



**The role of IGFBPs in the regulation of chondrocyte
metabolism *in vitro***

by

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Summary

Insulin-like growth factors (IGFs) and inflammatory cytokines (eg. IL-1 and TNF α) affect cartilage metabolism in opposite ways. While the growth factors stimulate the anabolic processes such as proteoglycan synthesis, the inflammatory cytokines increase the degradation rate of these cartilage macromolecules and suppress their synthesis through the mechanism which is not fully understood. The actions of IGFs in biological systems are modulated by locally produced IGF binding proteins (IGFBPs). Recent reports of elevated levels of IGFBPs in osteoarthritic (OA) and rheumatoid arthritic (RA) joints and findings that OA chondrocytes increase the production of IGFBPs have indicated that those factors could play a critical role in regulating the metabolism of cartilage-specific macromolecules in pathophysiological conditions. Therefore, I decided to investigate effects of the IGFs and inflammatory cytokines on IGFBPs produced by chondrocytes and the subsequent interplay of these factors on proteoglycan production *in vitro*. To do this, a primary culture of ovine articular chondrocytes was used as an *in vitro* experimental model system.

The initial results of this study have shown that, in basal conditions, ovine articular chondrocytes produce IGFBP-2 and a 24-kDa IGFBP whose mobility on the SDS gel corresponds to that of non-glycosylated IGFBP-4. Furthermore, the IGFs and inflammatory cytokines stimulated the appearance of IGFBP-5 in chondrocyte primary culture, although through different mechanisms of action. Thus, while IL-1 induced the expression of IGFBP-5 mRNA, IGF-I upregulated IGFBP-5 predominantly at the post-translational level by protecting the binding protein from proteolytic degradation. The inhibitor profile of the chondrocyte-derived IGFBP-5 proteolytic activity points to a serine protease whose activity is inhibited in the presence of IGF-I. The two mechanisms of IGFBP-5 regulation acted synergistically

when chondrocytes were co-incubated with IGF-I and IL-1, which resulted in a considerably increased concentration of the binding protein.

IGF-I was ten times less potent in stimulating proteoglycan synthesis in ovine articular chondrocytes than its analogues with reduced affinities for IGFBPs, des(1-3)IGF-I and LR³IGF-I, clearly demonstrating the negative effect of the locally produced IGFBPs on the IGF-I activity. A recombinant IGFBP-5 additionally suppressed the IGF-I-induced proteoglycan synthesis indicating that this binding protein contributes to the overall negative effect of the IGFBPs on the IGF-I bioactivity. These results imply that the upregulation of IGFBP-5 by IGF-I is a negative feedback mechanism by which the growth factor restricts its own bioactivity in cartilage. In addition, IL-1, which in the presence of IGF-I caused a substantial increase in IGFBP-5 protein level, significantly diminished IGF-I-stimulated proteoglycan synthesis whereas the activities of des(1-3)IGF-I and LR³IGF-I remained unaffected. This again demonstrates the negative effect of the endogenous IGFBPs and strongly suggests that the suppressive effect of IL-1 on IGF-I-induced proteoglycan production in ovine articular chondrocytes is mediated through increased levels of IGFBP-5. A high level of IGFBP-5 most likely contributes to the IGF-I sequestration thus preventing the growth factor from interacting with the receptors.

In conclusion, the IGFBP-5-mediated decrease in proteoglycan synthesis could be a relevant *in vivo* mechanism by which IL-1 exerts its catabolic effect and disturbs the balance between the synthesis and degradation of cartilage matrix macromolecules in pathological conditions.

Declaration

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

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

Findings of this PhD study have been published, submitted or are being prepared for publication as indicated below:

- Šunić D, Belford DA, McNeil JD, Wiebkin OW. (1995) Insulin-like growth factor binding proteins (IGF-BPs) in bovine articular and ovine growth-plate chondrocyte cultures - their regulation by IGFs and modulation of proteoglycan synthesis. *Biochim. Biophys. Acta*, 1245:43-48.
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- Šunić D, McNeil JD, Rayner TE, Andress DL, Belford DA. (1997) Suppression of insulin-like growth factor-stimulated proteoglycan synthesis in ovine articular chondrocytes by interleukin-1: the role of insulin-like growth factor binding protein-5. *Endocrinology* (submitted)
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Glossary

ACTH.....	adrenocorticotropic hormone
bFGF	basic fibroblast growth factor
BSA.....	bovine serum albumin
cAMP	adenosine 3',5'-cyclic monophosphate
cDNA	complementary DNA
CPC.....	cetyl-pyridinium chloride
deoxyCTP.....	deoxycytidine 5'-triphosphate
DMEM.....	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
ECL.....	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
FBS	foetal bovine serum
FPLC	fast protein liquid chromatography
FSH.....	follicle-stimulating hormone
GAG.....	glycosaminoglycan
GAPDH	glyceraldehyde phosphate dehydrogenase
IGF.....	insulin-like growth factor
IGFBP.....	insulin-like growth factor binding protein
IL-1	interleukin-1
IL-1Ra	IL-1 receptor antagonist
IRS-1	insulin receptor substrate-1
kDa.....	kilodaltons
LH.....	luteinizing hormone
MMP.....	matrix metalloprotease

mRNA	messenger ribonucleic acid
MSA	multiplication stimulating activity
NGF	nerve growth factor
OA.....	osteoarthritis
PAGE.....	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDGF.....	platelet-derived growth factor
PEG.....	polyethylene glycol
PGE ₂	prostaglandin E ₂
PMSF.....	phenylmethylsulfonyl fluoride
PSA	prostate-specific antigen
PtdIns 3'-kinase	phosphatidylinositol 3'-kinase
RA.....	rheumatoid arthritis
rbIGFBP-2.....	recombinant bovine IGFBP-2
RGD	Arginine-Glycine-Aspartate
rhIGFBP-5.....	recombinant human IGFBP-5
RIA.....	radioimmunoassay
SDS	sodium dodecyl sulphate
SH2	Src homology region 2
TBS	Tris-buffered saline
TGFβ	transforming growth factor β
TIMP.....	tissue inhibitor of matrix metalloproteases
TNFα.....	tumour necrosis factor α
TSH.....	thyroid-stimulating hormone
UTP.....	uridine 5'-triphosphate

Chapter 1

Introduction and Literature

Review



1.1. Introduction

Articular cartilage is a highly specialised tissue which covers the ends of the long bones and together with synovial fluid enables the low friction movement of the joints. Biomechanical properties of the cartilage are determined by cartilage-specific macromolecules. Predominant macromolecular components which build the cartilage matrix are proteoglycans and collagens. The proteoglycan component gives resistance to compression forces while collagen fibres provide cartilage with a tensile strength.

Cartilage matrix is synthesised and maintained by the highly specialised cartilage cells, chondrocytes. The majority of chondrocyte activities (e.g. metabolic activity, growth and differentiation) are regulated by growth factors and cytokines. In general terms, growth factors such as insulin-like growth factors (IGFs), transforming growth factor β (TGF β) and basic fibroblast growth factor (bFGF) increase anabolic processes while inflammatory cytokines, including the interleukins (ILs) and tumour necrosis factor α (TNF α), promote catabolism.

IGFs have been identified as anabolic factors which increase protein and deoxyribonucleic acid (DNA) synthesis in a variety of tissues acting either as a part of the endocrine system or in an auto/paracrine manner. More specifically, IGFs regulate the level of proteoglycans in cartilage by increasing their synthesis and suppressing their degradation.

In contrast to IGFs, IL-1 and TNF α increase the breakdown rate of proteoglycans. Moreover, they can also suppress IGF-stimulated proteoglycan synthesis. There is compelling evidence that the degenerative effects of IL-1 and TNF α (increased degradation and decreased synthesis of the matrix) lead to cartilage loss in inflammatory and degenerative arthritides.

The biological actions of IGFs are modulated locally by IGF binding proteins (IGFBPs) which have been shown to be produced by numerous cell types including

chondrocytes. The IGFBP family consists of seven homologous proteins that bind IGFs with high affinity. Although the specific physiological role of IGFbps is still unclear, they have the potential to diminish the activity of IGFs by preventing the growth factors from interacting with their receptors. The recent discovery of proteolytic activities capable of cleaving IGFbps further demonstrates the full complexity of IGF-IGFBP interactions. IGFBP proteases are believed to modulate IGF activity by modifying the affinity of IGFbps for IGFs and thereby controlling tissue levels of bioactive, "free" IGFs. The activities of those proteases can be regulated by IGFs and cytokines.

Although some very recent reports have shed more light on IGFBP biology in cartilage, data available about chondrocyte IGFbps still remain scarce. Little is known about their regulation and the way they modulate IGF actions within cartilage. As a result, I have focussed on studying the interactions of IGF axis members, namely IGFs and IGFbps, in chondrocytes.

Therefore, the aim of this thesis was to investigate the mechanism(s) of chondrocyte IGFBP regulation and to establish the role of the IGFbps in PG synthesis *in vitro*. The elucidation of the impact of inflammatory cytokines on the IGFbps and their effect on IGF bioactivity was also a part of this project.

1.2. Literature Review

1.2.1. Biochemical Aspects of Articular Cartilage

Articular cartilage is a constitutive part of synovial or diarthrodial joints. This highly specialised connective tissue enables the joint to absorb impact forces and provides a smooth, resilient surface for joint movement. Cartilage biomechanical characteristics are determined by the biochemical composition of its extracellular matrix. Hydrated matrix is, apart from water, composed mainly of collagen and proteoglycans while structural glycoproteins, lipids and inorganic components are present in minor amounts (Mankin and Brandt, 1989). Chondrocytes have the capacity to synthesise and degrade all the components of the cartilage matrix, thus determining biochemical properties of this connective tissue.

1.2.1.1. Collagens

Collagen constitutes over 60% of dry weight of articular cartilage (Table 1-1.). Molecules of collagen are organised into fibrils which are woven in a network thus providing cartilage with a potential to resist tensile forces and enabling cartilage to counteract the osmotic pressure of the highly hydrated proteoglycans.

There are at least 19 genetically distinct types of collagen which share more than 20 different polypeptide α chains (Seyer and Kang, 1989; Lethias *et al.* 1996; Myers *et al.* 1994). The typical collagen molecule has a highly repetitive sequence of more than 1000 amino acids. Glycine appears as almost every third residue while the other most frequent amino acids are proline, hydroxyproline and hydroxylysine (Traub and Piez, 1971; Ramachandran and Ramakrishnan, 1976). With its small size, glycine allows three α chains to wind around a common axis and form a triple helix which is additionally stabilised by interchain hydrogen bonds between hydroxyproline

residues. Hydroxylysine is involved in the formation of covalent bridges between the polypeptide α chains thus increasing the stability of the molecule.

Table 1-1. Biochemical Composition of Articular Cartilage

Total Weight	Percentage
Water	65-75%
Solids	25-35%
Dry Weight	
Inorganic	5%
Organic	95%
Collagens	53-67%
Proteoglycan	22-38%
Matrix proteins	5-15%
Hyaluronate	<1%
Lipids	<1%

Articular cartilage contains mainly type II collagen (85-90% of total collagen). The fibres of this structural protein appear to be positioned inside the cartilage in an organised way. Type II collagen interacts with proteoglycans and seems to associate with type IX collagen and other cartilage glycoproteins (Mankin and Brandt, 1989). Apart from type IX, other collagens including type V, X and XI are also present in cartilage. Types V and XI are located in the pericellular region around chondrocytes and function as an exocytoskeleton while type IX is dispersed throughout matrix (Gay *et al.*1981; Burgeson *et al.*1982). Type X collagen has also been found in the hypertrophic zone of growth-plate cartilage and is believed to play a role in calcification (Grant *et al.*1987; Schmid and Linsenmayer, 1985). Collagens of a

healthy cartilage are metabolically very stable. In pathological conditions such as osteoarthritis (OA) and rheumatoid arthritis (RA) their turnover rate is affected by increased degradation, mainly due to the increased activity of the matrix-specific proteases (Walakovits *et al.* 1992; Clark *et al.* 1993).

1.2.1.2. Proteoglycans

Proteoglycans show a high level of structural heterogeneity and, consequently, substantial differences in their functions have been demonstrated. Some, like heparan sulphate proteoglycans, are integral parts of the cell membrane and act as low affinity receptors for bFGF (Rapraeger *et al.* 1991; Yanagishita and Hascall, 1992) whereas decorin modulates the actions of TGF β (Boyd and Massague, 1989; Ruoslahti, 1989; Yamaguchi *et al.* 1990). Other proteoglycans take part in complex interactions between the cytoskeleton and the components of the immediate cell surroundings acting as receptors for interstitial matrix and thereby regulating cell-cell and cell-matrix adhesion (Rapraeger and Bernfield, 1983; Sanderson and Bernfield, 1988). However, it is not the scope of this thesis to give an overview of proteoglycan functions in general but rather to concentrate on the large structural proteoglycans which determine the structural and functional integrity of cartilage.

Structure and Function of Cartilage Proteoglycans

Proteoglycans comprise more than 30% of cartilage dry weight (Table 1-1.). These complex macromolecules are composed of glycosaminoglycan (GAG) chains covalently linked to a core protein (Rosenberg and Buckwalter, 1986). GAGs are long anionic, unbranched polysaccharides made of repeating disaccharide units. One of the two sugar residues of the disaccharide unit is always an amino sugar (N-acetylgalactosamine or N-acetylglucosamine) and is usually sulphated. GAGs can be distinguished by the sugar residues that they are made of, by their level of sulphation

and the position of sulphate groups and by the type of bond between the residues. Chondroitin 6-sulphate, chondroitin 4-sulphate, keratan sulphate and hyaluronate (Fig. 1-1.) comprise over 95% of articular cartilage GAGs, while other GAGs like dermatan sulphate, heparan sulphate and heparin are scarcely present in cartilage (Scott, 1991; Scott *et al.* 1991). Unlike other GAGs, hyaluronate is an exceptionally long molecule that is not sulphated and does not covalently bind to proteins. This atypical GAG is crucial in the biology of cartilage proteoglycans and its role will be discussed briefly later.

The primary structure of a core protein of the most abundant cartilage proteoglycan, chondroitin sulphate proteoglycan, reveals several distinct domains. Two globular domains at the N-terminus (G1 and G2) and the third globule at the C-terminus (G3) are separated by a long, serine-rich, stretch of keratan sulphate and chondroitin sulphate binding regions (Bourdon *et al.* 1986; Doege *et al.* 1987). The chondroitin sulphate proteoglycan monomer contains more than 100 chondroitin and about 50 keratan sulphate GAG chains covalently bound to the core protein and has a molecular mass of 3×10^6 Daltons (Heinegard and Sommarin, 1987).

In spite of their size, monomers of chondroitin sulphate proteoglycan rarely exist as separate entities. They usually associate with hyaluronate to form proteoglycan aggregates of an enormous size often exceeding 10^8 daltons (Fig. 1-1.). This aggregation is accomplished by a non-covalent association of G1 domain of the core protein with hyaluronate and stabilised by a link protein which binds to hyaluronate as well as to G1 domain (Hardingham and Muir, 1974; Heinegard and Sommarin, 1987). Due to GAGs inflexibility, proteoglycans occupy a large volume relative to their mass (Hascall and Hascall, 1981; Scott, 1992). Furthermore, carboxyl and sulphate groups of GAGs provide a high density of fixed, negative charge enabling proteoglycans to attract small, osmotically active, cations (e.g. Na^+) which in turn

attract water. Thus, hydrated proteoglycans create the environment of high osmotic pressure which enables cartilage to sustain compressive forces.

Although the biology of cartilage proteoglycans is still far from fully understood it is believed that proteoglycan function is not limited to providing hydrated space in the extracellular matrix. Since proteoglycans interact with the collagen fibres it is highly likely that those interactions are an additional factor which contributes to cartilage resistance to shear and compression forces (Poole *et al.* 1982). Moreover, it has been demonstrated that proteoglycans affect the formation of collagen fibrils *in vitro* (Toole and Lowther, 1968). Furthermore, proteoglycans can, to some extent, regulate the transport of nutrients, metabolites, growth factors, cytokines and hormones through cartilage by promoting diffusion of some molecules (e.g. glucose) while slowing or completely blocking the transport of others, depending on their size and charge (Hadler *et al.* 1982; Hadler, 1980). The upper limit of a diffusion through a healthy cartilage has been estimated to be around 65000 Daltons (Maroudas, 1974).

Proteoglycan Biosynthesis

The biosynthesis of proteoglycans begins with the core protein synthesis on the endoplasmic reticulum-bound ribosomes (Dorfman, 1981). Post-translational modification of the core protein is initiated in the endoplasmic reticulum lumen and continues in the Golgi apparatus by glycosylation of serine residues. GAG chains are assembled by glycosyl transferases, enzymes that elongate the polysaccharide chains by the addition of single sugar residues (Hardingham, 1986). Finally, epimerisation and sulphation of certain GAG sugar residues occur (Heinegård and Paulsson, 1984).

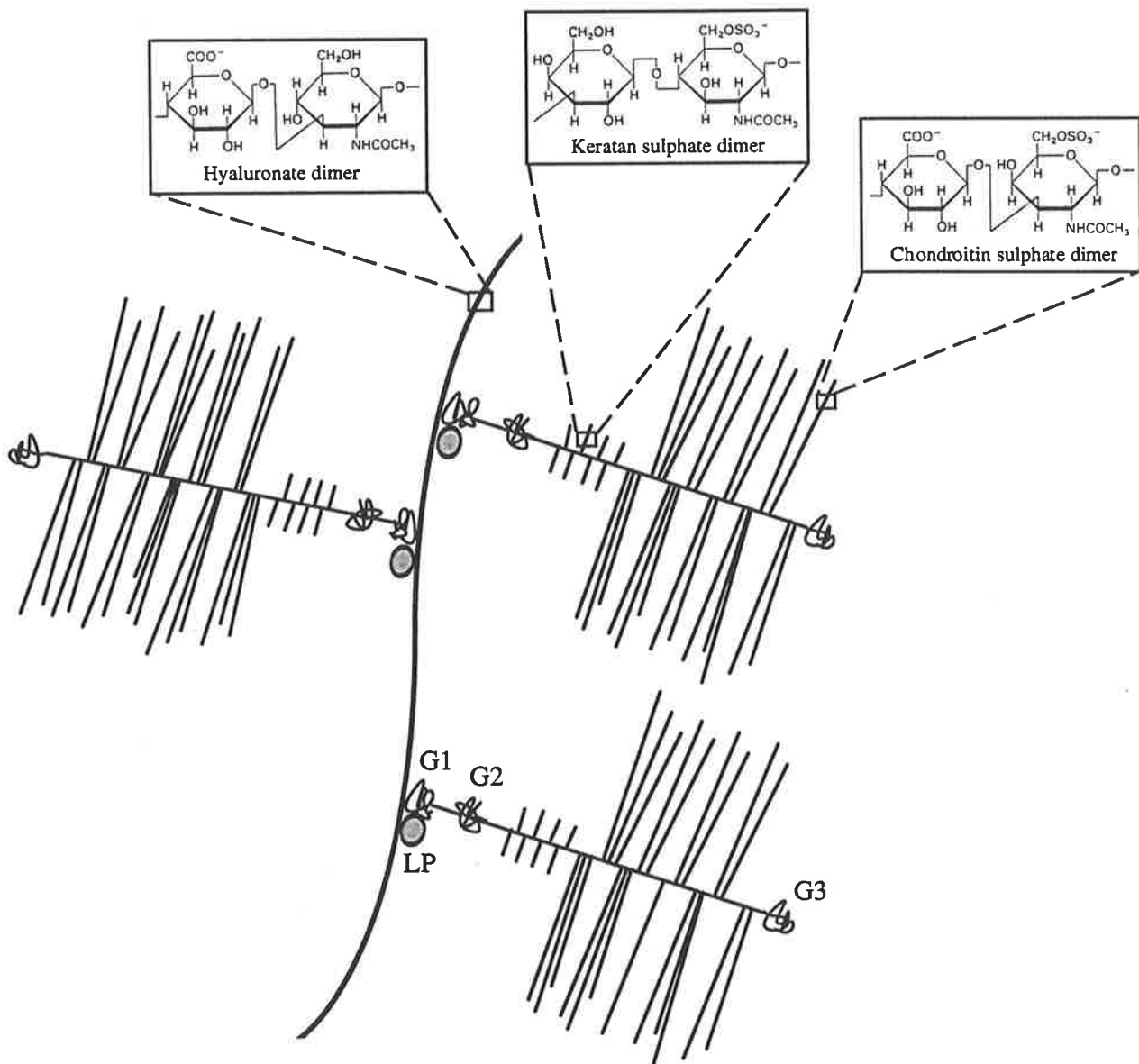


Fig. 1-1. Schematic illustration of cartilage large aggregating proteoglycan.

G1, G2 and G3 - core protein globular domains 1, 2 and 3, respectively.

LP - link protein.

Chondroitin sulphate dimer: D-glucuronic acid and N-acetyl-D-galactosamine

Keratan sulphate dimer: D-galactose and N-acetyl-D-glucosamine

Hyaluronate dimer: D-glucuronic acid and N-acetyl-D-glucosamine

Upon completion of post-translational processing and subsequent secretion into the cell environment, proteoglycan monomers aggregate with hyaluronate to form proteoglycan aggregates. Along with proteoglycan, the other components of the aggregate are synthesised in, what appears to be, a coordinated manner (Curtis *et al.*1992). Similarly to core protein, link protein is synthesised in the endoplasmic reticulum and the Golgi apparatus while hyaluronan synthesis takes place on the cell membrane (Prehm, 1990).

The synthesis of cartilage proteoglycans is regulated by different growth factors of which IGF-I is the most prominent. It has been shown that the synthesis of all proteoglycan aggregate components is increased by this growth factor (see section 1.2.3.2.).

Proteoglycans in Pathological Conditions of Cartilage

Proteoglycans, generally, have a higher turnover rate than collagens and it appears that the synthesis and degradation pathways of the two are distinct. It has been estimated that the half-lives of cartilage proteoglycans in normal cartilage vary between several days to more than a year (Mankin and Brandt, 1989). The balance between the rates of proteoglycan synthesis and proteoglycan degradation, by which healthy tissue maintains the integrity of cartilage, is disturbed in pathological conditions (e.g. OA). OA is characterised by increased degradation and decreased synthesis of proteoglycans. Although, at the early stage of the disease proteoglycan synthesis increases, at later stages the synthesis is diminished thus leading to a loss of proteoglycans from cartilage (Mankin *et al.*1971; Mankin *et al.*1981; Ryu *et al.*1984). Moreover, the structure of the newly synthesised proteoglycans in OA cartilage differs biochemically from that in normal tissue. The size and composition of GAG chains and proteoglycan monomers is altered as well as the ability of proteoglycans to form aggregates with hyaluronate (Bollet and Nance, 1966; Inerot *et al.*1978; Rizkalla *et al.*1992). Diminished proteoglycan concentration leads to a decreased elasticity of the

affected tissue and, on the other hand, to increased permeability of the matrix (Mankin and Brandt, 1989). The latter can, for example, contribute to increased diffusion of the degradative enzymes within cartilage which, in turn, may further affect the rate of proteoglycan degradation.

Enzymes responsible for cartilage proteoglycan degradation are mainly synthesised by chondrocytes, although synoviocytes and inflammatory cells can also be the source of these enzymes particularly in OA and RA (Case *et al.* 1989; Gravallesse *et al.* 1991; Pelletier *et al.* 1993). Proteoglycan breakdown is mediated by matrix metalloproteases (e.g. stromelysin), serine proteases and lysosomal enzymes like cathepsin B and D. *In vitro* and *in vivo* data show that the interglobular domain between G1 and G2 of the core protein is susceptible to proteolytic cleavage by stromelysin, cathepsin B and other proteases (Fosang *et al.* 1991; Fosang *et al.* 1992; Ilic *et al.* 1992; Flannery *et al.* 1992). Furthermore, lysosomal enzymes can degrade hyaluronan and other GAG chains (e.g. chondroitin 6-sulphate) (Howell and Woessner, 1980). Cytokines, in particular IL-1 α and TNF α , upregulate the activity of the degradative enzymes (Pelletier *et al.* 1993) thus increasing the degradation rate of proteoglycans. Although the physiological significance of enzymes with the potential to degrade aggrecan is not fully understood, it is assumed that they are essential for the matrix remodelling (Woessner, 1991; Lennarz and Strittmatter, 1991). These proteases are secreted as proenzymes and are subsequently activated in the cell surroundings (Matrisian, 1992). Activated forms are bound to specific inhibitors such as tissue inhibitor of matrix metalloproteases and α -2-macroglobulin which are also present in the extracellular matrix (Travis and Salvesen, 1983; Woessner, 1991). Once an imbalance between the enzymes and their inhibitors occur and the molar ratio changes in favour of the enzyme, proteoglycans are degraded and the matrix is damaged (Dean *et al.* 1989; Pelletier *et al.* 1990; Gravallesse *et al.* 1991; Lohmander *et al.* 1993).

