmon sperm DNA and in addition, 0.1 mg/ml tRNA was used with RNA probes. Blots were hybridised overnight with 1 x 10^6 cpm/ml ^{32}P-labelled probe in the above

solution at 42 °C for the DNA probe and 55 °C for riboprobes. The DNA probe was heat denatured before hybridisation.

After hybridisation blots were washed three times in 2 x SSC for 15 min at room temperature, incubated in 2 x SSC containing 1 μg/ml RNAse A for 30 min at room temperature before a final wash at 55-65 °C in 0.2 x SSC/0.1% (w/v) SDS for 30 min. Blots were then exposed to film at -80 °C.

2.2.15 Autoradiography and densitometry

Western ligand blots and Northern blots were wrapped in Saranwrap and exposed to Hyperfilm MP (Amersham) film at -80 °C using enhancing screens. Film was then developed for 3 min in developer and fixed for 3 min (Kodak). Autoradiographs were analysed by densitometry using either a Biorad 620 Video Densitometer and 1-D Analysis software (Herts, UK) or a Molecular Dynamics Personal Densitometer SI with Image-Quant software (Molecular Dynamics Ltd, Kensing, UK). The area under the peaks was measured, and expressed as arbitrary OD units normalised for control samples run on each gel.

Some Western ligand blots were exposed to a Molecular Dynamics PhosphoImager screen at room temperature and the image detected by a PhosphoImager 445SI and analysed by Image-Quant software. Quantification by densitometry or the PhosphoImager used bands within the linear range; the PhosphoImager screen has the advantage of a linear range 200 times greater than that of X-ray film.

CHAPTER 3: THE IGF AXIS IN CELLS OF THE SHEEP IMMUNE SYSTEM

3.1 Introduction

A role for GH and IGF-1 in the immune response has been demonstrated in several species but few studies have addressed the role of the IGFBPs. Little is known about the IGF axis within the sheep immune system therefore the production of IGF-1, type-1 IGF receptor, IGFBPs, and IGFBP-3 protease were examined in order to establish a role for the IGFs in the sheep immune response.

3.2 Materials and Methods

3.2.1 Isolation of cells

Blood and tissues were obtained from Finn-Dorset cross sheep by venepuncture or at slaughter. Thymus, peripheral lymph node and mesenteric lymph node mononuclear cells (MNCs) were prepared by density gradient centrifugation (Boyum 1968), using Lymphopaque (1.086 g/L, Nycomed UK, Birmingham, UK). Minced tissue was pushed through a wire mesh into RPMI, the cell suspension was pelleted, washed in RPMI at 300 x *g* for 10 min, before resuspending in RPMI, layering on a lymphopaque gradient and centrifuging for 30 min at 800 x *g*. The interface layer of cells was diluted 1/10 in RPMI, pelleted at 400 x *g* for 10 min, and then washed repeatedly at 200 x *g* for 10 min to minimise platelet contamination. Centrifugation at 200 x *g* pelleted white cells and left a platelet rich supernatant. Platelets were prepared from heparinised blood either from the interface layer of lymphopaque density gradients as above or directly from diluted blood (in EDTA containing

buffer) by centrifuging at 250 x *g* for 20 min to obtain a platelet rich supernatant, which was then centrifuged at 900 x *g* for 10 min to pellet platelets. For some platelet preparations Hepes buffer (100 mM Hepes, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM dextrose, 15 mM acetate, 2 mM EDTA pH 7.4) was used instead of RPMI.

Platelet-depleted defibrinated blood was centrifuged at 1600 x *g* for 20 min, and the buffy coat diluted 1/2 in RPMI before loading on a lymphopaque gradient. Peripheral blood MNC were obtained from the interface, washed and the red cells lysed by resuspending in prewarmed Tris-NH_4Cl (144 mM ammonium chloride, 17 mM Tris pH 7.2) for 15 sec, diluting in RPMI and washing cells twice at 300 x *g* for 10 min. Granulocytes were then isolated from the lymphopaque gradient by removing the ficoll layer, resuspending the pellet in RPMI, centifuging at 600 x *g*, then lysing RBC as above. Wrights stained cytocentrifuge preparations of the cell fractions were assessed by differential white blood cell counts.

3.2.2 Cell culture

Cells were cultured in Iscoves medium (Iscove's modification of Dulbeccos medium with bovine albumin, human transferrin and soy bean lecithin, ICN Biomedicals, High Wycombe, Bucks. UK) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (ICN Biomedicals) at 2 x 10^6/ml with Con A (Sigma C2575), PHA (Sigma L-9132), and IGF-1 where appropriate, at 37 °C in 95% air:5% CO_2. For some cultures Iscoves medium consisted of Iscoves modified Eagles medium (Northumbria Biologicals, Cramlington, Northumberland) supplemented

with 400 μg/ml soyabean lecithin, 400 μg/ml BSA, 1 μg/ml bovine transferrin (Sigma T5761), 100 u/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine.

Conditioned medium was prepared by harvesting medium from 24 well-plate cultures after 24, 48 and 72 h, which was then centrifuged at 300 x *g* for 10 min (to pellet cells) then 14,000 x *g* (to pellet cell debris), snap frozen and stored at -20 °C. Cell stimulation was measured in corresponding 96-well cultures by adding 1 μCi [^{3}H]thymidine for the last 4 h of culture, then harvesting on an automatic cell harvester onto filter paper discs. Discs were tranferred to vials and Emulsifier Safe scintillation fluid added, then counted on a 1600 TR Liquid Scintillation Analyser (Canberra Packard). [^{3}H]thymidine uptake assay in 96-well cultures was used to determine the appropriate concentration of mitogen to stimulate cells.

3.2.3 Solution phase assay for IGFBP activity

Conditioned media from thymus cell cultures (200 μl) were assayed as in Chapter 2. Unconditioned medium was assayed in parallel and binding to it was subtracted from the total bound radioactivity in conditioned medium to determine specific binding activity.

3.2.4 IGFBP protease activity

Conditioned media from unstimulated, PHA-stimulated, and Con A-stimulated thymus cell cultures were assessed for protease activity as described in Chapter 2. Samples (15 μl) were used and where appropriate incubation was in the presence of the protease inhibitors 10 mM EDTA, 2 mM (saturated) phenylmethylsulphonyl fluoride

(PMSF), 10 mM N-α-ρ-tosyl-l-lysine chloromethyl ketone (TLCK), 1 mg/ml leupeptin (Peptide Institute, Osaka, Japan) or 0.4 mg/ml Aprotinin.

3.2.5 Western ligand blotting

WLB was performed as described in Chapter 2. Conditioned medium from peripheral lymph node and thymus cultures were used neat on gels (20 μl), or lyophilised and the equivalent of 50 μl loaded on a track. IGFBP activity was diminished on storage (-20 °C) or after dialysis against TBS, possibly because of proteolysis although dialysis was carried out at 4 °C.

3.2.6 Western immunoblotting

Western blotting was performed as decribed in Chapter 2, and the blots were then immunostained with antisera to bovine IGFBP-2 (UBI) at a 1/1000 dilution.

3.2.7 [^{125}I]IGF-1 cell binding assay

Most assays were performed in RPM1 1640 containing 1% BSA 25 mM Hepes pH 7.4. Some assays of platelet binding used Hepes buffer (as above) containing 1% BSA and 1 mM EDTA. 400 μl of cell suspension and 50 μl of [^{125}I]IGF-1 (20-30,000 cpm) with 50 μl of unlabelled peptide where appropriate were incubated overnight at 4°C. Cells were then centrifuged at 400 x *g* for 10 min (1000 x *g* for platelets) twice and the pellets counted. Specific binding was calculated by subtracting nonspecifically bound radioactivity from the total bound. Nonspecific binding was determined as radioactivity bound in the presence of excess (1 μg/ml) unlabelled IGF-1 (Zhao *et al.* 1992).

3.2.8 Northern analysis

IGF-1 mRNA was measured in RNA isolated from lymph node, thymus, spleen, and liver as described in Chapter 2.

3.2.9 IGF-1 Radioimmunoassay (RIA)

This was carried out as described in Chapter 2. Conditioned medium (5 μl) from peripheral lymph node cultures were assayed after acid ethanol extraction against a standard curve ranging from 0.5-16 ng/ml which was diluted in Iscove's medium. WLB of conditioned medium from these cultures showed no evidence of IGFBPs, therefore conditioned medium was subsequently assayed again without extraction, allowing a greater volume (100 μl) to be used in the RIA.

3.3 Results

3.3.1 IGFBP production by cultured thymus cells

Conditioned media from thymus cells cultured with or without PHA or Con A were assayed for IGFBPs using a solution phase assay of [^{125}I]IGF-1 binding. IGFBP activity increased with time in unstimulated, PHA-stimulated and Con A-stimulated cultures. Both PHA- and Con A-stimulation significantly increased the IGFBP activity compared with unstimulated cultures (Fig 3.1A). Cell stimulation by PHA and Con A was verified by [^{3}H]thymidine uptake (Fig 3.1B). WLB of conditioned medium from stimulated cultures detected a single band of about 24 kDa, binding to which could be competed by unlabelled IGF-1 (not shown).

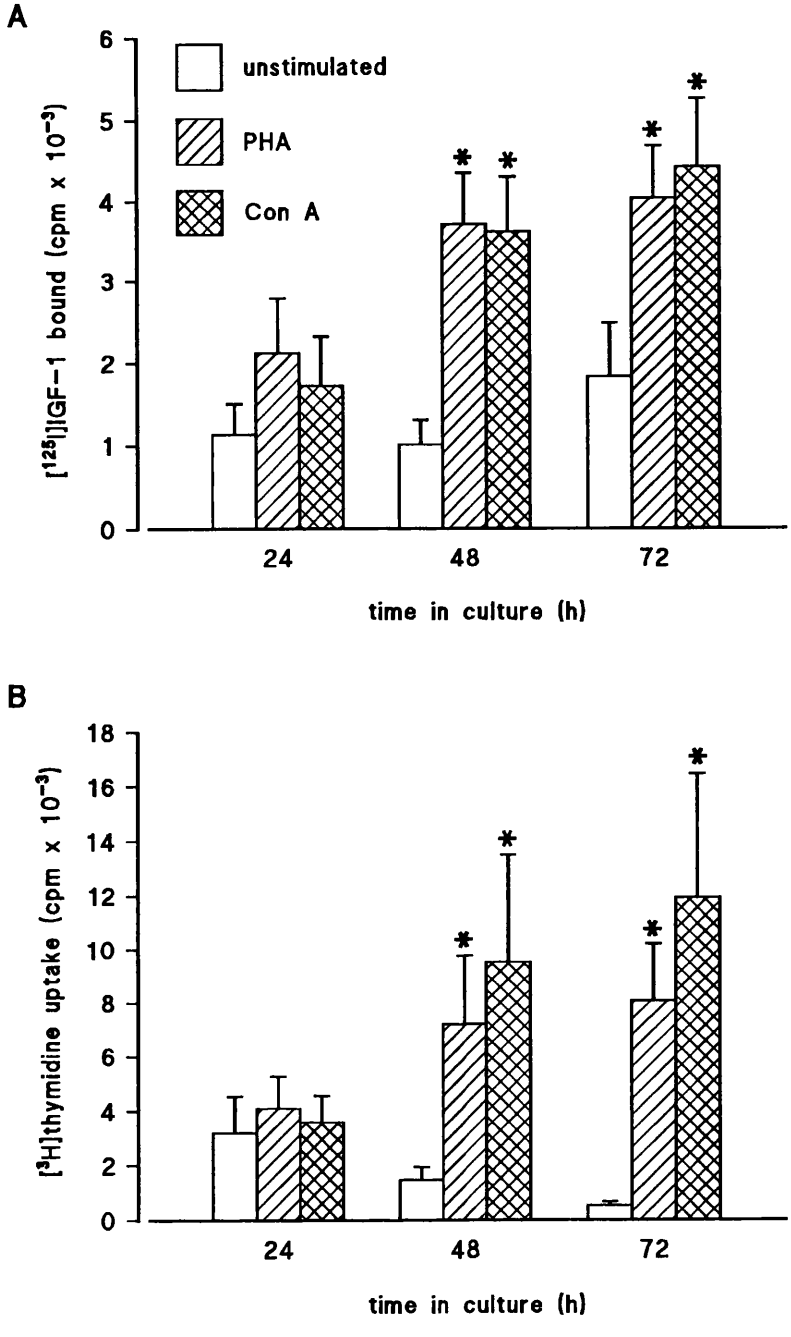


Fig 3.1 Production of IGFBP and [^{3}H]thymidine incorporation by thymus cell cultures. (A) Conditioned medium was collected at 24, 48 and 72h and IGFBP concentrations assayed by solution phase [^{125}I]IGF-1 binding. Results are expressed as the mean ± SEM (n = 6 for unstimulated and PHA; n = 5 for Con A). Data was analysed using Genstat REML (Residual Maximum Likelihood); *$P < 0.01$ compared with unstimulated cells. (B) [^{3}H]thymidine incorporation was measured by culturing cells for 24, 48 or 72h and adding 0.5 μCi [^{3}H]thymidine for the last 4h before harvest. Results are the mean ± SEM (n = 5). Data was analysed using Genstat ANOVA (Analysis of variance), *$p < 0.01$ compared with unstimulated cells.

3.3.2 IGFBP-3 Protease activity

IGFBP-3 protease activity in conditioned media from thymus cells cultured for 72 h with or without PHA or Con A was assessed using iodinated recombinant hIGFBP-3 as a substrate. Conditioned media from PHA-stimulated cultures degraded [^{125}I]hIGFBP-3 to lower molecular weight products; degradation could be inhibited markedly by PMSF, and to a lesser extent by Aprotinin and TLCK (Fig 3.2). Leupeptin and EDTA had little effect. Conditioned medium from unstimulated and Con A-stimulated cultures also degraded [^{125}I]hIGFBP-3 to lower molecular weight products (not shown). In the presence of unconditioned medium some of the radioactivity remained at the top of the gel but this was not seen with any other samples, including conditioned medium from unstimulated cells and buffer control.

3.3.3 [^{125}I]IGF-1 binding to cells

Specific binding was demonstrated on cells from the thymus and mesenteric lymph node, peripheral blood MNC, erythrocytes and platelets (Table 3.1). Binding to platelets was lower than to mesenteric lymph node or thymus cells, and binding to erythrocytes was very low. Specific binding was also demonstrated on granulocyte enriched preparations from defibrinated blood. Table 3.2 shows the proportion of granulocytes and MNC, and relative specific binding. Although granulocyte preparations were heavily contaminated with mononuclear cells (61%), specific binding was higher in the granulocyte preparations compared with the mononuclear cell preparation, which suggested that the granulocyte content was contributing to the specific binding.

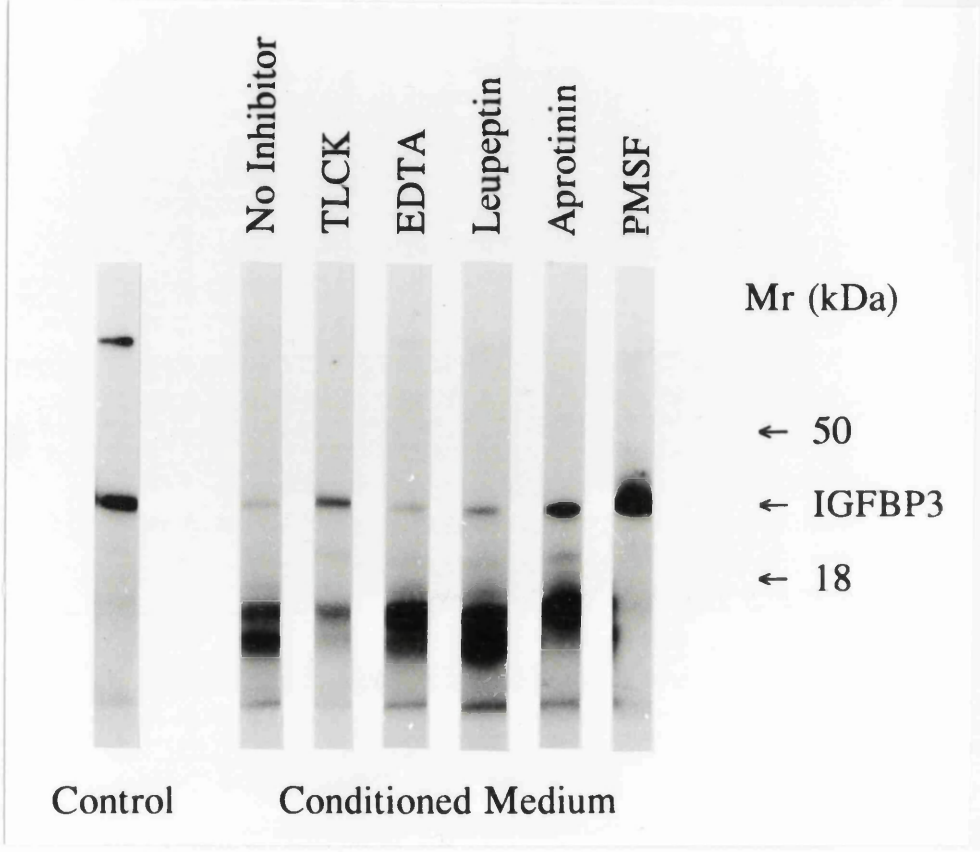


Fig 3.2 Proteolysis of [^{125}I]IGFBP-3 by conditioned medium from thymus cells cultured for 72 hours with PHA. Samples (12 μl) were incubated with 30,000 cpm recombinant non-glycosylated [^{125}I]hIGFBP-3 for 5h at 37°C. Incubations were conducted in the presence of 10 mM TLCK, 10 mM EDTA, 1 mg/ml Leupeptin, 0.4 mg/ml Aprotinin or 2 mM PMSF (saturated). The control sample is unconditioned medium. After incubation samples were separated by SDS-PAGE, the gel dried under vacuum and autoradiographed for 18 h.

	Specific [^{125}I]IGF-1 binding (fmol per 10^{10} cells)[a]
Mesenteric lymph node	4.73 ± 0.13 (3)
Thymus	4.64 ± 0.88 (5)
Peripheral blood MNC	2.42 ± 0.72 (10)
Platelets	0.80 ± 0.11 (9)
Erythrocytes	0.02 ± 0.02 (8)

[a]Data was normalised for the total radioactivity added.

Table 3.1 Comparison of [^{125}I]IGF-1 binding to cells from peripheral blood, mesenteric lymph node and thymus. Results are mean ± SEM, the number of animals is shown in parentheses.

	Specific [^{125}I]IGF-1 binding	percentage cell type	
	(fmol per 10^{10} cells)[a]	mononuclear	granulocyte
cell fraction			
mononuclear	1.43 ± 0.57	99.8 ± 0.0	0.0 ± 0.0
granulocyte	3.21 ± 0.12	61.9 ± 11.2	38.1 ± 11.6

[a] Data was normalised for the total radioactivity added.

Table 3.2 Comparison of [^{125}I]IGF-1 binding to cell preparations from defibrinated peripheral blood. Results are mean ± SEM for three animals.

The relative potencies of IGF-1, IGF-2 and insulin as competitive inhibitors of [^{125}I]IGF-1 binding to platelets were determined (Fig 3.3). Binding was inhibited 50% by 1.8 ng/ml IGF-1, 2.3 ng/ml IGF-2 and 4.4 μg/ml of insulin. Data from six IGF-1 dose response curves were analysed by the LIGAND program using a one site model (Fig 3.4) giving a Kd of 266 pM and about 40 binding sites per platelet.

3.3.4 IGF production

IGF-1 was undetectable (less than 0.5 ng/ml) in conditioned medium (24, 48, 72 h) from stimulated and unstimulated peripheral lymph node cells when either neat, or acid-ethanol extracted samples were analysed. Cell stimulation was verified by [^{3}H]thymidine uptake and Fig 3.5 shows their response to dilutions of polyclonal activators. In corresponding cultures for conditioned medium production, cells at 2 x 10^{6}/ml were stimulated with 0.5 μg/ml Con A and 5 μg/ml PHA (Fig 3.5A), and where 4 x 10^{6}/ml cells were used Con A was at 0.2 μg/ml and PHA at 1.56 μg/ml (see Fig 3.5B).

3.3.5 Effects of IGF-1 on cell stimulation

Con A and PHA stimulated uptake of [^{3}H]thymidine by peripheral blood MNC was enhanced by IGF-1 at 10 ng/ml ($p < 0.02$) (Fig 3.6A). The presence of IGF-1 did not affect [^{3}H]thymidine uptake by mesenteric lymph node cells (Fig 3.6B).

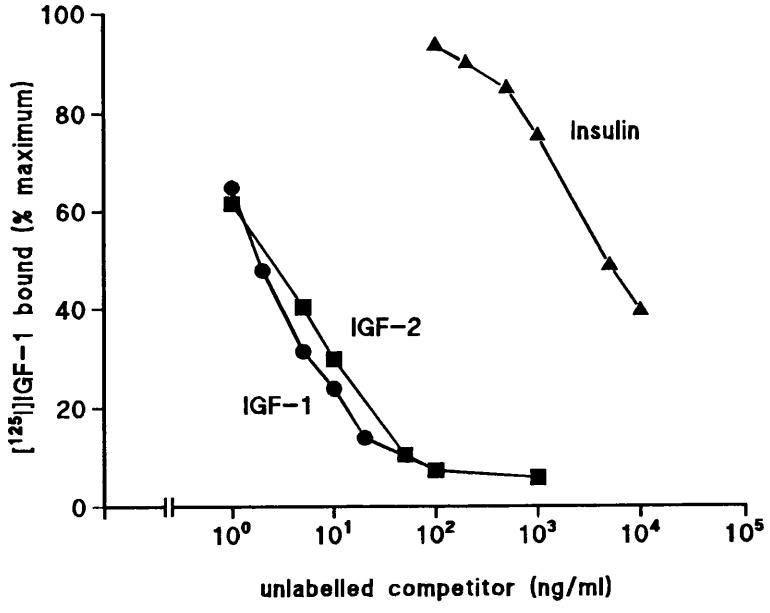


Fig 3.3 Inhibition of [^{125}I]IGF-1 binding to platelets by unlabelled peptides. Binding was expressed as a percentage of maximal specific binding determined in the absence of any competing unlabelled peptide, which was 9.5% of input radioactivity. Nonspecific binding (binding in the presence of 1 μg/ml unlabelled peptide) representing 11.25% of the maximum [^{125}I]IGF-1 bound was subtracted from the data. Results are mean values for six experiments.

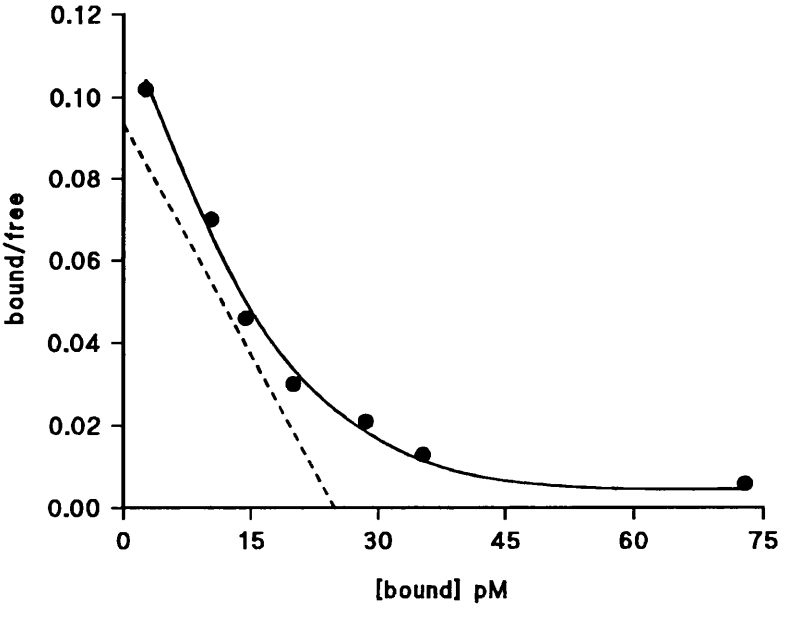


Fig 3.4 Scatchard analysis of [^{125}I]IGF-1 binding to platelets. Analysis was performed with the LIGAND program, a one site model was fitted to the pooled results from six experiments. The calculated number of receptor sites per cell was about 40 and the Kd was 266 pM.