1.2.1.3. Chondrocytes

Chondrocytes have the potential to synthesise and degrade all components of the cartilage matrix. They govern remodelling of the matrix in healthy tissue and probably take an active part in the pathological states of OA and RA by responding to external signals mediated by cytokines and growth factors.

Chondrocytes in vivo

Chondrocytes are, developmentally, closely related to fibroblasts and osteoblasts in that they all originate from mesenchymal tissue. In the developmental stages chondrocytes actively participate in skeletal growth by proliferation and the synthesis of the matrix of the growth-plate regions in the long bones. In contrast mature chondrocytes of articular cartilage are characterised by a low proliferative potential. Articular cartilage chondrocytes live solitary lives, rarely in cell clusters, scattered across cartilage with no direct contacts between the cells, completely surrounded by the extracellular matrix and occupying less than 2% of total cartilage volume (Stockwell and Meachim, 1979). It seems that chondrocytes are capable of organising the matrix in their immediate vicinity since specific receptors, including CD 44 and integrins, with a potential to bind various components of the extracellular matrix like hyaluronate, collagen and fibronectin have been identified on the cell surface (McCarthy and Toole, 1989; Holmvalld *et al.*1995; Salter *et al.*1996; Yonezawa *et al.*1996). Since articular cartilage is an avascular tissue, all nutrients, as well as external signals which regulate cell functions, reach chondrocytes by diffusion through the cartilage matrix. It has also been demonstrated that chondrocytes from articular cartilage utilise anaerobic glycolysis as a principal source of energy and have a low metabolic activity (Sledge, 1989). They are highly differentiated cells specialised in maintaining cartilage matrix. Synthesis of type II collagen and large

cartilage-specific proteoglycan are the main phenotypic characteristics of differentiated chondrocytes.

Chondrocytes in vitro

When grown *in vitro*, chondrocytes tend to de-differentiate and lose their cartilage-specific phenotype after prolonged culture and/or serial passages. Instead of type II collagen, they shift to the synthesis of type I and III collagens (von der Mark *et al.*1977; Benya *et al.*1977; Layman *et al.*1972). Moreover, large aggregating proteoglycan is decreased (Srivastava *et al.*1974). Fortunately, several strategies exist to maintain the production of cartilage-specific macromolecules which are biochemically indistinguishable from those in the tissue from which the chondrocytes were originally isolated. Chondrocytes grown in suspension culture (in a spinner bottle) continue to synthesise type II collagen (Norby *et al.*1977) and cartilage-specific proteoglycans (Srivastava *et al.*1974; Wiebkin and Muir, 1977). Growing chondrocytes in agarose cultures and alginate beads is another approach successful with respect to the maintenance of a cartilage-specific phenotype (Benya *et al.*1977; Guo *et al.*1989; Hauselmann *et al.*1992; Hauselmann *et al.*1994). However, primary cultures plated at high density ($1-2 \times 10^5$ cells/cm²) have proved to be the most useful system for studying chondrocyte biology *in vitro*. Bovine articular chondrocytes, for example, maintain the phenotypic stability for several weeks in high density primary monolayer culture (Kuettner *et al.*1982b; Kuettner *et al.*1982a). Upon cell adhesion to the surface of a culture dish, cell morphology changes from spherical to polygonal and no direct cell-cell contacts or intercellular junctions can be detected. The cells show extremely low proliferative activity and, consequently, stable population density over several weeks. In the first several days in culture, cells synthesise and organise extracellular matrix which continues to be maintained throughout the culture and whose major macromolecular components are identical to those of the parent tissue.

Thus, primary culture of high density monolayers has several advantages over other *in vitro* systems described above. Cells of a non-human origin can be obtained and manipulated easily resulting in cell populations that are relatively homogeneous. Large numbers of samples can be processed and most importantly, data obtained are relevant and reflect *in vivo* situations.

1.2.2. The Insulin-Like Growth Factor System

1.2.2.1. Short History

IGFs are anabolic polypeptides with the capacity to increase DNA and protein synthesis in a variety of cells. They were originally discovered in 1957 when Salmon and Daughaday found that the effect of growth hormone on ³⁵S-sulphate incorporation into cartilage was mediated by factors in serum, initially called sulphation factors and later, somatomedins. Independent of that study, Froesch *et al.* (1963) reported that serum contained factors with an insulin-like effect on adipocytes whose activity could not be suppressed by anti-insulin antibodies. The following decade brought the discovery of another serum factor also produced by liver cells in culture which exerted multiplication-stimulating activity (MSA) (Pierson and Temin, 1972; Dulak and Temin, 1973). The studies which followed showed that somatomedin C was identical to insulin-like growth factor-I (IGF-I) (Klapper *et al.* 1983) and MSA was later identified as insulin-like growth factor-II (IGF-II) (Marquardt *et al.* 1981).

Originally, IGF-I was regarded to be a part of the endocrine system, synthesised in the liver under the control of growth hormone and then transported by the circulation to the site of action in the peripheral tissues (Daughaday *et al.* 1972). Moreover, numerous cell types including muscle cells, epithelial cells, glial cells,

Leydig cells, fibroblasts, chondrocytes and bone cells have been shown to produce and secrete IGFs in their surroundings (D'Ercole *et al.*1984; Clemmons, 1989). Therefore, the original hypothesis has been modified and it is now postulated that IGF-I can act in an autocrine/paracrine manner as well.

Other components of the IGF system emerged soon after the discovery of IGFs. The actions of the IGFs are mediated through IGF-specific cell membrane receptors, principally the IGF-I receptor (type 1 IGF receptor) (Rechler and Nissley, 1985). The functional significance of the IGF-II receptor (type 2 IGF receptor), is still unclear since no transduction of the IGF signal through this receptor has been demonstrated. The IGF-I and insulin receptors show a high level of structural homology and the IGFs can bind to the insulin receptor although with substantially lower affinity than insulin. Another component of the IGF system which has been unveiled recently is the family of IGF binding proteins (IGFBPs) (Clemmons, 1992; Rosenfeld *et al.*1994). Due to their affinity for IGFs, IGFBPs have a potential to modify the actions of the growth factors by affecting their interactions with receptors. It is also believed that by forming complexes with IGFs in the circulation, IGFBPs prolong the half-lives of growth factors and suppress their insulin-like activity which would have otherwise resulted in hypoglycaemia (Zapf *et al.*1986).

1.2.2.2. Insulin-Like Growth Factors

Structure

Human IGF-I and II are single-chain polypeptides of 7.5 kDa, consisting of 70 and 67 amino acids, respectively, and sharing 65% sequence homology (Fig 1-2.). They are not only functionally, but also structurally, closely related to insulin showing more than 40% sequence identity with the A and B domains of the proinsulin molecule (Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b). IGF-I is

highly conserved across species showing only minor variations in primary structure and biological activity. The sequences of human, bovine, porcine and canine IGF-I molecules are identical, indicating the functional importance of this growth factor throughout evolution (see ref. in Fig 1-2.).

The recognition of the IGF-I receptor by the IGF-I molecule is, at least partly, determined by the aromatic residues in the positions Phe²³-Phe²⁵ in the B domain (Cascieri *et al.*1988) while the A domain residues Phe⁴⁹-Ser⁵¹ appear to be involved in the interactions with the IGF-II receptor (Cascieri *et al.*1989a). It has also been demonstrated that residues Pro²⁸-Arg³⁷ in the C region are involved in high affinity binding to the IGF-I receptor (Bayne *et al.*1989). The amino terminal region seems to be crucial for binding to the IGFBPs since truncated forms of IGF-I and II lacking the first three and six residues at the N-terminus, respectively, have been shown to have substantially reduced affinity for IGFBPs (Francis *et al.*1988; Francis *et al.*1993). Substitution of residues Glu³ and Glu⁶ in IGF-I and II, respectively, with positively charged arginine also reduces affinity of the resulting variants for the IGFBPs (Francis *et al.*1993; King *et al.*1992). Likewise, substitutions of residues in the B and A domains of the IGF-I molecule, such as residues Gln¹⁵ and Phe¹⁶ with Tyr and Leu, respectively, lead to a markedly reduced affinity for IGFBPs (Cascieri *et al.*1989b; Bayne *et al.*1988). A similar decrease in the affinity for IGFBPs is seen in the IGF-II molecule when Phe²⁶ is substituted with Ser or Leu and Phe⁴⁸-Ser⁵⁰ with the analogous insulin residues (Bach *et al.*1993b). Moreover, like the corresponding residues in the IGF-I molecule, the IGF-II residues Phe²⁶ and Phe⁴⁸-Ser⁵⁰ seem to be involved also in IGF-II and IGF-I/insulin receptor binding, respectively (Bach *et al.*1993b).

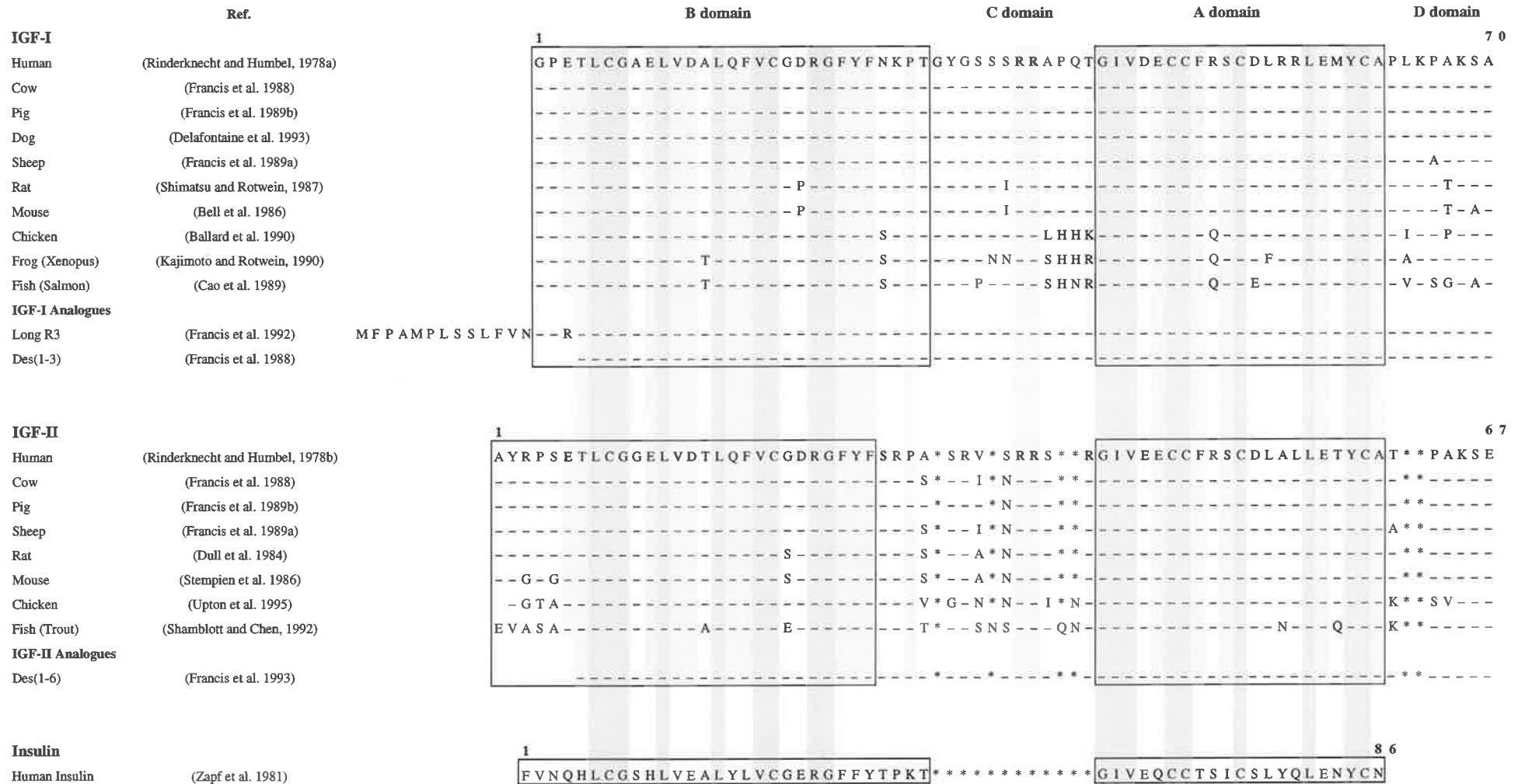


Fig. 1-2. Comparison of IGF-I, -II and insulin sequences.

Amino acid residues are shown using a one-letter code. Dashed lines indicate conserved residues compared to respective human molecules. Asterisks were inserted to allow for maximal alignment of the homologous regions. Shaded areas represent homologous regions of IGF-I, IGF-II and insulin molecules.

IGF Actions in vivo

Human IGF-I and II are the products of distinct genes located on chromosomes 12 and 11 respectively, with different regulatory elements in the promoter regions (Humbel, 1990). IGF-I and II genes are expressed, and corresponding proteins synthesised, in various tissues at all stages of life. Growth hormone is the major factor that regulates IGF-I expression in liver as well as in other tissues (Mathews *et al.* 1986; Nilsson *et al.* 1990). Apart from growth hormone, other hormones like estrogen, insulin, parathyroid hormone, ACTH, FSH and LH also have the potential to control the synthesis of IGF-I (Rutanen and Pekonen, 1990). IGF-II is less responsive to growth hormone stimulation than IGF-I and its regulation seems to be developmentally related, since factors like placental lactogen stimulate its synthesis (Adams *et al.* 1983).

IGFs affect physiology of various tissues and organs of all developmental stages including gut, muscles, brain, nervous system, skin, reproductive system, kidney, bone and cartilage. Thus, studies in hypophysectomised rats and genetically modified mice have confirmed previous assumptions that IGFs play a critical role in growth regulation. IGF-I has been reported to be almost as effective as growth hormone in promoting longitudinal bone growth in hypophysectomised rats (Schoenle *et al.* 1982; Guler *et al.* 1988; Hunziker *et al.* 1994). In addition, by using *in situ* immunohistochemistry and hybridisation, Nilsson *et al.* (1986; 1990) have found that IGF-I is upregulated in the proliferative zone of the growth-plate in hypophysectomised rats in response to growth hormone administration, indicating that IGF-I mediates the effect of growth hormone on longitudinal bone growth. This view has been challenged by Shinar *et al.* (1993) and Wang *et al.* (1995b) who detected abundant IGF-II expression but failed to detect IGF-I mRNA in rat and mouse growth-plate cartilage, respectively, in the early developmental stage. Delayed bone development and severe muscle dystrophy are characteristics of IGF-I knockout mice

(Powell Braxton *et al.*1993; Baker *et al.*1993), while mice with a nonfunctional IGF-II gene grow up to a substantially lower body weight than their normal counterparts during the embryonic phase (DeChiara *et al.*1990). Together with above mentioned studies, recent *in vitro* and *in vivo* findings that IGF-II expression is increased in foetal and early post-natal tissues have further supported the concept which regards IGF-II as an important factor in developmental processes (Delhanty and Han, 1993; Kou and Rotwein, 1993).

IGF Actions in vitro

The majority of IGF biological actions occur as a result of upregulated DNA, RNA or protein synthesis. IGFs exert their effect on a variety of cell types. The cells of the reproductive system, nerve cells, muscle cells, bone cells and cartilage cells are just a few examples of the cells which are targeted by IGFs. In reproductive tissues IGF-I enhances the actions of FSH on granulosa cells (Adashi *et al.*1988) and stimulates steroidogenesis in Leydig cells (Lin *et al.*1986). IGF-I also stimulates proliferation and maturation of oligodendrocytes into myelin-producing cells (McMorris *et al.*1993). Furthermore, IGF-I and II regulate myoblast differentiation in primary cultures of chicken embryonic cells (Schmid *et al.*1983). Osteoblast functions are affected by IGFs at all stages of differentiation. They regulate bone cell proliferation, differentiation and bone matrix production (Hock *et al.*1988). Similarly, IGFs stimulate proliferation of chondrocytes (Lindahl *et al.*1987; Vetter *et al.*1986) and enhance the synthesis of the cartilage matrix proteoglycans and collagen at all developmental stages (Trippel *et al.*1989; Ohlsson *et al.*1992; Hill *et al.*1992). In addition, IGFs negatively affect catabolic processes by inhibiting protein breakdown (Ballard *et al.*1980).

1.2.2.3. Insulin-Like Growth Factor Receptors

IGF-I and II bind to three distinct cell membrane receptors, the IGF-I receptor (type 1 IGF receptor), the IGF-II receptor (type 2 IGF receptor) and the insulin receptor. The primary structures of these receptors, which have been deduced from the corresponding cDNAs, show a substantial level of homology between the IGF-I and insulin receptors (>50%) while the IGF-II receptor is structurally unrelated to the other two receptors (Ullrich *et al.* 1985; Ullrich *et al.* 1986).

The physiological relevance of these receptors has been demonstrated by using specific anti-receptor antibodies with the potential to block IGF actions. Thus, the IGF-I receptor, which transduces metabolic and mitogenic effects of IGF-I, seems also to be able to mediate insulin signalling since an increase in DNA synthesis by insulin can be abolished by α IR-3, a monoclonal antibody against the IGF-I receptor (Flier *et al.* 1986; van Wyk *et al.* 1985). Furthermore, the same antibody can block the mitogenic effects of IGF-I and II in human fibroblasts, suggesting that the IGF-I receptor mediates the effects of both IGF-I and II (Conover *et al.* 1986). Moreover, the results of the same study indicated that the IGF-II receptor did not function as a transducer of the IGF-II signal. This finding corroborated results of a previous study which showed that an antibody against the IGF-II receptor inhibited binding of IGF-II to hepatoma cells but failed to affect IGF-II-stimulated DNA synthesis (Mottola and Czech, 1984). Although there is indirect evidence that the insulin receptor on adipocytes (Kato *et al.* 1993) and hepatoma cells (Gunn *et al.* 1977) mediates the actions of IGF-I, it seems that this receptor does not transduce the effects of IGF-I in other cell types *in vitro* and *in vivo* (Ballard *et al.* 1994).

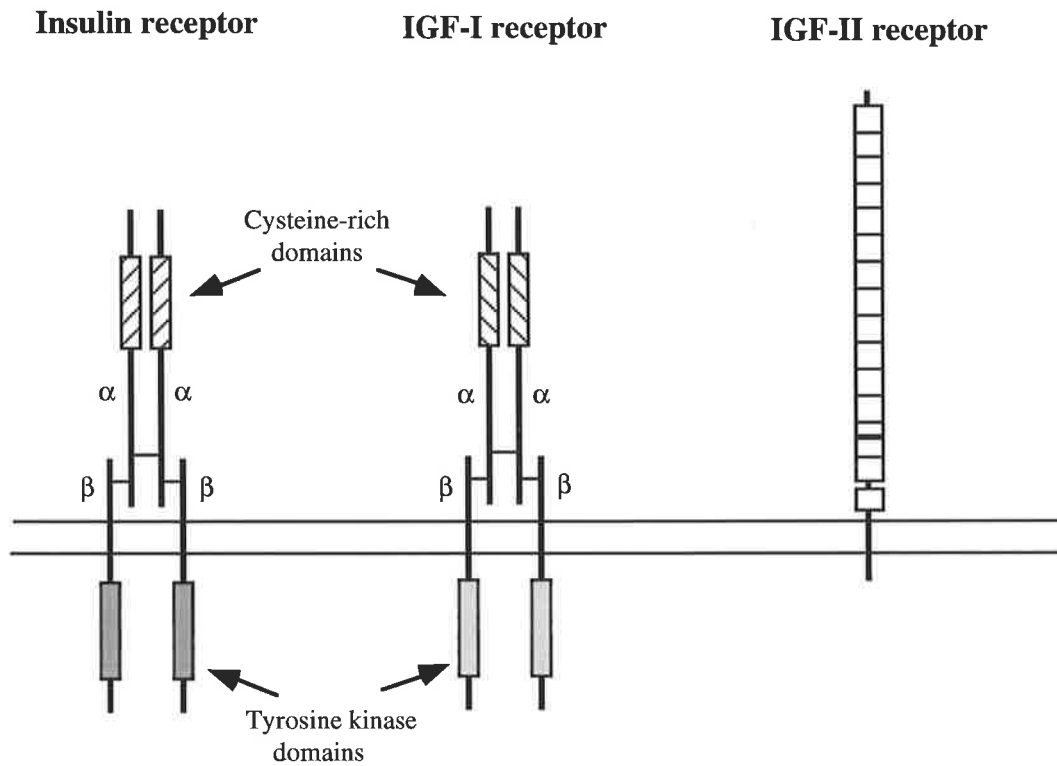


Fig. 1-3. Schematic illustration of the insulin receptor, IGF-I receptor and IGF-II receptor structures.

Relative binding affinities of the growth factors to the receptors
(Clemmons, 1989; Humbel, 1990):

insulin receptor: insulin \gg IGF-I \geq IGF-II

***IGF-I receptor:** IGF-I $>$ IGF-II \gg insulin

IGF-II receptor: IGF-II $>$ IGF-I (insulin does not bind)

* Dissociation rates of the growth factors from the IGF-I receptor
in Chinese hamster ovary cells: IGF-I, $K_d=1.5$ nM; IGF-II,
 $K_d=3$ nM; insulin, $K_d=100$ nM (Steele Perkins *et al.* 1988).

IGF-I and Insulin Receptors - Structure and Signal Transduction

Both the IGF-I receptor and the insulin receptor are >300 kDa glycosylated transmembrane heterotetramers composed of two α (130 kDa) and two β (90 kDa) subunits linked by disulphide bonds (Fig. 1-3.). $\alpha\beta$ dimers which constitute the $\alpha_2\beta_2$ heterotetramers arise by a posttranslational processing of a single chain of the preproreceptor precursor. The α subunits of the IGF-I and insulin receptors are positioned extracellularly and contain the ligand binding regions sharing 67% sequence homology while β subunits span across the membrane with the intracellular portions containing tyrosine kinase domains near the C-terminal regions (84% sequence homology) (Ullrich *et al.*1985; Ullrich *et al.*1986). The receptor intrinsic tyrosine kinase activity is essential for cellular responses to an IGF signal (Kato *et al.*1993).

Binding of a ligand to the α subunit activates the receptor, presumably by inducing conformational changes in the β subunit which in turn leads to activation of the tyrosine kinase and a cascade of phosphorylation events, including receptor autophosphorylation, phosphorylation of cellular substrates and subsequent activation of signalling pathways involved in the regulation of cell growth, metabolism and differentiation (Baron *et al.*1990; Baron *et al.*1992). A biochemical event that follows the tyrosine kinase activation and autophosphorylation is the phosphorylation of the insulin receptor substrate 1 (IRS-1). IRS-1 is a cytosolic protein which, upon tyrosine phosphorylation, functions as a docking molecule for phosphatidylinositol 3' kinase (PtdIns 3'-kinase) by interacting through its phosphorylated motif with SH2 domains of p85, a regulatory subunit of PtdIns 3'-kinase (Sun *et al.*1991). It has been demonstrated recently that IRS-1 is a common element in signal transduction through the IGF-I and insulin receptors (Myers *et al.*1993a). Furthermore, previous studies which have utilised a mouse fibroblast cell line (Yamamoto *et al.*1992b) and a solubilised IGF-I receptor immobilised on agarose beads (Yamamoto *et al.*1992a) demonstrated an association of IGF-I-activated IGF-I receptor and PtdIns 3'-kinase.