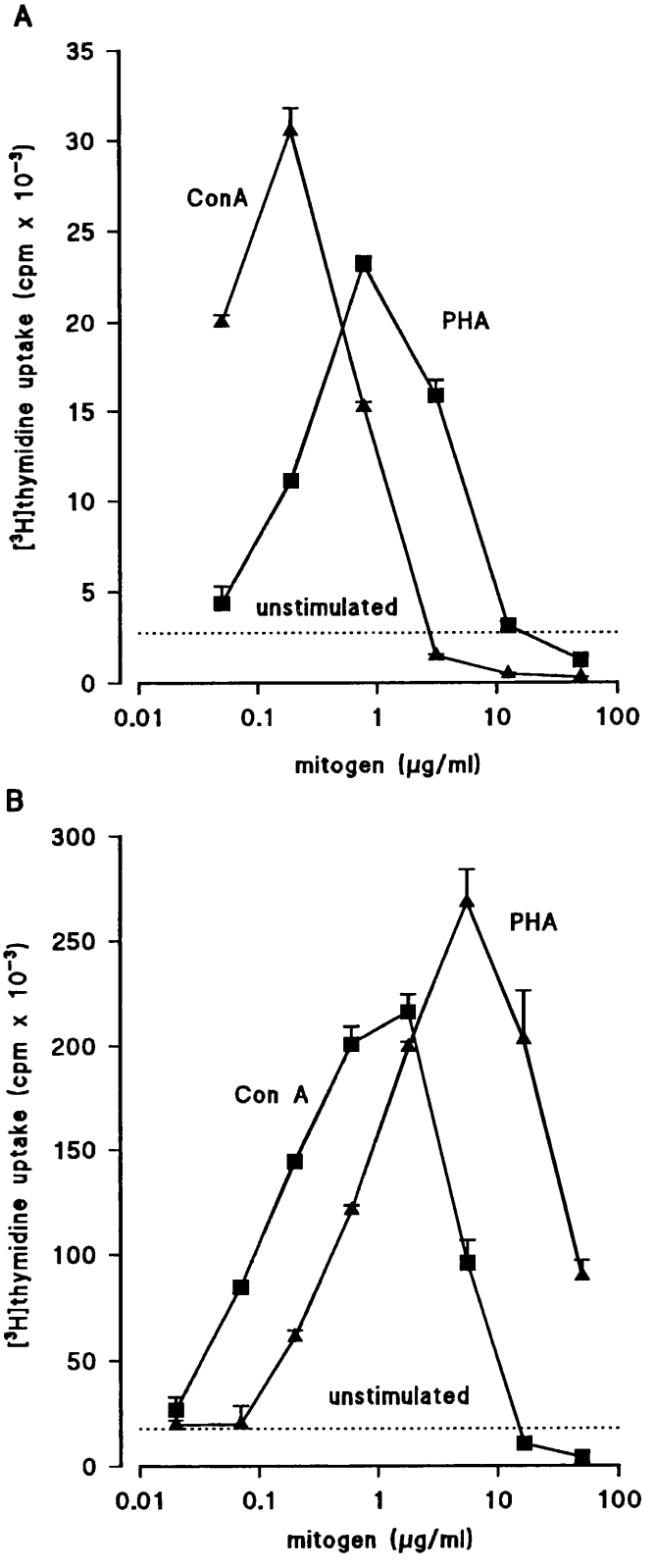


Fig 3.5 [^{3}H]thymidine incorporation into peripheral lymph node cell cultures. Cells were cultured at 2 x 10^6/ml (A) or 4 x 10^6/ml (B) for 72h, 0.5 μCi [^{3}H] thymidine was added for the last 4h before harvest. Results are the mean $\pm$ SEM for replicate wells.

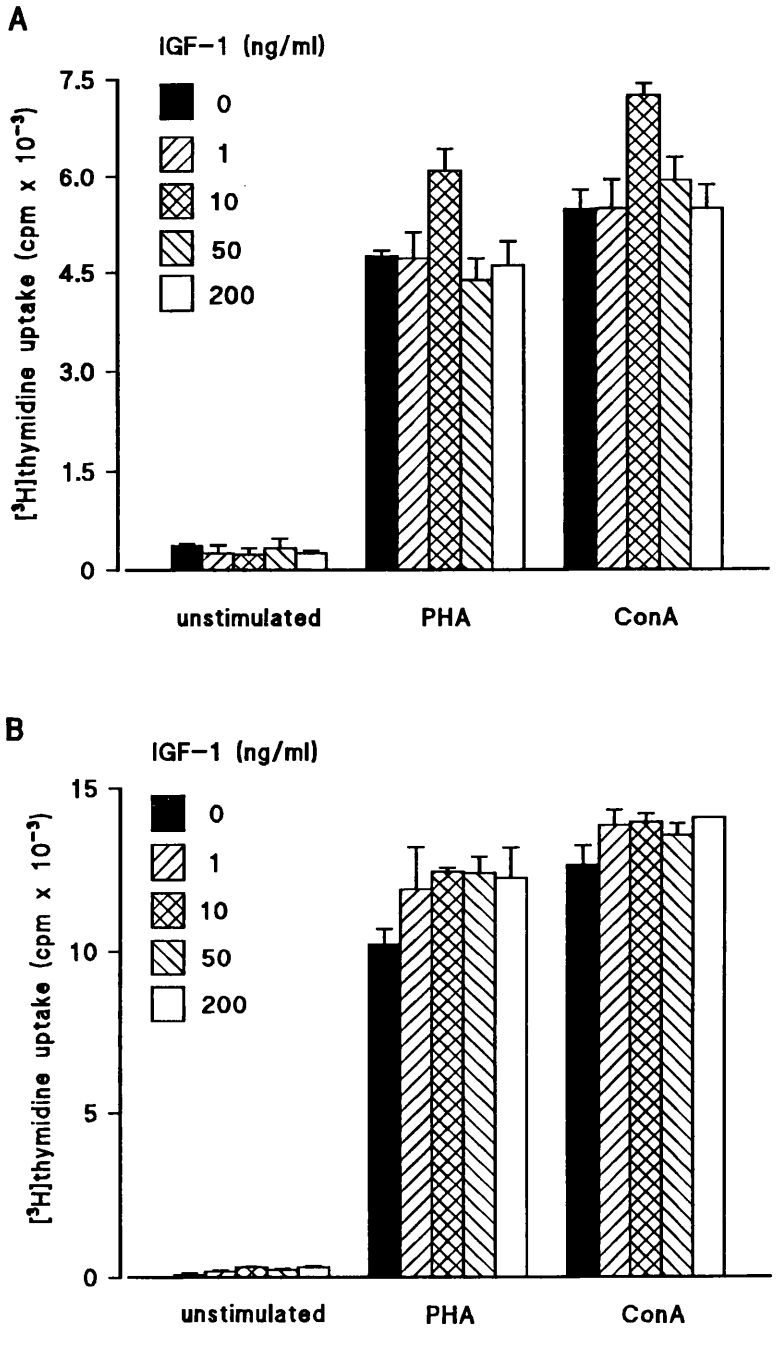


Fig 3.6 [^{3}H]thymidine incorporation into peripheral blood mononuclear cell (A) or mesenteric lymph node cell cultures (B). Cells were cultured at 2 x 10^6/ml with 2 μg/ml PHA or 0.5 μg/ml Con A for 72h, 0.5 μCi [^{3}H]thymidine was added for the last 4h before harvest. Results are the mean $\pm$ SEM for replicate wells.

3.3.6 Northern analysis

The major IGF-1 mRNA species detected in rat liver was at about 0.7-1.2 kb with minor bands 1.5 and 7.7 kb, whereas only the 7.7 kb species was detected in sheep liver. This probe hybridised to mRNA species of about 7.7 kb in sheep lymph node and thymus, and at 0.7-1.2 kb in sheep thymus (data not shown).

3.4 Discussion

Although there is increasing interest in the role of the IGFs in the immune system, few studies have investigated the role of the IGFBPs. This study provides evidence of IGFBP production by thymus cells. Furthermore PHA- and Con A-stimulation, verified by [^{3}H]thymidine uptake, significantly increased the IGFBP activity suggesting that IGFBP production is by the lymphoid cells. Presence of an IGFBP was confirmed by WLB analysis which showed specific binding to a single band of about 24 kDa. This IGFBP could not be detected by an antiserum to bovine IGFBP-2 (UBI), which crossreacts with sheep, though the low amount of IGFBP could explain this; the quantity of conditioned medium which can be run on a gel track is limited by the protein content, and Iscoves medium has 400 μg/ml BSA. Solution phase assay or WLB could not detect IGFBP in conditioned medium from peripheral lymph node cultures.

Baxter *et al.* (1991) found that IGF-1 levels in conditioned medium from rat spleen cultures were equivalent in untreated and acid-extracted samples, suggesting that little or no IGFBP was present. Spleen tissue from adult rats expresses IGFBP-2, and -4, and in juvenile rats expresses IGFBP-2, though the particular cell types expressing mRNA was not identified (Domene *et al.* 1994, Yakar *et al.* 1994).

Human peripheral blood cells express mRNA for several IGFBPs (Nyman & Pekonen 1993). Using PCR methods unstimulated cells were shown to express IGFBP-2 and -3, stimulated cells expressed in addition IGFBP-4 and -5, but IGFBP-1 mRNA was undetectable in either stimulated or unstimulated cells. However IGFBP protein in conditioned medium was difficult to detect by WLB in the presence of IGFBPs from the 10% FCS used in the culture medium. Cells cultured in the presence of 0.1% BSA instead of FCS produced only one IGFBP at 34 kDa by WLB (Nyman & Pekonen 1993).

Recently IGFBP protein production has been demonstrated from human-leukaemic blasts lines (Neely *et al.* 1991). Both T- and B-lines produced IGFBP-2 and -4, but none produced IGFBP-1, or -3. IGFBPs of 31 and 33 kDa were detected by anti Hec-1A antibody (raised against the products of Hec-1A endometrial carcinoma cells, which is known to detect IGFBP-3 and -2), but not by an antibody to IGFBP-3; the strong 31 kDa band was probably IGFBP-2, but the identity of the weaker 33 kDa band is unclear. Thus IGFBP protein can be detected in medium of lymphoid cells but levels are low. The thymus conditioned medium used in the present study is from a mixed culture of primary cells, and since only a portion of the cells will respond to stimulation, the cells producing IGFBP may be in small numbers.

Several IGF-1 mRNA species can be detected in rat liver, the proportions of which vary with the animals physiological state (Travers *et al.* 1993) and this cDNA probe detected IGF-1 mRNA expression in ovine thymus and lymph node. Expression of IGF-1 mRNA has previously been shown in rat spleen and thymus (Murphy *et al.* 1987). Using the highly sensitive PCR technique, PHA-stimulated human peripheral blood lymphocytes expressed both IGF-1 and IGF-2, though unstimulated cells expressed neither (Nyman & Pekonen 1993). Arkins *et al.* (1993)

also detected IGF-1 cDNA transcripts in a variety of mouse lymphoid tissues and cell lines after PCR amplification. However using Northern blotting or ribonuclease protection assay, IGF-1 mRNA was negligible in lymphoid cell lines and lymphoid tissues. In contrast, abundant transcripts were found in differentiated macrophages (but not pre-myeloid cells), suggesting that in haemopoietic cells it is the myeloid rather than lymphoid cells which express IGF-1. If this is also the case with sheep cells, then Northern blotting would not be expected to detect IGF-1 mRNA in the ovine lymphoid cells.

IGF-1 protein could not be detected by RIA in conditioned medium from ovine lymph node i.e. less than 0.5 ng/ml, even though cell stimulation was verified by [^{3}H]thymidine incorporation into corresponding cultures. Low levels of IGF production in lymphoid cells have previously been reported. IGF-1 was detected at less than 0.1 ng/ml and IGF-2 at less than 0.3 ng/ml, in conditioned medium from human leukaemic blast lines (Neely *et al.* 1991). Normal human B-lymphocytes produced no IGF, and although transformed cells produced no IGF-2, IGF-1 was detectable at 3 ng/ml and this was increased to 12 ng/ml when cells were GH stimulated (Merrimee *et al.* 1989). Baxter *et al.* (1991) detected IGF-1 production by rat spleen and thymus cells using immunocytochemistry, though on a per cell basis they produced 60 times less than cultured hepatocytes. Therefore since lymphocytic cells in other species seem to produce small amounts of IGFs, perhaps our methods were not sensitive enough. These small amounts may nevertheless have paracrine or autocrine effects in the local microenvironment of lymphoid tissue.

Conditioned medium from unstimulated and stimulated ovine thymus cultures contained an IGFBP-3 protease activity which was inhibited by protease inhibitors,

strongly suggesting the presence of a Ca^{2+}/Mg^{2+} independent IGFBP-3 serine protease. The presence of this protease may be responsible for the low level of IGFBP detected by WLB. Some of the radioactivity consistently remains at the top of the gel in the presence of unconditioned medium but not other samples, including the buffer control and conditioned medium from unstimulated cells. This suggests that Iscove's medium aggregates radiolabel and that this is inhibited in media from stimulated cells. IGFBP-3 protease has been detected previously in a variety of tissue homogenates and cell conditioned media, it has not been investigated in the ruminant immune system. It is possible that this protease activity present in conditioned medium may have rendered IGFBP-3 in these cultures undetectable by WLB; perhaps proteases active against other IGFBPs are also present.

IGF-1 receptors have been shown previously on a range of myeloid and lymphocytic cells. Only one study has been reported in ruminants and this showed IGF-1 receptors on bovine peripheral blood mononuclear cells, neutrophils and to a lesser extent on erythrocytes (Zhao *et al.* 1992). Here we show that IGF-1 receptors are present also on cells from normal ovine lymphoid tissue as we have demonstrated specific IGF-1 binding on cells from the thymus and mesenteric lymph node as well as peripheral blood MNC, platelets and erythrocytes, though binding to erythrocytes was very low. Preparations of granulocytes, while enriched for granulocytic cells, contained substantial numbers of MNC (Table 3.2). However since there is specific binding in both fractions, this would suggest that granulocytes are contributing to the specific binding seen, although this assumes that the MNC obtained from the interface layer (MNC fraction) are from the same population as the MNC in the granulocyte fraction.

The platelet, derived from haemopoietic stem cells, possesses many features of the classical inflammatory cell, e.g. phagocytosis, cytotoxicity and the release of mediators augmenting inflammation. Wound IGF levels are important for healing and the IGF-1 and IGFBP-3 stored in human platelet α-granules (Spencer *et al.* 1993) when released on aggregation would contribute to wound levels. Human platelets show enhanced aggregation in response to stimulants in the presence of IGF-1. As the platelet is anucleate, this demonstrates a metabolic effect of IGF-1 in the absence of its mitogenic effect (Motani *et al.* 1992). Platelets have a lower binding per cell, but they are in much greater numbers than MNC in blood and since both cell types settle at the interface of a lymphopaque density gradient, MNC were prepared from platelet-depleted defibrinated blood. Cell preparations from tissues were repeatedly washed at low speed to minimise platelet contamination; preparations were used only if platelet contamination was too low to make a measurable contribution to [^{125}I]IGF-1 binding. The IGF-1 binding was best characterised on platelets, which were therefore used for competitive binding studies. Competition by unlabelled polypeptides with potencies of IGF-1 > IGF-2 > > insulin suggests that [^{125}I]IGF-1 is binding to a type 1 IGF receptor since IGF-1 binding to a type 2 IGF receptor or to a cell surface associated IGFBP would not be competed by insulin (Massague & Czech 1982; Zapf *et al.* 1975). Platelet [^{125}I]IGF-1 binding was competed by IGF-2 at a concentration only slightly higher than IGF-1; this differs from the typical strong preference for IGF-1 (Massague & Czech 1982), but heterogeneity of IGF-1 receptors has been described and the rat thymus and mouse thymoma IGF-1 receptors bind IGF-1 and IGF-2 with equal affinity (Verland & Gammeltoft 1989). Scatchard analysis estimates of the Kd and the number of receptor sites are

comparable to values obtained from human platelets (Hartmann *et al.* 1989). The high affinity IGF-1 binding shown here (Kd 266 pM) is also consistent with the presence of a type 1 IGF receptor since IGF-1 binds with much lower affinity (Kd 0.4 μM to the type 2 IGF receptor (Tong *et al.* 1988).

The IGFs have previously been shown to have effects on a variety of immune responses, including the enhancement of cell proliferation in response to polyclonal activators. Here a preliminary assay showed enhancement of [^{3}H]thymidine uptake by ovine peripheral blood cells by IGF-1, but no significant effect on mesenteric lymph node cells. This discrepancy may reflect the differences in cell types from the two sources but in the absence of replication of these assays such conclusions cannot be drawn. There have been conflicting reports of IGF-1 effects on [^{3}H]thymidine uptake into cells of the immune system. In some studies IGF-1 has no effect possibly because monocytes or other stromal cells provide sufficient endogenous IGF-1. Here the ovine cells cultures contained stromal cells, therefore future assays should utilise adherent cell-depleted preparations.

Blocking of the endogenous IGF activity by antibodies to the type 1 IGF receptor antibody could be considered, as previously used by Roldan *et al.* (1989). However the antibodies to type 1 IGF receptors available at the time of study, such as the widely used anti-IR3, do not crossreact with sheep type 1 IGF receptors. Likewise anti-sera to IGF-1 were not available in sufficient quantities to block endogenous IGF-1, therefore the effect of IGF-1 on cell proliferation was not pursued until a better design was possible. This study provides evidence for the involvement of the IGF axis in the sheep immune response, particularly in relation to the thymus. These preliminary findings warrant further evaluation of the IGF axis

in the sheep immune response and in particular its assessment during the periparturient period when immunocompetence is compromised.

CHAPTER 4: PRODUCTION OF ANTIBODIES AGAINST SYNTHETIC PEPTIDE SEQUENCES OF IGFBP-2

4.1 Introduction

GH is well established as the major galactopoietic hormone in several species although its precise mode of action is still unknown. Systemic GH administration to lactating women (Milsom *et al.* 1992; Breier *et al.* 1993) and cows (Vicini *et al.* 1991) increased milk volume and serum IGF-1 concentrations while decreasing serum IGFBP-2 concentrations. A negative relationship between serum GH and IGFBP-2 concentrations has been shown in other studies (Zapf *et al.* 1990), and it has been suggested that serum IGFBP-2 may mediate IGF transport and tissue distribution (Klempt *et al.* 1992), therefore it is feasible that GH may enhance galactopoiesis by similtaneously increasing IGF-1 and reducing the concentrations of inhibitory IGFBPs to enhance IGF effects; thus IGFBP-2 was of particular interest in the study of the IGFs during lactation.

Few studies have investigated IGFBP structure-function relationships and therefore the major aim was to use synthetic peptide technology to make site directed anti-sera which may be used to investigate IGFBP-2 action, for example by modulating IGFBP-2 action in cell culture. A further aim was to obtain an antiserum suitable for use in radioimmunoassay to allow quantitation of IGFBP-2. Since these studies require anti-sera which recognise the native whole IGFBP-2 molecule, anti-sera must be directed against peptide sequences which are on the surface of the molecule. Although the 3-dimensional structure of the IGFBPs is unknown the choice of sequences was based on the likelihood of surface exposure; this approach is

obviously limited to the indentification of potential continuous epitopes, as identification of conformational epitopes would require knowledge of the 3-dimensional structure. In addition anti-IGFBP-2 antiserum could be used to identify denatured IGFBP-2 on western blots, although for this purpose the location of the epitopes in the whole molecule are not so important.

The peptide sequences were chosen from the deduced amino acid sequence of rat IGFBP-2 (Brown *et al.* 1989). The IGFBP-2 sequence of the rat was used to design peptides because a rat model was used to study the IGF axis in lactation, and because rat IGFBP-2 derived from Clone 9 hepatocyte cultures would be the likely source of IGFBP-2 to purify for use in culture systems. However antisera which crossreact with ruminant IGFBP-2 would enable this study to be extended to ruminants. Table 4.1 shows the rat peptide sequences chosen and the high amino acid sequence homology between the rat sequence and the corresponding bovine and ovine sequences, with amino acid differences shown in bold.

Peptides were chosen using the Jameson-Wolf index of antigenicity. Selection of suitable peptides depends on predictions of surface exposure, mobility, and antigenicity of the amino acid. Surface exposure correlates with hydrophilicity and mobility, polar and hydrophilic regions tend to be at the surface and are more mobile. IGFBP-2Ac[Tyr^{154}](154-171)NH_2 was chosen because it had previously been used successfully to raise an antiserum which recognised the whole molecule (Liu *et al.* 1993). This peptide also includes a putative short glycosaminoglycan (GAG) binding concensus sequence XXBBXBX (where B is a Lys or Arg group, and X is unidentified (underlined in Table 4.1)), which is likely to be of functional significance (Hodgkinson *et al.* 1994). The C-terminal peptide IGFBP-2(239-251)

rat IGFBP-2 peptide	species	sequence
(61-75)	rat	CYPNPGSEKPL**K**ALV
	bovine	CYPNPGSEKPL**H**ALV
	ovine	CYPNPGSEKPL**R**ALV
[Cys^{112}](97-112)	rat	**EDD**HSEGG**L**VENHVD[C]
	bovine	**GEE**HSEGG**L**VENHVD
	ovine	**GEE**HSEGG**Q**VENHVD
Ac[Tyr^{154}](154-171)NH_2	rat	[Y]L**S**LEEPKKLRPPPARTP
	bovine	L**S**LEEPKKLRPPPARTP
	ovine	L**G**LEEPKKLRPPPARTP
(72-185)	rat	CQQELDQVLERIST
	bovine	CQQELDQVLERIST
	ovine	CQQELDQVLERIST
(239-251)	rat	IQGAPTI**RGD**PEC
	bovine	IQGAPTI**RGD**PEC
	ovine	IQGAPTI**RGD**PEC

Table 4.1 Rat IGFBP-2 peptide sequences synthesised: comparison with different species. Amino acid differences between species are shown in bold, glycosaminoglycan binding consensus sequence is shown double-underlined, and the Arg-Gly-Asp sequence which binds the fibronectin receptor is shown in shadow. Sequences were obtained from Brown *et al.* 1989 (rat), Upton *et al.* 1990 (bovine), and Delhanty & Han 1992 (ovine).

was chosen to include the Arg-Gly-Asp (RGD) sequence which may bind to the fibronectin receptor (shadowed in Table 4.1). Most peptides were made with a cysteine at the N- or C-terminus to allow conjugation to the carrier protein using MBS (m-maleimidobenzoyl-N-hydroxy-succinimide ester).

4.2 Materials and Methods

4.2.1 Peptide synthesis

Peptides were synthesised by solid phase methods using small scale N-(9-fluorenylmethoxycarbonyl) (Fmoc) chemistry (Atherton *et al.* 1979) on an automatic 431A synthesiser (Applied Biosystems, Warrington, Cheshire, UK). Amino acids were obtained as their Fmoc N-terminal protected derivatives containing side chain protecting groups as appropriate (ABI or Nova-Biochem). Protecting groups were: t-butyl for Asp, Glu, Ser, Thr, Tyr; trityl for Cys, Asn, Gln; Pmc (2,2,5,7,8-Pentamethylchroman-6-sulphonyl) for Arg; Boc (tert-butoxycarbonyl) for His, Lys. Most peptides were synthesised with a free C-terminus using HMP resin (0.11-0.12g), and after attachment of the first amino acid, free sites on the resin were blocked with Benzoic anhydride (Aldridge). Peptides with an amidated C-terminal end were synthesised using Rink amide AM resin.

TFA was used to cleave the peptide from the resin and to deprotect amino acid side chains, and ethanedithiol and thioanisole scavengers are added to minimise side reactions and protect certain amino acids. One peptide (IGFBP-2Ac[Tyr^{154}](154-171)NH_2) was initially cleaved in the presence of silane as a scavenger instead of ethanedithiol, but was subsequently subjected to the cleaving/deprotection step used ethanedithiol.

After peptide synthesis the resin was placed in a flask on ice, to this was added 10 ml cooled (4 °C) cleavage mix consisting of 10 mls TFA, 0.5 ml distilled water, 0.5 ml thioanisole, 0.25 ml ethanedithiol. The flask was then removed from ice and allowed to warm room temperature, stoppered, and stirred for 90 min. The mixture was then filtered through glass wool (to retain resin) into about 100 ml of ice cold ether and allowed to stand for 5 min to precipitate the peptide. The ether peptide suspension was then centrifuged at about 700 x *g* for 5 min, the pellet was resuspended and washed 6 times in ether. The peptide was then dried in a vacuum dessicator, dissolved in a suitable solvent and freeze dried.

Peptides were analysed by reverse-phase HPLC on a C_8 analytical column (Brownlee) using a model 151A HPLC system (Applied Biosystems). Peptides were dissolved at about 1 mg/ml in 0.1% TFA and 100 μl applied to the column. Elution was over 20 min in acetonitrile/water gradient consisting of 0.1% TFA in H_2O (buffer A) and 70% acetonitrile in H_2O containing 0.035% TFA (buffer B) using 0-100% buffer B.

Peptide IGFBP-2[Tyr^{154}](154-171)NH_2 was obtained from Dr Nicholas Ling to use as a control for the IGFBP-2Ac[Tyr^{154}](154-171)NH_2 peptide we have synthesised here (identical except for an Ac at the N-terminus).

4.2.2 Amino acid analysis

This was performed on IGFBP-2Ac[Tyr^{154}](154-171)NH_2 by M-Scan, Ltd, Berks, UK, using a custom designed automatic amino-acid analyser. The sample was acid hydrolysed in constant boiling HCl at 110 °C for 24 h. Separation was on an Aminex

A8 Ion-exchange column followed by post-column derivatisation with Cadmium Ninhydrin.

4.2.3 Fast atom bombardment mass-spectroscopy (FAB-MS)

This was performed by M-Scan Ltd (for IGFBP-2Ac[Tyr^{154}](154-171)NH_2) or by Dr Su Chen, University of Warwick.

4.2.4 Conjugation methods

4.2.4.1 *Conjugation of peptides to haemocyanin using the heterobifunctional reagent MBS*

Haemocyanin (Sigma H1757) dissolved in 50 mM KPO_4 pH 8.0 was added to MBS in DMSO in a molar ratio of 1:40, mixed and then stirred at room temperature for 30 min. Unreacted MBS was then removed by immediately adding to a column pre-equilibrated with 50 mM KPO_4 pH 6.7, 1 ml fractions were eluted and the OD_{280} of the eluates measured. The MBS-activated haemocyanin was frozen at -20 °C until required. Peptides (5 mg) in PBS pH 7.5 were added to 5 mg MBS-activated haemocyanin in a carrier:peptide molar ratio of 1:2600, the pH was then adjusted to 7-7.5 with KOH, and mixed at room temperature for 3 h. Aliquots were stored at -20 °C.

4.2.4.2 *Conjugation of peptides to ovalbumin using the heterobifunctional reagent MBS*

Ovalbumin (Sigma A5503) dissolved in 50 mM KPO_4 pH 8.0 was added to MBS in DMSO in a ratio of 1:10, and stirred for 30 min room temperature. MBS-activated

ovalbumin was eluted from a Sephadex G25 column as above. 4.5 mg of MBS-activated ovabumin was added to 5 mg of peptide in PBS, and mixed for 3 h at room temperature (carrier:peptide molar ratio, 1:26), then aliquots were stored at -20 °C.

4.2.4.3 *Glutaraldehyde conjugation of peptides to ovalbumin*

1 mg ovalbumin and 1 mg peptide (molar ratio 1:22) were dissolved in PBS and mixed with glutaraldehyde (final concentration 0.05%). After stirring for 30 min at room temperature the conjugate was then frozen in aliquots at -20 °C until use.