Once activated, PtdIns 3'-kinase phosphorylates the D-3 position of phosphatidylinositol, a molecule which can, in a phosphorylated state, control certain aspects of cell growth and metabolism. Beyond the PtdIns 3'-kinase activation point, the signal transduction pathway becomes obscure and poorly understood. A possible downstream divergence of the IGF-I and insulin signalling pathways could explain the apparent differences in their receptor signalling.

It has been reported that a chimeric receptor consisting of extracellular insulin receptor domains and intracellular IGF-I receptor domains is 10 times more active than the wild-type insulin receptor in stimulating DNA synthesis in response to insulin (Lammers *et al.* 1989). Conversely, a chimeric IGF-I receptor in which the C-terminal domain was replaced by the corresponding insulin receptor domain responds to IGF-I stimulation with a biological activity which is characteristic for the insulin receptor (Tartare *et al.* 1994). These findings suggest that the C-terminal domain of the IGF-I and insulin receptors may play a crucial role in determining receptor specificity. Furthermore, there is evidence that hybrid IGF-I/insulin receptors exist *in vitro* and *in vivo*. Thus, placenta has been described as a source of the hybrid receptors whose affinity for insulin was 10 fold lower compared with the "wild-type" insulin receptor and were more responsive to IGF-I (Soos *et al.* 1993). Likewise, fibroblasts and hepatic cells have been shown to possess an IGF-I receptor hybrid which is fully functional and whose tyrosine kinases can be activated by both ligands, although IGF-I seems to be more potent than insulin in stimulating autophosphorylation of the β subunits (Moxham *et al.* 1989). This hybrid formation could be tissue specific and may be another mechanism by which IGF-I and insulin exert their distinct biological effects.

IGF-II Receptor

In contrast to the IGF-I and insulin receptors which belong to a class of cell membrane receptors with intrinsic tyrosine kinase activity, the IGF-II receptor lacks the kinase activity although it can serve as a substrate for a membrane-associated tyrosine kinase (Corvera *et al.* 1986). It is a monomer of 250 kDa whose sequence is identical to the mannose-6-phosphate receptor, a receptor which is involved in the transport of lysosomal enzymes (Morgan *et al.* 1987). IGF-II/mannose-6-phosphate receptor seems to be a multifunctional molecule with distinct binding sites for IGF-II and mannose-6-phosphate-labelled proteins (Tong *et al.* 1988). It consists of a long extracellular domain composed of 15 cysteine-rich repeat sequences and a short intracellular region which lacks intrinsic tyrosine kinase activity and is thought to mediate ion transport through interaction with a G protein (Nishimoto *et al.* 1989). The IGF-II receptor can be cleaved at the transmembrane domain resulting in a ~200 kDa water soluble form that can be detected in the circulation. This fragment has been shown to function as an IGF-II binding protein in rat serum (Kiess *et al.* 1987a).

Results of the studies with anti-IGF-II receptor antibodies suggest that the IGF-II receptor is not involved in mediating the IGF mitogenic effects. Moreover, Kiess *et al.* (1987b) showed that IGF-II degradation can be inhibited by more than 90% when anti-IGF-II receptor antibodies are used indicating the receptor involvement in the IGF-II degradative rather than signalling pathway. However, the physiological significance of the IGF-II receptor, in terms of transducing IGF signals, remains to be elucidated.

1.2.2.4. Insulin-Like Growth Factor Binding Proteins

An early observation that IGFs circulate in blood bound to high molecular weight complexes led to a discovery of the IGF binding proteins (IGFBPs) (Zapf *et al.* 1975). Seven members of the IGFBP family which have been discovered and characterised in

the recent years take part in different physiological processes and affect IGF actions *in vitro* and *in vivo*. For example, the combined plasma concentrations of IGF-I and II of 750 ng/ml or 100 nM would be sufficient to cause hypoglycaemia if the IGFs were able to interact freely with the IGF receptors. However, since more than 95% of the plasma IGFs are bound to the IGFbps, which bind IGFs with affinities that equal or are higher than the affinities of the IGF receptors (constants of association range from $4 \times 10^{11} \text{ M}^{-1}$ to $6 \times 10^9 \text{ M}^{-1}$), IGF-I and II do not exert a hypoglycaemic effect in normal conditions. Another role of the IGFbps is the protection of circulating IGFs from clearance. The half-life of IGFs complexed with the IGFbps is 10-15 hours compared to 10 minutes for free growth factors (Guler *et al.* 1989). Apart from plasma, IGFbps are found in other body fluids including amniotic fluid, cerebrospinal fluid and synovial fluid. Furthermore, various cell types produce and release IGFbps into conditioned medium when grown *in vitro*. IGFbps produced locally, at the site of action, can either diminish or potentiate IGF activity, depending on the type of cells used, the IGFbp involved and experimental conditions. Recent *in vitro* and *in vivo* discoveries of another regulatory element in the IGF system, IGFbp-specific proteases, demonstrate a further complexity of the system. In addition, it has been found that certain IGFbps may exert their biological activities independently of IGFs, acting through pathways which do not interfere with the other components of the IGF system.

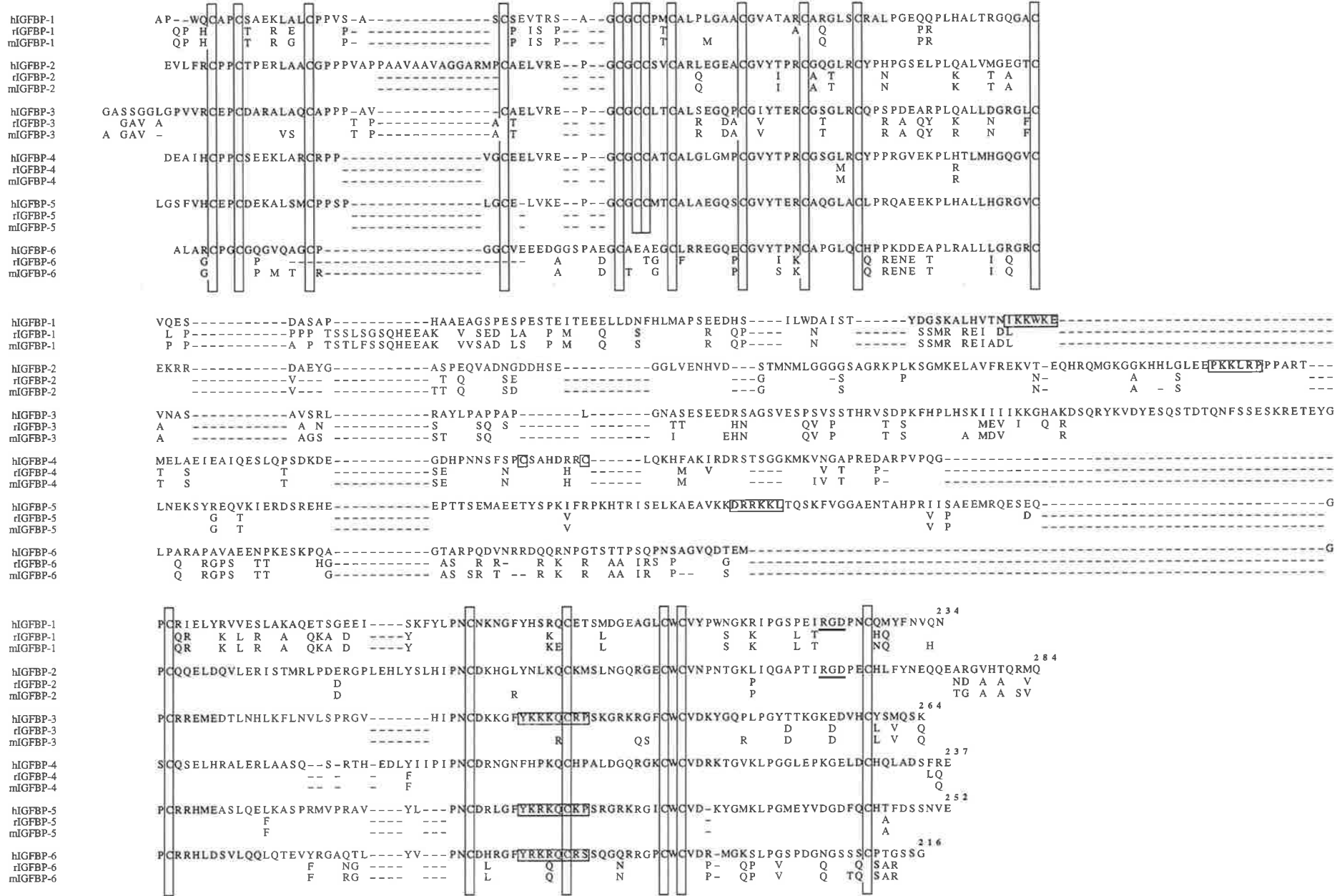


Fig. 1-4. Comparison of the amino acid sequences of the mature human, rat and mouse IGF1 binding proteins.

The sequences were obtained from Shimasaki et al. (1991) and Schuller et al. (1994). The amino acids are shown using the one-letter code. Only the residues that differ from the human sequences are shown for the rat and mouse IGF1 binding proteins. The absence of a residue is denoted by a dash. The sequences are aligned by cysteine residues. RGD domains are underlined and putative heparin-binding sites are shown as shaded boxes. The lengths of the human IGF1 binding proteins are indicated above the last C-terminal amino acid.

IGFBPs - Protein Structure and Post-Translational Modification

Complementary DNAs encoding the six human, rat and mouse IGFBPs have been cloned and the amino acid sequences of the corresponding proteins deduced (Fig. 1-4.) (Shimasaki *et al.*1991b; Brewer *et al.*1988; Shimasaki *et al.*1991a; Kiefer *et al.*1991; Schuller *et al.*1994). Comparison of the cloned sequences reveals a high degree of homology between the IGFBPs of a particular species as well as a high level of conservation across species. Thus, mouse IGFBP-1, -2, -3, -4, -5 and -6 show 73%, 89%, 81%, 93%, 97% and 73% homology with the respective human counterparts. The level of homology of the mouse IGFBPs is even greater with the rat IGFBPs ranging from 93% (IGFBP-3) to 99.6% (IGFBP-5) (James *et al.*1993). IGFBP-5 is the most conserved IGFBP in mammals as well as in non-mammalian vertebrates. Structurally, IGFBP-5 is closely related to IGFBP-3 and, to a lesser extent, to IGFBP-6. These three binding proteins share a similar heparin-binding domain at the carboxyl-termini of the molecules. The narrow range of the differences between the IGFBPs of a particular species (e.g. 45-60% similarity between the rat IGFBPs) indicates that their genes diverged from a single ancestral gene by successive gene duplications in a short period of time during evolution. All 18 cysteine residues, found almost exclusively in the N- and C-terminal regions of the IGFBP molecules, are conserved with the exception of IGFBP-4 which has two additional cysteine residues in the middle region and IGFBP-6 which lacks 2 cysteines in the N-terminal region (Fig. 1-4.). Carboxyl termini of IGFBP-1 and -2 contain the Arg-Gly-Asp (RGD) sequence which is known to be involved in cell adhesion and migration by facilitating binding of proteins to the cell membrane integrin receptors (Ruoslahti and Pierschbacher, 1987). Furthermore, all human IGFBPs except IGFBP-4 contain XBBXB and/or XBBBXXB motifs (B represents any basic amino acid, X is unassigned), well characterised heparin-binding consensus sequences, indicating that

the binding proteins have the potential to bind to heparin and other GAGs present in the extracellular matrix (Fig. 1-4., Table 1-2.).

Post-translational modification of the IGFBP polypeptides includes a signal sequence cleavage, phosphorylation and O- and/or N-glycosylation. A phosphorylated form of human IGFBP-1 secreted by hepatoma cells *in vitro* has a several fold higher affinity for IGF-I than its dephosphorylated form (Jones *et al.*1991). IGFBP-1 found in foetal serum and amniotic fluid is less phosphorylated compared with the IGFBP-1 in hepatoma cell conditioned medium suggesting that this binding protein becomes partially dephosphorylated *in vivo*. Moreover, a recent study demonstrated the ability of dephosphorylated IGFBP-1 to increase wound-breaking strength when used in combination with IGF-I while the phosphorylated protein was inactive (Jyung *et al.*1994). These findings imply that phosphorylation or dephosphorylation could be a mechanism which affects IGFBP biological activity *in vivo*. The significance of the binding protein glycosylation is still unknown. It appears that this post-translational mechanism does not affect the capability of IGFbps to alter IGF actions since recombinant IGFBP-3 synthesised in a prokaryotic expression system, and therefore non-glycosylated, has been shown to be equipotent in modulating the action of IGF-I when compared to its glycosylated counterpart expressed in an eukaryotic system (Conover, 1992).

Table 1-2. Structural, physico-chemical and biochemical characteristics of the human IGFBPs

Protein	Predicted mol.wt. (kDa)	Mobility on non-reduced SDS gels (kDa)	Glycosylation	Phosphorylation	RGD sequence	Heparin-binding domain		Relative affinities for IGF-I and II
						XBBXBX	XBBBXXBX	
hIGFBP-1	25.8	31	-	+	+	+	-	IGF-II ≥ IGF-I
hIGFBP-2	31.4	35	-	+	+	+	-	IGF-II > IGF-I
hIGFBP-3	28.7	39, 43	+	+	-	-	+	IGF-II > IGF-I
hIGFBP-4	26	24, 29	+	?	-	-	-	IGF-II ≥ IGF-I
			(N-glycosylated)					
hIGFBP-5	28.5	29, 31	+	+	-	+	+	IGF-II > IGF-I
hIGFBP-6	22.8	30	+	?	-	-	+	IGF-II >> IGF-I
			(O-glycosylated)					

Regulation of the IGFbps' Gene Expression

The relevance of the IGFbps in the early development has been demonstrated by detecting their gene expression during gestation and in early postnatal life. A recent study, utilising Northern blot analysis, showed that the mRNAs of mouse IGFBP-2, -3, -4 and -5 could be detected as early as day 11 of gestation (Schuller *et al.*1994). Later, during adult life, mouse IGFBP-1 is found to be expressed primarily in liver while the mRNAs of the other five mouse binding proteins are, apart from liver, detectable in kidney, lung, brain, spleen, heart, muscle, testis and ovary, although at different levels. This study corroborates the findings of a previous study which characterised a pattern of IGFBP expression during mouse and rat development by using *in situ* hybridisation (Cerro *et al.*1993). Similarly, tissue-specific expression of sheep IGFBP-2 has been observed in the foetus and postnatally with elevated mRNA levels in early gestation and decreased expression during maturation (Delhanty and Han, 1993). Moreover, the IGFbps seem to be expressed in a cell-type specific manner in certain tissues as demonstrated by *in vitro* studies of the male reproductive system. Cultured peritubular cells produce IGFBP-2 as revealed by Western ligand blot and Northern blot analyses while the Sertoli cells synthesise predominantly IGFBP-3 (Smith *et al.*1990). Skeletal development in mice is also characterised with cell- and developmental stage-specific expression of IGFBP-2, -3, -4, -5 and -6 during the process of chondro- and osteogenesis (Wang *et al.*1995b). The expressions of five rat IGFbps in primary cultures of osteoblasts vary depending on the developmental stage of the culture (Birnbaum and Wren, 1994). Proliferating cells express mainly IGFBP-2 and -5 whereas differentiated osteoblasts in the phase of matrix production and mineralisation show maximal expression of IGFBP-3, -4 and -6.

Taken together, these findings imply both a tissue-specific and an autocrine/paracrine role for the IGFbps in addition to an endocrine action for some

binding proteins such as IGFBP-1. They also demonstrate a differential expression of the IGFBPs which correlates with the developmental stages and the level of cell differentiation.

There is a wide range of factors capable of regulating IGFBPs at the level of gene transcription. Recently, the promoter regions of several IGFBP genes have been characterised and, as expected, showed a high level of conservation thus supporting the concept of their important role in the regulation of the binding protein gene expression. The first 470 bases upstream from the translation start sites of rat and human IGFBP-1 genes display 65% homology (Unterman *et al.*1992) while three dodecameric elements in the promoter regions of human, rat and mouse IGFBP-2 are fully conserved (Binkert *et al.*1992). A computer and functional analyses of the 5'promoter region of the rat IGFBP-1 gene have revealed the presence of c-jun/c-fos dimers (AP-1), insulin and glucocorticoid response elements (Unterman *et al.*1992; Goswami *et al.*1994). In addition, Duan and Clemmons (1995) and McCarthy TL *et al.* (1996) have identified a responsive element for transcription factor AP-2 in IGFBP-5 gene promoter region required for basal and cAMP-mediated IGFBP-5 transcription in human fibroblasts. The AP-2 site does not seem to be involved in mediating the inhibition of IGFBP-5 gene expression by cortisol (Gabbitas *et al.*1996).

Glucocorticoids differentially regulate IGFBP expression in human and rat osteoblast-like cells. While IGFBP-3, -4 and -5 mRNA levels decrease after treatment with cortisol and dexamethasone, steady-state levels of IGFBP-1 are, in contrast, increased (Okazaki *et al.*1994; Gabbitas *et al.*1996). Likewise, dexamethasone has been shown to increase the rat IGFBP-1 promoter activity ten fold as assessed by measuring the activity of luciferase reporter gene constructs in hepatoma cells (Goswami *et al.*1994). In the same study, insulin almost completely abolished the

dexamethasone-induced IGFBP-1 promoter activity indicating its potential to suppress the expression of IGFBP-1.

Recent demonstrations of the capacity of IGFs to regulate the IGFbps at the transcriptional and translational level were not unexpected since the ability of IGFs to alter the level of IGFbps *in vitro* and *in vivo* had been well documented (Clemmons, 1992). Like insulin, IGF-I and II downregulate the IGFBP-1 promoter activity and decrease the basal and cAMP/theophylline-stimulated IGFBP-1 mRNA levels in human hepatoma cells (Lee *et al.*1993). In rat thyroid follicular cells, IGFs and insulin upregulate the IGFBP-5 expression while TSH opposes the actions of the growth factors by diminishing basal levels of IGFBP-5 transcripts and attenuates the IGF-I induced increase in IGFBP-5 mRNA (Backeljauw *et al.*1993). Furthermore, IGF-I and II increase the level of IGFBP-5 transcripts in rat osteoblast-enriched cultures but have no effect on the IGFBP-2, -4 and -6 steady-state transcript levels (McCarthy *et al.*1994).

The same study has revealed that prostaglandin E₂ (PGE₂), an intracellular mediator of inflammation and a potent cAMP inducer, increases the transcript levels of IGFBP-3, -4 and -5 but has no effect on IGFBP-2 and -6 mRNAs. This finding is corroborated by a recent report which demonstrated the ability of PGE₂ to cause a time- and dose-dependant increase in IGFBP-5 promoter activity in rat osteoblast-enriched cells most likely through a cAMP-dependant pathway (Pash and Canalis, 1996). The ability of PGE₂ to upregulate IGFBP-5 mRNA indicates that the inflammatory cytokines, which increase PGE₂ levels in target cells, could affect IGFBP expression. However, a study reporting the inhibition of calcitriol-induced IGFBP-4 mRNA expression by TNF α in mouse osteoblasts demonstrated the inability of indomethacin, an inhibitor of PGE₂ synthesis, to prevent the negative effect of TNF α (Scharla *et al.*1994). This finding suggests that, at least in osteoblasts, the effect of TNF α on IGFBP expression is not mediated by

prostaglandins. In contrast to its negative effect on IGFBP-4 mRNA level in osteoblasts, TNF α increases IGFBP-3 mRNA in primary culture of porcine Sertoli cells (Besset *et al.*1996) while IL-1 also increases the transcription rate of IGFBP-3 in another cell type of the mammalian male reproductive system, namely rat Leydig cells (Wang *et al.*1995a). Several other reports demonstrate the potential of the inflammatory cytokines to alter the level the IGFBPs in the cell culture but the mechanism(s) of IGFBP regulation in those studies has not been established.

Interactions of the IGFBPs with Heparin and Other GAGs

The ability of GAGs to affect IGF-IGFBP high molecular weight complexes in serum has been documented previously (Clemmons *et al.*1983; Baxter, 1990). More recently, *in vitro* studies have demonstrated that heparin can inhibit formation of the IGF-I:IGFBP-5 complex (17-fold decrease in the affinity of IGFBP-5 for IGF-I) as well as IGF-I-IGFBP-3 complex, while it has no effect on the complexes between IGF-I and IGFBP-1, -2 or -4 (Arai *et al.*1994b). The same authors have shown that a synthetic peptide containing the putative heparin-binding domain of IGFBP-5 (²⁰⁵YKRRKQCKP²¹²) interferes with heparin's actions, indicating the functional relevance of that region in the IGFBP-5-heparin interactions. Other GAGs that contain O-sulphated 2 or 3 carbon atom of iduronic acid, like heparan sulphate and dermatan sulphate, also inhibit formation of the IGF-I:IGFBP-5 complex whereas hyaluronic acid, keratan sulphate and chondroitin sulphate have no effect. Heparin, heparan sulphate and dermatan sulphate can also inhibit proteolytic degradation of IGFBP-5 in fibroblast conditioned medium, and their inhibitory activity has been demonstrated to depend on O-sulphation of 2 and 3 carbon positions in the saccharide rings (Arai *et al.*1994a). Studies that followed confirmed the relevance of the long putative heparin-binding region in mediating the association of IGFBP-5 with fibroblast extracellular matrix (Parker *et al.*1996) and its involvement in the binding of

IGFBP-3 to heparin, heparan sulphate and dermatan sulphate (Fowlkes and Serra, 1996). Although the short heparin-binding consensus sequence was also active, it was less potent than the long region in mediating the association of the two binding proteins with the extracellular matrix and the GAGs. In addition, heparin has been shown to increase the stability of IGFBP-3 in rat Sertoli cell culture by inhibiting the association of the binding protein with cell surface proteoglycans (Smith *et al.* 1994). This finding implies that the binding of IGFBP-3 to cell membrane proteoglycans is mediated through the IGFBP-GAG interactions and that this may be a relevant mechanism by which IGFBPs associate with cell membranes and extracellular matrices, a process which may result in altered affinity for IGFs.

It is possible that conformational changes in the IGFBPs, induced upon binding to GAGs (Arai *et al.* 1994b), result in a decreased affinity of IGFBPs for IGFs thus increasing the availability of the growth factors for interactions with the receptors. Circulating and/or cell-associated GAGs could also mediate the transfer of IGFs across the capillary barrier. Furthermore, matrix and membrane bound IGFBPs could serve as extracellular pools of IGFs increasing the growth factor's concentration in the receptor micro environment or perhaps providing a slow release of IGFs thus keeping their availability for interactions with the receptors at steady levels. However, the physiological significance of these interactions remains unknown.

IGFBP Proteases

IGFBP proteases, the most recently discovered component of the IGF axis, affect the affinities of IGFBPs for IGFs thus regulating the bioavailability of the growth factors both in the circulation and at the cellular level. Limited proteolysis of IGFBPs is seen *in vitro* in cell conditioned media and *in vivo* in serum and synovial fluid. Apart from the IGFBP proteolytic activities that have been only partially characterised, several previously known enzymes including matrix metalloproteases (MMPs),

plasmin, prostate specific antigen (PSA) and cathepsin D have been demonstrated to cleave IGFBPs both *in vitro* and *in vivo*.

IGFBP proteolytic activity was originally discovered in human and rodent pregnancy sera where a limited proteolysis of IGFBP-3, the major IGF transport protein in the circulation, by calcium-dependant serine protease(s) was detected (Giudice *et al.* 1990; Fielder *et al.* 1990; Hossenlopp *et al.* 1990; Davenport *et al.* 1990). The structurally altered IGFBP-3 binds IGF-I with lower affinity than the intact protein resulting in higher levels of free IGF-I in pregnancy serum compared to non-pregnancy serum. In addition, proteolytic activities which cleaved a radiolabelled recombinant human IGFBP-3 but not IGFBP-1 were found in sera of post-operative patients and patients with different types of cancer (Frost *et al.* 1993). Although the significance of these findings is unclear it is assumed that the IGFBP proteolytic activity in the circulation could result in increased tissue availability of IGFs. Similarly to IGFBP-3, IGFBP-5, and to a lesser extent IGFBP-4, are partially degraded in pregnancy serum and amniotic fluid by a cation dependant serine protease (Claussen *et al.* 1994). At least part of the IGFBP-3 proteolytic activity in serum can be attributed to MMPs (principally MMP-1 and MMP-3) since the IGFBP-3 degradation by serum can be almost completely inhibited with antibodies against MMP-1 and MMP-3 or with tissue inhibitor of metalloproteases (TIMP-1) (Fowlkes *et al.* 1994b). Furthermore, MMPs have been found responsible for IGFBP-3 degradation in human dermal fibroblast culture, generating several low molecular weight fragments ranging from 14-26 kDa (Fowlkes *et al.* 1994a). The N-terminal sequence analysis of the IGFBP-3 breakdown products revealed that MMPs cleaved within the mid-region of the binding protein molecule, a domain which shows little homology with the other IGFBPs. Prostate specific antigen (PSA), a serine protease of the kallikrein family found in seminal plasma and produced by prostate epithelium and implicated in prostate cancer, is another IGFBP-3 protease capable of generating

low molecular weight fragments of IGFBP-3 with decreased affinity for IGFs. When added to prostate epithelial cell culture PSA abolishes inhibitory effects of IGFBP-3 on IGF-induced mitogenesis indicating its possible involvement in normal and malignant prostate growth (Cohen *et al.*1994a). Nerve growth factor (NGF) γ -subunit, a structurally related peptide to PSA, also shows IGFBP proteolytic activity but unlike PSA, which cleaves primarily IGFBP-3 and -5, it is less selective, cleaving IGFBP-3, -4, -5 and -6 (Rajah *et al.*1996). Cathepsin D, urokinase and tissue plasminogen activators are other well characterised enzymes with demonstrated capability to cleave IGFBP-3 (Frost *et al.*1993; Lalou *et al.*1994). Grimes and Hammond (1994) have demonstrated that the degradation of IGFBP-3 in porcine ovarian granulosa cell culture by a serine protease similar to plasminogen/plasmin activity is inhibited by IGFs, probably via binding of the growth factors to the binding protein.

An analogous role of IGFs in protecting IGFBP-5 from proteolytic degradation has been demonstrated in a variety of cells including granulosa cells (Fielder *et al.*1993a), fibroblasts (Camacho Hubner *et al.*1992; Nam *et al.*1994), osteosarcoma cells (Conover and Kiefer, 1993) and chondrocytes (Matsumoto *et al.*1996b). Characterisation of the IGFBP-5 proteolytic activity in dermal fibroblasts showed that a cation-dependant serine protease, similar to kallikreins, was responsible for the IGFBP-5 cleavage to 17-, 20- and 22-kDa non-IGF-I binding fragments (Nam *et al.*1994). Similarly, Kanzaki *et al.* (1994) found that a serine protease of approximately 67-160 kDa, as assessed by gel filtration, degraded IGFBP-5 into 18- and 20-kDa fragments in a bone cell culture. More recently, Thrailkill *et al.* (1995) have shown, by using IGFBP-5 substrate zymography and protease inhibitors including TIMP1 and MMP-specific antibodies, that MMP-1 and -2 and an unidentified 97-kDa protease degrade IGFBP-5 into 14- and 22-kDa fragments in differentiating murine osteoblasts.

While IGFBP-3 and -5 are, generally, protected from the proteolysis by IGF-I and II, IGFBP-4 degradation is, in contrast, initiated or enhanced by the IGFs. The IGF-modulated IGFBP-4 proteolytic activities have been found in fibroblasts (Conover *et al.* 1993), decidual cells (Myers *et al.* 1993b), osteosarcoma and bone cells (Kanzaki *et al.* 1994), neuroblastoma cells (Cheung *et al.* 1994) and smooth muscle cells (Parker *et al.* 1995). The sites in IGFBP-4 cleaved by the proteolytic activities from fibroblast/osteoblast-like cells and neuroblastoma cells have recently been identified by Conover *et al.* (1995) and Chernausek *et al.* (1995), respectively. Proteases from fibroblasts and osteoblast-like cells cleave at residue 135, whereas a neuronal cell protease cleaves at position 120. Both cleavage sites are located in the mid-region of the IGFBP-4 molecule, a region that is not homologous with other IGFBPs, which could explain the specificity of the protease for IGFBP-4. The same authors have demonstrated, using IGFBP-4 degradation fragments and IGFBP-4 mutants resistant to proteolysis, that a limited proteolysis of IGFBP-4 which results in the binding protein fragments with reduced affinity for IGFs is a relevant mechanism by which a cellular response to IGFs (e.g. DNA synthesis) can be modulated. An intriguing study by Fowlkes *et al.* (1995) showed the inhibitory effect of exogenous IGFBP-3 on IGFBP-4 proteolysis in MC3T3-E1 osteoblast culture. The addition of IGFBP-3 to conditioned medium almost completely inhibited IGFBP-4 degradation, whereas IGF-I and -II abolished the effect of IGFBP-3 in a dose-dependant manner returning the proteolytic activity to the original values. The authors postulated that IGFs stimulated proteolysis of IGFBP-4 indirectly, by binding to IGFBP-3 and displacing it from its complex with the protease, a mechanism which may account for IGF stimulatory effect on IGFBP-4 proteolysis seen in cell systems which, unlike MC3T3-E1 osteoblasts, produce IGFBP-3.

Thus, apart from the regulation of IGFBP gene expression which is mediated through IGF receptors, IGFs can also regulate the level of the binding proteins

through a post-translational, non-receptor mediated mechanism, primarily by affecting the activity of IGFBP proteases.

IGFBPs and IGF Actions

Although some recent findings show the ability of IGFBPs to exert their activity independently of the IGF system, it is believed that IGFBPs function primarily as local modulators of IGF activity by affecting the availability of the growth factors to their receptors. The proposed mechanism by which locally produced IGFBPs modulate IGF activity in target tissues is based on different affinities of IGFs for the receptors and binding proteins. IGFBPs, whose affinity for IGFs is higher than the affinity of the IGF receptors, are believed to sequester IGFs by forming IGFBP-IGF complexes. Once complexed with IGFBPs, IGFs are unable to interact with the receptors and, consequently, there is no transduction of the IGF signal. When the affinity of IGFBPs for IGFs is decreased by limited proteolysis or through interactions with GAGs, IGFs are freed from the complex able to interact with and stimulate the IGF receptors.

Soon after IGFBPs were discovered it became obvious that their effect on IGF activity was composite and, sometimes, even diametrically opposed. Early studies of Zapf *et al.* (1979) and Drop *et al.* (1979) showed an inhibitory activity of impure IGFBP preparations isolated from plasma and amniotic fluid, respectively, on IGF actions. In contrast, Elgin *et al.* (1987) found that the binding protein isolated from human amniotic fluid potentiated the activity of IGF-I but not insulin on porcine aortic smooth muscle cells and chicken and mouse embryo fibroblasts. A study that followed, by De Mellow and Baxter (1988) demonstrated the ability of IGFBP-3 purified from human serum to either inhibit or enhance the biologic response of human skin fibroblasts to IGF-I depending on the experimental conditions. Co-incubation of IGF-I and IGFBP-3 resulted in an inhibition of IGF-I-stimulated ^3H -

thymidine incorporation while, in contrast, preincubation of the cells with the binding protein caused the potentiation of the IGF-I effect by increasing the maximal rate of DNA synthesis. Similarly, Conover (1992) reported that a preincubation of bovine fibroblasts with recombinant human IGFBP-3 enhanced the cellular responsiveness to IGF-I in a time-dependant manner. The exogenous IGFBP-3 which associated with the fibroblast monolayer during the preincubation period was processed in 72 hours to low molecular weight forms which had a 10-fold lower affinity for IGF-I compared to intact IGFBP-3 in solution. It appears that fibroblast-associated IGFBP-3 could also modify the IGF-I receptor-mediated signalling independently of its ability to bind IGFs, since the bioactivity of growth factors with reduced or no affinity for IGFbps such as [QAYL] IGF-I and insulin, respectively, was also potentiated by preincubation with IGFBP-3. IGFBP-3 can also potentiate IGF-I activity in other cells including kidney and skin fibroblasts (Blum *et al.* 1989), osteoblasts (Ernst and Rodan, 1990) and breast carcinoma cells (Chen *et al.* 1994). Likewise, IGFBP-5 has also been shown to potentiate IGF activity in osteoblast cultures (Andress and Birnbaum, 1992; Andress *et al.* 1993). A carboxyl terminus-truncated IGFBP-5 (23 kDa), with reduced affinity for IGFs derived from human osteoblast-like cells, potentiated the IGF-I and II-stimulated ³H-thymidine uptake by mouse osteoblast-like cells. The stimulatory effect of IGFBP-5 on IGF activity was attributed to its reduced affinity for IGF-I and the ability of the binding protein to associate with osteoblast monolayers. Moreover, the 23 kDa IGFBP-5 stimulated DNA synthesis in the absence of IGFs indicating its ability to act independently of the IGF system, via a pathway which does not require IGF receptor activation. Furthermore, IGFBP-5 associated with the fibroblast extracellular matrix has been shown to potentiate growth stimulatory activity of IGF-I while the same binding protein in solution is without effect (Jones *et al.* 1993a).

Numerous reports have described the inhibitory effects of IGFbps on IGF bioactivity. Early studies have shown that preparations of IGFbps purified from human decidua (Ritvos *et al.*1988), endometrium (Rutanen *et al.*1988) and bovine kidney cells (Ross *et al.*1989) inhibit the biological activities of IGFs most likely by sequestering the growth factors and preventing them from interacting with the receptors. The subsequent use of pure, recombinant IGFbps demonstrated the potential of most IGFbps to diminish or completely block the activity of IGFs. Thus, IGFBP-1 completely inhibits the mitogenic action of IGF-I on breast cancer cells (Figuroa *et al.*1993) while the IGF-regulated proliferation rate of intestinal epithelial cells is increased after blocking the synthesis of IGFBP-2 by an antisense IGFBP-2 cDNA construct, indicating an inhibitory effect of the endogenous IGFBP-2 (Corkins *et al.*1995). IGFBP-3 can prevent the IGF-I-induced receptor down-regulation and cell desensitisation in bovine fibroblasts (Conover and Powell, 1991). The IGFBP-3 inhibition of IGF activity in prostate epithelial and carcinoma cells is disrupted by IGFBP-3 proteases which cleave this binding protein into low mol. weight fragments that have reduced affinity for IGFs (Cohen *et al.*1994b; Angeloz Nicoud and Binoux, 1995). Similarly, intact IGFBP-4 has been shown to suppress the IGF-I-stimulated aminoisobutyric acid uptake by bovine fibroblasts (Conover *et al.*1993; Conover *et al.*1995) and IGF-I-induced ³H-thymidine incorporation in neuronal cells (Chernausek *et al.*1995), whereas the proteolysed forms of IGFBP-4 have no effect on the IGF bioactivity in these systems. Yet further evidence of IGFBP inhibitory activity is provided by Conover and Kiefer (1993) who have found that the endogenous IGFBP-5 suppresses IGF-I but not des(1-3)IGF-I-stimulated proliferation of osteosarcoma cells.

Independent Actions of IGFbps

The IGF-independent actions of some IGFbps have been reported recently. Thus, IGFBP-1 is able to stimulate the migration of Chinese hamster ovary cells through the interaction of its RGD sequence with the $\alpha_5\beta_1$ integrin receptor, an activity unaffected by the presence of IGF-I (Jones *et al.* 1993b). IGFBP-1 has also been implicated in IGF-I folding in the study by Hober *et al.* (1994). These authors found that IGFBP-1 assisted in the formation and maintenance of correct disulphide bonds in the IGF-I molecule *in vitro*, a process that could play a relevant role in regulating the stability and half-life of IGF-I *in vivo*.

Growth inhibition of mouse Balb/c fibroblasts transfected with the IGFBP-3 cDNA could not be reversed by the addition of insulin indicating that, at least partly, IGFBP-3 acted through an IGF-independent pathway (Cohen *et al.* 1993). In line with this finding is the report by Oh *et al.* (1993a) that the inhibition of breast cancer cell growth and DNA synthesis by exogenous IGFBP-3 is not preventable by either wild type IGFs or IGF analogues with reduced affinity for IGFbps. Moreover, IGFBP-3 has been shown to associate with specific cell surface proteins which may act as receptors mediating the inhibitory effect of this binding protein (Oh *et al.* 1993d). In addition, a plasmin-induced 16-kDa proteolytic fragment of IGFBP-3, which lacks affinity for IGFs, can inhibit mitogenic activity of IGF-I and insulin in fibroblasts, suggesting an IGF-independent mechanism of action (Lalou *et al.* 1996).

A 23-kDa osteoblast-derived IGFBP-5 truncated at the carboxyl terminus appears to enhance osteoblast mitogenesis by interacting with IGFs and also by an IGF-independent mechanism since it is able to stimulate the ^3H -thymidine uptake even in the absence of the growth factors (Andress and Birnbaum, 1992).

1.2.2.5. IGF Analogues

IGF analogues have proved to be a very useful tool in characterising IGF activity and establishing the individual roles of the system components. Substitution, deletion or/and addition of amino acid residues is usually used to modify the wild type IGFs in a way that enables the resulting analogues to escape interactions with the IGF-BPs or/and IGF receptors. The primary structures of some IGF analogues are shown in Fig. 1-2.

One of the variants with reduced affinity for IGF-BPs, des(1-3)IGF-I, has been isolated from different sources including human brain (Sara *et al.* 1986) and bovine colostrum (Francis *et al.* 1988). It lacks the first three amino acids at the N-terminus and, as a result, has substantially reduced affinity for IGF-BPs. Due to a lower affinity for IGF-BPs, des(1-3)IGF-I is more potent in biological systems than IGF-I. For example, half-maximal stimulatory doses of the truncated form are 8-10 fold lower than those of the intact IGF-I (Francis *et al.* 1988). Recent reports describing proteolytic activities in rat serum (Yamamoto and Murphy, 1994) and tissues (Yamamoto and Murphy, 1995) capable of converting IGF-I to des(1-3)IGF-I suggest that this truncated form of IGF-I is not merely an artefact of the extraction procedure, but rather a factor that may play a relevant role at the site of IGF action *in vivo*. Des(1-6)IGF-II is an IGF-II equivalent of the IGF-I truncation. It has also a reduced affinity for IGF-BPs and higher biological potency than its wild type precursor, IGF-II (Francis *et al.* 1993). LR³IGF-I is another IGF-I variant which has an extension of 12 amino acids of the porcine growth hormone at the N-terminus and an arginine residue instead of the glutamate in position 3, modifications that result in virtually no affinity of this IGF-I analogue for IGF-BPs (Francis *et al.* 1992).

Other mutants often used in the IGF studies include [Gln³,Ala⁴,Tyr¹⁵,Leu¹⁶]IGF-I ([QAYL]IGF-I) and [QAYL]IGF-II, both with decreased affinity for IGF-BPs (Bayne *et al.* 1988; Conover and Kiefer, 1993). On the other hand, [Ser²⁴]IGF-I and

[1-27,Gly₄,38-70]IGF-I retain high affinity for IGFBPs but have 16- and 30-fold reduced affinity, respectively, for the IGF-I receptor (Cascieri *et al.*1988; Oh *et al.*1993b). Similarly, [Leu²⁷]IGF-II has a high affinity for IGFBPs and the IGF-II receptor but 100-fold reduced affinity for the IGF-I receptor, while [QAYL][Leu²⁷]IGF-II has reduced affinity for both the IGFBPs and the IGF-I receptor (Beukers *et al.*1991; Bach *et al.*1993a; Oh *et al.*1993c).

1.2.3. The IGF System and Cartilage

IGFs have been shown to regulate the growth and metabolism of articular cartilage chondrocytes. Indeed, stimulation of sulphate incorporation into cartilage was the first detected biological activity of IGFs. Apart from IGFs, the other components of the IGF system have also been detected in cartilage and surrounding tissues.

1.2.3.1. IGF Receptors on Chondrocytes

The existence of the IGF-specific receptors has been demonstrated on both the growth-plate and articular cartilage chondrocytes. Trippel *et al.* (1983; 1988) have detected the presence of the IGF-I and IGF-II receptors on bovine growth-plate and articular chondrocytes in suspension. Furthermore, Watanabe *et al.* (1985) have characterised a specific IGF-I binding activity on high density, primary monolayers of bovine articular chondrocytes and found that IGF-II and insulin were 15- and 1000-fold less potent, respectively, than IGF-I in displacing the ¹²⁵I-labelled IGF-I from the monolayers. The binding sites were down-regulated by IGF-I, insulin and growth hormone. A recent study by Middleton *et al.* (1996), using *in situ* hybridisation, has revealed the expression of the IGF-I receptor in human articular chondrocytes. The level of the IGF-I receptor mRNA was elevated in OA cells. In contrast, Tardif *et al.*

(1996) reported no change in the expression of the IGF-I receptor in OA chondrocytes. The same study has also revealed a slightly decreased expression of the IGF-II receptor in diseased chondrocytes compared to healthy cells.

1.2.3.2. Metabolic and Mitogenic Actions of IGFs in Articular Cartilage

IGFs contribute to the maintenance of the steady-state metabolism of articular cartilage by regulating extracellular matrix synthesis. Moreover, IGF-I has been identified as the major stimulatory factor in serum (McQuillan *et al.* 1986) and synovial fluid (Schalkwijk *et al.* 1989) of cartilage proteoglycan synthesis since the activities of those biological fluids were completely inhibited by a monoclonal antibody directed against IGF-I. However, a contribution of IGF-II to the proteoglycan-stimulating activity can not be completely excluded as the antibody cross-reacts to a certain extent with this growth factor as well.

In vitro studies have confirmed that IGFs maintain the chondrocyte differentiated phenotype by stimulating the synthesis and decreasing the breakdown of cartilage-specific macromolecules. IGF-I increases the synthesis of proteoglycan core proteins, stimulates elongation of chondroitin sulphate chains, promotes their sulphation and furthermore, it enhances the synthesis of the link protein and hyaluronan (Froesch *et al.* 1976; Kilgore *et al.* 1979; Silbergeld *et al.* 1981; Curtis *et al.* 1992). Although the upregulation of the enzymes which take part in the synthesis of the proteoglycan components has been postulated as a likely mechanism of the IGF-stimulated proteoglycan synthesis, the pathways through which IGFs control proteoglycan production remain obscure. IGF-I also increases the synthesis and matrix incorporation of another articular cartilage-specific macromolecule, type II collagen (Xu *et al.* 1996).

A study by Luyten *et al.* (1988) showed that IGF-I was far more potent than IGF-II in increasing the proteoglycan synthesis and decreasing proteoglycan catabolism in bovine articular cartilage explants. The authors concluded from these results that the cell response to the IGFs was mediated through the IGF-I receptor. Nevertheless, since IGF-II has a generally higher affinity for IGFbps than IGF-I, the contribution of locally produced IGFbps to different potencies of the IGFs can not be excluded (see Table 1-2.). In the same study, the level of endogenous IGFs in cartilage explants decreased with time indicating that the chondrocytes themselves synthesise little or no IGFs *in vitro*. Likewise, Middleton *et al.* (1996) found no substantial IGF-I mRNA expression in human articular cartilage using *in situ* hybridisation.

The higher potency of IGF-I than IGF-II has also been seen in assays of articular chondrocyte proliferation. Vetter *et al.* (1986) found that IGF-I was significantly more potent than IGF-II in stimulating the clonal growth of human adult chondrocytes plated at low density while insulin and growth hormone had no effect. Similarly, rabbit articular chondrocytes seeded at low density proliferate more readily when exposed to IGF-I than to IGF-II (Froger Gaillard *et al.* 1989).

1.2.3.3. IGFbps in Cartilage

Similar to many other cell types, chondrocytes produce IGFbps *in vitro*. A study by Froger-Gaillard *et al.* (1989) revealed the presence of several IGFbps in medium conditioned by rabbit articular chondrocytes. The level of the 30-kDa and low molecular weight forms was increased by IGF-I, IGF-II and insulin while growth hormone had no effect. Likewise, bovine chondrocytes of different developmental stages release IGFBP-2, -3 and other, unidentified, IGFbps into culture medium when grown at high density in primary culture (Olney *et al.* 1993). In the same study, IGF-I, and to lesser extent, IGF-II increased the level of virtually all IGFbps and, moreover, induced the appearance of a 31-kDa form. In spite of those advances in the

study of chondrocyte-derived IGFBPs, the mechanism(s) involved in IGFBP regulation by IGFs in chondrocytes remains unclear.

Schiltz *et al.* (1993) demonstrated that the exogenous IGFBP-4 can inhibit basal and IGF-mediated DNA synthesis in explant culture of chick embryonic pelvic cartilage. In addition, IGFs complexed with IGFBPs seem to be less potent than free IGFs in stimulating proteoglycan synthesis in bovine articular cartilage explants (Tesch *et al.* 1992). Taken together, these findings suggested that, like in other tissues, IGF actions in cartilage are modulated by locally produced IGFBPs.

1.2.3.4. IGFBPs in Pathological Conditions

IGFBPs are upregulated in the pathological conditions of articular cartilage such as OA and RA. Moreover, several studies have suggested a possible involvement of IGFBPs in the pathophysiological processes in cartilage.

Joosten *et al.* (1991) have demonstrated a non-responsiveness of murine arthritic cartilage to IGF-I in terms of proteoglycan synthesis in spite of the presence of functionally unaltered IGF receptors. This finding was followed by studies of Dore *et al.* (1994) and Tardif *et al.* (1996) who reported an increased number of IGF-I binding sites and elevated IGFBP production by human OA chondrocytes, respectively, accompanied by the chondrocyte's non-responsiveness to IGF-I stimulation. Olney *et al.* (1996) also reported increased levels of components of the IGF system, including IGF-I, IGFBP-3 and -5 and proteolytic activities against these binding proteins in OA cartilage cultured *in vitro*.

Further evidence of altered IGFBP levels in diseased cartilage has recently been provided by several investigators who found elevated levels of some IGFBPs in synovial fluids of OA and RA patients (Matsumoto *et al.* 1996a; Fernihough *et al.* 1996; Kanety *et al.* 1996). Although the IGF-I levels are increased as well, Fernihough *et al.* (1996) have estimated that the IGFBP-3 to IGF-I molar ratio

increases from 0.86 in healthy subjects to almost 1.5 in synovial fluids of the OA and RA patients, indicating that a decreased level of IGF-I is available to interact with the IGF receptors in the arthritic joint.

1.2.4. Inflammatory Cytokines in Cartilage

Cytokines are a heterogeneous group of proteins which act in autocrine/paracrine fashion or systemically affecting and/or governing various physiological and pathophysiological processes including the immune response, haematopoiesis, tissue repair and wound healing, processes related to atherosclerosis and inflammatory reactions. Interleukin-1 (IL-1) and tumor necrosis factor α (TNF α) are the most prominent cytokines involved in the mediation of inflammation in RA and the late stages of OA.

1.2.4.1. The Effects of IL-1 on Cartilage Metabolism

IL-1 is a 17.5-kDa protein which exists in two forms, IL-1 α and IL-1 β , consisting of 159 and 153 amino acids, respectively, and encoded by distinct genes (Dinarello, 1991). They both bind to the same receptors, although with different affinities, and exert the same biological activities. IL-1 has been implicated in inflammatory and degenerative processes associated with cartilage matrix depletion in RA and OA. Tyler (1985) has shown that the IL-1 isolated from medium conditioned by pig mononuclear leukocytes decreases the synthesis of the aggregating proteoglycan in pig articular cartilage explants. Further studies have revealed the ability of IL-1 to inhibit the production of other cartilage-specific macromolecules including types II and XI collagens *in vitro* (Lefebvre *et al.* 1990; Xu *et al.* 1996). Likewise, the exposure of human chondrocytes to IL-1 results in suppressed

expression of types II and XI collagens and increased types I and III collagens, indicating a dedifferentiation of the cells (Goldring *et al.* 1988). A further aspect of the catabolic activity of IL-1 is its ability to increase degradation of cartilage proteoglycans and type II collagen *in vitro* (Saklatvala *et al.* 1984; Xu *et al.* 1996). Although IL-1 increases the level of intracellular inflammatory mediators such as PGE₂ in chondrocytes, it appears that the effects of the cytokine on the metabolism of matrix macromolecules are mediated by distinct pathway(s) (Arner and Pratta, 1989). The IL-1-induced degradation of cartilage matrix macromolecules is a consequence of increased proteolytic activity in cartilage. IL-1 has been shown to stimulate the production of collagenase in chondrocytes and synoviocytes (Lefebvre *et al.* 1990; Dijkgraaf *et al.* 1995). Furthermore, the activities of other matrix metalloproteases and serine proteases, which contribute to matrix depletion, are also upregulated by this cytokine. Thus, IL-1 increases the activity of stromelysin (Gowen *et al.* 1984) and tissue- and urokinase-type plasminogen activators (Campbell *et al.* 1988) in human articular chondrocytes. Although the effect of IL-1 on the protease inhibitors in cartilage is still unclear it seems that, at least *in vitro*, IL-1 downregulates metalloprotease inhibitors like TIMP1 which results in a further disruption of the enzyme:inhibitor balance and degradation of cartilage (Martel Pelletier *et al.* 1991; Lum *et al.* 1996).