4.2.4.4 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide hydrochloride (EDC) conjugation of peptide to ovalbumin

5 mg of peptide was added to a final concentration of 10mg/ml EDC in H_2O and gently agitated at room temperature for 10 min. 4 mg of ovalbumin was then added (molar ratio 1:28), and the mixture stirred at room temperature (in foil) for 2h. The conjugate was then dialysed for 24 h against 5 L of distilled water containing 0.01% 2-mercaptoethanol.

4.2.5 Immunisation of rats

Three rats were immunised with each peptide. Peptide-haemocyanin (MBS) conjugates were emulsified in complete Freunds adjuvant (FCA, Sigma F-4258) (1 part immunogen in PBS:1 part FCA:1 part incomplete Freunds adjuvant). 300 μl of emulisified conjugate containing 100 μg peptide was injected subcutaneously over 3 sites. Booster injections of conjugate containing 100 μg peptide were administered in incomplete Freunds (FIA) after 2 and 4 weeks, and a tail bleed was taken at 10

days after the second boost. A third boost consisting of free peptide in FIA was given at 8 weeks, and a blood sample taken 10 days later. After a 6-week interval animals were boosted again using a peptide-ovalbumin (glutaraldehyde) conjugate in FIA as above, and a test bleed taken 10 days later.

4.2.6 Immunisation of rabbits

Peptide-ovalbumin (MBS) conjugate, or peptide-ovalbumin (carbodiimide) conjugate containing 500 μg of peptide was emusified in FCA (1 part conjugate in PBS: 1 part FCA), and 1 ml given subcutaneously over 4 sites to Dutch rabbits. Three booster injections of conjugate in FIA were given at approximately 3-week intervals, and 10 days after boosting a test bleed was taken from the ear vein.

4.2.7 Antiserum to IGFBP-2 whole molecule

Antiserum raised to IGFBP-2 purified from MDBK cells (which crossreacts with sheep, rat, and human IGFBP-2) was purchased from TCS Biologicals Ltd, Bucks, UK. Antiserum to rat IGFBP-2 synthetic peptide fragment [Tyr^{137}](118-137), amino acid sequence GGSSAGRKPPKSGMKELAVY, coupled to ovalbumin was a gift from Dr Nicholas Ling, the Whittier Institute, La Jolla, CA.

4.2.8 ELISA of anti-IGFBP-2 peptide responses

Immulon II plates (Dynatech) were coated by adding 1μg peptide per well in 100 μl PBS pH 7.4, and incubating at 4 °C for 18 h. The plate was then washed 3 times in PBS/0.5% Tween 20, flooded with buffer, and incubated at room temperature for 30 min, then frozen at −20 °C until required.

Sera were diluted in PBS/0.05% Tween 20, and serial dilutions made using a multichannel pipette. 100 μl of primary antisera diluted in PBS/Tween was incubated for 90 min at room temperature, washed 4 times (brief soak between the last two washes). 100 μl of a 1/1000 dilution alkaline phosphatase conjugated anti-rabbit or anti-rat IgG secondary antibody was added as appropriate, incubated for 90 min, then washed 5 times (brief soak between the last 2 washes). 200 μl of 1 mg/ml nitrophenyl phosphate in alkaline phosphate buffer (100 mM glycine pH 10.3, 1 mM $ZnCl_2$, 1 mM $MgCl_2$) was added and incubated in the dark, and the OD_{405} measured at 20 min intervals on a Titertek twinreader (Flow Laboratories).

4.2.9 Western blotting

This was carried out as in Chapter 2. Bovine sera (15 μl) or Clone 9 conditioned media (150 μl) per gel was separated by SDS-PAGE and blotted onto nitrocellulose. Dilutions of anti-peptide sera (200 μl) were incubated for 60 min in a Biometra "miniblot" apparatus. After washing, alkaline phosphatase conjugated secondary antibody was incubated with the blot in a plastic container.

4.2.10 Immunoprecipitation of peptides

Anti-IGFBP-2[Tyr^{157}](118-137) positive control serum, rabbit anti-peptide serum or normal rabbit serum, were incubated overnight at room temperature with 17,000 cpm [^{125}I]IGFBP-2AcTyr154](154-171)NH_2 or 25,000 cpm [^{125}I]IGFBP-2[Tyr^{154}](154-171)NH_2 in a 300 μl volume. Anti-rabbit IgG precipitating antibody in 8% PEG/10

mM EDTA/RIA buffer was added for 6 hours at room temperature. Tubes were then spun at 1700 x *g* for 30 min, the supernatant was tipped off and the pellet counted.

4.2.11 Immunoprecipitation of IGFBP-2 pre-bound to IGF-1

Clone 9 conditioned medium (5μl) was incubated with about 20,000 cpm [^{125}I]IGF-1 overnight, then dilutions of anti-peptide antibody were added and incubated overnight. Anti-rabbit IgG secondary antibody with 8% PEG/EDTA in RIA buffer was added for 6 hours at room temperature. Tubes were then spun at 1700 x *g* for 30 min, the supernatant was tipped off and the pellet counted.

4.3 Results

4.3.1 FAB-MS Analysis and reverse phase HPLC

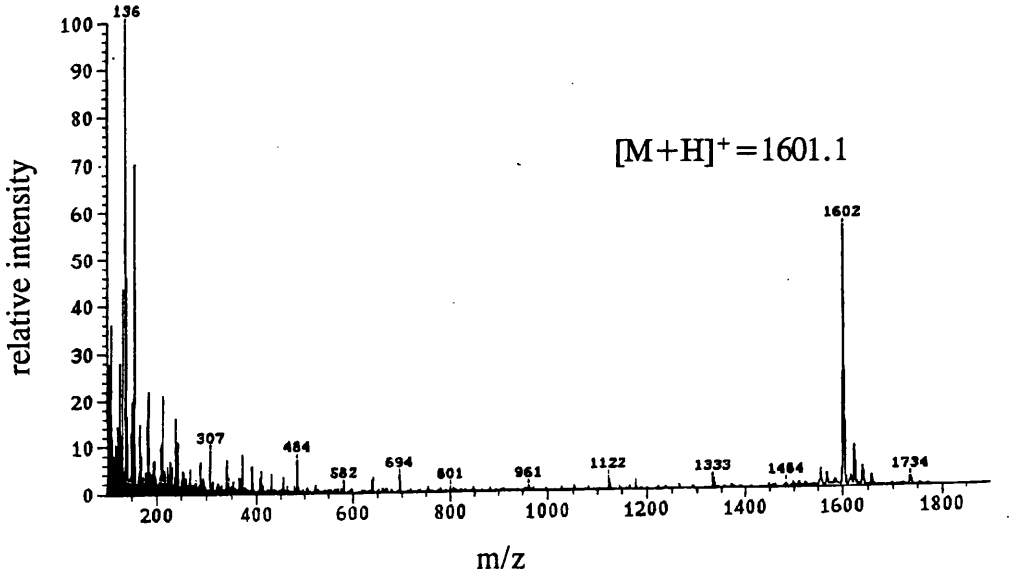
HPLC analysis of the peptides showed one major peak and some minor peaks as expected of unpurified peptides, however Mass Spectroscopy analysis was not currently available and therefore peptides IGFBP-2(61-75), IGFBP-2(172-185), IGFBP-2(239-251) and IGFBP-2[Cys^{112}](97-112) were used for rat immunisations without further analysis. FAB-MS analysis of these peptides was subsequently performed by Dr Su Chen. Fig 4.1A, C and D shows IGFBP-2(61-75), IGFBP-2(172-185), and IGFBP-2(239-251) have signals corresponding to the appropriate molecular ion, though there are many other signals present; when such a complicated mixture is analysed it is impossible to assign molecular ions to all the signals detected. IGFBP-2[Cys^{112}](97-112) showed no appropriate signal at the molecular ion, but there was a peak at 2010 Da which could represent incompletely deprotected peptide, therefore this peptide was "recleaved", but the losses during the

recleaving/deprotection were too great for the peptide to be used therefore it was not further analysed (Fig 4.1B).

Amino acid analysis of IGFBP-2Ac[Tyr^{154}](154-171)NH_2 peptide showed the experimentally determined ratio of amino acids correlated well with the expected ratio (Table 4.2). FAB-MS analysis of IGFBP-2Ac[Tyr^{154}](154-171)NH_2 peptide showed a molecular ion $[M+H]^+$ at 2133.6 m/z corresponding to a protonated molecule of the expected peptide sequence (Fig 4.1E). Other sequence ions were also present including the doubly charged molecular ion $[M+H]^{++}$ at m/z 1067.2. FAB-MS analysis using glycerol/thioglycerol and meta-NitroBenzylAlcohol matrices produced similar profiles. Several other potential molecular ions were present, at about 266 Da higher than expected and at 2173.6, signals at about 163 Da lower than these possibly reflect loss of tyrosines. Signals in the 2000-2600 Da range may arise from incomplete deprotection of various amino acid protecting groups, therefore this peptide was taken through the cleavage/deprotection step again, and subsequently analysed by FAB-MS. Fig 4.1F shows the signals in the 2000-2600 Da range in the original preparation have decreased and the 2134.5 m/z $[M+H]^+$ signal has increased in amplitude. Reverse phase HPLC profiles of IGFBP-2Ac[Tyr^{154}](154-171)NH_2 show the increased homogeneity after the second cleavage/deprotection step; the profile is similar to that of IGFBP-2[Tyr^{154}](154-171)NH_2.

On the basis of the FAB-MS profiles peptides IGFBP-2(61-75), IGFBP-2(172-185), IGFBP-2(239-251), and a mixture of recleaved IGFBP-2Ac[Tyr^{154}](154-171)NH_2/IGFBP-2[Tyr^{154}](154-171)NH_2 were conjugated to ovalbumin for rabbit immunisations.

A



B

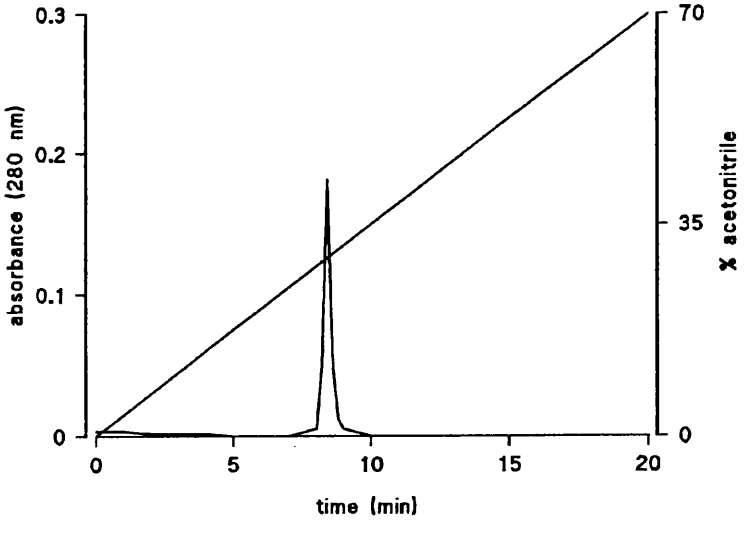
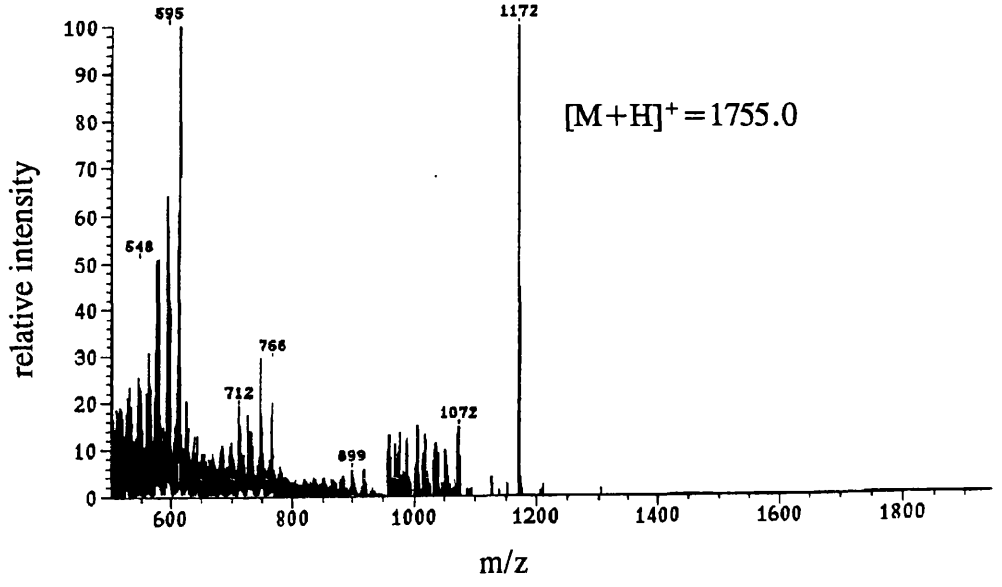


Fig 4.1A FAB-MS (A) and reverse-phase (C_8) HPLC (B) analysis of synthesised IGFBP-2(61-75) peptide.

A



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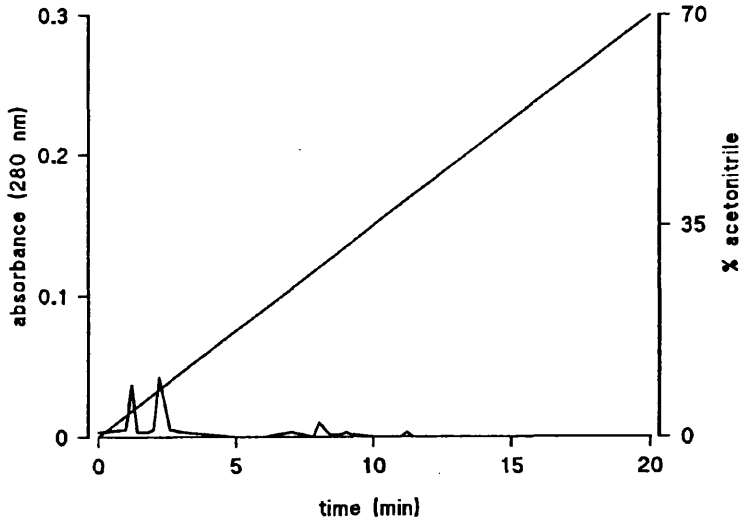
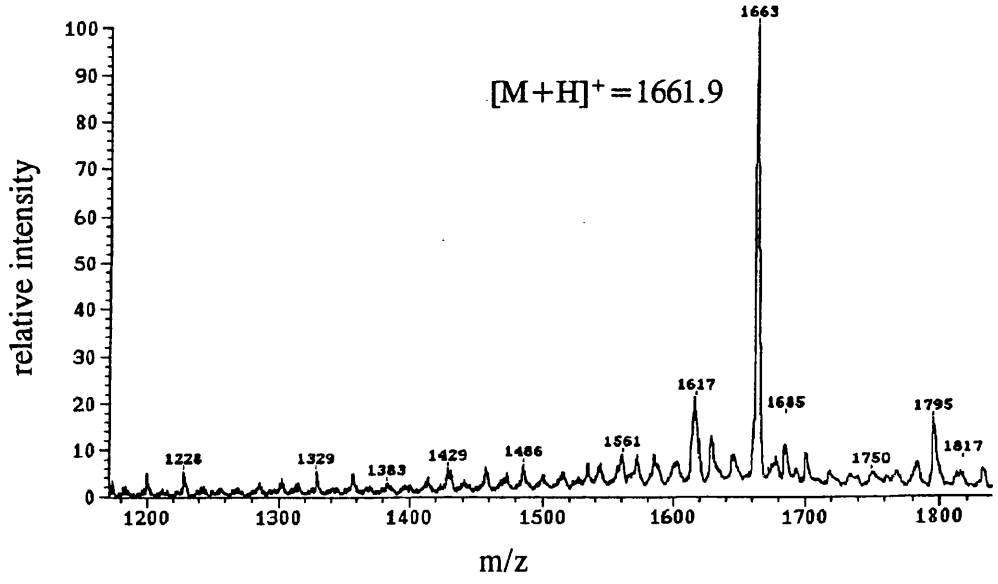


Fig 4.1B FAB-MS (A) and reverse-phase (C_8) HPLC (B) analysis of synthesised IGFBP-2[Cys^{112}](97-112) peptide.

A



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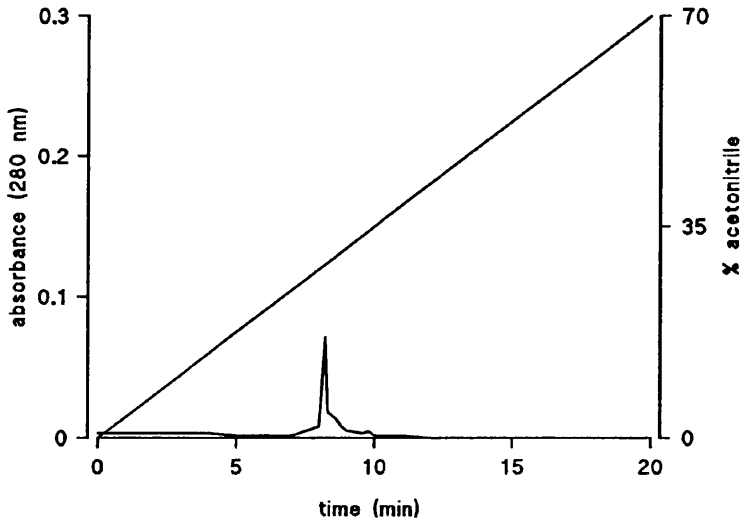
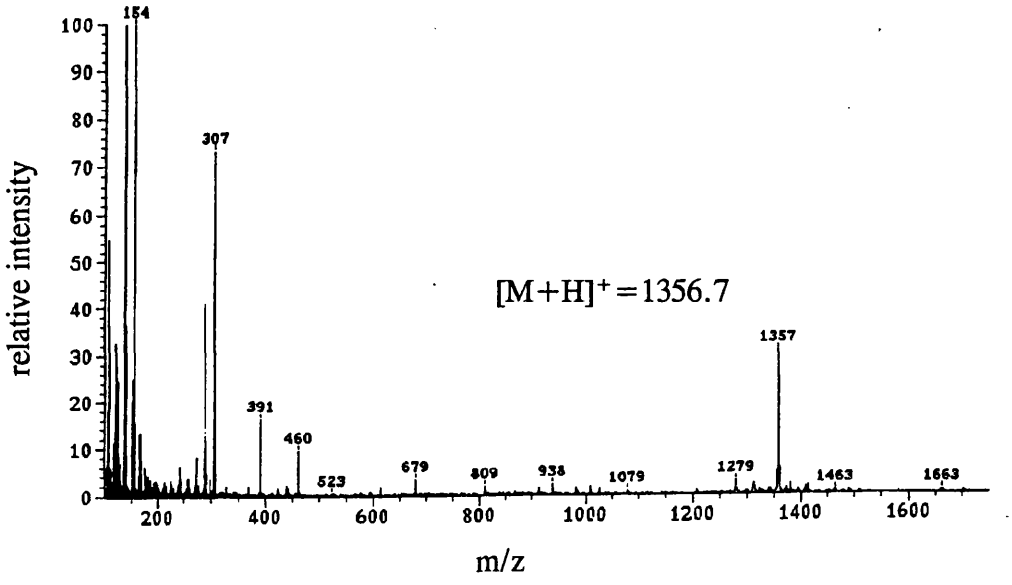


Fig 4.1C FAB-MS (A) and reverse-phase (C_8) HPLC (B) analysis of synthesised IGFBP-2(172-185) peptide.

A



B

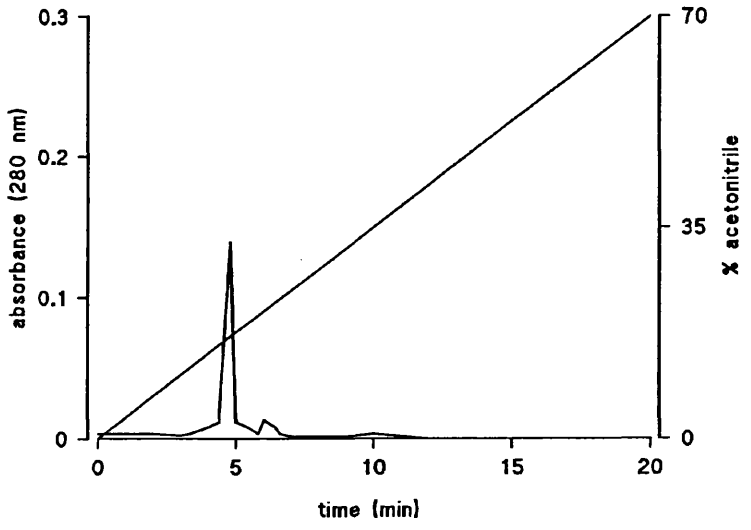
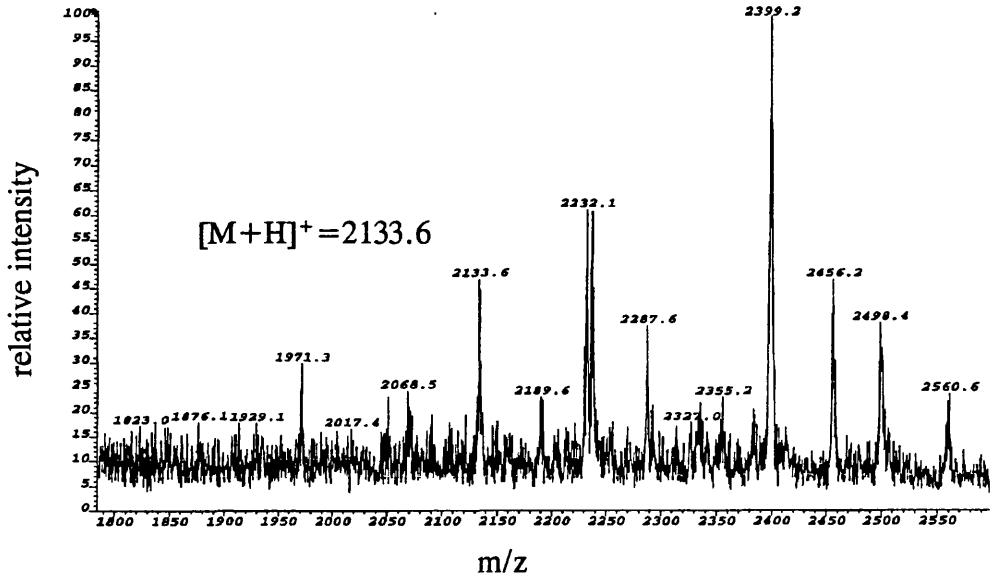


Fig 4.1D FAB-MS (A) and reverse-phase (C_8) HPLC (B) analysis of synthesised IGFBP-2(239-251) peptide.

A



B

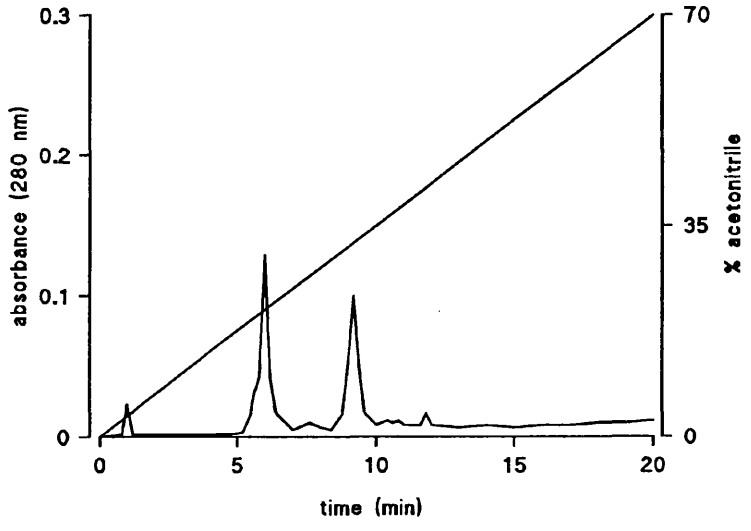
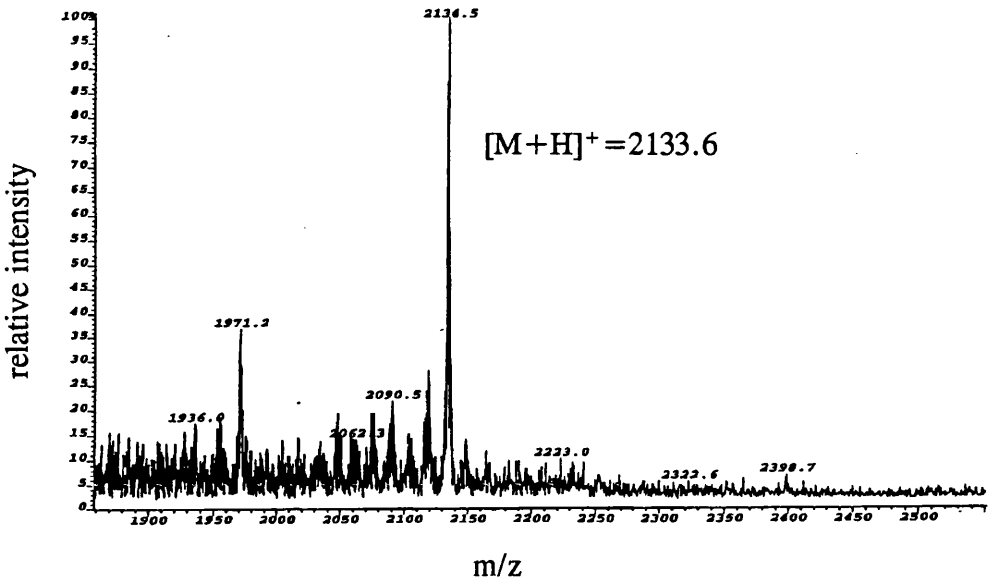


Fig 4.1E FAB-MS (A) and reverse-phase (C_8) HPLC (B) analysis of synthesised IGFBP-2Ac[Tyr^{154}](154-171)NH_2 peptide after one cleavage step.