A recent *in vivo* study by Caron *et al.* (1996) has clearly demonstrated the damaging role of IL-1 in experimentally induced OA in dogs. The authors successfully protected cartilage in the experimental animals against the development of OA lesions by intra-articular injections of recombinant IL-1 receptor antagonist (IL-1Ra), a naturally occurring inhibitor of IL-1 activity. In addition, collagenase expression in cartilage was significantly reduced in IL-1Ra treated animals compared to controls, suggesting that the IL-1-stimulated level, and presumably activity, of this metalloprotease was, at least in part, responsible for cartilage degradation.

1.2.4.2. The Effects of TNF α on Cartilage Metabolism

Similar to IL-1, TNF α appears to be heavily involved in the pathological processes that affect articular cartilage. Its *in vitro* effects include inhibition of the synthesis of matrix macromolecules such as aggrecan and types II and XI collagens and potentiation of cartilage matrix resorption, by increasing the activity of cartilage-degrading enzymes (Saklatvala, 1986; Lefebvre *et al.*1990; Campbell *et al.*1990). *In vivo* studies have demonstrated increased synovial fluid (Holt *et al.*1992) and serum (Manicourt *et al.*1993) levels of TNF α in RA patients compared to healthy individuals. This, together with previous findings that showed increased levels of TNF α in RA synovial membrane, indicates a possible involvement of this inflammatory cytokine in cartilage destruction.

A study by Elliot *et al.* (1993) has provided clinical evidence of the regulatory role of TNF α in RA. Treatment of RA patients with a chimeric monoclonal antibody against TNF α resulted in reduced clinical symptoms of the disease including a decreased swollen joint count, increased grip strength and lower serum levels of C-reactive protein and rheumatoid factor.

1.2.4.3. The Effects of IL-1 and TNF α on the IGF System

The ability of the inflammatory cytokines to interfere with the IGF system both *in vitro* and *in vivo* has been well documented. Thus, IL-1 affects the IGF axis in the male reproductive system. The expression of IGF-I mRNA in rat Leydig cells is inhibited *in vitro*, as well as *in vivo*, by IL-1 β (Lin *et al.*1992). In addition, IL-1 β increases the expression of IGFBP-3 without affecting IGFBP-2 and -4 mRNA levels in rat Leydig cells suggesting that this is a likely mechanism by which this cytokine inhibits IGF-induced steroidogenesis (Wang *et al.*1995a). Osteoblasts also respond to

TNF α *in vitro* by decreasing the expression of IGF-I and IGFBP-4 mRNAs (Scharla *et al.* 1994). The inability of indomethacin to reverse the inhibitory effects of TNF α suggests that the cytokine's actions in osteoblasts are not dependent on prostaglandins. Furthermore, infusion of TNF α to rats induces tissue- and organ-specific changes in IGF-I and IGFBP-1 levels (Fan *et al.* 1995).

Until recently, evidence of the inflammatory cytokines' involvement in regulation of IGFBPs and other components of the IGF system in cartilage was almost non-existent. However, two recent reports have demonstrated the ability of both IL-1 and TNF α to increase the level of IGFBPs in chondrocyte cultures. Matsumoto *et al.* (1994) have shown that IL-1 β increases the concentration of a 41-kDa IGFBP (presumably IGFBP-3) in culture medium conditioned by rat chondrocytes. The authors also reported increased levels of IGF-I in the medium without any effect on IGF-I mRNA expression, and increased number of IGF-I receptors as a consequence of the chondrocytes' exposure to IL-1 β , although it remains unclear whether the elevated binding protein level interfered with the analysis by affecting the RIA for IGF-I and ¹²⁵I-IGF-I cell binding assay, respectively. IGFBP-3 protein levels are also increased in human articular chondrocyte primary cultures in response to stimulation of the cells with IL-1 α or TNF α (Olney *et al.* 1995). In addition, a metalloprotease which cleaves IGFBP-3 is upregulated by IL-1 α . However, the mechanism(s) of IGFBP regulation by the inflammatory cytokines in chondrocytes still remains to be elucidated. Moreover, the question of how this regulation reflects on the IGF bioactivity in cartilage remains unanswered.

Chapter 2

Materials and Methods

2.1. Materials

Ovine and bovine hocks of adult animals were supplied by MetroMeat (Old Noarlunga, SA, Australia). Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS) and penicillin/streptomycin (5000 U/ml, 5000 µg/ml) were purchased from Cytosystems (Castle Hill, NSW, Australia). Collagenase (type IA, 305 U/mg), hyaluronidase (340 U/ml), and bovine serum albumin (BSA, RIA grade) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cetyl pyridinium chloride (CPC) was obtained from Ajax Chemicals (Auburn, NSW, Australia). Sodium ³⁵S-sulphate (5mCi/ml, aqueous solution) was purchased from DuPont (Boston, MA, USA). Dialysis membrane with molecular weight cut off of 3,500 Daltons was used for dialysis of conditioned media samples and was purchased from Spectra/Por (Laguna Hills, CA, USA).

Recombinant human IGFs were supplied by GroPep Pty. Ltd. (Adelaide, SA, Australia). Recombinant bFGF, TGFβ and PDGF were purchased from Austral Biologicals (San Ramon, CA, USA). Recombinant human IL-1α was a gift from Hoffmann La Roche (Nutley, NJ, USA). The specific activity of IL-1α was 3 x 10⁸ units/mg. Recombinant human TNFα was obtained from Genzyme (Cambridge, MA, USA). Recombinant human IGFBP-5 was kindly provided by Dr. Dennis Andress (Department of Veteran Affairs, Medical Center, Seattle, WA, USA). Rabbit anti-human IGFBP-1, -2, -3, -4 and -5 antisera were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). The nitrocellulose membrane used for Western blotting was a product of Schleicher & Schuell (Dassel, Germany). The enhanced chemiluminescence (ECL) detection system and sodium ¹²⁵I-iodide (100 mCi/ml) were purchased from Amersham (Castle Hill, NSW, Australia). Protease inhibitors [EDTA, Na salt; 1,10-phenanthroline; phenylmethylsulfonyl (PMSF);

aprotinin; antipain; soybean trypsin inhibitor and benzamidine, HCl] were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant human tissue inhibitor of matrix metalloproteases 1 (TIMP1) was a gift from Dr. Andrew Docherty, Celltech Ltd., Berkshire, UK. Unless otherwise stated, all buffers and solutions were made up in water purified by Milli-Q Ultrapure Water System, manufactured by Millipore Corporation (Bedford, MA, USA).

2.2. Cell Culture

Articular cartilage from ovine or bovine metacarpophalangeal joints was aseptically dissected and incubated in DMEM containing 1 mg/ml collagenase, 1 mg/ml hyaluronidase, 10% FBS, 150 U/ml penicillin, 150 µg/ml streptomycin and 2.5 µg/ml fungizone overnight at 37°C. Isolated chondrocytes were washed twice with phosphate-buffered saline (PBS) (0.8% NaCl, 0.02% KCl, 0.092% Na₂HPO₄, 0.02% KH₂PO₄, pH 7.4) and resuspended in DMEM supplemented with 10% FBS (v/v), 150 U/ml penicillin, 150 µg/ml streptomycin and 2.5 µg/ml fungizone. Cells were plated at a density of 2.5×10^5 cells/cm² in 24-well plates (Falcon, Becton Dickinson & Co., Lincoln Park, NJ, USA) for detection of IGFBPs, in 24- or 48-well plates (Costar, Cambridge, MA, USA) for determination of proteoglycan synthesis and in 6-well plates (Falcon) for total RNA isolation and cultured at 37°C in humidified atmosphere of 5% CO₂. After reaching confluence, monolayers were washed with PBS and incubated in serum-free medium for 24 h. Serum-free medium was then replaced with the medium containing 0.01% (w/v) BSA and the growth factors or cytokines and the chondrocytes were cultured for additional 48 h. Conditioned medium was collected and stored at -20°C for subsequent IGFBP

analysis while cell-layers were used for proteoglycan synthesis assay or total RNA extraction.

2.3. SDS-PAGE and Western Ligand Blotting and Immunoblotting

Conditioned medium samples (1 ml) were dialysed overnight at 4°C against 0.1 M acetic acid and vacuum dried. Samples were reconstituted in 30 µl of SDS sample buffer (0.0625 M Tris, 2% SDS, 5% glycerol, 0.001% bromophenol blue, pH 6.8) and 10 µl subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western ligand blot analysis as described by Hossenlopp *et al.* (1986). Briefly, samples were heated at 65°C for 15 minutes prior to electrophoresis under non-reducing conditions on 4% stacking and 12.5% separating, SDS-polyacrylamide, gel at 15 mA/gel for 2-3 hours (compositions of buffers and gels are shown in Appendix I). Electrophoresis was performed on small size gels (8 cm wide x 5 cm high x 1.5 mm thick) using Hoefer Mighty Small gel apparatus. Separated proteins were electroblotted onto the nitrocellulose membrane at 0.8 mA/cm² for 1 hour using the Pharmacia Multiphor II Nova Blot transfer system (Pharmacia LKB, Uppsala, Sweden). The membranes were washed in 1% Triton X-100 (v/v) for 30 minutes, blocked with 1% BSA (w/v) for 2 hours, equilibrated in 0.1% Tween-20 (v/v) and probed overnight with 5 x 10⁵ cpm ¹²⁵I-IGF-II per membrane. Finally, the membranes were extensively washed in 0.1% Tween-20 and autoradiography was performed by exposing the membranes to the X-ray film for 2-4 days at -80°C.

Conditioned medium samples for Western immunoblotting were prepared and processed in the same way as those for Western ligand blotting, although, due to the low sensitivity of some anti-IGFBP antibodies used, 20 µl (0.66 mEq) samples were

subject to SDS PAGE and electroblotting. The immunoblotting was performed using the enhanced chemiluminescence (ECL) detection system supplied by Amersham. The nitrocellulose membranes were blocked for 2 h at room temperature in Tris-buffered saline-Tween (TBS-T) (20 mM Tris base, 137 mM NaCl, pH 7.6; 0.1% Tween-20) containing 3% BSA, washed in TBS-T and incubated overnight at 4°C with anti-IGFBP polyclonal antibodies diluted in TBS-T to the indicated concentrations. The membranes were washed in TBS-T and incubated 1 h at room temperature with goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate (Dakopatts A/S, Denmark) at a final dilution of 1:2000, followed by extensive rinsing. The nitrocellulose was then treated with the ECL reagents according to the manufacturer's instructions and the reactive bands were visualised by autoradiography.

2.4. Extracellular Matrix Preparation

Ovine articular chondrocytes were cultured as described above. Extracellular matrix of the confluent monolayers which were exposed to IGF-I (0-100 ng/ml) was prepared by keeping the plates on ice and using ice-cold buffer solutions. The cell-layers were rinsed with PBS, incubated for 10 minutes with 0.5% Triton X-100 in PBS, pH 7.4 and then for 5 minutes in 25 mM ammonium acetate, pH 9.0. The resulting extracellular matrix of each well was rinsed in PBS, dissolved in 25 µl of SDS sample buffer and analysed by SDS-PAGE and Western ligand blotting using ¹²⁵I-IGF-II as a tracer.

2.5. RNA Extraction and Northern Blotting

Total RNA was extracted from cultured cells using RNazol B kit (Biotech Laboratories Inc., Houston, TX, USA), following the protocol supplied by the manufacturer. The isolated RNA was quantitated spectrophotometrically and equal amounts of RNA (15 µg/lane) were loaded and size fractionated on a 1% agarose-formaldehyde gel, transferred to a Hybond nylon membrane (Amersham, Castle Hill, NSW, Australia) and cross-linked by UV light (compositions of buffers and gels are shown in Appendix I). To confirm the integrity, equal loading and even transfer of the RNA, the gel was stained with ethidium bromide and ribosomal RNAs were visualised before and after the transfer. The probe used for IGFBP-5 mRNA detection, derived from 640-bp *EcoRI-BamHI* restriction fragment of the rat IGFBP-5 cDNA (kindly provided by Dr. J. D'Ercole, The University of North Carolina, Chapel Hill, NC, USA), was labelled with [α -³²P]deoxyCTP using the Amersham Mega Prime random primer labelling kit (Amersham, Castle Hill, NSW, Australia). The hybridisation was performed at 42°C for 16 h in 5 x SSPE, 45% formamide, 5 x Denhardt's solution, 0.2% SDS and 100 µg/ml salmon sperm DNA. After hybridisation, the membranes were washed at 42°C with 2 x saline-sodium citrate (SSC), 0.1% SDS, 1 mM EDTA and then in 0.5 x SSC, 0.1% SDS, 1 mM EDTA and bound radioactive material was visualised by autoradiography. Glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA was detected using an [α -³²P]UTP-labelled complementary RNA (cRNA) probe that was prepared from a 400 bp fragment of the rat GAPDH cDNA using Promega riboprobe kit (Promega, Madison, WI, USA) and T7 RNA polymerase. The membranes were stripped off the IGFBP-5 probe and rehybridised with the GAPDH probe at 65°C for 16 h, washed in succession with 3x, 2x, 0.5x and 0.1 x SSC buffer at 65°C and autoradiographed.

2.6. Iodination of IGFs and IGFBP-5

Recombinant human IGF-I and II were ^{125}I -labelled by the chloramine-T method as described by Van Obberghen Schilling and Pouyssegur (1983). Briefly, 10 μg IGF-I or II was dissolved in 50 μl 10 mM HCl and 50 μl 0.5 M sodium phosphate pH 7.5, was added. The iodination reaction was started by the addition of 1 mCi sodium ^{125}I -iodide and 20 μl 0.4 mg/ml chloramine-T. The reaction was stopped after 1 minute by the addition of 20 μl 0.6 mg/ml $\text{Na}_2\text{S}_2\text{O}_5$. After 5 minutes of incubation, the reaction mixture was diluted with 0.3 ml chromatography running buffer (50 mM sodium phosphate pH 6.5, 150 mM NaCl, 2.5 g/l BSA) and fractionated through a Sephadex G50 column in order to separate ^{125}I -IGF from unreacted Na^{125}I and other reaction products. Radioiodinated IGF was identified in a γ counter by a shorter retention time than Na^{125}I and its insolubility in 10% (w/v) trichloroacetic acid. Fractions containing radioactivity which was > 99% insoluble were pooled and aliquoted. IGFs were iodinated to a specific activity of 50-100 $\mu\text{Ci}/\mu\text{g}$ protein.

Recombinant human IGFBP-5 was ^{125}I -iodinated using the same, slightly modified, method. The chloramine-T reaction was restricted to only 30 seconds and the Sephadex G50 fractions were kept in siliconized tubes on ice during processing. IGFBP-5 was radioiodinated to a specific activity of approx. 50 $\mu\text{Ci}/\mu\text{g}$ protein.

2.7. Purification of Endogenous IGF-BPs by Size-Exclusion Chromatography

Confluent ovine articular chondrocytes were incubated with 100 ng/ml IGF-I for 48 hours and the resulting conditioned medium was pooled and dialysed overnight at 4°C against 0.1 M acetic acid. Dialysed medium (20 ml) was vacuum dried and reconstituted in 200 µl of FPLC running buffer (Appendix I) and purified by FPLC on a Superose-12 column, HR 10/30 (Pharmacia LKB, Uppsala, Sweden) using ice-cold running buffer. The flow rate was 0.5 ml/min and one fraction per minute was collected.

Detection of the IGF-I- and IGF-BP-peak in the FPLC eluate by RIA for IGF-I

FPLC fractions (50 µl portions) were analysed by the IGF-I radioimmunoassay (RIA) kit obtained from GroPep Pty. Ltd. (Adelaide, SA, Australia) according to the manufacturer's instructions. GroPep's rabbit anti-IGF-I polyclonal antibody, Dako's rabbit γ globulin (IgG) and an anti-rabbit γ globulin (IgG) from Silenius Laboratories Pty.Ltd. (Victoria, Australia) were used. PEG 6000 was used to precipitate ¹²⁵I-IGF-I-primary-secondary antibody complex. Briefly, FPLC samples or reference standards were mixed with 250 µl of RIA buffer (Appendix I), 50 µl of ¹²⁵I-IGF-I (approx. 20,000 cpm) and 50 µl of 1/80,000 diluted anti-IGF-I polyclonal serum. The assay tubes were incubated at 4°C for 18 hours. The rabbit γ globulin diluted to 1/200 (25 µl) and anti-rabbit γ globulin diluted to 1/20 (50 µl) were added and samples incubated for 30 minutes at 4°C. One ml of cold 6% (w/v) PEG 6000 in 0.15 M NaCl was added, samples incubated for a further 10 minutes on ice and centrifuged at 4000 g for 30 minutes at 4°C. The supernatants were aspirated and the radioactivity of the pellets counted in a γ detector. The precipitated radioactivity was

plotted against the concentration of the unlabelled IGF-I and unknown sample concentrations were then calculated from the standard curve.

Fifty μ l portions of the FPLC fractions assumed to contain IGFBPs were analysed by Western ligand blotting to confirm the presence of the binding proteins. The FPLC fractions containing IGFBPs were pooled and dialysed for 4 hours at 4°C against 0.1 M acetic acid, aliquoted and vacuum dried. Pellets containing purified chondrocyte IGFBPs were subsequently used in the protease activity assay as a source of potential substrates.

2.8. IGFBP-5 Proteolytic Assays

Size-Exclusion Chromatography-Purified Endogenous IGFBPs as Substrates

Confluent ovine articular chondrocytes were cultured in basal, serum-free, conditions for 48 hours and the conditioned medium was collected. Pellets of chondrocyte-derived IGFBPs purified by FPLC (see section 2.7.) were dissolved in the conditioned medium and incubated at 4 or 37°C for different periods of time. The reaction was stopped by the addition of 4x SDS sample buffer. The samples were analysed by SDS-PAGE and Western ligand blotting and the signal was detected by autoradiography.

Unlabelled Recombinant Human IGFBP-5 as a Substrate

Confluent ovine articular chondrocytes were cultured under basal conditions for 48 hours and conditioned medium was either collected and 1 ml/well transferred to a

new, cell-free, 24-well plate (previously rinsed in 0.1% BSA) or left in the original wells with the cell-layers. Recombinant hIGFBP-5 was added (25 ng/well) and incubated for further 24 hours at 37°C in the presence or absence of IGF-I, des(1-3)IGF-I or LR³IGF-I. At the end of the incubation period, conditioned media (1ml) were dialysed overnight at 4°C against 0.1% acetic acid, vacuum dried, reconstituted in 30 µl SDS buffer and 10 µl was analysed by SDS-PAGE and Western ligand blotting using ¹²⁵I-IGF-II as a tracer.

¹²⁵I-Iodinated Recombinant Human IGFBP-5 as a Substrate

Cell-Free Conditions

¹²⁵I-IGFBP-5 (10000-30000 cpm) was incubated with 20 µl reaction mixture, containing medium conditioned by ovine articular chondrocytes grown under basal conditions (DMEM with 20 mM Hepes, 0.01% BSA) and various protease inhibitors, at 37°C for 6 and 24 hours. Protease inhibitors were added to the medium at the following concentrations: EDTA, 5 mM; 1,10-phenanthroline; 1 mM, PMSF, 5 mM; aprotinin, 1 mg/ml; soybean trypsin inhibitor, 2 mg/ml; benzamidine, HCl, 10 mM; antipain, 4 mM; TIMP 1, 20 µg/ml and heparin 20 µg/ml. The reaction was stopped by the addition of 4x SDS sample buffer (1/4 final volume) and samples were separated by SDS-PAGE. Gels were fixed for 30 minutes in 50% methanol (v/v), 10% acetic acid (v/v), washed 30 minutes in 5% methanol (v/v), 7% acetic acid (v/v), equilibrated 30 minutes in 10% glycerol (v/v), 10% acetic acid (v/v), vacuum dried at 80 °C and exposed to X-ray film overnight.

Incubation with Cell-Layers

Confluent ovine articular chondrocytes were cultured for 48 hours in basal conditions in 48-well plates. Approximately 250,000 cpm of ¹²⁵I-IGFBP-5 was added to each well together with various protease inhibitors. The concentrations of

protease inhibitors were as follows: EDTA, 5 mM; 1,10-phenanthroline, 1 mM; PMSF, 5 mM; aprotinin, 1 mg/ml; soybean trypsin inhibitor, 2 mg/ml; benzamidine, HCl, 10 mM; TIMP 1, 20 µg/ml and heparin, 20 µg/ml. After 24 hours of incubation at 37°C, 20 µl aliquots were taken and the reaction was stopped by the addition of 1/4 final volume of 4x SDS sample buffer. Samples were separated by SDS-PAGE. Gels were fixed for 30 minutes in fixative buffer (50% methanol, 10% acetic acid), washed 30 minutes in washing buffer (5% methanol, 7% acetic acid), equilibrated 30 minutes in equilibration buffer (10% glycerol, 10% acetic acid), vacuum dried at 80 °C and exposed to X-ray film overnight.

2.9. Proteoglycan Synthesis Assay

Proteoglycan production was estimated by measuring the incorporation of [³⁵S]-sulphate into newly synthesised glycosaminoglycans, the sulphated polysaccharide component of proteoglycans. Confluent chondrocytes were treated with various factors for 48 h and exposed to 3 µCi [³⁵S]-sulphate/ml for the final 24 h. The cell-layers were solubilised in 0.2 M NaOH (2 hours at 37 °C) and the samples were subsequently neutralised by the addition of 3 volumes of PBS. Proteoglycans were precipitated by the addition of cetyl-pyridinium chloride (CPC). Prior to addition of an equivalent amount of 1% CPC to the neutralised cell-layer lysate, hyaluronic acid (5 µg/ml final concentration) was added to enhance the precipitation of proteoglycans. The samples were incubated for 2 hours at 37 °C and centrifuged at 1300 g for 10 minutes. Precipitates were washed twice with CPC and resuspended in scintillation fluid. The radioactivity was counted in a Beckman LS 6000 LL liquid scintillation counter.

2.10. Cell Counting

Cell-layers were treated with 1% trypsin for 15 minutes at 37 °C. The reaction was stopped by the addition of 5 volumes of DMEM containing 10% FBS. Cells were centrifuged for 10 minutes at 1000 g and washed twice in PBS. Finally, cells were resuspended in PBS, 20 µl aliquots were mixed with the equivalent amount of trypan blue and viable cells were counted in a haemocytometer.

2.11. Densitometry

A quantitative analysis of positively stained bands on the Western blots and Northern blots was performed by scanning laser densitometry on UltraScan XL, Gel Scan XL(2.1), Pharmacia LKB.

2.12. Statistical analysis

Statistical analysis was performed using the Student's t-test. The results were considered significantly different at $P < 0.05$.

Chapter 3

IGFBP Production by Ovine and Bovine Articular Chondrocytes

3.1. Introduction

Synthesis of IGFBPs is a characteristic of many cell types including chondrocytes (see Chapter 1). Previous studies have shown that chondrocytes isolated from rabbit (Froger Gaillard *et al.*1989) and bovine (Olney *et al.*1993) articular cartilage and grown in primary culture produce and release several binding proteins including IGFBP-2, -3 and unidentified 24-kDa and 29-31-kDa forms. Furthermore, IGFs have been found to be capable of regulating the level of IGFBPs produced by articular chondrocytes (Froger Gaillard *et al.*1989; Olney *et al.*1993).

The aim of this set of experiments was to find whether ovine articular chondrocytes produce IGFBPs, to characterise them and to establish which factors regulate their level. Primary culture of ovine articular chondrocytes was used as the experimental model in this study for reasons stated in Chapter 1, paragraph "Chondrocytes *in vitro*". Sheep articular cartilage was chosen as the principal source of chondrocytes since little, if anything, was known about IGFBPs in cartilage of this species and because the material was readily available. In addition, a thorough knowledge of the IGFBP biology in ovine chondrocytes *in vitro* would contribute to a better understanding of the IGFBP role *in vivo*, in normal, as well as, in pathophysiological processes like those seen in the sheep OA model, which has been extensively used in studying degenerative processes in cartilage (Ghosh *et al.*1993). Bovine articular chondrocytes were used in order to confirm that this phenomenon was not isolated to a single species and for the subsequent biological activity assay.

3.2. Results

3.2.1. Detection and Identification of IGFBPs in Ovine Articular Chondrocyte Culture

3.2.1.1. The Effect of IGF-I on IGFBPs

IGFBP production in the primary culture of ovine articular chondrocytes in response to IGF-I was measured. Confluent chondrocytes were incubated in the presence or absence of IGF-I and IGFBPs in the conditioned medium were analysed as described in *Materials and Methods*. Under basal conditions (serum-free medium, containing 0.01% BSA), ovine articular chondrocytes secreted 35- and 24-kDa IGFBPs into the conditioned medium as assessed by Western ligand blot analysis using ^{125}I -IGF-II as a tracer (Fig. 3-1). Treatment of the cells with 1-100 ng/ml IGF-I resulted in the appearance and a dose-dependant increase of a 29/31-kDa IGFBP in the medium. As shown in figure 3-2, by using Western immunoblotting and anti-IGFBP-1, -2, -3, -4 and -5 polyclonal antibodies, the 35-kDa band was identified as IGFBP-2, while the IGF-I-regulated 29/31-kDa doublet was identified as IGFBP-5. The 24-kDa band did not stain positively with any of the five anti-IGFBP polyclonal antibodies used.

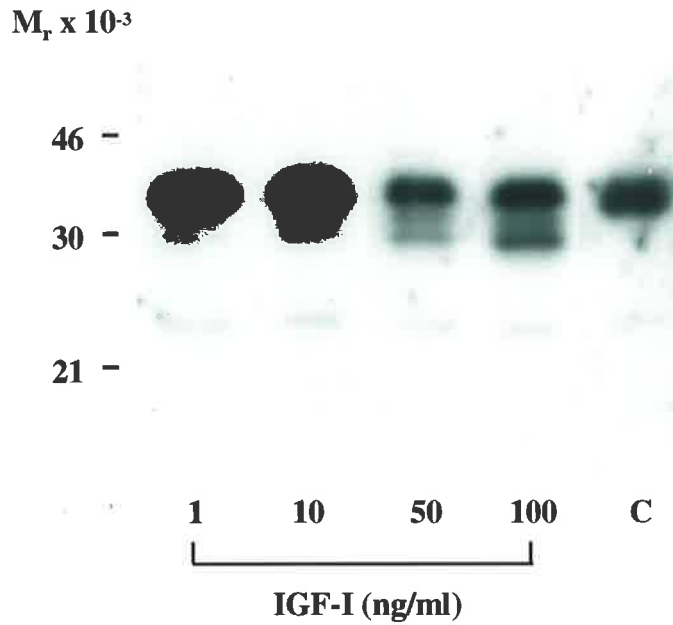


Fig. 3-1. IGF-BPs secreted into the medium conditioned by ovine articular chondrocytes upon exposure to IGF-I. Confluent chondrocytes were cultured for 48 hours in the presence or absence of indicated concentrations of IGF-I. Conditioned media were dialysed, dried, reconstituted in SDS buffer, separated by SDS PAGE and analysed by Western ligand blotting using 5×10^5 cpm ^{125}I -IGF-II per membrane. The membrane was exposed to X-ray film for 3 days. C, control (no IGF-I). The positions of molecular weight markers are indicated on the left.

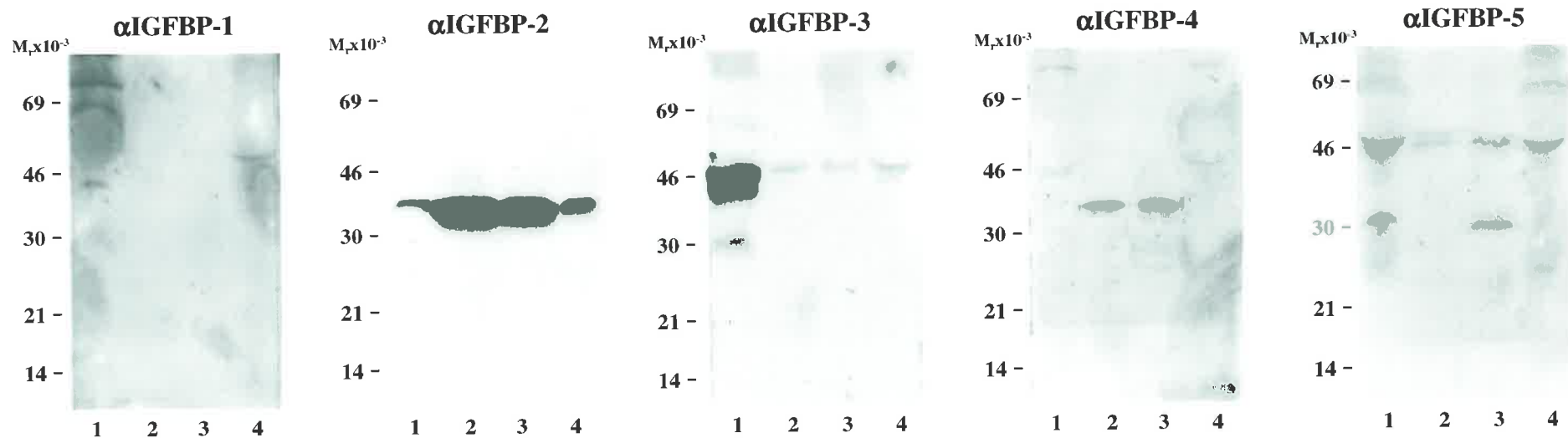


Fig. 3-2. Identification of ovine articular chondrocyte IGFbps by Western immunoblotting. Confluent chondrocytes were cultured for 48 hours in the presence or absence of 50 ng/ml IGF-I. Resulting conditioned media were dialysed, dried, reconstituted in SDS buffer, separated by SDS PAGE and analysed by Western immunoblotting using anti-IGFBP polyclonal antibodies at dilution rates as follows: anti-IGFBP-1 (1:500), anti-IGFBP-2 (1:2000), anti-IGFBP-3 (1:250), anti-IGFBP-4 (1:250) and anti-IGFBP-5 (1:500). Bound primary antibody was detected using goat anti-rabbit horse-radish peroxidase conjugate and the ECL system. The signal was detected by exposing the membranes to X-ray film for 1 minute. Human and ovine plasma samples were used as positive controls. Lane 1, human plasma (2 μ l diluted in 10 ml SDS buffer); lane 2, control, no IGF-I; lane 3, IGF-I (50 ng/ml), lane 4, ovine plasma (2 μ l diluted in 10 ml SDS buffer). The positions of molecular weight markers are indicated on the left.

3.2.1.2. The Effect of bFGF, TGF β , PDGF and the Inflammatory Cytokines on IGFBPs

Apart from IGF-I, other growth factors including bFGF, TGF β and PDGF were used in order to determine whether the IGFBP-5 regulation was IGF-specific and to establish whether they had any effect on the other IGFBPs produced by ovine articular chondrocytes. As shown in figure 3-3, Western ligand blot analysis has revealed no change in the level of IGFBPs in the conditioned medium compared to control after 48-hour exposure of chondrocytes to up to 100 ng/ml bFGF, 50 ng/ml TGF β or 100 ng/ml PDGF.

To determine whether inflammatory cytokines can affect the ovine articular chondrocyte IGFBPs, confluent monolayers were exposed to various doses of IL-1 α or TNF α for 48 hours and the IGFBPs secreted into the conditioned medium analysed by Western ligand blotting. IL-1 α or TNF α increased the 29/31-kDa IGFBP in a dose-dependant manner reaching the maximum stimulatory effect at 200 u/ml and 10 ng/ml, respectively (Fig. 3-4). The doublet was subsequently confirmed to be IGFBP-5 by Western immunoblotting (Fig. 3-5).

3.2.1.3. The Combined Effect of IGF-I and the Inflammatory Cytokines on IGFBP-5

In order to examine the combined effects of IGF-I and the inflammatory cytokines on the IGFBP-5 level, chondrocytes were cultured for 48 hours in the presence of either factor alone as well as with IGF-I co-incubated with either IL-1 α or TNF α . The resulting conditioned media were analysed by Western immunoblotting using the anti-IGFBP-5 polyclonal antibody and the density of positively stained bands of IGF-I- and IL-1 α -treated samples was determined as described in *Materials and Methods*.

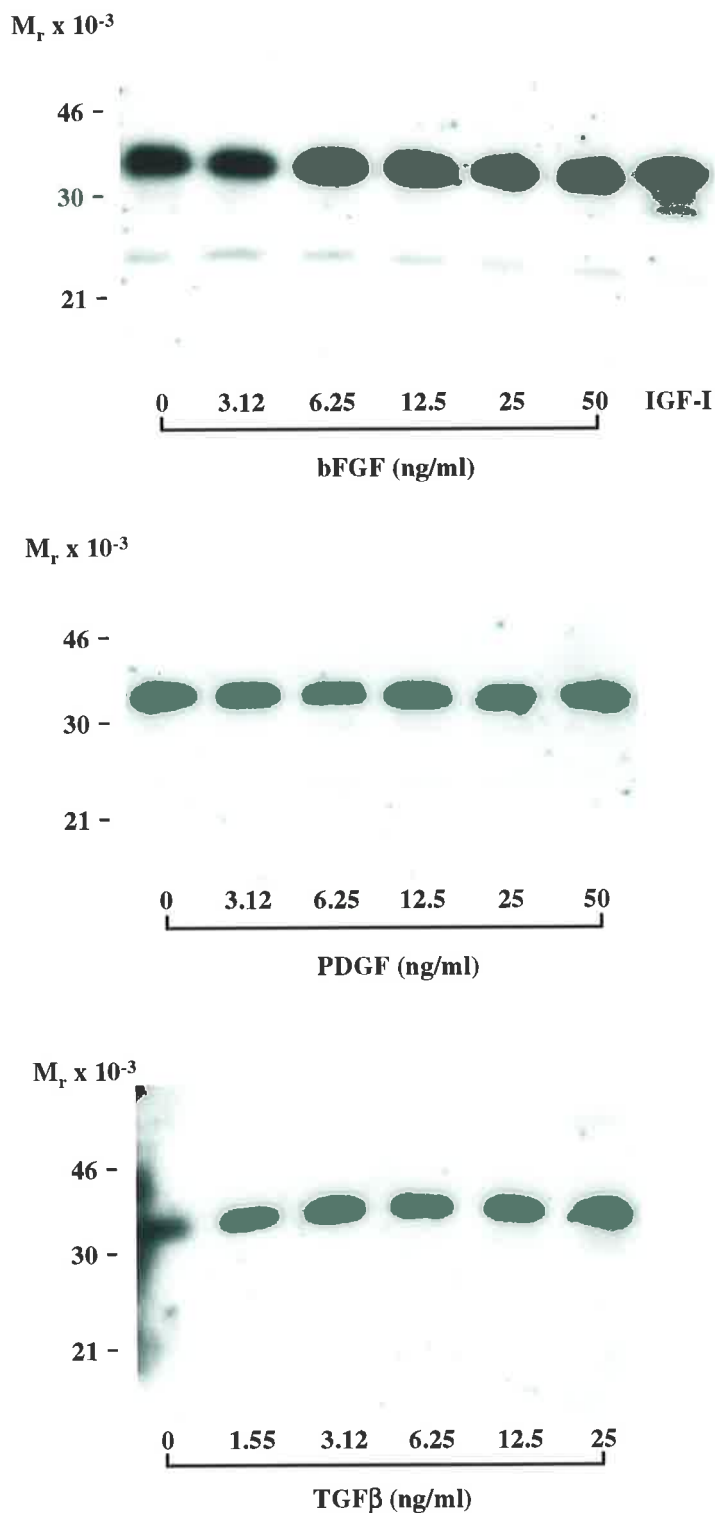


Fig. 3-3. The effect of various growth factors on IGF-BPs in ovine articular chondrocyte culture. Confluent chondrocytes were cultured for 48 hours with indicated doses of bFGF, PDGF or TGF β . Conditioned media samples were dialysed, dried, reconstituted in SDS buffer, separated by SDS PAGE and analysed by Western ligand blotting using 5×10^5 cpm ^{125}I -IGF-II per membrane. The membranes were exposed to X-ray film for 4 days. Medium conditioned by chondrocytes which were exposed to 50 ng/ml IGF-I was used as a positive control. The positions of molecular weight markers are indicated on the left.

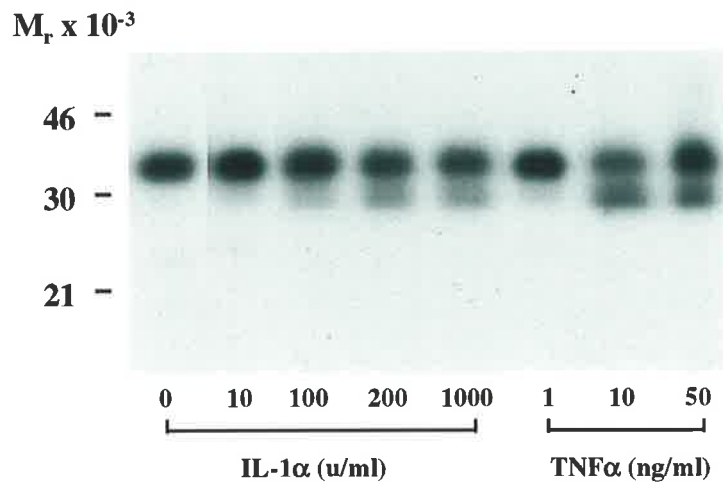


Fig. 3-4. The effect of the inflammatory cytokines on IGF-BPs in ovine articular chondrocyte culture. Confluent chondrocytes were cultured for 48 hours with indicated doses of IL-1 α or TNF α . Conditioned media samples were dialysed, dried, reconstituted in SDS buffer, separated by SDS PAGE and analysed by Western ligand blotting using 5×10^5 cpm ^{125}I -IGF-II per membrane. The membranes were exposed to X-ray film for 4 days. The positions of molecular weight markers are indicated on the left.

As shown in figure 3-5, separate incubations of chondrocytes with 50 ng/ml IGF-I and 1000 u/ml IL-1 α caused a 9-fold and 4.5-fold stimulation of the IGFBP-5 doublet, respectively, while co-incubation of the same concentrations of IGF-I and IL-1 α resulted in a 33-fold increase of IGFBP-5 over basal levels. This synergistic effect of IGF-I and IL-1 α suggested that these two factors acted through different mechanisms which complemented each other in the stimulation of IGFBP-5 production. Similarly, co-incubation of 50 ng/ml IGF-I and 10 ng/ml TNF α resulted in substantially increased level of IGFBP-5 in conditioned medium (Fig. 3-5c), indicating a synergism in the actions of those two factors as well.

3.2.1.4. IGFbps in the Extracellular Matrix of Ovine Articular Chondrocytes

Since it has been reported that IGFBP-5 could associate with fibroblast extracellular matrix (Jones *et al.*1993a) and because the majority of IGFbps, including IGFBP-5, possess heparin-binding consensus domains, the presence of IGFbps in extracellular matrix of ovine articular chondrocytes was investigated. Confluent chondrocytes were incubated with IGF-I (0-100 ng/ml) for 48 hours and extracellular matrices prepared as described in *Materials and Methods*. IGFbps were detected by Western ligand blotting. As shown in figure 3-6, only after prolonged exposure, a 30-kDa and 43-kDa faint ¹²⁵I-IGF-II-binding bands were detected in basal conditions as well as in IGF-I-treated cultures. The intensity of the bands remained unchanged throughout all conditions indicating that IGF-I did not have any effect on the extracellular matrix-derived ¹²⁵I-IGF-II-binding activity.

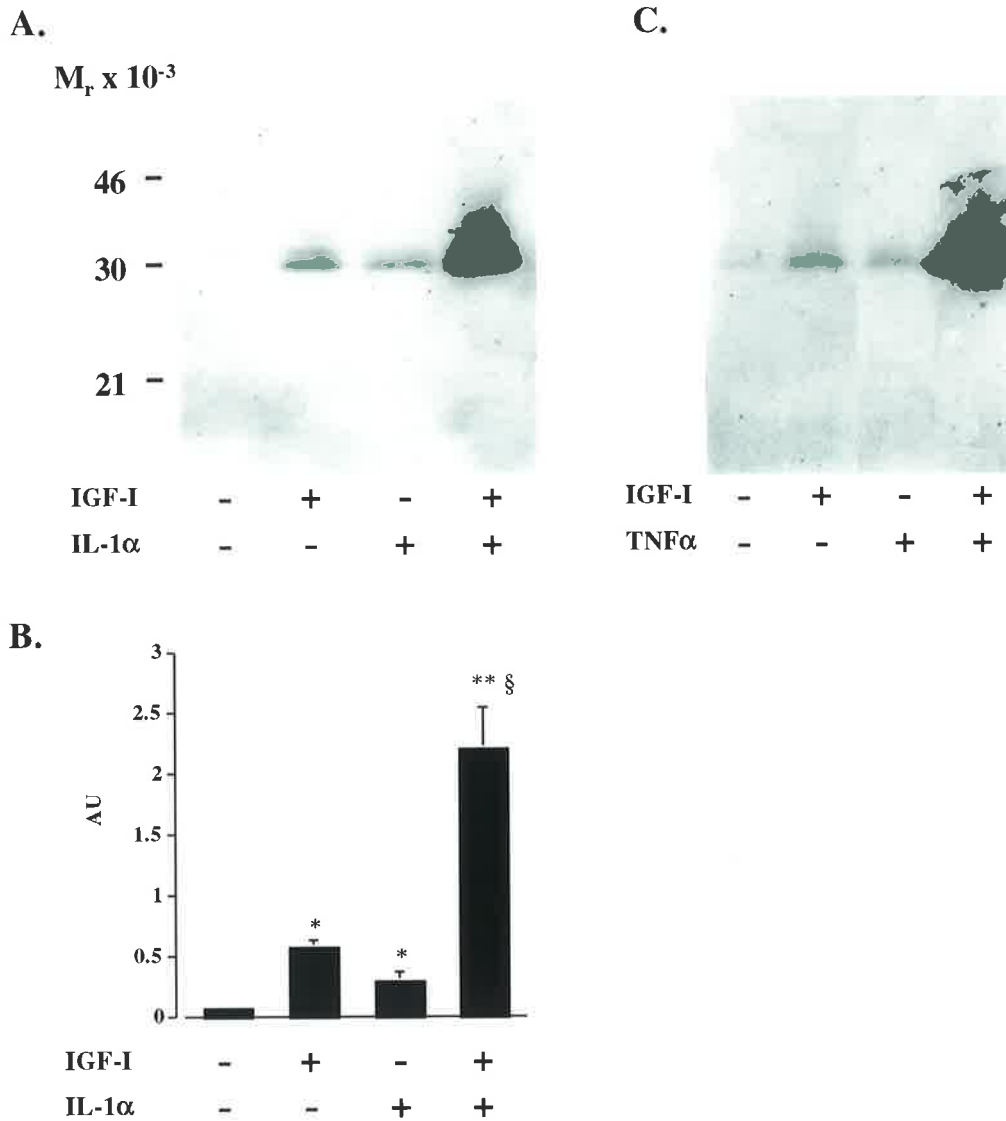


Fig. 3-5. The effect of co-incubation of IGF-I and IL-1 α or TNF α on the IGF-BP-5 level in ovine articular chondrocyte conditioned medium. Confluent chondrocytes were cultured for 48 hours with 50 ng/ml IGF-I in the presence or absence of 1000 u/ml IL-1 α or 10 ng/ml TNF α . A. and C. Conditioned medium samples were dialysed, dried, reconstituted in SDS buffer, separated by SDS PAGE and analysed by Western immunoblotting using the anti-IGFBP-5 polyclonal antibody. The chemiluminescence signal was detected by autoradiography after 1 minute exposure of the membranes to X-ray film. The positions of molecular weight markers are indicated on the left. B. Densitometric analysis of anti-IGFBP-5 stained bands. Densitometry was performed only on IGF-I- and IL-1 α -treated cultures. Data are expressed as arbitrary absorbency units (AU). The columns represent the means \pm standard errors of four samples from three separate experiments. *P<0.05, **P<0.005, compared to control; \S P<0.05, compared to the sum of IGF-I and IL-1 α effects.

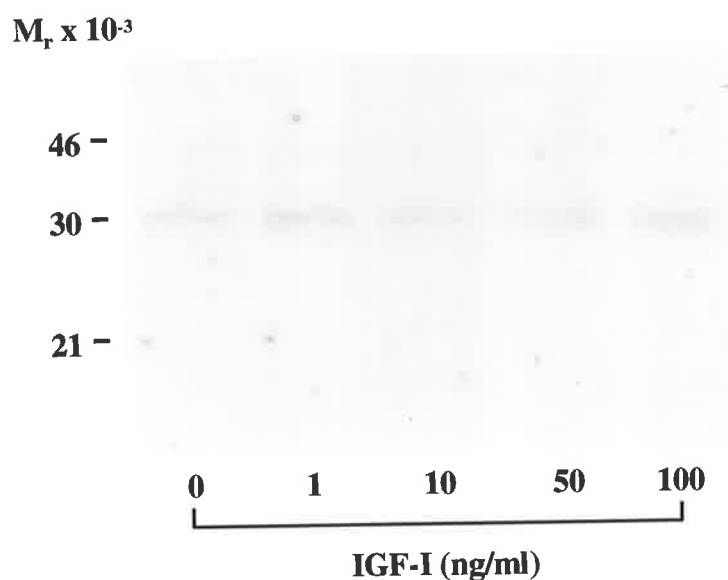


Fig. 3-6. IGF-BPs in extracellular matrix of ovine articular chondrocytes. Confluent chondrocytes were cultured for 48 hours in the presence of 0, 1, 10, 50 or 100 ng/ml IGF-I. Cell-layers were washed, treated with 0.5% Triton X-100 and 25 mM ammonium acetate, washed and extracellular matrices dissolved in SDS buffer. Samples were subjected to SDS PAGE and analysed by Western ligand blotting using 5×10^5 cpm ^{125}I -IGF-II per membrane. The membrane was exposed to X-ray film for 3 days. The positions of molecular weight markers are indicated on the left.

3.2.2. Detection of IGFBPs in Bovine Articular Chondrocyte Culture: the Effect of IGF-I

To determine whether the effect of IGF-I on IGFBPs was species-specific or whether it could be extrapolated to species other than ovine, bovine articular chondrocytes, isolated and cultured in the same manner as their ovine counterparts, were exposed for 48 hours to IGF-I. Conditioned media samples were analysed by SDS-PAGE and Western ligand blotting as described in *Materials and Methods*. In contrast to ovine chondrocyte culture, IGFBPs could not be detected under basal conditions in the absence of the growth factors in bovine chondrocyte culture (Fig. 3-7). Nevertheless, IGF-I stimulated the appearance of a 29/31-kDa IGFBP doublet in a dose-dependant manner, an effect which was seen in ovine articular chondrocytes as well.