A

IGFBP-2Ac[Tyr^{154}](154-171)NH_2



B

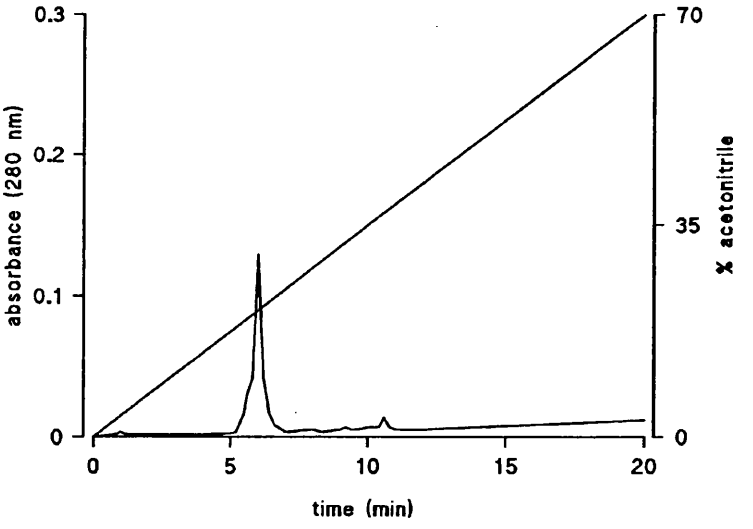


Fig 4.1F FAB-MS (A) and reverse-phase (C_8) HPLC (B) analysis of synthesised IGFBP-2Ac[Tyr^{154}](154-171)NH_2 peptide after a second cleavage step.

Amino acid	found ratio	theoretical ratio
Lys	1.85	2
Thr	0.95	1
Ser	0.90	1
Glu	1.80	2
Pro	4.90	5
Arg	1.87	2
Ala	1.00	1
Leu	2.65	3
Tyr	0.73	1

Table 4.2 Amino acid analysis of IGFBP-2 Ac[Tyr^{154}](154-171)NH_2.

4.3.2 ELISA of rat antisera raised against IGFBP-2 peptides

Fig 4.2 shows the antisera responses to homologous peptide after immunisation with peptide-haemocyanin conjugates, initial boosting twice with peptide-haemocyanin conjugate, then boosting once with either free peptide (closed symbols) or peptide-ovalbumin conjugate (open symbols). Non-immune rat serum (NRS) showed a low background response against all the peptides demonstrating the specificity of the anti-peptide response. Fig 4.2 demonstrates the variation in response between rats given the same immunogen. In addition, boosting affected the response of the individual animals differently; the titre increased in some animals and was decreased in others.

4.3.3 Western Blots of rat antisera against rat IGFBP-2

None of the antisera bound either reduced or non-reduced rat IGFBP-2 (Clone 9) (results not shown). Anti-IGFBP-2[Tyr^{137}](118-137) serum was used as a positive control which reacted strongly. No binding was detected against reduced bovine serum (dry cow) either using anti-bIGFBP-2 sera as a positive control.

4.3.4 ELISA of rabbit antisera raised against IGFBP-2 peptides

All anti-peptide sera bound homologous peptide but did not cross react with non-homologous peptides, except for a slight crossreaction of anti-IGFBP-2(61-75) serum with IGFBP-2(239-251) peptide (Fig 4.3).

4.3.5 Western blots of rabbit sera against rat IGFBP-2 peptides

Antisera were tested against both reduced and non-reduced rat IGFBP-2 (Clone 9). There was no binding to IGFBP-2 except with the positive control anti-IGFBP-

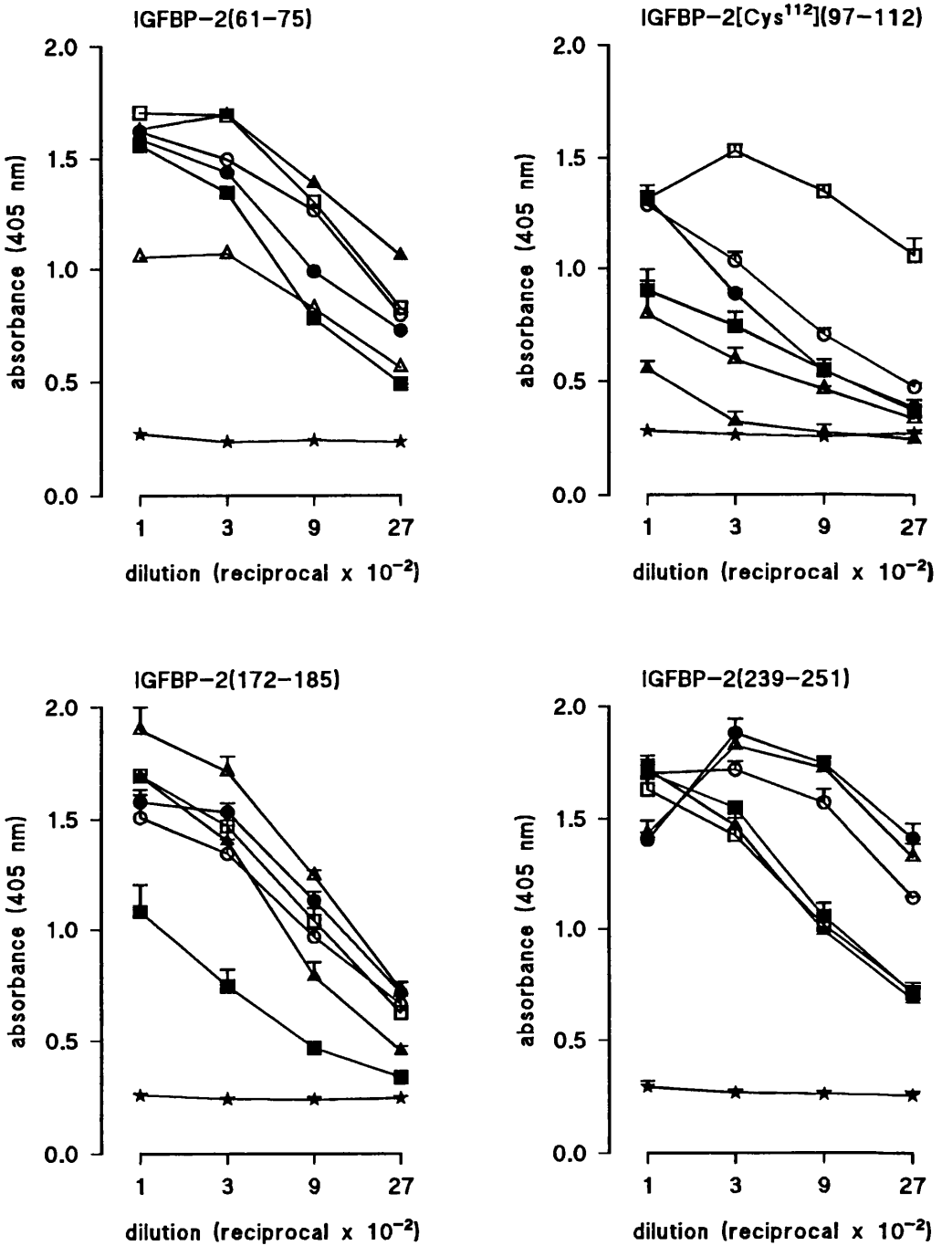


Fig 4.2 ELISA of rat anti-IGFBP-2 peptide antiserum responses against the homologous peptide. Individual responses of three rats are shown after boosting with free peptide (filled symbols) and peptide-ovalbumin conjugate (open symbols). The non-immune rat serum response is shown for each peptide (★). Results are absorbances after 20 min incubation with substrate.

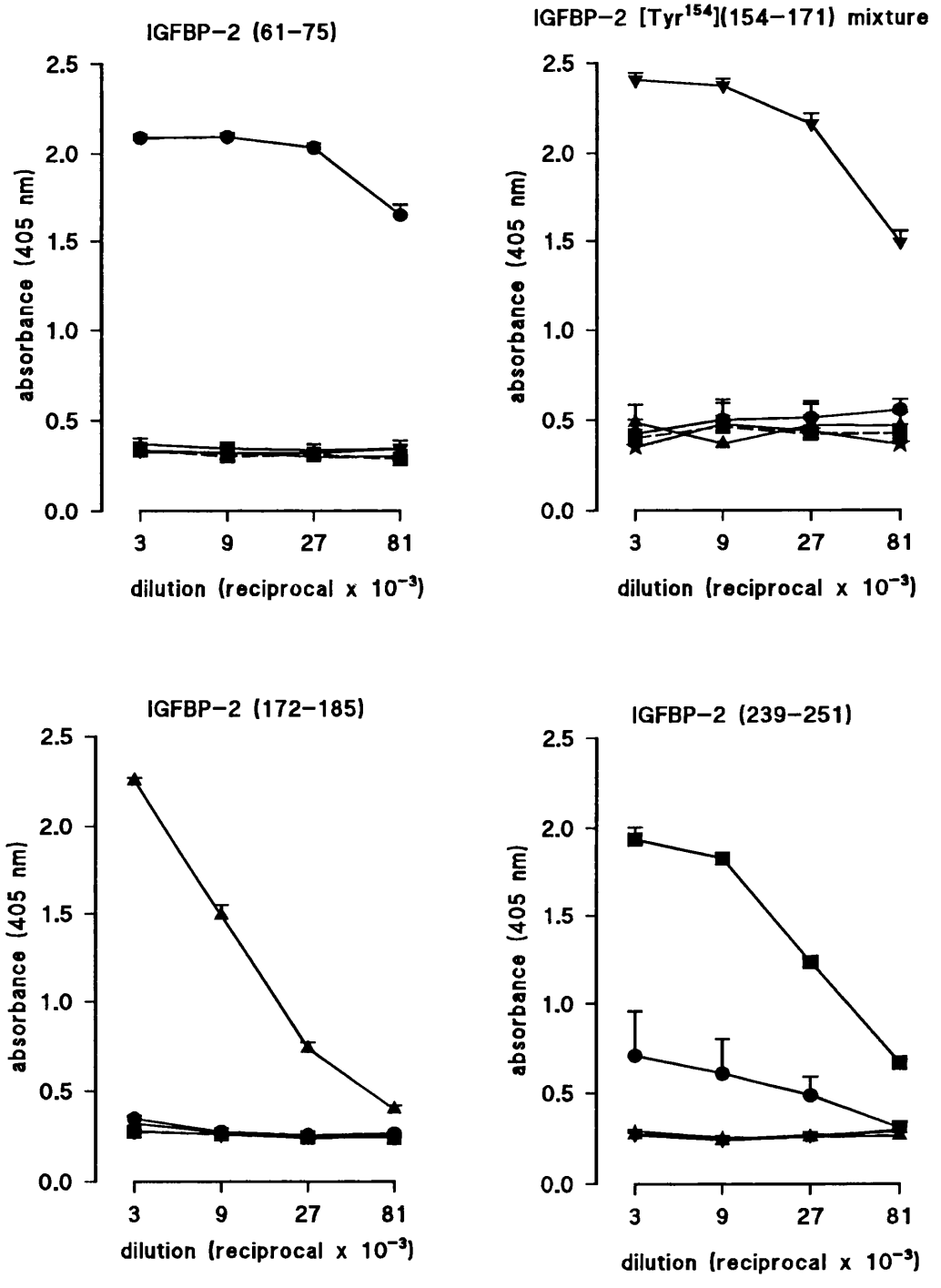


Fig 4.3 ELISA of rabbit anti-IGFBP-2 peptide antiserum responses against homologous and non-homologous peptides. Anti-IGFBP-2(61-75) (●); anti-IGFBP-2(172-185) (▲); anti-IGFBP-2[Tyr^{154}](154-171)NH_2 mixture (▼); anti-IGFBP-2(239-251) (■). Results are absorbances after 40 min incubation with substrate.

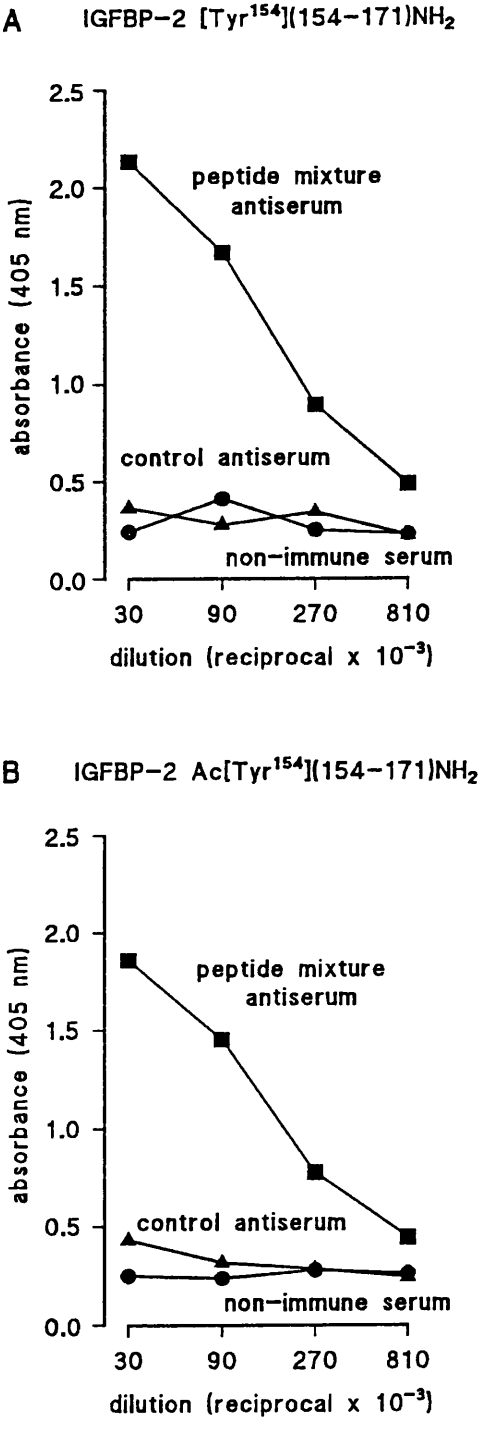


Fig 4.4 ELISA of rabbit IGFBP-2[Tyr^{154}](154-171)NH_2 mixture antiserum responses against the individual peptides. (A) IGFBP-2[Tyr^{154}](154-171)NH_2 and (B) IGFBP-2Ac[Tyr^{154}](154-171)NH_2 was coated on the plates. Control antiserum is anti-IGFBP-2[Tyr^{137}](118-137). Results are absorbances after 20 min incubation with substrate.

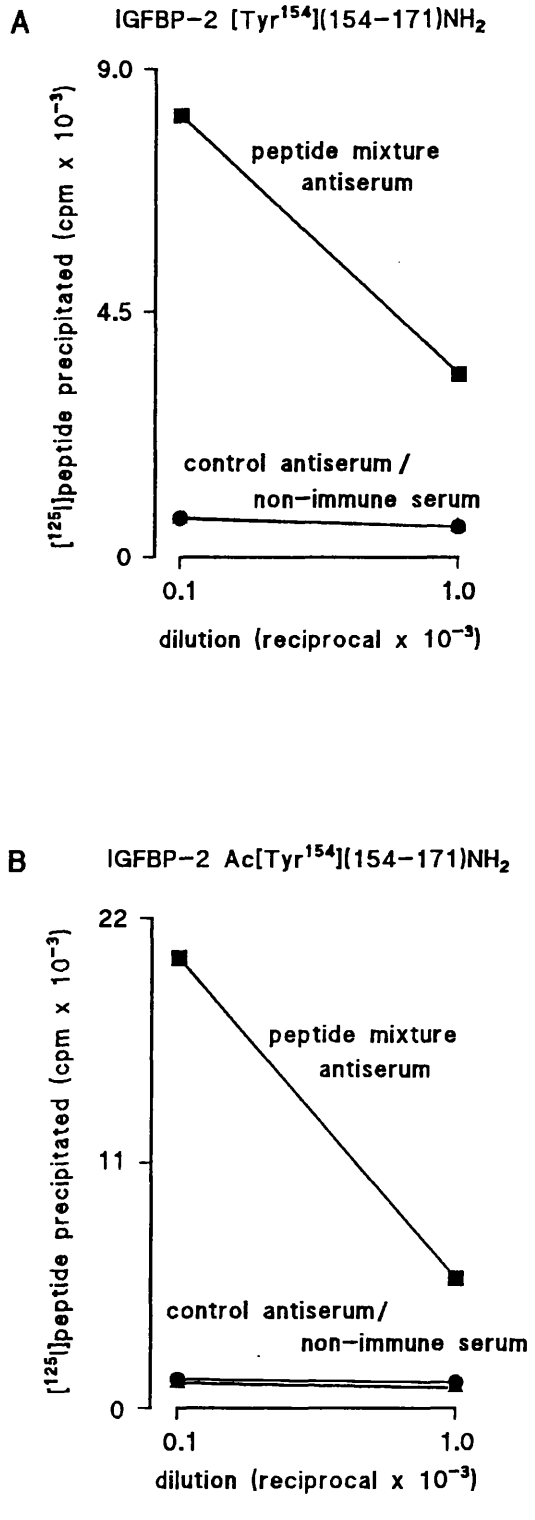


Fig 4.5 Immunoprecipitation of [^{125}I]IGFBP-2 peptides by rabbit IGFBP-2[Tyr^{154}](154-171)NH_2 mixture antiserum. (A) IGFBP-2[Tyr^{154}](154-171)NH_2 and (B) IGFBP-2Ac[Tyr^{154}](154-171)NH_2 were incubated with anti-peptide antiserum and precipitated with anti-Ig secondary antibody. Control antiserum is anti-IGFBP-2[Tyr^{137}](118-137).

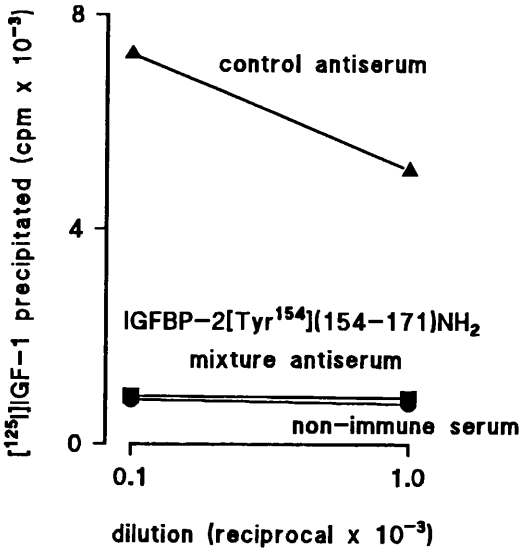


Fig 4.6 Immunoprecipitation of IGFBP-2 prebound to [^{125}I]IGF-1. Rat IGFBP-2 (Clone 9) was preincubated with [^{125}I]IGF-1 followed by incubation with rabbit anti-IGFBP-2 peptide serum, non-immune serum or control antiserum (anti-IGFBP-2[Tyr^{137}](118-137)) and precipitated with anti-Ig secondary antibody.

2[Tyr^{137}](118-137) serum. A faint band was detected with anti-IGFBP-2(172-185) at a higher molecular weight than that of IGFBPs (results not shown).

4.3.6 ELISA response of an antiserum raised to IGFBP-2Ac[Tyr^{154}](154-171)NH_2/IGFBP-2[Tyr^{154}](154-171)NH_2 mixture against the individual peptides

Antiserum to the mixture recognised both peptides by ELISA equally well (Fig 4.4).

4.3.7 Immunoprecipitation of [^{125}I]IGFBP-2 peptide with anti-peptide antisera

Fig 4.5 shows the immunoprecipitation of both radiolabelled IGFBP-2Ac[Tyr^{154}](154-171)NH_2 peptide and IGFBP-2[Tyr^{154}](154-171)NH_2 by antisera raised against a mixture of these peptides, but normal rabbit serum and control anti-IGFBP-2[Tyr^{137}](118-137) did not precipitate either peptide.

4.3.8 Immunoprecipitation of IGFBP-2 (Clone 9) prebound to [^{125}I]IGF-1

Control anti-IGFBP-2[Tyr^{137}](118-137) serum precipitated whole molecule IGFBP-2, whereas anti-IGFBP-2[Tyr^{154}](154-171)NH_2 mixture and non-immune rabbit serum were ineffective (Fig 4.6).

4.4 Discussion

Several antisera were raised which specifically bind homologous peptides by ELISA and in at least one case can precipitate peptide in solution. However none of these anti-sera showed detectable binding to the whole IGFBP-2 molecule either native in solution or denatured on western blots. Rats were initially chosen because they were available in greater numbers than rabbits thus allowing several animals to be

immunised with each peptide, since individual variation in response between animals was expected. Immunised rats were boosted with free peptide or peptide attached to a different carrier to avoid the strong carrier response from detracting from the anti-peptide response, but titres by ELISA were not improved nor did the antisera bind in western blotting.

Rabbit anti-IGFBP-2 peptide sera which bound IGFBPs in western blots were subsequently reported, and this approach was adopted along with the IGFBP-2 peptide sequence used in that study (Liu *et al.* 1993). The limited number of rabbits available could have been utilised in two ways, either one animal per peptide for several peptides or several animals for fewer peptides. The former was chosen firstly because antisera to different regions of the molecule were desired, and secondly in the absence of the 3-D structure of IGFBP-2 the surface epitopes were unknown but based only on predictions.

There are several reasons why anti-peptide antisera may not bind the protein. Peptide IGFBP-2[Cys^{112}](97-112) was used for rat immunisations but subsequently shown not to contain the appropriate peptide, therefore it is not surprising that the serum does not react with the protein, but peptides IGFBP-2(61-75), IGFBP-2(172-185), and IGFBP-2(239-251) used for immunisation of rats and rabbits were shown by mass spectroscopy to contain the appropriate peptide. However the peptide preparations were not purified and would contain other peptides. It is feasible that some of the antibody response to immunisation with crude peptides may be directed against these contaminants, thus detracting from the desired response.

It is possible that the antisera bind to the peptide sequences chosen but these sequences, in spite of the predictions of surface exposure, may not be on the surface of the molecule or are otherwise inaccessible to the antisera. However anti-peptide sera tend to react well with the denatured form of the protein. Therefore the failure of anti-peptide sera to bind either reduced and non-reduced IGFBP-2 on a western blot was disappointing, although partial renaturation of blotted proteins on nitrocellulose has been described and the orientation of protein on the nitrocellulose may mask some peptide sequences.

Most peptides were conjugated to carrier protein using MBS. A cysteine residue was included at one end of the peptide sequence to foster conjugation through this end residue thus avoiding altering the required sequence. However most conjugation methods can involve more than one residue in the coupling and the peptides antigenicity may be altered by conjugation.

Some of the above considerations concerning the failure of anti-peptide sera to bind protein do not apply to IGFBP-2[Tyr^{154}](154-171)NH_2: it has been successfully used to raise an antiserum which binds peptide in solution and IGFBP-2 on western blots, though unfortunately it was unknown if this antiserum binds the native molecule (Liu *et al.* 1993). A mixture of IGFBP-2 Ac[Tyr^{154}](154-171) and peptide IGFBP-2[Tyr^{154}](154-171) obtained from Dr N. Ling was used for immunisations, and the resulting antiserum bound both peptides equally well by ELISA. Difficulty in obtaining Bis-benzidine prevented the use of diazotised benzidine for carrier conjugation as used by Liu *et al.* (1993) and therefore carbodiimide was used. Therefore although mass spectroscopy and amino acid analysis confirmed the identity of IGFBP-2Ac[Tyr^{154}](154-171)NH_2, the difference

in the conjugation methods used here and in Liu *et al.* (1993) may have resulted in different configurations of the peptide in the two conjugates and hence different antibody responses. Also, as mentioned above, even if identical peptide-carrier conjugates were prepared inter-animal variation in responses is expected.

In the absence of purified IGFBP-2 to iodinate for immunoprecipitation studies, an indirect assay was used in which IGFBP-2 was precomplexed to [^{125}I]IGF-1 before adding antisera to IGFBP-2 peptides. This indirect method has the advantage of avoiding denaturation of IGFBP-2 by radiolabelling; the antiserum must bind the native molecule to modulate the biological function of the IGFBP, although such antisera are difficult to produce.

The positive control antiserum to rat IGFBP-2 synthetic peptide fragment [Tyr^{137}](118-137) used here is of a higher titre than the anti-IGFBP-2[Tyr^{154}](154-171)NH_2 used in Liu *et al.* (1993). The anti-IGFBP-2[Tyr^{154}](154-171)NH_2 used in Liu *et al.* (1993) can detect 50 ng of IGFBP-2 by WLB, but in the absence of this antiserum or purified IGFBP (the IGFBP-2 content of the Clone 9 conditioned medium used is unknown), it is impossible to compare the reactivity of our anti-IGFBP-2[Tyr^{154}](154-171)NH_2 with that in Liu *et al.* (1993)

Blum *et al.* (1993) used rhIGFBP-2(176-190)(LEEPKKRPPPART) to produce an anti-peptide antiserum which bound the protein. The surface exposure of this peptide was confirmed by the ability of the antiserum to bind native rhIGFBP-2 in solution. Interestingly, IGFBP-2 from rat or sheep did not cross react even though this sequence is homologous in all three species. This sequence is incidentally very similar to the peptide IGFBP-2[Tyr^{154}](154-171)NH_2) ([Y]LSLEEPKKLPPPARTP). Thus a sequence which on the basis of secondary structure calculations is believed

to be on the surface of the molecule failed to bind rat IGFBP-2. Although there is extensive IGFBP-2 amino acid homology between species, their secondary structure may differ: if the conformation of a peptide and its surrounding residues differs between species, its antigenicity and access to an antibody may be altered. In addition, post-translational modifications of proteins may affect antiserum binding, and although no potential O- or N-linked glycosylation sites have been described for human or rat IGFBP-2, the phosphorylation state of IGFBP-2 from different sources has not been studied.

In conclusion, although antisera to several IGFBP-2 peptides were successfully raised, antisera which bind the protein were as expected more difficult to obtain. Given the inter-animal variation in response and difficulty in precisely controlling conjugation, future immunisation strategy would require the use of more than one animal per peptide and more than one conjugation method for each peptide.