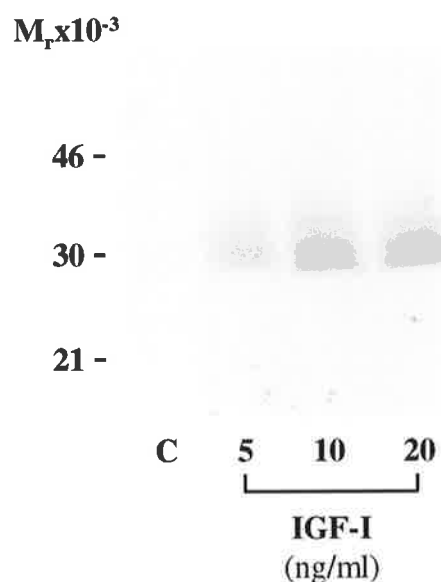


Fig. 3-7. IGF-BPs secreted into the medium conditioned by bovine articular chondrocytes upon exposure to IGF-I. Confluent chondrocytes were cultured for 48 hours in the presence of 0, 5, 10, or 20 ng/ml IGF-I. Conditioned media were dialysed, dried, reconstituted in SDS buffer, separated by SDS PAGE and analysed by Western ligand blotting using 5×10^5 cpm ^{125}I -IGF-II per membrane. The membrane was exposed to X-ray film for 4 days. C, control (no IGF-I). The positions of molecular weight markers are indicated on the left.

3.3. Discussion

Ovine articular chondrocytes were shown to secrete several IGFbps. When grown in basal, serum-free conditions, chondrocytes released a 35- and 24-kDa IGFbp into the culture medium. Exposure to IGF-I resulted in a dose-dependant increase in the appearance of a 29/31-kDa IGFbp doublet. Using anti-human IGFbp polyclonal antibodies, the 35-kDa IGFbp was identified as IGFbp-2, while the IGF-I-regulated 29/31-kDa doublet reacted positively with the anti-IGFbp-5 antibody. The identity of the 24-kDa band was not positively revealed since it did not react with any of the five anti-IGFbp antibodies available (anti-IGFbp-1, -2, -3, -4 and -5). Nevertheless, it is possible that the 24-kDa band represents IGFbp-4 since its mobility on the SDS gel corresponds to that of non-glycosylated IGFbp-4 and because the anti-human polyclonal antibody used in this study which cross-reacts poorly with ovine IGFbp-4 may not recognise minor amounts of the ovine binding protein. This band is highly unlikely to be IGFbp-6 since the naturally occurring form of this binding protein in human fibroblast culture was reported to migrate as a 32-34 kDa doublet on non-reducing SDS gels (Martin *et al.*1994). However, the possibility that the 24-kDa band is a low molecular weight IGFbp breakdown product can not be excluded.

Bovine articular chondrocytes did not release detectable amounts of IGFbps when cultured under basal conditions. Nevertheless, similar to ovine articular chondrocytes, bovine chondrocytes responded to IGF-I by increasing the level of a 29/31-kDa IGFbp doublet in a dose-dependant manner. Although the doublet has not been positively identified, its mobility and a doublet-like appearance on the SDS gel are typical of IGFbp-5 (Kiefer *et al.*1992; Camacho Hubner *et al.*1992; Conover and Kiefer, 1993; Andress and Birnbaum, 1991).

These findings are consistent with previous studies which demonstrated the production and IGF-driven regulation of various IGFFBPs by rabbit and bovine articular chondrocytes, including IGFBP-2, -3, 24- and 29-31-kDa forms (Froger Gaillard *et al.* 1989; Olney *et al.* 1993). At the time IGFBP-5 regulation by IGF-I in ovine articular chondrocyte culture was being elucidated in this study, Matsumoto *et al.* (1996b) reported IGFBP-5 production and upregulation by IGFs in primary culture of rat articular chondrocytes.

Other growth factors that stimulate chondrocyte anabolism including bFGF, TGF β and PDGF (Verschure *et al.* 1994; Hill *et al.* 1992) did not affect the IGFBP-5 level in medium conditioned by ovine articular chondrocytes indicating that the regulation of this binding protein was IGF-specific. It also appears that the levels of the other two IGFFBPs produced by ovine articular chondrocytes remain unaffected by these growth factors.

The inflammatory cytokines used, IL-1 α and TNF α , increased the level of IGFBP-5 in ovine articular chondrocyte culture in a dose-dependent manner, showing the maximum activity at 200 u/ml and 10 ng/ml, respectively. Although the ability of IL-1 and TNF α to increase the secretion of IGFBP-3 has been shown in rat (Matsumoto *et al.* 1994) and human (Olney *et al.* 1995) articular chondrocyte cultures, this is the first time that the upregulation of IGFBP-5 by these two cytokines has been demonstrated. Moreover, it took place in the same system in which IGF-I was also shown to stimulate the IGFBP-5 level. Therefore, the next logical step was to consider how would chondrocytes respond to a simultaneous exposure to these factors. The co-incubation of IGF-I with IL-1 α or TNF α resulted in a substantial increase of IGFBP-5 protein level in the conditioned medium. Thus, the combined effect of IGF-I and IL- α significantly exceeded the sum of their separate effects by 2.5-fold, indicating a synergism of their mechanisms of action. The effects of IGFs and the inflammatory cytokines on cartilage metabolism are usually directed in

opposite directions, with the former increasing anabolic processes and the latter stimulating catabolism. Therefore, the ability of those factors to affect one of the regulatory components of the IGF system (IGFBP-5) *in vitro* in the same way and, moreover, to do that synergistically, comes as a surprise. The mechanisms of the regulation of IGFBP-5 by those factors and the possible implications on the biology of the extracellular matrix are addressed in the Chapters that follow.

Since IGFBP-5 possesses heparin-binding consensus domains, it was assumed that this binding protein could associate with heparin-like structures of glycosaminoglycans in the chondrocyte-derived extracellular matrix. Therefore, the presence of IGFBPs in extracellular matrix of ovine articular chondrocytes was investigated. Only minor amounts of a 30- and 43-kDa IGFBPs were found bound to extracellular matrix produced by the chondrocytes. The higher molecular weight form is most likely to be IGFBP-3 as its mobility on the non-reduced SDS gel is characteristic for 38/43-kDa IGFBP-3 doublet (Conover *et al.* 1990; Camacho Hubner *et al.* 1992). The identity of the 30-kDa IGFBP is less certain since several binding proteins, including IGFBP-1, -5, -6, glycosylated IGFBP-4 and a IGFBP-3 proteolytic fragment, that has a low affinity for IGFs and can be detected by Western ligand blot analysis, have been reported to migrate in a 30-kDa region on the SDS gel under non-reducing conditions (Hossenlopp *et al.* 1990; Lalou and Binoux, 1993). According to the literature, of those proteins, only IGFBP-5 and IGFBP-3 proteolytic fragments would have the structural prerequisites to associate with the matrix (Hodgkinson *et al.* 1994; Fowlkes *et al.* 1994a). However, proteolytic fragments of IGFBP-3 reported to contain putative heparin-binding consensus sequences are not likely to be found in the 30-kDa region since their calculated molecular masses are below 20,000 (Fowlkes *et al.* 1994a; Fowlkes and Serra, 1996). The level of the ¹²⁵I-IGF-II-binding bands associated with extracellular matrix remained unchanged irrespective of whether they were treated with IGF-I or not. This may be due to a

general inability of IGF-I to affect those binding proteins in ovine articular chondrocyte culture. It is also possible that the *in vitro* formed chondrocyte extracellular matrix possesses relatively small number of IGFBP-binding sites which are fully occupied in basal conditions. Therefore, any subsequent increase in the level of IGFBPs upon exposure of chondrocytes to IGF-I, similar to that seen in the conditioned medium, would not be reflected in the extracellular matrix. The lack of binding sites for IGFBP-5 would not be surprising since Arai *et al.* (1994b; 1994a) have demonstrated that IGFBP-5 interacts readily with heparan and dermatan sulphate but not with the glycosaminoglycans that prevail in articular cartilage: chondroitin sulphate, keratan sulphate and hyaluronic acid.

Chapter 4

Mechanisms of IGFBP-5 Regulation in Primary Culture of Ovine Articular Chondrocytes

4.1. Introduction

In the previous chapter, it has been demonstrated that IGF-I and the inflammatory cytokines increase the level of IGFBP-5 in culture medium conditioned by ovine articular chondrocytes. Moreover, the data suggest that IGF-I and the inflammatory cytokines regulate this binding protein through different pathways. Therefore, in order to elucidate those mechanisms, a new set of experiments has been performed and the results are reported here.

IGFBP-3, -4 and -5 are regulated by IGFs at the transcriptional and post-transcriptional level. The latter usually involves the regulation of proteolytic activity produced by various cell types. Thus, the transcriptional, receptor-dependant, upregulation prevails in granulosa (Adashi *et al.*1994) pituitary (Fielder *et al.*1993b) and thyroid follicular cells (Backeljauw *et al.*1993). On the other hand, IGFBP-5 is regulated by IGFs predominantly through a non-receptor-mediated mechanism in cells of the same developmental origin as chondrocytes, such as fibroblasts (Camacho Hubner *et al.*1992; Nam *et al.*1994) and osteoblasts (Conover *et al.*1993; Conover and Kiefer, 1993). Those connective tissue cells produce IGFBP-5 proteases whose activities are inhibited in the presence of IGFs which results in elevated levels of the binding protein.

The recent report by Wang *et al.* (1995a) describing the IL-1-stimulated expression of IGFBP-3 mRNA in Leydig cells represents, so far, the only instance of a defined mechanism of IL-1-driven IGFBP regulation. Although previous studies have demonstrated the potential of IL-1 to upregulate IGFBPs in chondrocyte cultures, in particular IGFBP-3 (Matsumoto *et al.*1994; Olney *et al.*1995), little is known about the mechanism(s) by which these cytokine-driven regulations occur.

IGF structural analogues and insulin are very useful tools in determining mechanisms of IGF actions including the regulation of IGFBPs. They can provide an

indication of whether the IGF activity is receptor mediated or not and can be used to determine the importance of IGF-IGFBP interactions. Available analogues were used, namely IGF-I and II analogues with substantially reduced affinity for IGFBPs including des(1-3)IGF-I, LR³IGF-I and des(1-6)IGF-II. The affinity of des(1-3)IGF-I for IGFBPs is substantially reduced compared to IGF-I (50-300 fold), while the other IGF-I mutant, LR³IGF-I, has virtually no affinity for the binding proteins (1000 fold reduced affinity compared to IGF-I) (Francis *et al.*1992; Francis *et al.*1993). Similarly, the IGF-II N-terminus truncated analogue, des(1-6)IGF-II, has a 150-300 fold reduced affinity for IGFBPs compared to the wild type form (Francis *et al.*1993). All analogues used retain high affinity for their respective receptors. Also, insulin which does not bind to IGFBPs can exert its activity through the IGF-I receptor and, at supraphysiological concentrations, can reach the same level of maximum activity as IGF-I.

4.2. Results

4.2.1. Effects of Various IGFs and Insulin on the IGFBP-5 Level in Ovine Articular Chondrocyte Culture

Ovine articular chondrocytes were exposed to the wide range of IGF molecules and insulin in order to determine whether the IGF-induced appearance of IGFBP-5 in culture medium was a receptor dependant event. Confluent chondrocytes were incubated with 50 ng/ml of IGF-I, II, des(1-3)IGF-I, LR³IGF-I or des(1-6)IGF-II and 10 µg/ml of insulin for 48 hours, conditioned media were collected and the IGFBP-5 level analysed by SDS-PAGE and Western ligand blotting. As shown in Fig. 4-1, IGF-I and II increased the level of IGFBP-5 as compared to basal conditions with the latter being more potent at the given dose. In contrast to the native IGFs, LR³IGF-I and insulin had no effect on IGFBP-5. Des(1-3)IGF-I showed an intermediate effect on IGFBP-5, it was less potent than IGF-I and II but more potent compared to LR³IGF-II and insulin. Interestingly, des(1-6)IGF-II was as effective as its wild type precursor, IGF-II.

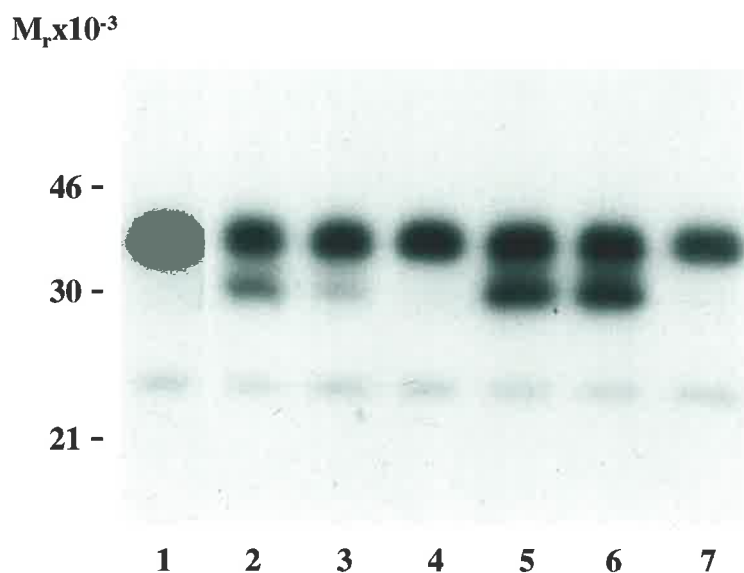


Fig. 4-1. Effects of IGFs and insulin on IGFBP-5 production by ovine articular chondrocytes. Confluent chondrocytes were cultured for 48 hours in the presence or absence of 50 ng/ml of indicated IGFs or 10 μ g/ml of insulin. Conditioned media were dialysed, dried, reconstituted in SDS buffer, separated by SDS PAGE and analysed by Western ligand blotting using 5×10^5 cpm 125 I-IGF-II per membrane. The membrane was exposed to X-ray film for 4 days. Lane 1, control (no growth factors), lane 2, IGF-I, lane 3, des(1-3)IGF-I, lane 4, LR³IGF-I, lane 5, IGF-II, lane 6, des(1-6)IGF-II, lane 7, insulin. The positions of molecular weight markers are indicated on the left.

4.2.2. Quantitative Analysis of IGFBP-5 Stimulation by IGFs and Insulin

In order to quantitatively analyse the differences between the growth factors, chondrocyte cultures were exposed to various concentrations of the above mentioned IGFs ranging from 1 to 200 ng/ml and to insulin (0.1 to 10 μ g/ml) for 48 hours. IGFBP-5 was detected by SDS-PAGE and Western ligand blot analysis. The intensities of the IGFBP bands on autoradiographs from several experiments were quantified on a laser densitometer and the mean and standard error values calculated. The level of IGFBP-5 is expressed as a ratio of the sum of the intensities of the 29- and 31-kDa bands (IGFBP-5) and the intensity of the 35-kDa band (IGFBP-2). The results are shown in Fig. 4-2. IGFBP-2 was chosen as the reference point since the intensities of the bands varied considerably between the X-ray films due to different times of exposure and because the level of IGFBP-2 remained unaffected by the IGFs and insulin. The wild type IGFs and their truncated analogues stimulated the appearance of the IGFBP-5 doublet in a dose-dependant manner with different potencies. IGF-II and des(1-6)IGF-II were found to be more potent than IGF-I and des(1-3)IGF-I in stimulating IGFBP-5, respectively. The differences were statistically significant at concentration of 50 and 100 ng/ml. LR³IGF-I and insulin did not affect the level of the IGFBP-5 doublet at the concentrations up to 100 ng/ml and 10 μ g/ml, respectively. These results indicate the receptor-independent nature of the regulation of chondrocyte-derived IGFBP-5 by IGFs and point to its dependency on IGF-IGFBP interactions.

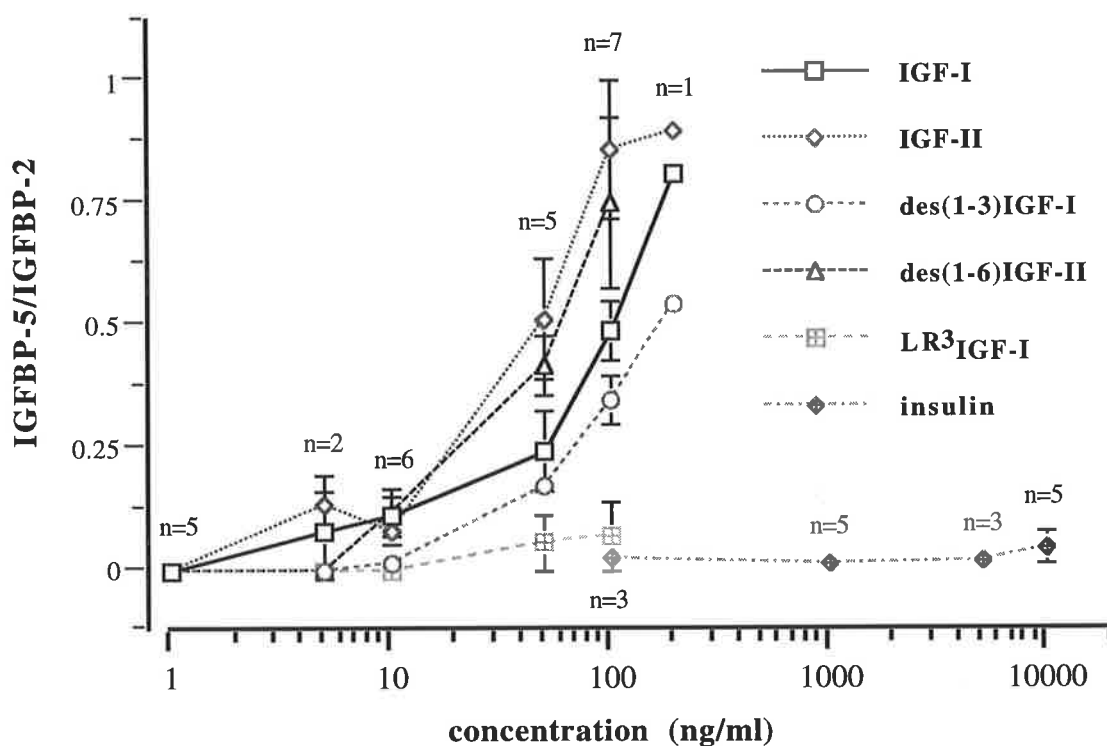


Fig. 4-2. Quantitative analysis of effects of various IGFs and insulin on IGFBP-5 production by ovine articular chondrocytes. Confluent chondrocytes were cultured for 48 hours with 1, 5, 10, 50, 100 and 200 ng/ml of indicated IGFs or 0.1, 1, 5 and 10 $\mu\text{g/ml}$ of insulin. Conditioned media samples were dialysed, dried, reconstituted in SDS buffer, separated by SDS PAGE and analysed by Western ligand blotting using 5×10^5 cpm ^{125}I -IGF-II per membrane. The membranes were exposed to X-ray film for 3-6 days and IGFBP bands quantified by laser densitometry. The symbols represent means \pm SEM of the ratio of the intensities of the 29- and 31-kDa bands (IGFBP-5) and the intensities of the 35-kDa band (IGFBP-2) obtained from the indicated number of cultures (n).

4.2.3. Effects of IGF-I and IL-1 α on the Expression of IGFBP-5

mRNA in Ovine Articular Chondrocytes

As demonstrated in the previous chapter, the level of IGFBP-5 in primary cultures of ovine articular chondrocytes is elevated by both IGF-I and IL-1 α . Moreover, co-incubation of IGF-I and IL-1 α results in a synergistic increase of IGFBP-5 indicating different mechanisms of stimulation of the binding protein by those two factors. To determine if IGFBP-5 is transcriptionally regulated, the level of IGFBP-5 mRNA was measured by Northern blotting after exposure of the cells to IGF-I and/or IL-1 α . Confluent chondrocytes were incubated for 48 hours with or without 50 ng/ml IGF-I or 1000 u/ml IL-1 α or both factors together. Total RNA was isolated from the cell-layers, separated by electrophoresis on the agarose/formaldehyde gel, electro transferred to a nylon membrane which was then hybridised with the ³²P-labelled rat IGFBP-5 cDNA probe. The rat cDNA probe was used to detect IGFBP-5 mRNA since no ovine IGFBP-5 cDNA probe was available at the time Northern blot analysis was performed. The rat GAPDH RNA probe was used to detect the expression of the GAPDH gene. The hybridised IGFBP-5 and GAPDH mRNAs were detected by autoradiography, quantified, and IGFBP-5/GAPDH ratios calculated. As shown in Fig 4-3, IL-1 α increased the expression of IGFBP-5 6.0-kilobase mRNA by 4.5-fold. In contrast, neither the basal level of IGFBP-5 mRNA expression nor the IL-1 α -stimulated transcript levels were significantly affected by IGF-I. This clearly demonstrated that IGF-I and IL-1 α utilised distinct pathways in the regulation of ovine articular chondrocyte IGFBP-5.

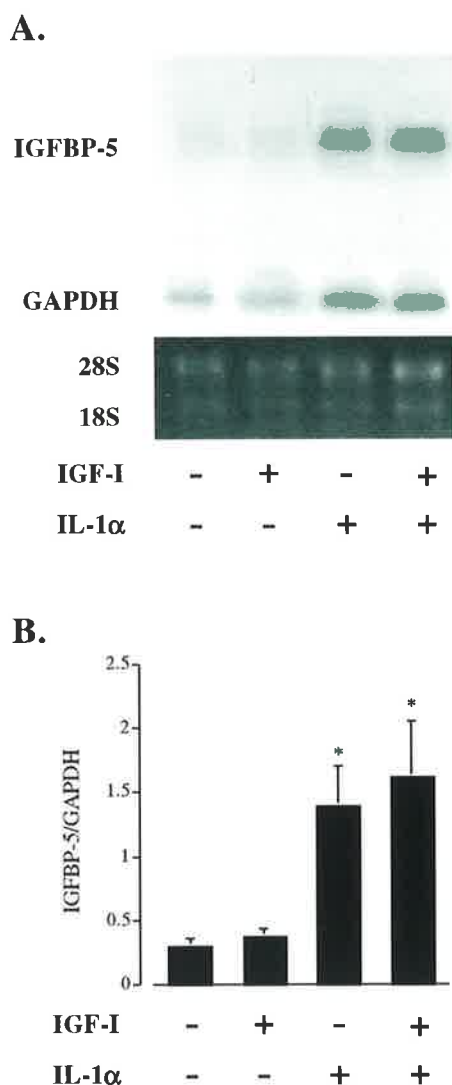


Fig. 4-3. Effects of IGF-I and IL-1 α on ovine articular chondrocyte IGFBP-5 mRNA expression. Confluent chondrocytes were cultured for 48 h in the presence or absence of 50 ng/ml IGF-I or 1000 u/ml IL-1 α or both factors together. Total RNA was isolated and the Northern blot analysis was performed using [32 P]-labelled rat IGFBP-5 cDNA probe as described in *Materials and Methods*. A. A representative Northern blot with IGFBP-5 and GAPDH mRNAs is shown. The ethidium bromide stained 18S and 28S ribosomal RNAs are shown below. B. Quantitative analysis of IGFBP-5 mRNA expression. Results shown are the means \pm SEM of IGFBP-5/GAPDH mRNA ratios from three cultures. * $P < 0.05$, compared to control.

4.3. Discussion

The previous studies of the regulation of IGFBPs in cartilage have demonstrated that IGF-I and II can increase the level of some IGFBPs in chondrocyte cultures but have not defined the mechanism(s) involved (Froger Gaillard *et al.*1989; Olney *et al.*1993).

Only recently, Matsumoto *et al.* (1996b) have shown that IGFBP-5 is regulated by IGFs at both transcriptional and post-transcriptional level in rat articular chondrocyte monolayers. They found that the transcriptional regulation was a predominant mechanism of the IGF-induced rise of IGFBP-5 level although the inhibition of the IGFBP-5 protease(s) which was dependant on the interactions between IGFs and IGFBPs was also involved.