CHAPTER 5: THE ROLE OF IGFBPS IN THE RAT MAMMARY GLAND

5.1 Introduction

Although PRL is the major galactopoietic hormone in the rat, GH plays an important role especially when serum PRL concentrations are low (Madon *et al.* 1986). The existence of functional GH receptors on mammary epithelial cells has not been demonstrated although there is evidence of GH receptor protein in rat mammary epithelial cells (Lincoln *et al.*1990). The effects of GH have therefore been considered to be indirect, via either nutrient partitioning or IGF-1 since the mammary gland does possess IGF-1 receptors and IGFs are potent mitogens for mammary cells *in vitro*. A rat model has previously been established using a specific anti-GH serum, in conjunction with bromocriptine to ablate PRL, to study the role of GH and PRL in lactation. This model was used to investigate the ability of hormones to reinitiate milk production in animals where GH and PRL have been ablated (Flint *et al.* 1992), and test the ability of IGFs to mimic GH action in reinitiating milk production (Flint *et al.* 1994); the present study forms part of this investigation. There is little known about the role of IGFs and the IGFBPs in the involuting mammary gland, and the aim of the present study was to assess the IGFBP profiles and their hormonal control in serum, mammary gland and milk of rats in which involution had been initiated either by litter removal or by ablation of lactogenic hormones.

5.2 Materials and methods

5.2.1 Endocrine manipulation of rats

5.2.1.1 *Effects of bromocriptine and anti-GH treatment in combination with GH, IGF-1 and IGF-2 on serum IGFs and IGFBP-3*

Lactating rats were treated on day 14 of lactation by administration of bromocriptine (500 μg/injection) or sheep anti-rGH γ-globulin (110mg/injection) or a combination of both. In addition they received the following treatments: recombinant bovine GH (Monsanto, St Louis, MO, USA; 0.5 mg/injection), recombinant IGF-1 (Ciba-Geigy, Friebourg, Switzerland; 0.2 mg in 500 μl 25% polyvinylpyrrolidone (PVP)) or recombinant bovine IGF-2 (Monsanto: 0.15 mg in 500 μl 25% PVP). Control rats received carrier solutions and non-immune γ-globulin. All treatments were administered twice daily for 2 days.

5.2.1.2 *Effects of PRL, GH or a combination IGF-1 and IGF-2 bound to hIGFBP-3, after pretreatment with bromocriptine and anti-rGH, on serum IGF-1 and IGFBP-3*

Lactating rats were treated on day 14 of lactation by administration of bromocriptine (500 μg/injection) or sheep anti-rGH γ-globulin (150 mg/injection) to inhibit lactation. Hormonal ablation treatment was administered subcutaneously (s.c.) twice daily for 3 days. Hormone replacement treatment was administered on the third day and comprised 1 mg bGH s.c. in 0.5 ml $NaHCO_3$ (0.1 mol/l), 1mg oPRL, 0.25 mg IGF-1 precomplexed to 0.8 mg hIGFBP-3 in 1 ml PBS containing 0.1% BSA or 0.25 mg IGF-1 plus 0.2 mg IGF-2 precomplexed to 0.8 mg hIGFBP-3; control rats received PBS injections. Doses were administered s.c. as 0.2, 0.2 and 0.1 ml at

1400 and 2200 h on day 3 and 1000 h on day 4. At 1200 h on day 4 all dams were killed by cervical dislocation, a blood sample obtained and mammary tissue removed and frozen in liquid nitrogen.

5.2.1.3 *Effects of bromocriptine and anti-GH treatment or litter removal on the concentration of IGFBPs in milk and serum*

Lactating rats were treated at day 12-14 of lactation by administration of bromocriptine, anti-GH or a combination of both as described above. Control rats received non-immune sheep γ-globulin (150 mg/injection). Treatments were given twice daily for 2 or 4 days and after 48 or 96h treatment all dams had their litters removed for a further 4 h to allow milk to accumulate. Additional animals had their litters removed at time 0 and milk was allowed to accumulate over 48 h. Dams were anaesthetised with 0.3 ml sodium pentobarbitol (60 mg/ml Sagatal, RMB Animal Health, Dagenham, UK) after which the dam received 1 U oxytocin (Intervet UK, Cambridge, UK) to induce milk ejection and allow milk to be removed by gentle pressure. The dam was then killed by cervical dislocation and a blood sample obtained. Milk samples were frozen in liquid nitrogen and stored at -20 °C until use.

5.2.1.4 *Effects of oestradiol treatment, teat-sealing, or litter removal in combination with PRL, GH, progesterone, corticosteroid, or anti-IGF-1 treatment, on serum IGF-1 and IGFBPs in milk*

Lactating rats were treated on day 14 of lactation by daily administration of 17-β-oestradiol (100 μg in safflower oil/injection). Another group of animals had teats on one side sealed with tissue glue and the pup number reduced to 6 (one per unsealed

gland). After treatment for 2 days litters were removed from these groups for 4 h to allow milk to accumulate before milking and blood sampling as described above.

Further groups of animals had litters removed and received one of the following treatments for two days: rbGH (1 mg in 20% PVP/injection) twice daily; PRL (1 mg in 20% PVP/injection) twice daily; hydrocortisone (1 mg in safflower oil/injection) once daily; progesterone (5 mg in safflower oil/injection) once daily; anti-IGF-1 serum (3 ml concentrated in a 1 ml volume/injection) twice daily. After treatment the animals were milked and blood samples taken as described above.

5.2.2 Collection of mouse milk

A lactating mouse was treated at day 11 of lactation by removal of its litter for 48 h, and a second mouse had its litter removed for 4 h to allow milk to accumulate. The dams were then anaesthetised with a mixture of hypnorm and hypnovel after which the dam received 100 mU oxytocin to induce milk ejection and allow milk to be removed by gentle pressure.

5.2.3 IGF RIA

Serum IGF-1 concentrations were measured as described in Chapter 2. Serum IGF-2 concentrations were measured by Dr F. Buonomo as descibed in Buonomo *et al.* (1988).

5.2.4 Solution phase assay for IGFBPs

This was performed as described in Chapter 2. The non-linearity of this assay did not seem to be caused by tracer limitation as increasing the counts 10-fold did not

affect the percentage binding of tracer, therefore a pool of milk from animals whose litters had been removed for 48 h was used in a standard curve to compare the IGFBP content in different milk samples. Competitive binding assays were carried out in the presence of unlabelled IGF-1, IGF-2, or IGF-1 analogues as described in the results.

5.2.5 Western ligand blotting

This was carried out as in Chapter 2. For most assays 1 μl of serum was used per gel track. Milk was diluted 1/10 in PBS or TBS and in some assays centrifuged at 100,000 x g for 20 min in a compressed air-driven high speed centrifuge, the supernatant was then removed and the pellet resuspended in buffer to the original volume. Casein micelles were disrupted by mixing equal volumes of milk with 200 mM EDTA pH 7.0 before centrifugation as above, then all fractions were adjusted to the initial volume. Some conditioned medium samples from mammary epithelial cell cultures (see 5.2.12) were dialysed against distilled water overnight at 4 °C, freeze dried, and dissolved in a sixth of the original volume with distilled water.

5.2.6 Size exclusion chromatography of milk

Protection of the size exclusion column required defatting of milk and removal of particulate matter by centrifugation. Centrifugation also precipitated micelles containing casein in association with calcium phosphate and thus greatly reduced the protein content. Milk was diluted 1/4 in PBS or 50 mM Tris HCl pH 7.5, centrifuged at 20 min at 13,500 x g, and the infranatant was filtered through a 0.22 μm filter. Filtrate (10 μl) was incubated overnight at 4 °C with 5 x 10^5 cpm

[^{125}I]IGF-1 in a final volume of 500 μl PBS/0.1% tween. 100 μl of this was injected into a Superose 12 high performance size exclusion column. The column was run at a flow rate of 0.5 ml/min in PBS/0.1% tween, and fractions were collected at 1 min intervals and counted in a gammacounter. Calibration markers were, β-amylase (200 kDa, Vo), BSA (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.3 kDa), IGF-1 (7.5 kDa), tyrosine (180 Da).

5.2.7 Deglycosylation of IGFBP

To assess the N-glycosylation of the IGFBP in a pool of milk from dams whose litters had been removed for 48 h, normal rat serum was used as a source of glycosylated IGFBP-3 (positive control), and Clone 9 conditioned medium as a source of non-glycosylated IGFBP-2 (negative control). Milk (0.1-0.4 μl), serum (1 μl), or Clone 9 conditioned medium (10 μl) was incubated with 0.5 U of N-glycanase for 20 h at 37 °C in a final volume of 10μl containing 50 mM EDTA, 2 mM PMSF, 1% Triton, 0.05% SDS and 12.5 % glycerol. Control samples without N-glycanase were incubated in the same buffer at 37 °C or 4 ° C for 20 h. After incubation 5 μl of 5-times concentrated SDS sample buffer added and the samples analysed by WLB.

5.2.8 Western Immunoblotting

This was carried out as in Chapter 2 using antisera to IGFBP-5 and IGFBP-2 (UBI). Dilutions of Clone 9 conditioned medium (positive control for IGFBP-2) and milk were analysed by WLB and quantities which yielded bands of equal intensity were used for immunoblotting.

5.2.9 Northern Analysis

Northern blot analysis of mammary RNA for IGFBP mRNA was carried out as described in Chapter 2.

5.2.10 *In Situ* Hybridisation

Frozen sections of rat mammary gland obtained from control lactating animals or after litter removal for 48 h were probed for IGFBP mRNA. Sections were dehydrated in 100% ethanol twice for 5 min then fixed in 2% formaldehyde in 100 mM Tris HCl, 50 mM EDTA pH 8.0 for 5 min. The sections were rinsed in distilled water for 5 min and the tissue permeabilised in 10 μg/ml proteinase K, 50 mM EDTA, 100 mM Tris HCl pH 8.0 for 30 min at 37 °C. After fixation in 2 % formaldehyde solution, sections were rinsed in water, then in 100 mM triethanolamine pH 8.0 for 2.5 min and acetylated for 10 min at room temperature in 100 mM triethanolamine pH 8.0, 0.0025% (v/v) acetic anhydride. The sections were rinsed briefly in 2x SSC and dehyrated in a graded ethanol series (50%, 70%, 95%, 100%, 100%) for 3 min each, dried under vacuum and stored dessicated at -20 °C.

Anti-sense and sense RNA probes were prepared by incubating 1 ng of template DNA, 200 μCi ^{35}S UTP, 50 mM GTP/CTP/ATP, 20 U RNA polymerase, 20 U ribonuclease inhibitor, 5 mM dithiothreitol (DTT), 2 mM spermidine, 10 mM NaCl, 6 mM $MgCl_2$, 40 mM Tris HCl pH 7.5 in a final volume of 20 μl at 37 °C for 2 h. DNase I (1 U) was then added at 37 °C for 15 min, then the reaction was stopped by adding 100 mM EDTA pH 8.0 and the labelled probe was separated from incorporated label using a Sephadex G-50 column.

Sections were prehybridised overnight at 55 °C in a humidified chamber in 100 μl hybridisation buffer consisting of 0.5 mg/ml tRNA, 0.5 mg/ml Torula yeast tRNA, 10 mM DTT, 50% formamide, 10% dextran sulphate, 0.2 mg/ml Ficoll 400, 0.2 mg/ml PVP, 0.2 mg/ml BSA, 1 mM EDTA, 300 mM Na Cl, 10 mM Tris HCl pH 8.0. Sections were then hybridised with 1 x 10^7 cpm ^{35}S RNA probe in 70 μl hybridisation buffer under a coverslip overnight at 55 °C in a humidified chamber.

After hybridisation the coverslips were soaked off in 4 x SSC then treated with 20 μg/ml RNase A in 500 mM Na Cl, 1 mM EDTA, 10 mM Tris HCl pH 8.0 for 30 min at 37 °C. The sections were then washed as follows: twice in 2 x SSC, 1 mM DTT for 10 min at room temperature; once in 0.5 x SSC for 20 min; once in 0.1 x SSC/1 mM DTT at 65 °C for 60 min; rinsed once in 0.1 x SSC/1 mM DTT. Sections were dehydrated for 3 min in each of: 50% ethanol in 0.1 x SSC/1 mM DTT; 70% ethanol in 0.1 x SSC/1 mM DTT; 95% ethanol; 100% ethanol. After vacuum drying for 30 min at room temperature the sections were exposed to β-max Hyperfilm for 3 weeks to estimate the extent of hybridisation. The sections were subsequently coated in Kodak NTB-2 emulsion diluted 1/2 with water and exposed at 4 °C and developed (Kodak), counterstained, dehydrated and mounted then examined under dark and light field microscopy.

5.2.11 Preparation of mammary gland acini

Lactating rats at day 12-14 of lactation were treated with anti-rGH γ-globulin and bromocriptine, or litter removal as described above. Mammary gland tissue was removed and rinsed in medium 199 and weighed. Tissue was minced with scissors and incubated at 2.5 g/20 ml digestion mixture (1mg/ml collagenase (Worthingtons

CL3), 0.15 mg/ml hyaluronidase (Boehringer), 2 mg/ml BSA in Medium 199 with an additional 1 mg/ml glucose) at 37 °C for 95 min in a water bath with vigorous pipetting at 15 min intervals. The mixture was then sieved through a tea strainer, centrifuged for 10 min at 400 x *g*, and washed at 100 x *g* for 6 min three times in medium 199 containing 20 μg/ml DNase. The pellet was then suspended in 10 ml of medium 199 (containing 100 U/ml penicillin, 100 μg/ml streptomycin) and plated at 1 ml per well in a 12 well plate. An aliquot was retained for DNA measurement as described in Chapter 2. After culture the acini and conditioned medium were harvested by centrifuging at 600 x *g* for 10 min.

5.2.12 Preparation and culture of mammary epithelial cells

Mammary epithelial cells were prepared from mice on day 15-16 of pregnancy (Wilde *et al.* 1991) and plated at 3-5 x 10^5 cells/cm^2 in 3.5 cm culture wells coated with Engelbreth-Holm-Swarm (EHS) matrix. Cells were cultured in Medium 199/Ham's F12 (50:50, v/v) containing insulin (5 μg/ml), hydrocortisone (1 μg/ml), triiodothyronine (0.65 ng/ml), epidermal growth factor (10 ng/ml) and PRL (1 μg/ml). Foetal calf serum (10% v/v) was present for the first day of culture to promote attachment, thereafter medium was changed daily with medium without FCS. In some samples cultures were treated on day 5 with 2.5 mM EGTA for 20 min, washed with culture medium and returned to culture for 24 h before the next harvesting.

5.3 Results

5.3.1 Effects of bromocriptine and anti-GH treatment in combination with GH, IGF-1 and IGF-2 on serum IGFs and IGFBP-3 in lactating rats

Bromocriptine treatment did not produce any changes in serum IGF-1, IGF-2, or IGFBP-3. However Anti-GH treatment decreased serum IGF-1, and IGF-2 levels below control lactating values although IGFBP-3 levels were unaffected. Combined treatment led to significant reductions in IGF-1, IGF-2, and IGFBP-3 levels, and GH therapy prevented all of these effects (Table 5.1).

5.3.2 Effects of PRL, GH or a combination of IGF-1 and IGF-2 bound to hIGFBP-3, after pretreatment with bromocriptine and anti-rGH, on serum IGF-1 and IGFBP-3

Both GH and IGF-1 increased serum IGF-1 levels compared with those of animals given bromocriptine and anti-rGH treatment, but only GH treatment increased rat IGFBP-3 levels. In contrast PRL treatment did not affect serum IGF-1 or IGFBP-3 levels (Table 5.2). There are discrepancies in the absolute IGF-1 concentrations in Table 5.1 and Table 5.2 for the treatments common to both studies (combined anti-rGH and bromocriptine treatment with or without GH replacement), although the relative differences are similar. These differences may be because the two assays were carried out 18 months apart using different primary antibody batches or alternatively they may reflect real differences in the serum IGF-1 levels between the groups of rats used.

	IGF-1	IGF-2	IGFBP-3
	(ng/ml)	(ng/ml)	(arbitrary units)
Control	421 ± 15	21.8 ± 1.5	0.98 ± 0.07
Bromocriptine	474 ± 22†	21.8 ± 3.8†	1.17 ± 0.06
Anti-rGH	270 ± 15**	15.0 ± 1.5*	1.04 ± 0.17
Br + anti-rGH	226 ± 8**	15.0 ± 1.5*	0.55 ± 0.08*
Br + anti-rGH + GH	728 ± 22**†	42.1 ± 6.8**†	1.42 ± 0.28†
Br + anti-rGH + IGF-1	330 ± 15*†	15.0 ± 1.5*	0.73 ± 0.07
Br + anti-rGH + IGF-2	218 ± 15**	26.3 ± 3.7†	ND

Table 5.1 Effects of bromocriptine (Br) and anti-rGH treatment in combination with GH, IGF-1 and IGF-2 on serum IGF-1 and IGFBP-3 in lactating rats. Lactating rats were treated at day 14 of lactation as shown; all treatments were administered for 2 days. IGF concentrations were determined by RIA and IGFBP-3 by ligand blotting. Values are means ± SEM for n=6 observations per group. *$p<0.01$, **$p<0.001$ compared with control values, †$p<0.01$ compared with Br + anti-GH treated (Student's unpaired t-test). ND= not determined.

	IGF-1	rat IGFBP-3	rhIGFBP-3
	(ng/ml)	(arbitrary units)	(μg/ml)
Treatments anti-rGH and bromocriptine plus:			
saline	120.3 ± 15.0	2.1 ± 0.3	UD
PRL	135.3 ± 22.5	3.3 ± 0.5	UD
GH	413.5 ± 82.7**	6.7 ± 0.9***	UD
PRL + GH	518.8 ± 135.3**	6.1 ± 0.5***	UD
IGF-1 + IGFBP-3	203.0 ± 30.0	3.1 ± 0.8	23.8 ± 5.2
IGF-1 + IGF-2 + IGFBP-3	210.5 ± 30.0	1.7 ± 0.2	9.2 ± 5.2

Table 5.2 Effects of various hormonal treatments, given in three divided doses over the final 22 h of the experiment, on serum IGFs, IGFBPs and solution phase [^{125}I]IGF-1 binding after pre-treatment for 48 h with bromocriptine and anti-rGH. IGF-1 and rhIGFBP-3 levels were determined by RIA, rat IGFBP-3 levels were determined by western ligand blotting. Values are means ± SEM for n=5 observations per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with saline-treated group; (Student's unpaired t-test). UD= undetectable.

	IGFBP-3	28-32 kDa	24 kDa
		(arbitrary units)	
Control	240 ± 3	68 ± 3	30 ± 3
Litter removal (48h)	363 ± 31*	100 ± 9*	53 ± 2***
Br + anti-GH (48h)	114 ± 14*	33 ± 4***	28 ± 4
Br + anti-GH (96h)	145 ± 26*	43 ± 6**	33 ± 4

Table 5.3 Effects of bromocriptine (Br) and anti-rGH treatment or litter removal on serum IGFBPs determined by ligand blotting. Values are means ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with control values (Student's unpaired t-test; $n=5$ observations).

5.3.3 Effects of bromocriptine and anti-rGH treatment or litter removal on serum IGFBPs detected by western ligand blotting

The levels of all serum IGFBP bands were increased by litter removal compared with lactating rats. Combined treatment with bromocriptine and anti-rGH decreased both the IGFBP-3 and 28-31 kDa bands but the 24 kDa band (IGFBP-4) was unaffected by hormonal ablation; the length of treatment had no effect (Table 5.3).

5.3.4 Effects of bromocriptine and anti-rGH treatment or litter removal on IGFBP levels in milk measured by solution phase [^{125}I]IGF-1 binding

Solution phase [^{125}I]IGF-1 binding to milk was substantially greater after litter removal than after hormone ablation treatment for 48 or 96 h (Fig 5.1).

5.3.5 Competitive inhibition of [^{125}I]IGF binding to milk from animals whose litters have been removed

Competitive inhibition of radiolabeled IGF-1 and -2 binding to 0.5 μl milk (50-100 μg protein) demonstrated a preferential binding of IGF-2 (Fig 5.2). Insulin and Long-R_3-IGF-1 did not compete even at high concentrations suggesting that binding was to an IGFBP rather than to a type 1 IGF receptor. Comparison of the ability of high concentrations of three IGF-1 analogues to compete for [^{125}I]IGF-1 binding showed Long-IGF-1 competed better than R_3-IGF-1, and Long-R_3-IGF failed to compete (Fig 5.3).

5.3.6 Size exclusion chromatography of milk

IGFBPs in milk obtained after litter removal formed principally a small molecular weight complex with IGF-1 at about 40 kDa and there was some evidence of a larger molecular weight complex at about 100 kDa; complexes were essentially absent in control milk (Fig 5.4).

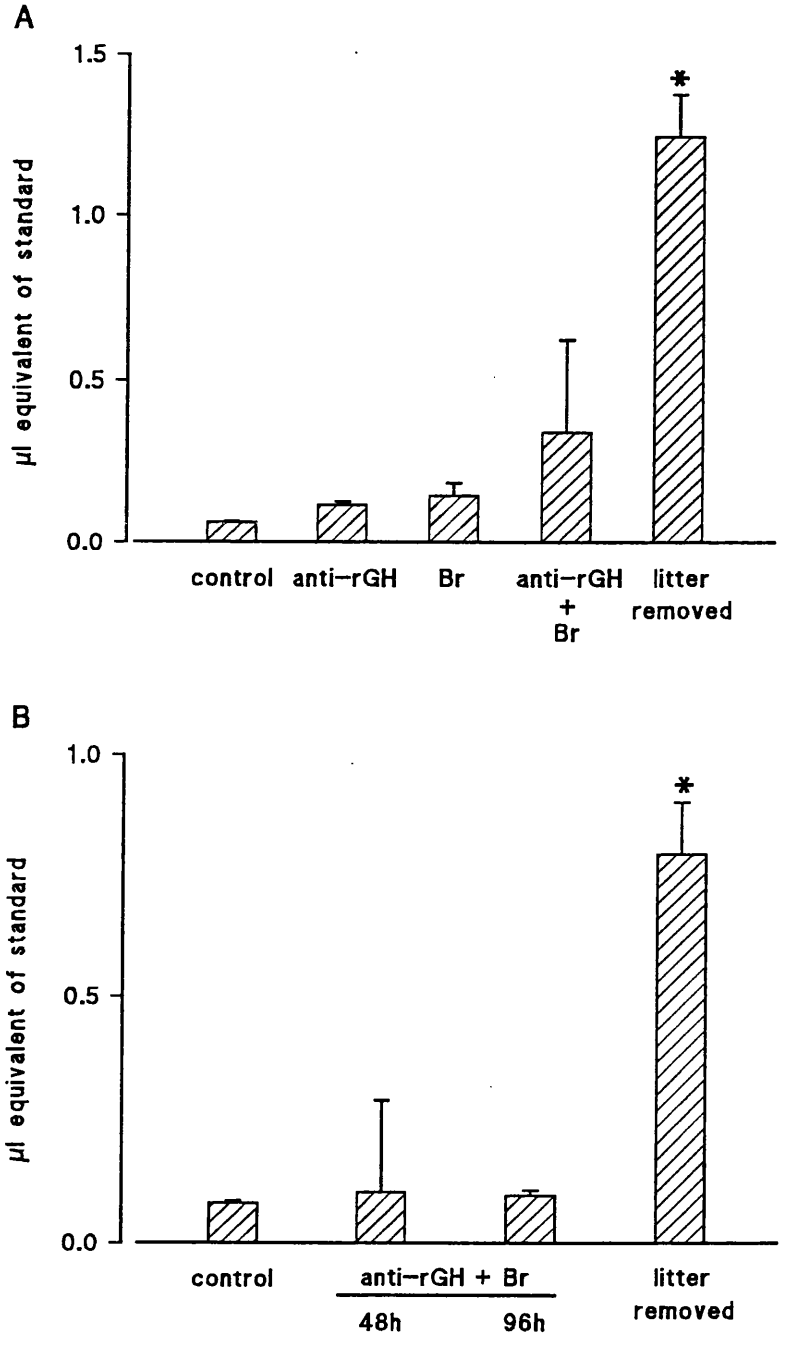


Fig 5.1 Effects of bromocriptine (Br) and anti-rGH treatment or litter removal on IGFBPs in milk. IGFBPs were measured by solution phase [^{125}I]IGF-1 binding to milk from hormonally manipulated lactating rats. (A) Comparison of treatment by hormonal ablation and litter removal for 48 h. (B) Comparison of hormonal ablation treatment for 24 and 48 h. Values are means ± SEM for 3-8 animals (A) or 5 animals (B), *$p < 0.001$ compared with control (analysis of variance).

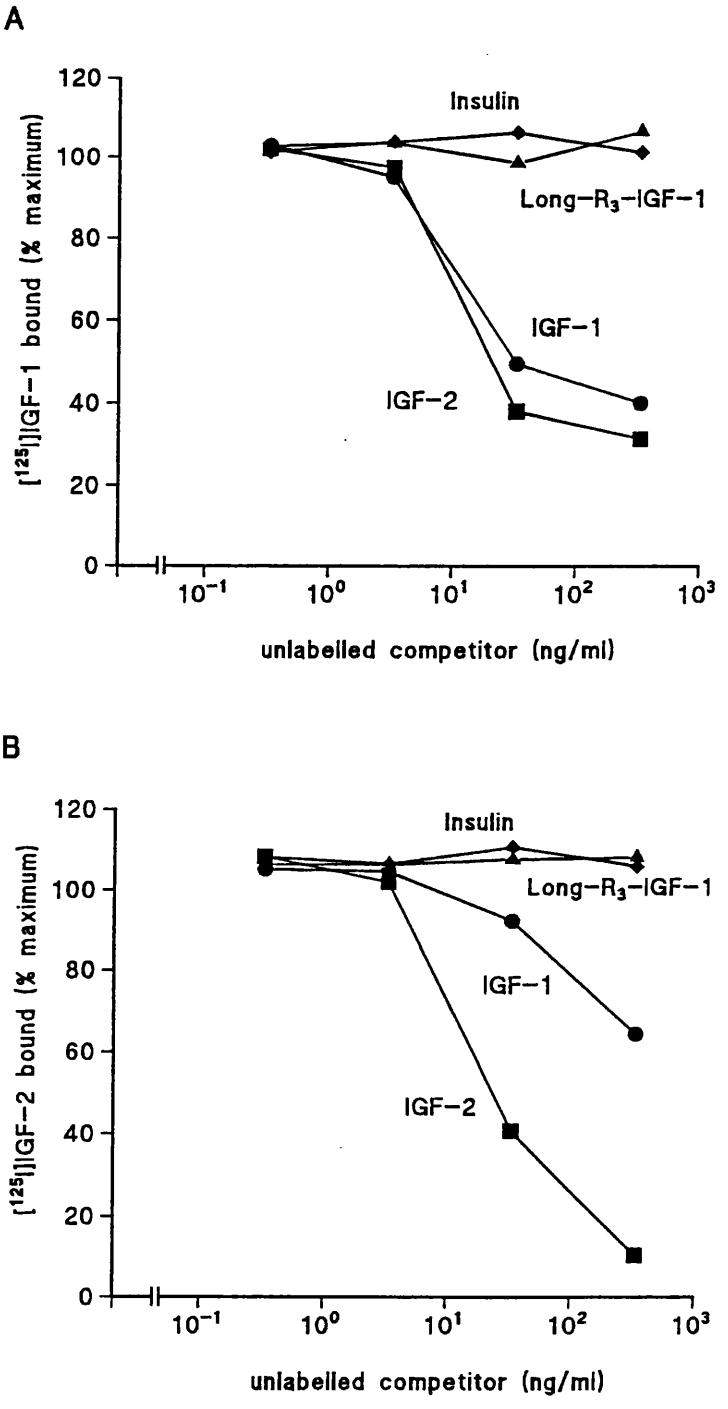


Fig 5.2 Comparison of the ability of IGFs and insulin to inhibit solution phase [^{125}I]IGF-1 (A) and [^{125}I]IGF-2 (B) binding to milk from lactating rats whose litters had been removed for 48 h. Pooled milk was incubated with radiolabelled IGF in the presence of competitors, and bound and free hormone were separated by charcoal adsorbtion.

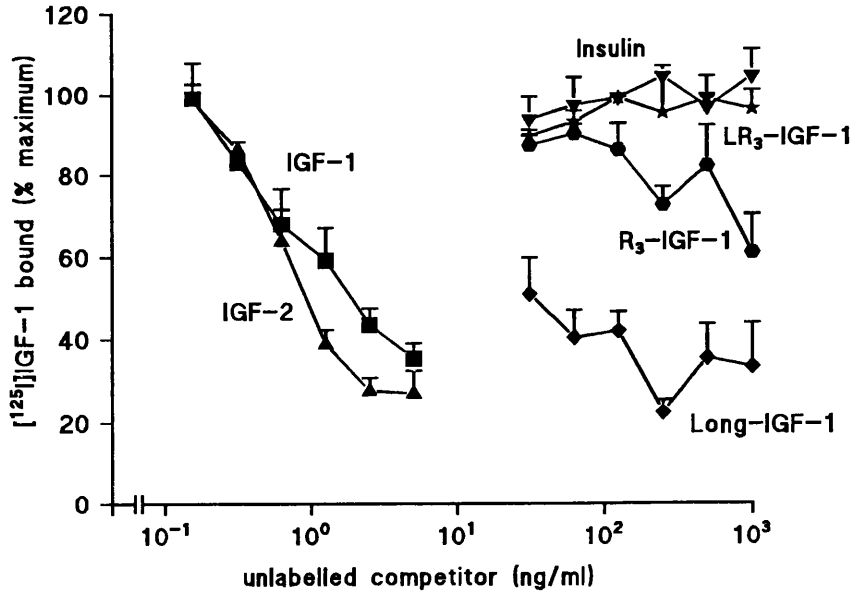


Fig 5.3 Comparison of the ability of IGF analogues to inhibit solution phase [^{125}I]IGF-1 binding to milk from lactating rats whose litters had been removed for 48 h. Pooled milk was incubated with [^{125}I]IGF-1 in the presence of competitors, and bound and free hormone were separated by charcoal adsorbtion. Values are means ± SEM for 3 replicate assays.

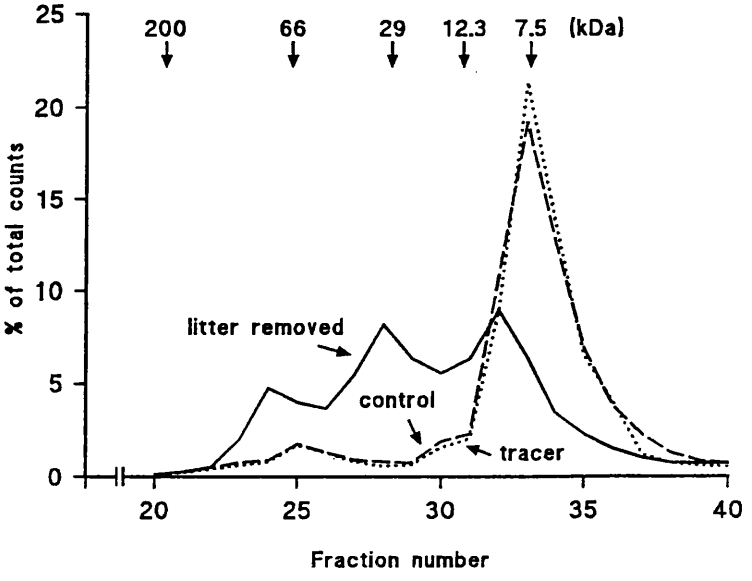


Fig 5.4 Size exclusion chromatography of milk. Diluted and filtered milk (10 μl) was incubated overnight at 4 °C with 5 x 10^5 cpm [^{125}I]IGF-1 in a final volume of 500 μl PBS/0.1% tween. 100 μl of this was injected into a Superose 12 high performance size exclusion column. The column was run at a flow rate of 0.5 ml/min in PBS/0.1% tween, and fractions were collected at 1 min intervals and counted in a gammacounter. Calibration markers were as indicated, β-amylase (200 kDa, Vo), BSA (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.3 kDa), IGF-1 (7.5 kDa).

5.3.7 Western ligand blot analysis of fractionated milk from rats whose litters have been removed for 48 h

WLB analysis of milk obtained after litter removal confirmed the presence of a major IGFBP band of about 30 kDa band. In some samples additional less intense bands of about 39-43 kDa and >200 kDa were detected all of which could be competed by unlabelled IGF-1. In contrast IGFBPs were poorly detectable in milk from control or hormonal ablated lactating rats (results not shown). WLB analysis of high speed centrifugation fractions of diluted milk from animals whose litters had been removed showed that the IGFBP was partitioned to the pellet fraction (Fig 5.5A). However disruption of casein micelles by EDTA treatment partitioned the IGFBP to the supernatant fraction (Fig 5.5B). A pool of milk which had been stored at -20 °C for several months was used for EDTA assays which may account for the less striking partitioning of IGFBP to the pellet fraction compared with the use of fresh milk samples in Fig 5.5A.

5.3.8 Deglycosylation and immunoblotting of milk from rats after litter removal

The molecular weight of the IGFBP in milk was unaffected by N-glycanase treatment (Fig 5.6). In addition IGFBPs were unaffected by the incubation temperature since there was no difference in control samples at 4°C and 37 °C (not shown). The IGFBP in milk migrates slightly in front of IGFBP-2 and was not recognised by an antiserum to bovine IGFBP-2 which crossreacts with rat IGFBP-2, however it was recognised by an antiserum to IGFBP-5 (Fig 5.7).

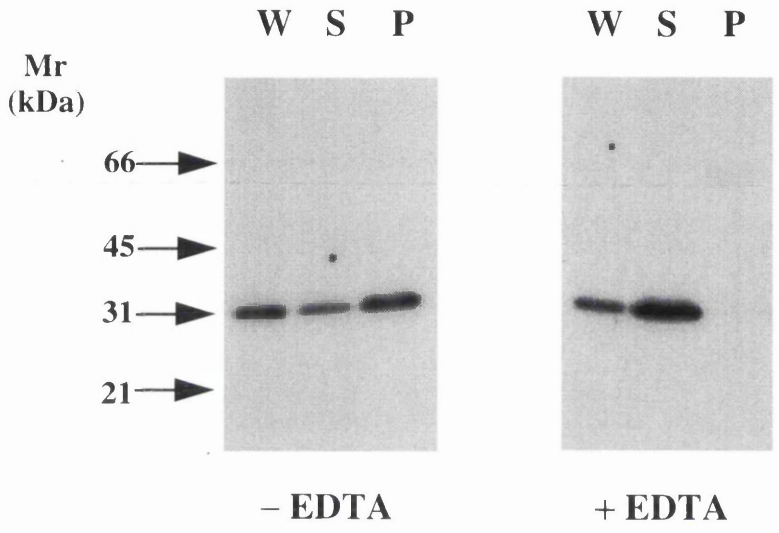


Fig 5.5a Western ligand blot analysis of fractionated milk from lactating rats whose litters had been removed for 48 h. A full length example of one of the blots (pool (1)) used in Fig 5.5.

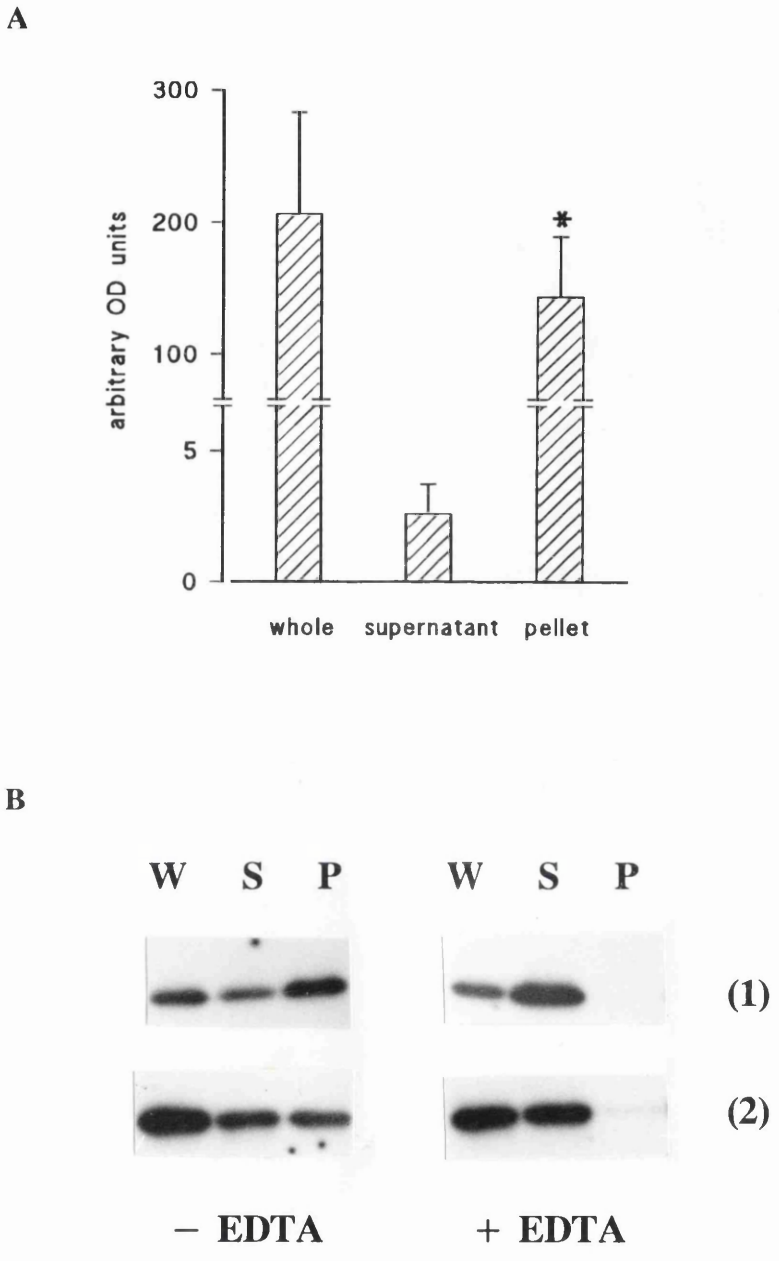


Fig 5.5 Western ligand blot analysis of fractionated milk from lactating rats whose litters had been removed for 48 h. (A) Quantitation of centrifuged milk fractions (0.1 μl equivalent). Values are means $\pm$ SEM for $n=5$ observations, $^*p<0.05$ compared with the supernatant fraction. (B) Effect of EDTA on the partitioning of milk IGFBP. Milk samples from two separate pools (1) and (2), were diluted in 100 mM EDTA before fractionation. Samples in (A) were from freshly collected milk but samples in (B) were from milk pools stored for several months; this difference may account for the discrepency in partitioning of IGFBP between pellet and supernatant fractions.

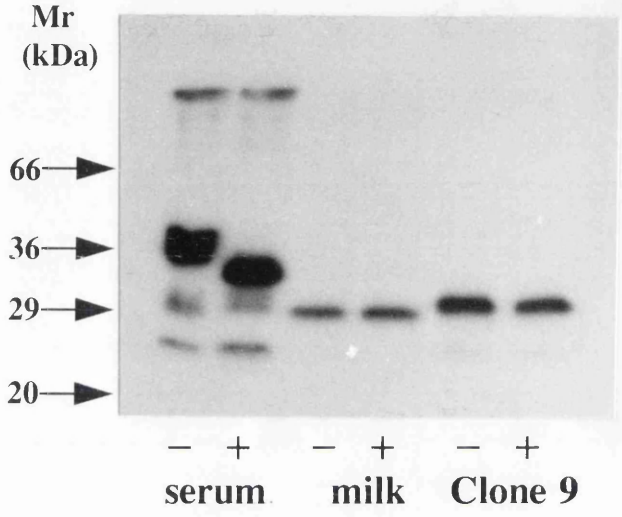


Fig 5.6 Deglycosylation of milk, serum and IGFBP-2 by N-glycanase. Western ligand blot of milk from lactating rats whose litters were removed 48 h (0.1-0.4 μl), pooled normal rat serum (1 μl), or 10 μl clone 9 conditioned medium incubated with (+) or without (−) 0.5 unit of N-glycanase for 20 h at 37 °C in a final volume of 10 μl containing 50 mM EDTA, 2 mM PMSF, 1% Triton, 0.05% SDS and 12.5 % glycerol. Representative result of 3 separate assays.

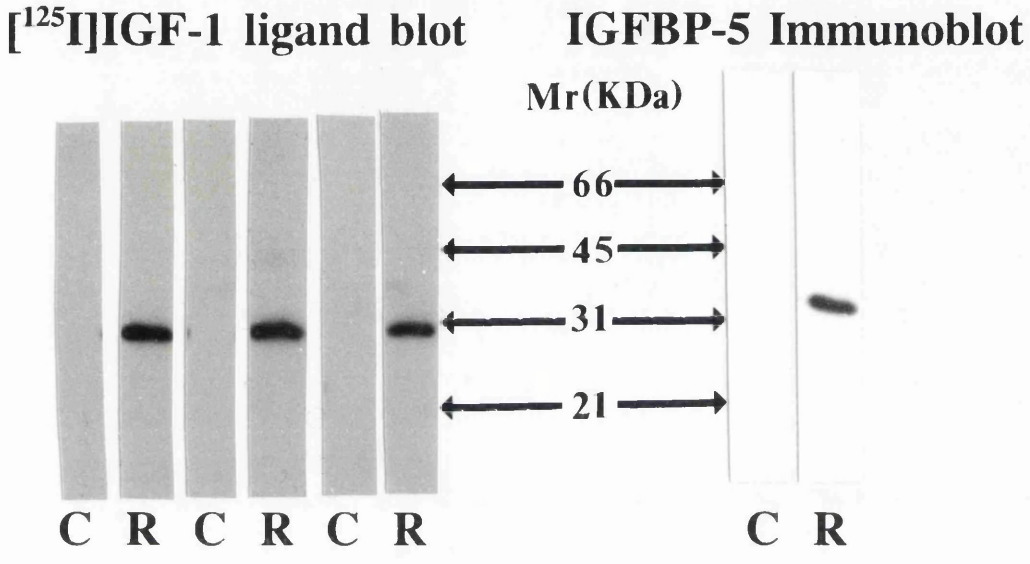


Fig 5.7 Immunoblotting of milk from lactating rats whose litters had been removed for 48 h. Milk (0.1μl) was Western ligand blotted with [^{125}I]IGF-1 or immunoblotted with an antiserum to IGFBP-5. C=control, R=litter removed.

5.3.9 Effects of oestradiol treatment, teat-sealing, or litter removal in combination with PRL, GH, progesterone, corticosteroid, or anti-IGF-1 treatment, on serum IGF-1 and IGFBPs in milk

Oestradiol treatment had no effect on IGFBP concentrations in lactating rats. Likewise lactating animals with half their glands sealed had IGFBP levels from the suckled glands similar to those of controls animals, but milk obtained from sealed glands had significantly increased IGFBP levels (5-10 fold), whereas litter removal increased IGFBP levels even further (100 fold). Concurrent PRL treatment reduced the level of the 29-30 kDa IGFBP in milk, whilst in contrast anti-IGF-1, GH, or hydrocortisone treatment in conjunction with litter removal did not affect milk IGFBP levels (Table 5.4).

Serum IGF-1 levels increased after litter removal compared with lactating controls, whereas lactating animals with half their glands sealed had serum IGF-1 levels similar to those of lactating controls. PRL treatment in conjunction with litter removal increased serum IGF-1 levels even further when compared with litter removal alone. IGF-1 levels in anti-IGF-1 treated animals could not be determined because anti-IGF-1 antibody in the serum samples interfered in the IGF-1 RIA (Table 5.4).

5.3.10 Northern analysis of IGFBP mRNA in mammary glands of lactating rats after combined bromocriptine and anti-rGH treatment or litter removal

Preliminary Northern analysis detected IGFBP-4 and -5 mRNA in mammary gland after litter removal at day 10 of lactation for 24 or 48 h (Fig 5.8); IGFBP-2 was also detectable, IGFBP-1 and -3 mRNA was undetectable but IGFBP-6 mRNA levels

	serum IGF-1 (ng/ml)	milk IGFBP (arbitrary units)
Control	188 ± 8	1.25 ± 0.42
Control + oestradiol	290 ± 44*	1.12 ± 0.31
Control: unsealed	200 ± 27	1.94 ± 0.34
: sealed		11.60 ± 3.32†
Litter removal (LR)	393 ± 73	81.61 ± 28.83
LR + PRL	590 ± 71‡‡	17.08 ± 2.97‡
LR + anti-IGF-1	ND¥	76.55 ± 11.06
LR + GH	ND	83.54 ± 19.69
LR + Progesterone	ND	67.54 ± 6.35
LR + Hydrocortisone	ND	96.14 ± 16.5

Table 5.4 Effects of oestradiol treatment, teat-sealing, or litter removal in combination with PRL, GH, progesterone, corticosteroid, or anti-IGF-1 treatment, on serum IGF-1 and IGFBPs in milk. Values are mean ± SEM for n=5-7 observations per group. ND= not determined, ¥= not determined because the administered anti-IGF-1 antibody interferes in the IGF-1 RIA. †p= <0.01 compared with sealed (Student's paired t-test). *p <0.01 compared with control; ‡p <0.02, ‡‡p <0.001 compared with litter removal (Student's unpaired t-test).

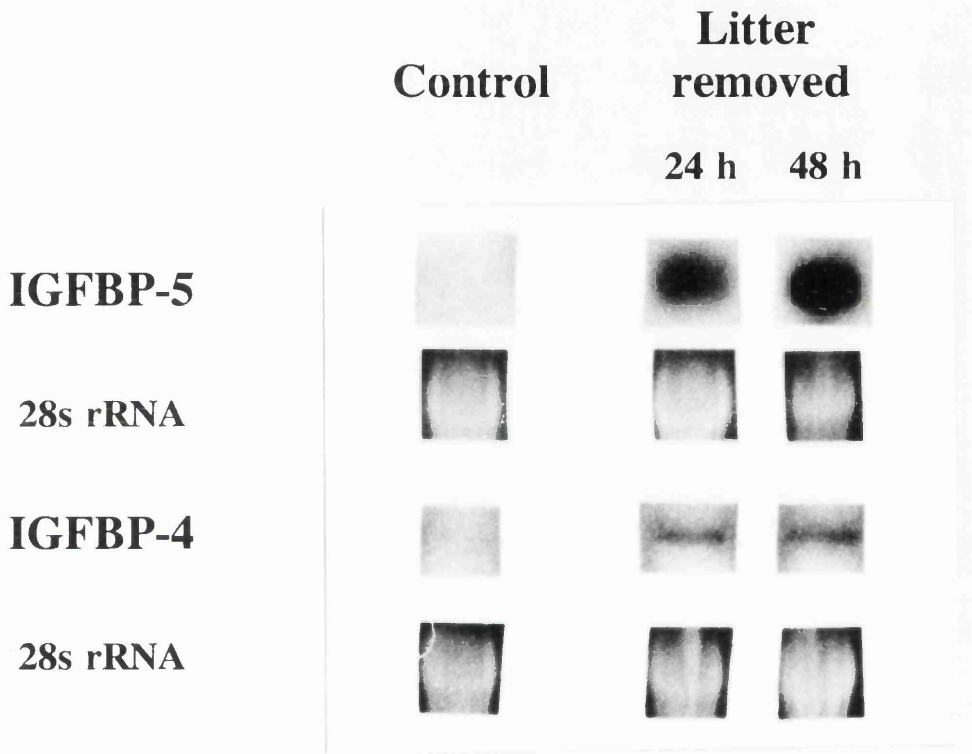


Fig 5.8 Northern analysis of IGFBP mRNAs in mammary gland from lactating rats whose litters were removed for 24 or 48 h. 20 μg samples of total RNA were separated on agarose gels, blotted and hybridised with RNA probes for IGFBP-4 and -5. The RNA was stained with ethidium bromide before blotting and the 28s rRNA species shown beneath the northern blot.

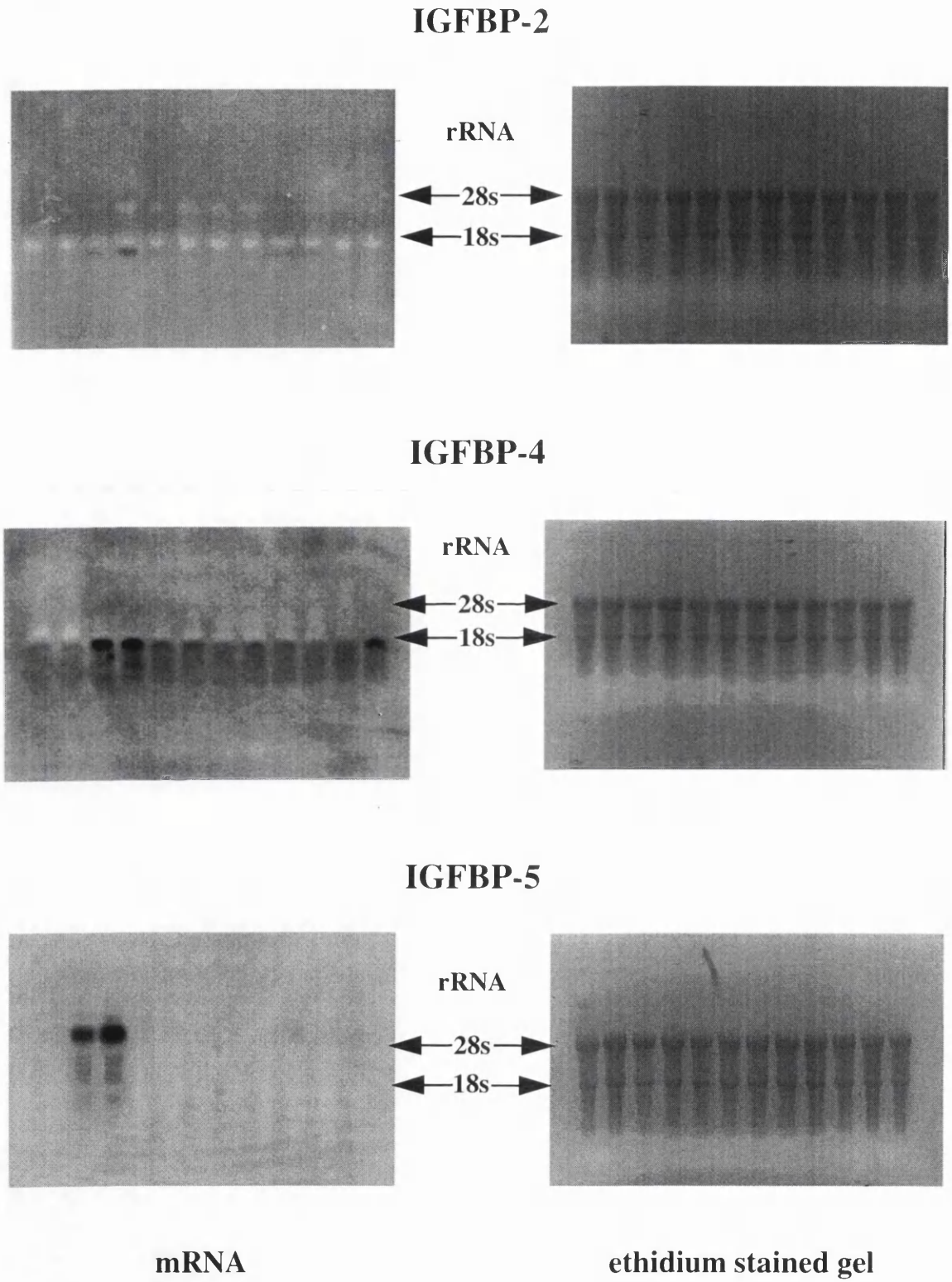


Fig 5.9a Northern analysis of IGFBP mRNAs in mammary gland from hormonally manipulated rats. Full length examples of the Northern blots used in Fig 5.9.

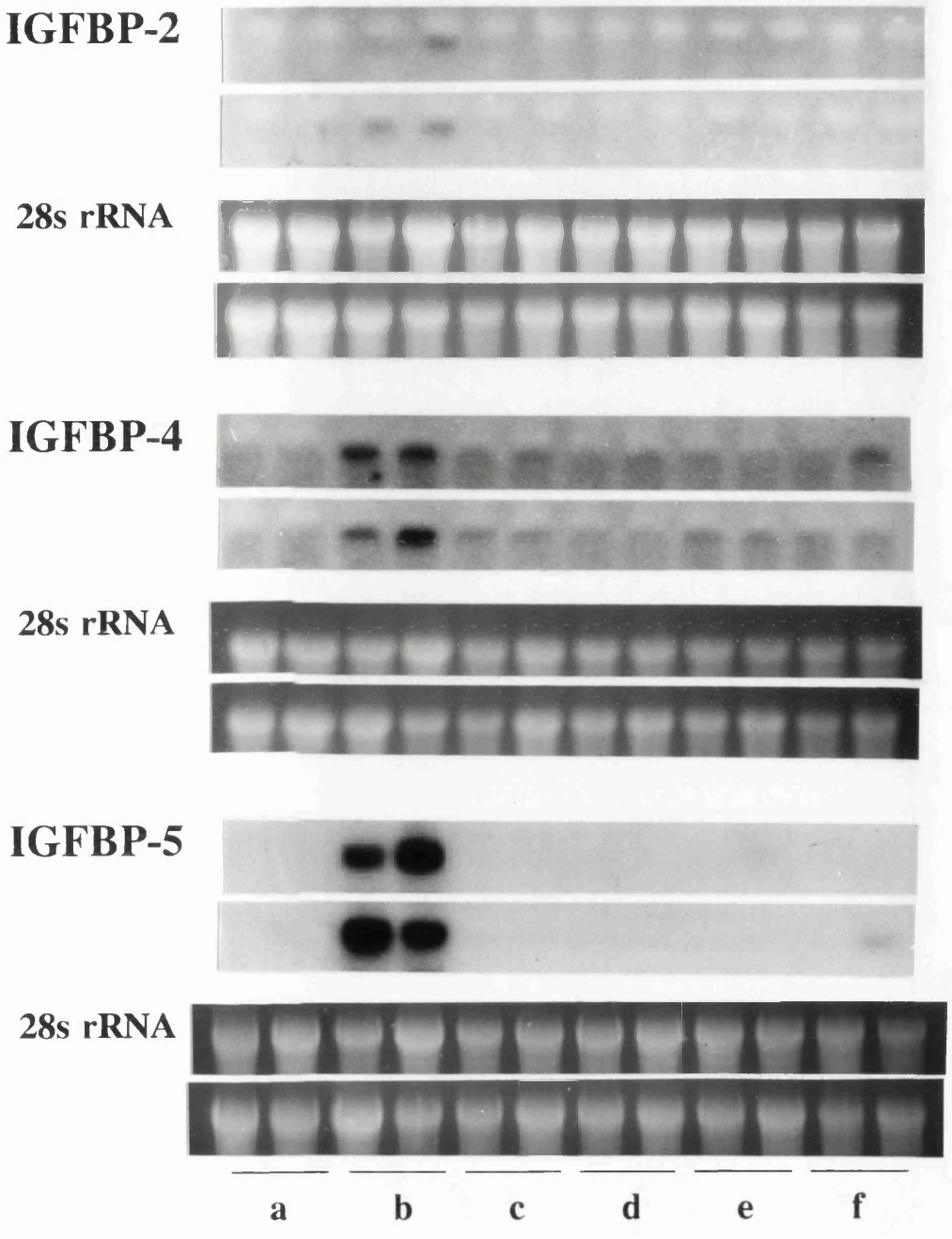


Fig 5.9 Northern analysis of IGFBP mRNAs in mammary gland from hormonally manipulated lactating rats. (a) control (b) litter removal for 48 h (c) Bromocriptine (Br) plus anti-GH for 48 h (d) Br plus anti-GH for 48 h with PRL for 22 h (e) Br plus anti-GH for 48 h with GH for 22 h (f) Br plus anti-GH for 96 h. 20 μg samples of total RNA were separated on agarose gels, blotted and hybridised with RNA probes for IGFBP-2, -4, and -5. The RNA was stained with ethidium bromide before blotting and the 28s rRNA species shown beneath the northern blot.

	IGFBP-2	IGFBP-4	IGFBP-5
	(arbitrary units)		
Control	-15.6 ± 13.4	32.5 ± 8.8	-43.4 ± 55.4
Litter removed	74.0 ± 27.5*	165.9 ± 37.9*	2481.8 ± 706.6*
Br + anti-GH (48h)	-16.6 ± 7.7	47.5 ± 6.2	-48.8 ± 59.7
Br + anti-GH (48h) + PRL	-34.2 ± 7.1	30.8 ± 12.8	44.3 ± 16.8
Br + anti-GH (48h) + GH	-21.6 ± 9.1	60.5 ± 17.2	52.0 ± 7.6
Br + anti-GH (96h)	-33.2 ± 10.4	54.3 ± 7.3	58.7 ± 41.8

Table 5.5 Northern analysis of rat mammary gland IGFBP mRNA. Values are means ± SEM derived from densitometry of blots normalised for 28s bands. Subtraction of the average background for each blot renders some values negative. *$p < 0.05$ compared with control values (Student's unpaired t-test; n=4 observations).

Fig 5.10 Detection of IGFBP-1 to -6 mRNA in rat mammary gland using *in situ* hybridisation. Frozen sections of mammary gland probed with antisense [^{35}S]IGFBP cRNA and exposed to β-max Hyperfilm to estimate the extent of hybridisation.

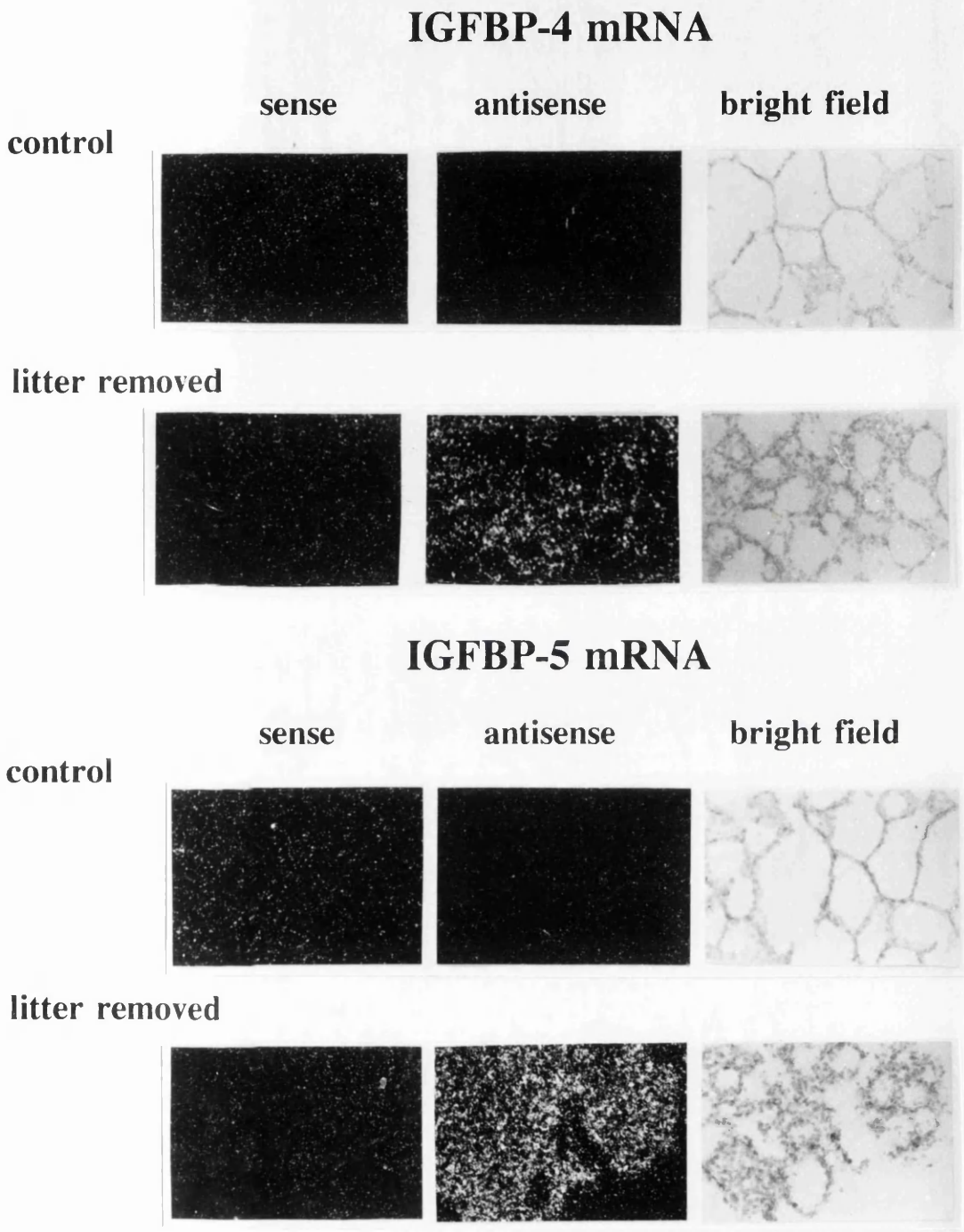


Fig 5.11 Detection of IGFBP-4 and -5 mRNA in rat mammary gland using *in situ* hybridisation. Frozen sections of mammary gland were probed with sense or antisense [^{35}S]IGFBP cRNA, then exposed to photographic emulsion to detect hybridisation. Sections were developed, counterstained, dehydrated and mounted then examined under dark and bright field microscopy.

could not be assessed because there was no positive control used. In contrast IGFBP mRNA was undetectable in control lactating gland.

Mammary glands from dams whose litters had been removed for 48 h at day 14 of lactation expressed IGFBP-2, -4 and -5 mRNA but mRNA was barely detectable or absent in glands from other treatments (Table 5.5). mRNA species detected were of about 1.8 kb for IGFBP-2, 1.3 kb for IGFBP-4, and 5.5 kb for IGFBP-5 (Fig 5.9).

5.3.11 *In situ* hybridisation analysis of rat mammary gland in lactation and after litter removal

Preliminary autoradiographic detection showed specific labelling with antisense probe to IGFBP-2, -4, and -5, but not IGFBP-1, -3, or -6 in mammary gland after litter removal. IGFBP-2 mRNA was also detectable in the control lactating gland. Subsequent detection of signals (x 200 magnification) using emulsion showed that gland obtained after litter removal expressed IGFBP-4, and -5 mRNA but these mRNAs were undetectable in glands from control lactating animals. In contrast IGFBP-2 was detectable in both control and involuting gland.

5.3.12 IGFBPs in mouse milk and mammary epithelial cell culture conditioned medium

WLB analysis of milk from a mouse whose litter had been removed for 48 h detected an IGFBP of the same mobility as that detected in rat milk from involuting glands. This IGFBP was undetectable in milk from control lactating mouse milk (Fig 5.12A). Unconditioned culture medium containing 10% FCS contained several

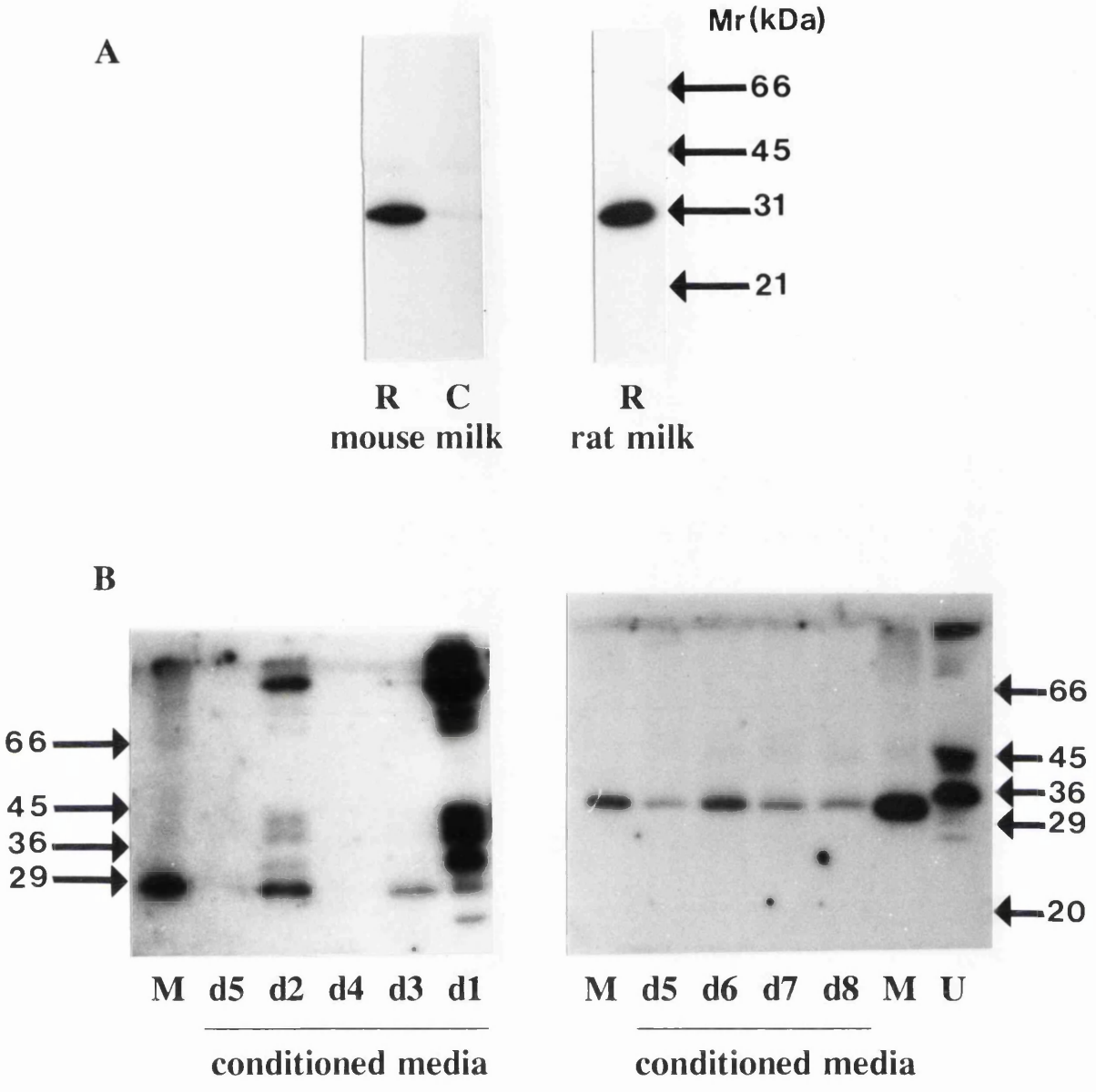


Fig 5.12 Western ligand blot analysis of mouse mammary epithelial cell culture conditioned medium and mouse milk. Mammary cells from pregnant mice were cultured on EHS matrix with lactogenic hormones. (A) Mouse milk (0.9 μl) from lactating (C) or involuting (R) gland or rat milk from involuting gland (0.1 μl). (B) Conditioned medium from two separate experiments: 1) 20 μl conditioned medium from days 1-5 2) dialysed concentrated conditioned medium (300 μl equivalent) from days 5-8, treated with EGTA on day 5. U is 10 μl unconditioned medium containing 10% FCS, M is rat milk (0.1 μl) from involuting glands.

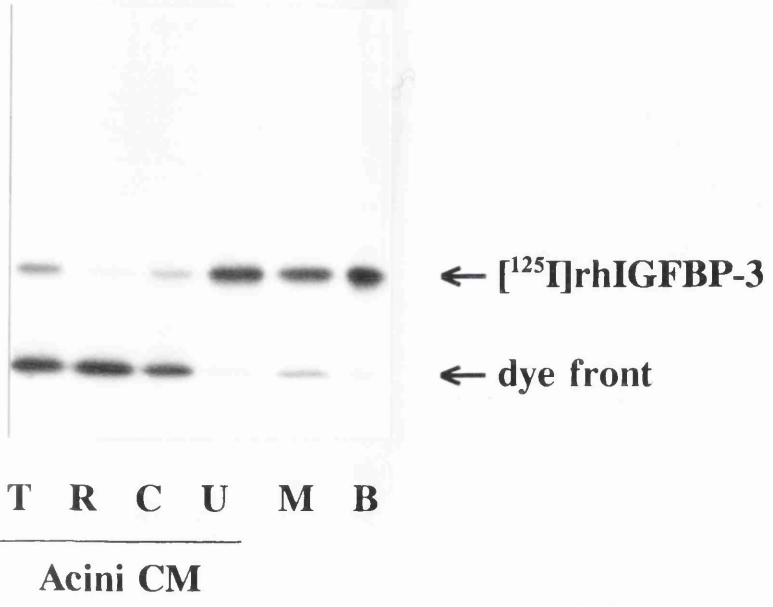


Fig 5.13 Proteolysis of [^{125}I]IGFBP-3 by conditioned medium from mammary cells. Mammary acini from treated lactating rats were incubated for 4 h at 37 °C, pregnant mouse mammary epithelial cells were cultured on EHS matrix with lactogenic hormones. Conditioned medium samples (20 μl) were incubated with 30,000 cpm recombinant non-glycosylated [^{125}I]hIGFBP-3 for 5 h at 37°C. Acini samples: T is treatment with anti-rGH and bromocriptine for 48 h, R is litter removal for 48 h, C is control, U is unconditioned medium. M is mammary epithelial cells culture, B is buffer control. After incubation samples were separated by SDS-PAGE, the gel dried under vacuum and autoradiographed for 18 h.

IGFBPs thus confounding the detection of mammary cell-derived IGFBPs at day 1-5. However the major IGFBP at day 1-5 has the same mobility as the IGFBP detected in rat milk from involuting glands. A similar IGFBP was detected at day 5-8 of culture when exogenous IGFBPs from FCS are absent from the medium.

5.3.13 IGFBPs in mammary acini conditioned medium

Conditioned medium from acini degraded [^{125}I]rhIGFBP-3 to lower molecular weight products which migrated with the dye front suggesting the presence of a protease (Fig 5.13). WLB detected the presence of a faint IGFBP band of about 31 kDa (the same mobility as rat IGFBP-2) in all samples and an additional band of 29/30 kDa in samples after litter removal (data not shown).

5.4 Discussion

This study was undertaken as part of an investigation into the role of IGFs as mediators of the galactopoietic effects of GH. Systemically administered IGF failed to mimic the ability of GH to reinitiate milk production in PRL and GH deficient rats even when administered complexed with IGFBP-3 (Flint *et al.* 1992; Flint *et al.* 1994). The IGFBPs are major modulators of IGF action therefore the elucidation of the role of the IGFs in lactation requires investigation of the IGFBPs involved. In this study we provide information on systemic and mammary gland IGFBP production and their hormonal control during lactation and involution.

The decrease in serum IGF-1 levels induced by anti-GH treatment of lactating rats was not as dramatic as that previously seen in treated young male rats (Madon *et al.* 1986), suggesting IGF-1 levels during lactation are not under such stringent

GH control as demonstrated in other states. Likewise, despite a 50% reduction in serum IGF-1 anti-GH treatment did not alter IGFBP-3 levels contrasting with the markedly reduced serum IGFBP-3 in young rats induced by anti-GH treatment (Palmer *et al.* 1993). Serum IGF-1 levels correlate with IGFBP-3 levels in a range of conditions and Gargosky *et al.* (1994) demonstrated that GH directly regulates serum IGF-1 in rats but serum IGFBP-3 is primarily regulated by IGF-1. The dissociation of serum IGF-1 and IGFBP-3 levels has been reported in another catabolic state: in endotoxin-treated fasted rats, serum GH and IGF-1 decreased but serum IGFBP-3 remained similar to control levels (Fan *et al.* 1993).

During lactation serum IGF-1 concentrations (Travers *et al.* 1993), serum IGFBPs (Donovan *et al.* 1991) and hepatic IGFBP-3 mRNA (Marcotty *et al.* 1994) are reduced. Although neither anti-GH or bromocriptine treatment alone affected serum IGFBP-3 levels, combined treatment decreased serum IGFBP-3. Furthermore, administration of GH, but not PRL, to PRL and GH deficient rats could restore serum IGFBP-3 levels. These data suggest that serum IGFBP-3 is under the dual control of GH and PRL during lactation. Dual control by GH and PRL has been shown for other parameters of galactopoiesis such as Acetyl Co-A Carboxylase activity and glucose transporter activity (Barber *et al.* 1992; Fawcett *et al.* 1991).

IGFBPs were poorly detectable in milk from lactating and hormone deficient rats although in some samples (data not shown) IGFBPs were detected of about 38-42 kDa, 29-30 kDa, and 24 kDa. Donovan *et al.* (1991) described IGFBPs in rat milk at 38-42 kDa (IGFBP-3), 29 kDa (IGFBP-2), 28 kDa (IGFBP-1), 24 kDa (IGFBP-4) and in some samples a 27 kDa proteolytic fragment of IGFBP-3. These IGFBPs were detected in the whey fraction of milk, in contrast with the IGFBP found in milk after litter removal which segregates with the casein pellet fraction.

Size exclusion chromatography of milk from control lactating rats showed no IGFBP complexes although Donovan *et al.* (1995) demonstrated IGFBPs in association with ternary complexes. However the failure to detect IGFBPs in control milk probably reflects the quantities of milk used.

Whereas serum IGF-1 and IGFBP-3 are low when involution is initiated by hormonal ablation, litter removal induced an increase in serum IGF-1 and IGFBP levels reflecting a return to maternal anabolism. The difference in physiological state induced by the two models of involution are also reflected in IGFBPs in milk.

The substantial solution phase [^{125}I]IGF binding to milk after litter removal is a measure of IGFBP concentration. Competition of [^{125}I]IGF-1 binding by IGF-1 and IGF-2 with roughly equal affinities is consistent with binding to IGFBPs or type 1 receptors. However since insulin did not compete for radioligand binding even at high concentrations IGF-1 binding was not to the Type 1 IGF receptor. Likewise IGF-1 analogues which have low affinity for IGFBPs but reasonable affinities for the Type 1 IGF receptor, failed to compete for ligand binding even at concentrations which would bind to the receptor. The ability of analogues to compete was in the order Long-IGF-1 > R_3-IGF-1 > Long-R_3-IGF-1, which correlates with their ability to bind IGFBPs from L6 myoblasts previously shown by Francis *et al.* (1992). Size exclusion chromatography showed [^{125}I]IGF-1 binding was mainly in small molecular weight complexes suggesting binding to IGFBPs rather than to IGF receptors. There is evidence of [^{125}I]IGF-1 binding to a high molecular weight band in WLB, although this was not as prominant in size exclusion chromatography. This binding may correspond to soluble (or membrane bound?) type 2 IGF receptor which has

previously been detected in bovine colostrum (Skaar *et al.* 1991). Since the majority of IGFBP in milk after litter removal is associated with the particulate fraction there is the possibility that the IGFBP in supernatant fraction used in HPLC analysis is not representative of that found in whole milk although the major IGFBP in milk after litter removal is detectable in both fractions by WLB.

Samples for deglycosylation were SDS-denatured but reduction was not compatible with subsequent detection by WLB, therefore N-glycanase treatment partially deglycosylated serum IGFBP-3 to about 37 kDa as described by Donovan *et al.* (1991); reduction is necessary for complete deglycosylation of glycosylated rhIGFBP-3 (Sommer *et al.* 1991). Immunoblotting identified the IGFBP in milk after litter removal as IGFBP-5 although immunoidentification had proved difficult; several commercially available antisera raised against recombinant human proteins failed to detect the IGFBP and although some of these antisera have known crossreactivity with rat proteins, we did not have positive controls for rat IGFBP-5 and -6 proteins.

IGFBP-5 in bone is bound tightly to mineralised calcium phosphate in bone (hydroxapatite, $3Ca_3(PO_4)_2.Ca(OH)_2$) (Mohan 1993). IGFBP-5 in milk associates with the particulate fraction containing micelles composed of casein polypeptides in association with calcium phosphate nanoclusters. However it is unknown if these two interactions are analogous, and whether the association in milk is an artefact or has some physiological significance.

Preliminary WLB data from the mouse showed an IGFBP in milk obtained after litter removal for 48 h which had a similar mobility to that detected in rat milk from involuting glands. An IGFBP of this mobility was also detected in conditioned

medium from mouse mammary epithelial cell cultures although IGFBPs in FCS-containing medium confound the detection of mammary-derived IGFBPs during the first few days of culture. Apoptosis has been described in mammary cell cultures around day 2 when mammospheres are forming and around day 8 when mammospheres degenerate (Quarrie 1996). This IGFBP in conditioned medium may be IGFBP-5 associated with apoptosis, but this correlation has not yet been established and would require a parallel study of IGFBPs and apoptosis in the same cultures.

The IGFs and IGFBPs in milk may be produced in the gland or enter via the paracellular route. Likewise IGFBPs produced in the gland may not be secreted into the milk. However, after litter removal tight junctions are leaky allowing IGFs and IGFBPs to enter milk from serum by a paracellular route, in addition to the normal secretory route via the epithelial cells. IGFBP-2, -4 and -5 mRNA expression was detected in glands after litter removal but was undetectable in glands from lactating or hormone-deficient rats. Thus milk IGFBP-5 is probably produced in the gland. High background binding of the [^{32}P]cRNA probes used may have masked all but strong signals, thus accounting for the discrepancy with Donovan *et al.* (1995) who demonstrated IGFBP-2 and IGFBP-4 mRNA expression by lactating rat mammary gland. However *in situ* hybridisation analysis showed IGFBP-2 mRNA expression in lactating glands and confirmed the expression of IGFBP-2, -4 and -5 mRNA, but not IGFBP-1, -3, and -6, after litter removal.

IGF-1 has been suggested as an anti-apoptotic factor in several studies (Raff 1992; Rodriguez-Tarduchy *et al.* 1992; Drago *et al.* 1991; Sell *et al.* 1995) and if it had such a role in maintaining mammary epithelial cells during lactation it would

be necessary to abrogate this effect during involution particularly since serum IGF-1 concentrations increase after litter removal. The function of mammary gland IGFBP-5 thus may be to sequester IGF-1 from its receptor thereby attenuating the IGF-1-mediated survival signal.

Recently Guenette & Tenniswood (1995) also showed induction of IGFBP-5 expression during involution of mammary glands after litter removal and their *in situ* hybridisation studies showed expression was localised to the epithelial cells. Similarly IGFBP-5 expression was induced during involution of the prostate after hormonal withdrawal. However in contrast with our Northern blot analysis IGFBP-2 mRNA was unchanged in either tissue during involution (Guenette & Tenniswood 1995). Possibly the sensitivity of the different techniques account for this discrepancy.

A role for IGFBPs in apoptosis has been postulated within the intraovarian IGF system. Most follicles never ovulate and become atretic at different stages of development by an apoptotic mechanism. Healthy follicles have high IGF-1, high FSH, and low IGFBP-4 and -5 levels and the opposite is true of atretic follicles. Liu *et al.* (1993) showed *in vitro* that IGFBP-4 and -5 could block the ability of FSH to stimulate oestradiol and progesterone production. IGF-1 is synergistic with FSH in stimulating granulosa cells and is stimulatory to IGFBP-5 expression (Adashi *et al.* 1994). In contrast FSH is not only inhibitory to the expression of IGFBP-4 and -5 but induces a protease which degrades these IGFBPs (Liu *et al.* 1993). These data suggest IGF-1 availability may be an important determining factor in follicular development. The complex balance of inhibitory and stimulatory influences on IGFBPs may determine IGF-1 availability and thus regulate apoptosis.

The development and function of the mammary gland is a well-studied example of the bidirectional exchange of information between cells and the ECM. Maintenance of the differentiated state is dependent on the basement membrane which induces, under hormonal control, the production of milk proteins. During mammary involution the withdrawal of endocrine stimuli induces degradation of basement membrane by proteases causing the loss of cells by an apoptotic mechanism; extensive remodelling occurs and the gland returns to a resting state.

Talhouk *et al.* (1992) demonstrated the coordinated expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) regulate epithelial function during involution although the signals involved in regulating basement membrane integrity are not elucidated. Although IGFBP-5 is abundant after litter removal there is little evidence of IGFBP-4 by WLB despite increased IGFBP-4 mRNA expression in the gland. This may be explained by proteolysis of IGFBP-4 since proteolysed IGFBPs are poorly detectable by WLB (Liu *et al.* 1993). Involuting mammary glands express several MMPs such as stromelysin (Lefebrve *et al.* 1992), tissue plasminogen activator (tPA), and uPA (Strange *et al.* 1992). Foulkes *et al.* (1994) showed degradation of IGFBPs by MMP-1 (intestinal collagenase) and MMP-3 (stromelysin). TIMP-1 or antisera to MMP-1 and -3 could prevent the degradation of rhIGFBP-3 by pregnant rat serum, although TIMP-1 was previously found to be inactive against human pregnancy serum IGFBP-3 (Frost *et al.* 1993). In addition enzymes other than MMPs such as plasmin can proteolyse IGFBP-3 and this also is increased during involution.

IGFBP-5 is however easily detected by WLB suggesting the proteases in the involuting gland are inactive against this IGFBP or it is protected from proteolysis.

Kanzaki *et al.* (1994) demonstrated that IGF-2 protected IGFBP-5 from proteolysis, but enhanced IGFBP-4 proteolysis. In addition the association of IGFBP-5 with extracellular matrix protects it from proteolysis (Arai *et al.* 1994): association with the particulate fraction of milk, or cell surfaces within the gland may thus protect it from proteolysis in the mammary gland.

The increase in IGFBP-5 levels after litter removal correlated with decreased total mammary DNA and the increase in DNA laddering indicative of apoptosis (Quarrie *et al.* 1995). PRL and GH ablation increased DNA laddering although to a lesser extent than litter removal whilst concurrent PRL treatment maintained DNA in anti-GH and bromocriptine treated rats. Travers *et al.* (1996) established that PRL is responsible for maintenance of the integrity of mammary cells. The maintenance of DNA levels by PRL is in agreement with previous studies demonstrating the anti-apoptotic effect of PRL during involution (Sheffield & Kotolski 1992). However neither GH nor IGF treatment of GH- and PRL-deficient rats inhibited DNA laddering, although GH did alter the pattern of fragmentation resulting in much higher molecular weight fragments (Quarrie *et al.* 1995). Feng *et al.* (1995) demonstrated local inhibition of mammary apoptosis by implants of corticosteroid and progesterone but not by PRL. This agrees with the earlier study of Ossowksi *et al.* (1979) who postulated that PRL inhibited involution indirectly by sensitising the dam to other factors. PRL levels during lactation maintain the corpus luteum thus maintaining high progesterone concentrations and the effects of the manipulation of PRL is therefore confounded to some extent by altered progesterone levels. The use of ovariectomised rats could help differentiate PRL and progesterone effects. Hormone ablation and litter removal induce different physiological states: litter

withdrawal induces milk stasis whereas after hormone ablation the gland is empty of milk. Sealing of half the dams teats has shown that milk stasis can also induce apoptosis in the sealed gland in the absence of endocrine changes (Quarrie *et al.* 1996). Consistent with our hypothesis IGFBP-5 levels increased in sealed mammary glands compared with their unsealed contralateral counterparts.

Because high systemic IGF-1 concentrations after litter removal correlated with increased IGFBP-5 production after litter removal, it was possible that systemic IGF-1 was the stimulus for mammary IGFBP-5 expression since IGF-1 stimulates IGFBP-5 production in bone cells (Dong & Canalis 1995) and in the ovary (Adashi *et al.* 1994). However in our study GH treatment of hormonally-ablated animals did not increase the levels of IGFBP in milk or expression of IGFBPs within the gland despite raised systemic IGF-1 concentrations. Similarly PRL treatment in combination with litter removal decreased milk IGFBP-5 but raised serum IGF-1 further still, suggesting systemic IGF-1 is not the stimulus for mammary gland IGFBP-5 expression during involution. Dams with half their teats sealed had serum IGF-1 concentrations similar to that of control lactating dams, but sealed glands had increased milk IGFBP-5 levels. In contrast oestrogen, corticosteroid, or progesterone treatment did not affect milk IGFBP-5 levels. However alterations in milk volume induced by PRL thus diluting milk IGFBP-5 concentrations cannot be ruled out, therefore IGFBP-5 mRNA analysis is necessary to investigate this possibility. Thus systemic PRL and milk accumulation are correlated with both IGFBP-5 expression and apoptosis in the mammary gland.

Strong evidence that IGF-1 contributes to the maintenance of lactation is provided by the inhibition of mammary involution in transgenic animals expressing

human des(1-3)IGF-1 under the control of rat WAP promotor (Hadsell *et al.* 1996). A conclusion of the study of Hadsell *et al.* (1996) is that a WAP-based transgene can have effects on the tissue despite their secretion into milk. A 32 kDa IGFBP was produced in the glands of transgenic animals; although this IGFBP was unidentified it may be an IGFBP produced in response to IGF-1 stimulus, but which is ineffective in abrogating the IGF-1 stimulus because it binds poorly to des(1-3)IGF-1. In contrast overexpression of IGF-1 in lactating rabbit mammary gland under the control of the α_{s1}-casein promotor had no effect on mammary development or lactation; possibly overexpressed IGF-1 is bound to IGFBPs limiting its availability to receptors (Brem *et al.* 1994). However Neuenschwander *et al.* (1996) also described the inhibition of mammary involution in transgenic mice overexpressing IGF-1 and IGFBP-3; presumably IGFBP-3 might enhance IGF-1 action in this situation although direct effects of IGFBP-3 cannot be ruled out.

The ability of locally overexpressed IGF-1 to inhibit involution contrasts with the failure of systemic IGF to do so. Systemic IGF-1 administration also failed to mimic GH action in the maintenance of lactation which implies that the exogenous IGF-1 cannot reach the mammary gland and mimic local production, even when administered as IGF-1 analogues which would not bind to any inhibitory IGFBPs which may be present (Flint *et al.* 1994). This suggests that GH has effects other than raising serum IGF-1 levels. Acid labile subunit (ALS) is reported to be in excess thus enabling IGF-1 administered to rats to form ternary complexes (Lewitt *et al.* 1993), GH regulates ALS production (Gargosky *et al.* 1994) therefore ALS in the hormone deficient rats may be limiting to ternary complex formation. Alternatively, Rutishauser *et al.* (1993) showed treatment with GH but not IGF-1

induced serum IGFBP-3 proteolytic activity in hypophysectomised rats, thus systemic administration of GH and IGF-1 may have different effects on IGF availability. In addition since insulin alters IGFBP-1 transport (Bar *et al.* 1990) other hormones such as GH may also affect IGF and IGFBP transport.

However Flint & Gardner (1994) subsequently demonstrated the ability of GH to stimulate milk production when implanted directly in the mammary gland. GH may thus have direct effects on mammary cells (although functional receptors have not been demonstrated) or indirectly via stromal cells. GH may stimulate local production of IGFs by stromal cells such as adipocytes which do possess GH receptors. Adipocytes also produce IGFBPs (Beattie & Vernon 1995; Peter *et al.* 1993) some of which are GH regulated and which might thus alter local IGF availability.

Evidence against a role for local IGF-1 in galactopoiesis comes from Marchotty *et al.* (1994) who showed low IGF-1 mRNA expression in the rat lactating mammary gland compared with pregnancy. Interestingly GH replacement treatment of PRL and GH deficient rats raised the serum IGF-2 concentration although IGF-2 is considered to be GH-independent. Local IGF-2 levels may be the important IGF-stimulus and although systemic IGF-2 levels in rats are low GH administration may regulate local IGF-2. In addition GH administration may modulate type 1 or 2 IGF receptors. In the ewe, there are high levels of IGFBP-5 and type 2 IGF receptors in atretic ovarian follicles but low levels in healthy follicles. Serum withdrawal from ewe granulosa cell cultures induced apoptosis and increased levels of type 2 IGF receptors and membrane-associated IGFBPs (Monget *et al.* 1995). IGF-1 treatment of serum-free cultures increased cell viability and

density, and decreased type 2 IGF receptors whilst increasing IGFBP-5 in the medium (Monget *et al.* 1995). In the mammary gland, GH could thus modulate apoptosis not only by increasing local IGF production, but by decreasing type 2 IGF receptor expression. The study of involuting mammary gland using *in situ* hybridisation and immunocytochemistry is required to identify the local expression of IGFs, their receptors and their regulation.

The mechanism of action of IGFs and their IGFBPs at the cellular level is not fully elucidated however the evidence for IGF-1 as a survival factor in other cell types, and the analogy with IGFBP-5 production in the prostate and ovarian IGF system, support the hypothesis that IGF-1 maintains lactation and IGFBP-5 is a primary regulator of mammary gland involution; a causal role of IGFBP-5 in mammary gland involution remains to be established.

CHAPTER 6: FINAL GENERAL DISCUSSION

The aim of this study was the investigation of the IGF axis in two areas important to animal health and hence lactational performance. There had been few studies of IGFBPs in either lactation or in animal health and disease. Here we have shown evidence supporting a role for IGFBPs in both lactation and the immune system.

During pregnancy and lactation the maternal metabolism adapts under hormonal control to meet the increased energy requirements and in some species the mother enters a catabolic state. In some species systemic IGF-1 decreases and IGFBP-3 proteolysis increases thus altering IGF availability. Serum IGF-1 concentrations in goats fall dramatically at parturition and remain low in early lactation. Similarly in critical illness, cancer, and septicaemia, GH and IGFBP-1 levels rise but IGF-1 and IGFBP-3 are low and IGFBP-3 protease activity is increased (Bentham *et al.* 1993). Immunosuppression is a characteristic of hypercatabolic states and since the IGFs have immunomodulatory effects on the immune system it is conceivable that the IGF system could contribute to this immunosuppression.

The contribution of systemic and local IGF and IGFBPs are unclear and systemic IGF levels may not reflect local levels. For example endotoxin treatment of rats reduces IGFBP-3 in the liver but not in peripheral tissues. Under the influence of the circulating hormonal milieu of lactation the mammary gland fully differentiates whereas the thymus involutes: at weaning the mammary gland regresses and the thymus regenerates. The IGFs have stimulatory effects on both mammary and immune cells therefore differential cellular reactivity and the complex mixture of systemic and local factors must control local IGF effects. If IGFBP-5 abrogation

of the anti-apoptotic effect of IGF-1 were hypothesised as a common mechanism during involution then local control mechanisms would be required to regulate this effect.

To define the role of the IGFs the analysis of systemic and local IGF-IGFBP production is required at both the mRNA and protein levels. *In situ* hybridisation and immunocytochemistry studies would determine the sites of synthesis and cellular localisation of IGF and IGFBP expression. Post translational modifications of IGFBPs, their proteolysis and location could clearly alter their biological function.

The immune system of the sheep has all the elements of the IGF axis, type 1 IGF receptors, IGF-1, IGFBP, and IGFBP protease production; unfortunately IGFBP nucleic acid probes were unavailable when these studies were undertaken therefore IGFBP mRNA levels were not determined. Recently Li *et al.* (1996) showed the production of a 25 kDa IGFBP-4 by murine thymus, spleen and mature myeloid cells but not by lymphoid cells. This implies that the IGFBP detected in sheep thymus cultures may be produced by stimulated macrophages in the cultures. Although these are clearly only preliminary and descriptive observations, they support a role for IGFs in the sheep immune response and form a basis to study the interactions of the immune and endocrine systems in ruminants.

Thymus regression during pregnancy is maintained into lactation. The rate of thymic regeneration during lactation is inversely correlated with the number of young and the number of functional nipples (Grégoire 1947a; Grégoire 1947b). Oestrogen induces thymic regression, but sex steroids decline in lactation and thymic involution is maintained in spayed animals thus multiple factors are involved; involution is induced by a complex interaction of neural, endocrine and immune

factors. Lymphoid and myeloid cells possess PRL and GH receptors and both hormones have immunomodulatory effects on haemopoietic tissues (Hooghe *et al.* 1993). For example PRL administration antagonises the suppression of mitogen-induced lymphocyte proliferation caused by corticosteroids (Bernton *et al.* 1992). However PRL administration failed to maintain thymic involution in rats after litter removal (Grégoire 1947b). GH enhances thymulin production by thymic epithelial cells via IGF-1 (Timsit *et al.* 1992) and therefore it is feasible that alterations in IGFs or IGFBPs in the microenvironment during lactation may contribute to thymic regression.

A similar decline in B-lymphopoiesis in pregnancy was recently identified and pregnancy hormones were shown to influence lymphopoiesis. Oestrogen is a negative regulator of B-lymphopoiesis and progesterone dramatically reduces the amount of oestrogen required to deplete B-lineage precursors in non-pregnant mice (Kincade *et al.* 1994). Lactation prolongs the regeneration of B-cell precursors (Medina *et al.* 1993) however the effect of post partum hormones on B-lymphopoiesis has not been well studied. IGF-1 enhances B-lymphopoiesis *in vivo* (Jardieu *et al.* 1994) and *in vitro* (Landreth *et al.* 1992). Bone marrow stromal cells produce IGF-1 and IGFBPs in culture (Abboud *et al.* 1991), and osteoblasts produce IGFs, IGFBPs and IGFBP-proteases (Kanzaki *et al.* 1994). Recently Arkins *et al.* 1995 showed that colony-stimulating factors induced expression of IGF-1 mRNA during differentiation of cultured murine bone marrow myeloid cells. In addition IGFBP-3 could inhibit cell proliferation implying that locally produced IGF-1 is a haemopoietic growth factor. Within this microenvironment the IGF axis may be shared between the cells maintaining bone cell and blood cell production.

It is unclear however how thymic involution and compromised B-cell lymphopoiesis in pregnancy and lactation contributes to the apparent immunosuppression. During pregnancy there is a suppression of cell-mediated responses but humoral immunity is spared. In mid pregnancy the cortex shrinks and many cortical thymocytes die, but the medulla expands and forms structures called medullary epithelial cell rings (MERs) around thymocytes, and although these shrink in late pregnancy the thymulin-producing epithelial cells remain. Glucocorticoids are powerful immunosuppressants and are potent inducers of apoptosis in lymphoid cells; this contrasts with their ability to inhibit apoptosis locally in the mammary gland (Feng *et al.* 1995). Hormones and growth factors can have different effects on haemopoiesis and on mature cell function, for example corticosteroids deplete pre-B cells but do not affect mature B-cells. In addition low-dose corticosteroid can stimulate cells *in vitro* rather than induce apoptosis. Thus the effects of glucocorticoids could depend on its concentration and the state of differentiation of cells.

The immunosuppression of late pregnancy and lactation has implications for the welfare of the mother and is likely to have deleterious effects on milk production and consequently on the young dependent on the milk. There have been many studies of the immunosuppression of pregnancy in rodents but fewer have studied ruminants that may have adopted different strategies to allow the mother to host an allogeneic foetus. The interactions of the lactogenic and galactopoietic hormones with IGFs in ruminant immune cells thus require further study.

Studies of hormonally manipulated lactating rats provide evidence of hormonal control of IGFBPs in lactation and suggested a role for IGFBP-5 in the

involution process. Both PRL and GH regulate circulating IGFBP-3, and IGFBP-5 expression in the involuting mammary gland is regulated by PRL and local factors induced by milk accumulation. To examine in detail the role of IGFBPs in the mammary gland an *in vitro* system is required. Preliminary assay of IGFBPs in conditioned medium from short term rat acini cultures suggested these were not suitable because of proteolytic activity against rhIGFBP-3; this protease may have been derived from the digestion mixture and could have rendered other IGFBPs undetectable by WLB. IGFBPs have however been previously detected by WLB in conditioned media from cultured bovine lactating and non lactating mammary gland explant and acini (Campbell *et al.* 1991). Explant and acini culture may therefore be useful to assess IGFBP production in hormonally manipulated animals. Primary cultures of mammary epithelial cells attached to reconstituted basement membrane form multicellular (mammosphere) structures that differentiate, become polarised and secrete milk components vectorially (Aggeler *et al.* 1991). Both rodent and ruminant mammosphere culture systems exist thus enabling the study of IGFs, IGFBPs, and IGFBP-proteases in differentiated mammary cells. Preliminary data suggests that mouse mammary epithelial cell cultures produce IGFBPs, including a 29-30 kDa IGFBP that was also in milk from involuting glands.

IGFBP-5 has been associated with apoptosis of mammary, prostate and ovarian follicles suggesting it may have a central role in regulating apoptosis in a variety of tissues. Several approaches could be used to establish a causal role for IGFBP-5 in mammary involution, such as the ability of recombinant IGFBP-5 administration to initiate involution in lactating animals. Recombinant IGFBPs may also be used in *in vitro* culture systems to investigate their mechanism of action

during lactation and involution. Recombinant IGFBP-5 and mammary-derived IGFBP-5 may differ in their posttranslational modifications (with possible consequences for their biological effects) therefore this would require characterisation *in vitro*. An alternative strategy would be the use of antisense oligonucleotides to block endogenous IGFBP-5 production. In addition, antisera to IGFBPs used with IGFs and IGFBPs could help elucidate the role of the IGFBPs.

Evidence for IGFBP-5 involvement in involution in commercially important species must be established. There are clearly great species differences in the reproductive strategy of different animals and extrapolation between species must be exercised with caution. In addition, mammary gland structure differs between species: unlike the rat, in ruminant mammary glands the milk collects in the cisternal space therefore proteins newly synthesised during early involution may be diluted. Assessment of mammary gland IGFBP mRNA is therefore particularly important in ruminants to determine IGFBP expression. Preliminary WLB analysis of milk from pigs detected a 29-30 kDa IGFBP in milk obtained after litter removal for 48 h but not in control milk.

Proteases are important in the remodelling process and some proteases in remodelling tissue proteolyse IGFBPs. The increase in IGFBP-4 mRNA expression in involuting mammary gland requires further investigation and suggests further complexity to the situation. This may be similar to the intraovarian IGF-IGFBP system where there are coordinate rises in IGFBP-5 and IGFBP-4 and their proteases (Adashi *et al.* 1994). Currently several studies shown the complexity of the IGF-IGFBP mediated regulation of IGFBP-4 proteases (Kanzaki *et al.* 1994; Donnelly & Holly 1996). Proteolysis of IGFBPs may render them enhancing or inhibitory to IGF

action: a 23 kDa proteolysed IGFBP-5 in bone potentiated IGF action (Andress & Birnbaum 1992), but fibroblast IGFBP-5 potentiated IGF action only when intact and a 21 kDa proteolysed form had no potentiating activity (Jones *et al.* 1993a). Although the IGFBP-5 detected in milk from involuting gland is largely intact its association with casein micelles may protect it from proteolysis. However if IGFBP-5 is secreted within the tissue it may not be thus protected; this is difficult to ascertain because of contamination of tissue by milk, but could be assessed using conditioned medium from the apical surfaces of mammosphere cultures.

Commercial bovine herds are normally concurrently pregnant and lactating and are dried off late in lactation; in contrast the rats in this study were non-pregnant and weaned in peak lactation. Optimal milk production in ruminants is achieved if there is a short dry period between lactation and parturition (Dias & Allaire 1982). Tissue remodelling is a complex process particularly during concomitant pregnancy where growth of ductal cells overlaps with apoptosis of differentiated epithelium. There is the possibility that the involution process induced by abrupt litter removal in peak lactation may be an emergency measure and therefore involution may not result in optimum remodelling for subsequent lactation cycles.

Although there is major cell loss during involution in rodents the process is not well elucidated and there may be carry-over of cells to subsequent cycles similar to that described in ruminants (Holst *et al.* 1987; Hurley 1989). The contribution of IGFs in the remodelling process is unclear. We have postulated an inhibitory role for IGFBP-5 on IGF action in apoptosis, which contrasts with its enhancing roles in fibroblasts (Andress & Birmbaum 1992) and bone (Jones *et al.* 1992). IGFs are mitogenic and differentiation factors for a wide range of cell types and are anti-apoptotic for several

cell types. IGFs are also motogenic for several cell types, and IGFBPs attached to the ECM could sequester IGFs in the microenvironment and provide a haptotactic stimulus. IGFs may be required for the survival of epithelial cells for subsequent lactation cycles, or for the motility and growth of stromal cells during remodelling; thus an alternative role for IGFBPs in involuting mammary gland may be the protection and delivery of IGF. In addition IGFBP-5 can have IGF-independent actions on cell proliferation (Andress *et al.* 1993).

The gland is most susceptible to infection during the early dry period and least susceptible in the mid-dry period. There is considerable interest for animal welfare in prolonging lactation and therefore further elucidation of the process of involution could be very important economically. To help define the involvement of the IGF axis in pregnancy and lactation, mammary gland IGFBP expression could be compared in the different species during pregnancy, lactation, concomitant pregnancy and lactation, and after multiple cycles of lactation.

IGFBP-5 is the most highly conserved IGFBP between species (James *et al.* 1993) and is widely expressed in adult rat (Shimasaki *et al.* 1991) and mouse tissues (Kou *et al.* 1994). The mouse IGFBP-5 gene is tightly linked to the IGFBP-2 gene and the gene and promoter has been characterised (Kou *et al.* 1995). Green *et al.* (1994) described tissue and stage specific expression of IGFBP-2 and -5 in the embryo and proposed a role for these IGFBPs in development. Richers & Wood (1995) recently described IGFBP-5 as the most highly expressed IGFBP in mammary tissue during pregnancy. IGFBP-5 expression was located exclusively in the epithelium of the mammary ducts, while IGFBP-2 was expressed in both epithelium and myoepithelium but levels were highest in the ductal epithelium.

It is thus likely that tissue-limited and developmentally regulated transcription factors control IGFBP-5 expression. The expression pattern of IGFBP-5 is similar to that of AP-2 in rat embryogenesis (Green *et al.* 1994). Duan & Clemmons (1995) showed that the high expression of transcription factor AP-2 in fibroblasts is partly responsible for their constitutively high expression of IGFBP-5.

IGFs are both differentiation and mitogenic factors for myoblasts and it is unclear how the switch between these responses occurs. IGF-1 induces myogenin expression (Florini *et al.* 1991) and during myoblast differentiation the expression of IGF-2, type 2 IGF receptor and IGFBP-5 increases (James *et al.* 1993). Similarly IGF-1 (Canalis & Gabbitas 1995) and bone morphogenic protein-2 (BMP-2) induces IGFBP-5 in differentiating osteoblasts. Although IGF-1 and IGF-2 act through the type 1 IGF receptor, overexpression of this receptor inhibits differentiation in L6 myoblasts (Quinn *et al.* 1993). IGFBP-5 binds to the cell surface and has a higher affinity for IGF-1 than the type 1 IGF receptor thus sequestering IGF-1; therefore IGFBP-5 may play a pivotal role for in determining IGF action.

The IGFs are important in mammary gland development and therefore a model such as undifferentiated cells from non pregnant animals in matrix culture could be used to study the influence of IGFBPs in differentiation and induction of milk protein synthesis.

In conclusion, the main findings of these studies support a role for the IGF axis in two areas important to animal performance, lactation and the immune system. The immune system of the sheep has all the elements of the IGF axis: type 1 IGF receptors, IGF-1, IGFBP, and IGFBP protease. Further study of the role of these factors in regulation of the immune system in sheep is clearly worthwhile.

The rat mammary gland, in which involution has been induced by litter removal, expresses IGFBP-2, -4 and -5 mRNA, and high levels of IGFBP-5 are detected in milk. Milk accumulation in sealed glands increased IGFBP-5 levels in milk, while PRL treatment of animals whose litters had been removed decreased the levels of IGFBP-5 despite milk accumulation. Serum IGF-1 concentrations did not correlate with increased IGFBP expression. Both PRL and GH have important roles in maintaining mammary cell function during lactation. PRL and GH may exert their effects by modulating the IGF-1 survival signal for mammary epithelial cells: in this model, GH induces IGF-1 production (possibly produced locally within the mammary gland stroma) while PRL suppresses the expression of IGFBP-5. During involution the withdrawal of PRL permits the production of IGFBP-5 which then abrogates the anti-apoptotic effects of IGF-1. We thus postulate that IGFBP-5 is an initiator of cell death in the involuting mammary gland and the direct demonstration of the survival effects of IGF-1, and a causal role for IGFBP-5 in involution are required to support this hypothesis.

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