The results that I obtained using ovine articular chondrocytes grown in primary culture and exposing them to the wild type IGFs, IGF mutants with reduced affinities for IGFBPs and insulin showed substantial differences in potencies of those factors in increasing IGFBP-5 concentrations in conditioned media. The order of potencies of the IGFs and insulin was as follows: IGF-II \geq des(1-6)IGF-II > IGF-I > des(1-3)IGF-I \gg LR³IGF-I, insulin. At 100 ng/ml, IGF-I, des(1-3)IGF-I and LR³IGF-I were 40%, 65% and 95% less potent, respectively, than IGF-II. Similar to LR³IGF-I, insulin did not significantly affect IGFBP-5 concentration even at the supraphysiological concentrations (up to 10 μ g/ml). As the IGF-I analogues used retain high affinity for the IGF-I receptor (Francis *et al.*1992) and since insulin, at supraphysiological concentrations, can exert its activity through the same receptor, the results obtained suggest that the IGF-stimulated increase of IGFBP-5 in ovine articular chondrocytes was not, or at least not predominantly, mediated through the IGF-I receptor. Furthermore, the potencies of IGF-I, IGF-II, IGF-I analogues and insulin correlated well with their affinities for IGFBPs (Ballard *et al.*1987; Francis *et*

*al.*1992; Francis *et al.*1993; Ross *et al.*1989), including IGFBP-5 (Clemmons *et al.*1992; Conover and Kiefer, 1993), suggesting that a direct IGF-IGFBP interaction was involved in the IGFBP-5 regulation.

In spite of the fact that IGF-II and, surprisingly, des(1-6)IGF-II were more effective than IGF-I, the IGF-II receptor is unlikely to be involved. If that was the case des(1-6)IGF-II would probably be more potent than IGF-II due to the inhibitory effect of chondrocyte endogenous IGFBPs.

Interestingly, des(1-6)IGF-II was almost as potent as IGF-II in stimulating IGFBP-5 in culture medium conditioned by ovine articular chondrocytes. It is possible that, in spite of its generally low affinity for IGFBPs from different species, des(1-6)IGF-II still binds to ovine IGFBP-5 with substantial affinity. In this regard des(1-3)IGF-I has been shown to bind with relatively high affinity to human IGFBP-3 (Forbes *et al.*1988), bovine IGFBP-3 and human IGFBP-4 (Clemmons *et al.*1992), and ovine 28-30-kDa IGFBP and IGFBP-3 (Lord *et al.*1991). On the other hand, it is possible that the regions of IGF molecules other than IGFBP binding regions are also involved in IGFBP-5 regulation in which case the differences in affinities for IGFBPs between IGFs and their analogues would not be crucial in determining their potencies for IGFBP-5 stimulation.

Since no ovine IGFBP-5 cDNA probe was available at the time Northern blot analysis was performed, the rat cDNA probe was used to detect IGFBP-5 mRNA. It was anticipated that the rat probe would hybridise to the ovine IGFBP-5 mRNA as IGFBP-5 is a highly conserved molecule with an extraordinary degree of interspecies sequence identity. Thus, mouse and rat IGFBP-5 show 99.6% protein sequence identity, while human IGFBP-5 is 97% identical to mouse and rat molecules (James *et al.*1993; Schuller *et al.*1994). Even though the sequence of the ovine IGFBP-5 is still unknown and a greater variation in the DNA sequences compared to the protein sequences would be expected because of the redundancy of the genetic code it is

highly likely that the ovine binding protein possesses significant sequence identity to other mammalian IGFBP-5 molecules and is therefore detectable by the corresponding probes.

Northern blot analysis revealed a low level of IGFBP-5 gene expression in ovine articular chondrocytes which was only marginally increased when the cells were incubated with IGF-I. This finding indicates that the IGFBP-5 regulation at the transcriptional level is not a principal mechanism of IGFBP-5 stimulation by IGF-I. In contrast, IL-1 α increased the level of IGFBP-5 mRNA by 4.5-fold over the basal level clearly pointing to a transcriptional nature of the IGFBP-5 upregulation by this cytokine. In contrast to the synergism of the effects of IGF-I and IL-1 α on the IGFBP-5 protein level (Chapter 3), the co-incubation of IGF-I with IL-1 α did not have any significant additional effect on the IL-1-stimulated IGFBP-5 mRNA expression. The marginal increase in the IL-1-induced IGFBP-5 mRNA expression upon exposure of the cells to IGF-I can certainly not account for the substantial increase in the IGFBP-5 protein level seen when IGF-I and IL-1 α are co-incubated. Neither can the minimum increase of the IGFBP-5 transcription by IGF-I explain the considerable rise in IGFBP-5 protein level induced by the same growth factor. These discrepancies imply that there is an additional mechanism which is responsible for the IGF-I-driven upregulation of IGFBP-5 in ovine articular chondrocytes.

Previous studies showed that connective tissue cells, including fibroblasts and osteoblasts, produce IGFBP-5 proteases (Camacho Hubner *et al.* 1992; Nam *et al.* 1994; Conover *et al.* 1993; Conover and Kiefer, 1993). Moreover, IGFBP-5 that is also produced by those cells, is upregulated by IGFs primarily through a receptor independent pathway which includes the inhibition of the proteolytic activities. Similarly, Matsumoto *et al.* (1996b) have found that a post-translational mechanism of IGFBP-5 regulation by IGF-I and II in rat articular chondrocytes involved the inhibition of the IGFBP-5 proteolytic activity. These data, together with the findings

described in the current and the previous chapter, point to a protection of IGFBP-5 from proteolytic degradation as the likely mechanism by which IGF-I increases the level of this binding protein in ovine articular chondrocyte cultures. This possibility is investigated further in the following chapter.

Chapter 5

Characterisation of the IGFBP-5 Proteolytic Activity in Ovine Articular Chondrocyte Culture

5.1. Introduction

As demonstrated in the previous chapters, IGFs and inflammatory cytokines stimulate IGFBP-5 in ovine articular chondrocyte culture utilising different pathways. Thus, while IL-1 acts at the gene level by increasing the transcription of IGFBP-5 mRNA, the activity of IGF-I is less well defined and appears to be a combination of post-translational and to much lesser extent, transcriptional mechanisms.

As reviewed in Chapter 1, IGFbps, including IGFBP-5, are regulated at the post-translational level by a variety of proteases. IGFs, in turn, either potentiate or diminish the activities of those proteases. There are several reports showing that IGFBP-5 undergoes a limited proteolysis in cultures of many cell types including fibroblasts and osteoblasts (Camacho Hubner *et al.*1992; Conover and Kiefer, 1993; Thraillkill *et al.*1995). The results obtained so far have revealed that IGFBP-5 proteases fall into two groups: cation-dependant proteases that belong to the family of matrix metalloproteases and serine or serine-like proteases (Nam *et al.*1994; Kanzaki *et al.*1994; Thraillkill *et al.*1995). Furthermore, IGFs can regulate IGFBP-5 at the post-translational level by inhibiting the activities of those proteases. The interference of IGFs with the IGFBP-5 proteolytic activities is characterised by a receptor non-involvement and direct IGF-IGFBP interactions.

The IGFBP-5 production data obtained in this study using the wild type IGFs, their analogues and insulin, together with data from the literature, point to a possibility that ovine articular chondrocytes produce a protease which cleaves IGFBP-5 and whose activity could be inhibited by IGFs. Therefore, the presence and nature of IGFBP-5 proteolytic activity in ovine articular chondrocyte culture were investigated by utilising chondrocyte endogenous IGFbps and exogenous recombinant IGFBP-5 as potential substrates and by using various protease inhibitors.

5.2. Results

5.2.1. Purification of Ovine Articular Chondrocyte IGFbps by Size-Exclusion Chromatography

To obtain a preparation of chondrocyte-derived IGFbps for subsequent assessment of their stability in a cell-free system, ovine articular chondrocytes were incubated with 100 ng/ml IGF-I and IGFbps were purified from the conditioned medium by size-exclusion chromatography on Superose-12 column. The rationale for this approach was as follows: (i) cells had to be exposed to IGF-I to increase the level of IGFBP-5, which was otherwise undetectable by Western analysis and (ii) the subsequent removal of IGF-I from the IGFBP preparation was necessary in order to avoid interference of the growth factor with the IGFBP stability assay. Therefore, the purification of chondrocyte endogenous IGFbps was performed in dissociative conditions (pH~2.0) and Superose-12 column was chosen since it was expected to efficiently separate molecules of 7.5 kDa and 20-40 kDa in size. Fractions containing IGFbps were detected by radioimmunoassay (RIA) for IGF-I, as IGFbps interfere with the RIA for IGF-I by binding and hence displacing radioactively labelled IGF-I from the complex with the anti-IGF-I antibody. Therefore, as shown in Fig. 5-1, the analysis of the fractions by IGF-I RIA resulted in two peaks of "IGF-I activity". Moreover, the baseline separation of the two peaks was achieved. The higher molecular weight peak (fractions 27-32) contained IGFbps, while the second peak of activity represented genuine IGF-I. Portions of the fractions that constituted the IGFBP peak were analysed by Western ligand blotting to confirm the presence of the IGFbps. As shown in Fig. 5-1, IGFbps detectable by Western ligand blotting were eluted in fractions 28-30. The fractions containing IGFbps were pooled and processed as described in Chapter 2 (Materials and Methods) and subsequently used

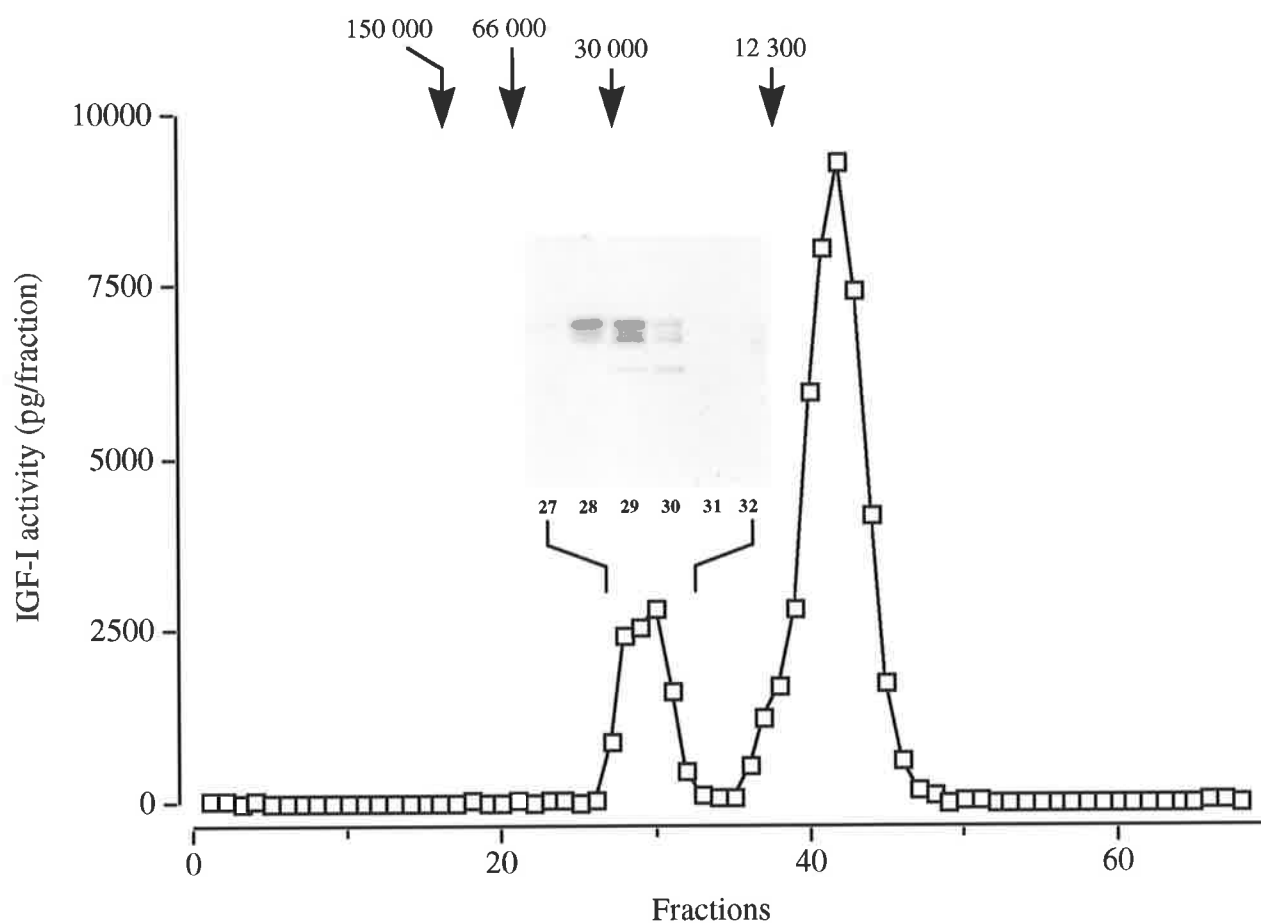


Fig. 5-1. Purification of IGF-BPs from medium conditioned by ovine articular chondrocytes. Confluent chondrocytes were cultured for 48 h in the presence of 100 ng/ml IGF-I, culture medium collected, dialysed overnight at 4°C against 0.1 M acetic acid and vacuum dried. The pellet was reconstituted in running buffer and IGF-BPs separated by FPLC size-exclusion chromatography under dissociative conditions on a Superose-12 column. 50 µl portions of the FPLC fractions were analysed by the IGF-I RIA and the results expressed as total amount of IGF-I per fraction. Individual fractions of the high molecular weight peak were also analysed by Western ligand blotting and the results are shown aligned to the corresponding fractions. The positions of the molecular weight markers are indicated at the top of the graph.

for testing the stability of the IGFBPs in the presence of ovine articular chondrocyte conditioned medium.

5.2.2. Stability of FPLC Purified Chondrocyte IGFBPs in the Presence of Ovine Articular Chondrocyte Conditioned Medium

To investigate the possibility that ovine articular chondrocytes produce a soluble IGFBP proteolytic activity that specifically cleaves IGFBP-5, chondrocyte IGFBPs purified by size-exclusion chromatography were incubated in a cell-free system with medium conditioned by ovine articular chondrocytes cultured under basal, serum-free, conditions and their stability was assessed by Western ligand blotting. As shown in Fig. 5-2, a 24-hour incubation at 4°C with the conditioned medium did not produce any change in the concentrations of chondrocyte IGFBPs whereas the incubation at 37°C resulted in a decreased level of IGFBP-5 while the levels of the other two chondrocyte IGFBPs remained unaffected.

In order to evaluate the effect of IGF-I on the stability of IGFBP-5, this growth factor was used at the concentration of 100 ng/ml that corresponded to an estimated IGFBP-5:IGF-I molar ratio of 1:15 at which IGF-I substantially increased the level of the binding protein in culture. IGF-I partially inhibited the disappearance of IGFBP-5 in this cell-free system indicating its protective role on IGFBP-5 protein levels (Fig. 5-2). This was the first evidence of IGF-I protection of IGFBP-5 from degradation and it also demonstrated that the protective effect of IGF-I did not require interaction with receptors. Some instability of IGFBP-5 was also observed when the endogenous IGFBPs were incubated with a non-conditioned culture medium (DMEM, 0.01% BSA), thus pointing to a possibility that the proteolytic activity was partially co-purified with chondrocyte-derived IGFBPs.

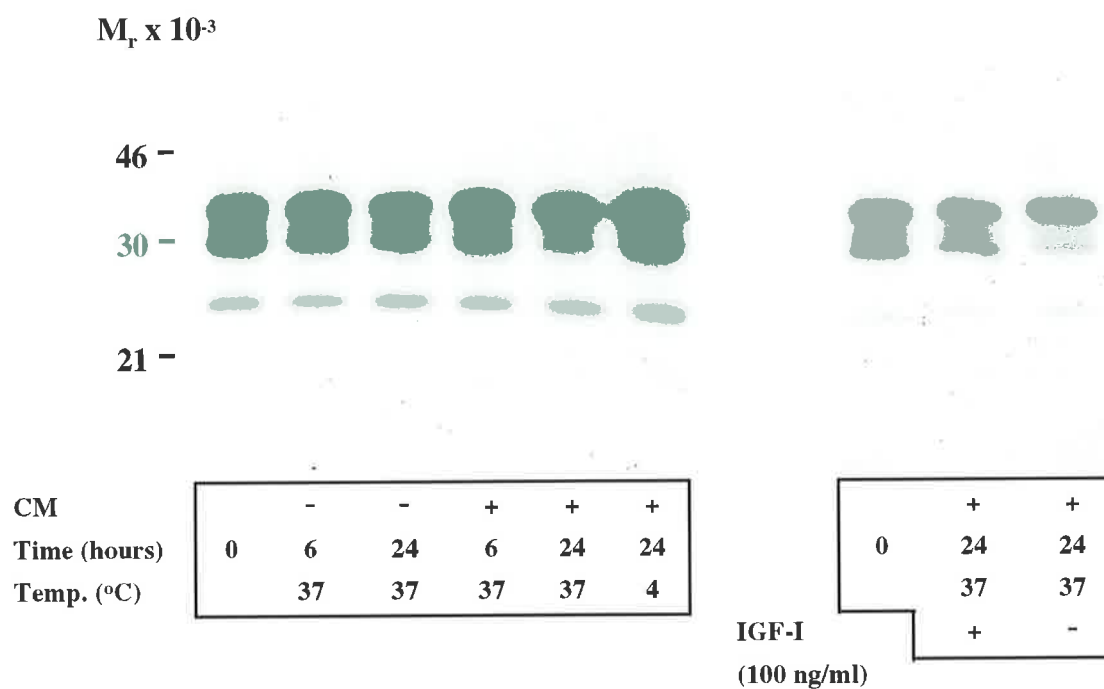


Fig. 5-2. Stability of the chondrocyte endogenous IGF-BPs. Chondrocyte IGF-BPs purified by size-exclusion chromatography were incubated in indicated conditions in the presence or absence of medium conditioned (CM) by ovine articular chondrocytes. Reactions were stopped by the addition of SDS buffer and Western ligand blot analysis performed. The positions of molecular weight markers are indicated on the left.

5.2.3. Protection of Recombinant Human IGFBP-5 by IGFs in Ovine Articular Chondrocyte Culture

To further characterise the stability of IGFBP-5 in ovine articular chondrocyte culture, recombinant human IGFBP-5 (rhIGFBP-5) was used as a potential substrate for chondrocyte-derived IGFBP-5 proteolytic activity. The stability of unlabelled rhIGFBP-5 was determined in the presence or absence of ovine articular chondrocytes and the protective effect of IGF-I and its analogues was assessed.

Briefly, confluent chondrocytes were grown in basal conditions for 48 hours and conditioned medium was either collected and 1 ml/well transferred to a new, BSA-rinsed, 24-well plate (cell-free system) or left in the original wells with the cell-layers. Recombinant hIGFBP-5 was added (25 ng/well) and incubated for further 24 hours at 37°C in the presence or absence of the IGFs. At the end of the incubation period, conditioned media were dialysed, vacuum dried, reconstituted in SDS buffer and analysed by SDS-PAGE and Western ligand blotting.

As shown in Fig. 5-3a and b, the addition of 25 ng/ml rhIGFBP-5 increased the intensity of the 29/31-kDa IGFBP doublet which was previously almost undetectable in the medium conditioned by chondrocytes grown under basal conditions. After a 24-hour incubation at 37°C in the cell-free system with conditioned medium alone, intact rhIGFBP-5 could not be detected by Western ligand blotting (Fig 5-3a). The presence of 100 ng/ml IGF-I in the incubation mixture resulted in a reduction of the disappearance of IGFBP-5. In contrast, des(1-3)IGF-I and LR³IGF-I were not protective at the concentration of 100 ng/ml.

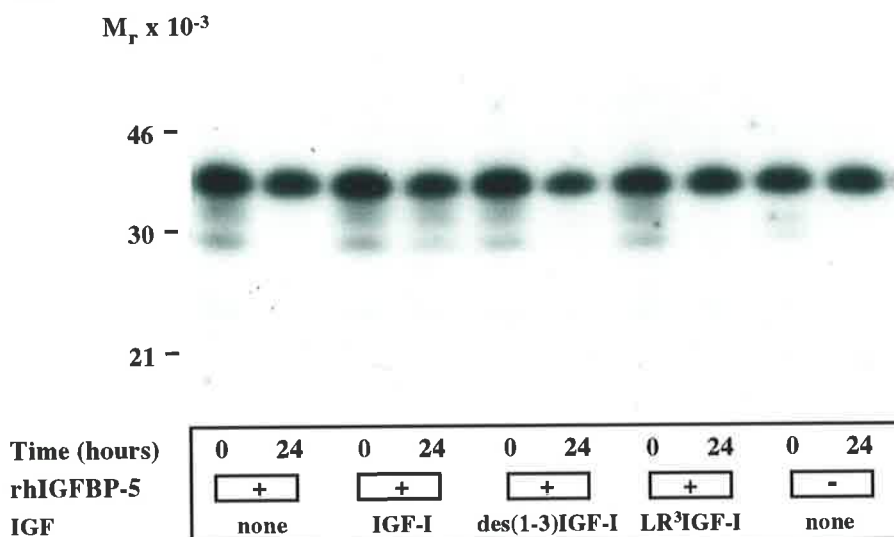
Similarly to the cell-free system, the exogenously added rhIGFBP-5 (25 ng/ml) almost completely disappeared after 24 hour incubation with the cell culture in the absence of IGFs (Fig. 5-3b). Incubation of rhIGFBP-5 with the cell culture in the presence of 100 ng/ml IGF-I resulted in an increased level of the 29/31-kDa IGFBP

doublet at the end of the incubation period. It was probably a result of the IGF-I protection of IGFBP-5, both exogenously added at the beginning and endogenously produced by the chondrocytes during the incubation. Interestingly, des(1-3)IGF-I was almost as effective as the wild type IGF in protecting IGFBP-5 from disappearance and even when rhIGFBP-5 was incubated in cell culture with 100 ng/ml LR³IGF-I a minor amount of the 29/31-kDa doublet was still visible on the Western ligand blot after the 24-hour incubation.

To assess whether there was a substantial association of IGFBP-5 with the cell-layer which would contribute to the disappearance of the binding protein from the culture medium, cell-layers were solubilised in SDS buffer and the total IGFBP contents were analysed by Western ligand blotting. As shown in Fig. 5-3b, after the 24-hour incubation, the levels of cell-layer-associated IGFBP-5 in basal conditions and IGF-I-treated cultures were much lower than the levels of IGFBP-5 at the beginning of the incubation period in culture media of the corresponding wells (basal conditions - first lane, IGF-I-treated culture - third lane). Given that only one third of the total IGFBP contents of the media were analysed and shown in Fig. 5-3, as opposed to the whole IGFBP contents of the corresponding cell-layers, it seems that the cell-layers did not considerably interfere with the IGFBP-5 disappearance by retaining the binding protein.

As anticipated, the levels of IGFBP-2 and the 24-kDa IGFBP were slightly increased by the end of the incubation period, as a result of the continuous production of those two binding proteins by chondrocyte cultures during the incubation.

A.



B.



Fig. 5-3. Stability of the rhIGFBP-5 in medium conditioned by ovine articular chondrocytes in the presence (B) or absence (A) of the cell-layers. The rh IGFBP-5 (25 ng/ml) was incubated for 24 hours at 37°C either in the cell-free system with 1 ml of medium conditioned by chondrocytes grown under the basal conditions or with the chondrocyte cell-layers (medium was not changed) in the presence or absence of 100 ng/ml of indicated IGFs. At the end of the incubation period, media were collected, dialysed, vacuum dried and reconstituted in SDS buffer. Samples (1/3 of their final volumes) were analysed by SDS-PAGE and Western ligand blotting. The second to last and the last lane of the blot shown in B section represent total IGFFBPs recovered from the cell-layers that were, during the 24-hour incubation, exposed to the basal conditions or IGF-I, respectively. The positions of molecular weight markers are indicated on the left.

5.2.4. Proteolysis of ^{125}I -Labelled rhIGFBP-5 in Primary Culture of Ovine Articular Chondrocytes

Recombinant hIGFBP-5 radioactively labelled with ^{125}I was used as a potential substrate in order to confirm the presence and activity of the IGFBP-5 protease in the primary culture of ovine articular chondrocytes and to detect the degradation products. A range of inhibitors, including protease inhibitors, IGFs and IL-1 α , was tested to characterise the nature of the proteolytic activity. The protease inhibitors used included inhibitors of metallo- and metal-activated proteases that were either non-specific inhibitors, in the form of chelators of divalent cations (EDTA, 1,10-phenanthroline), or a naturally occurring tissue inhibitor of matrix metalloproteases (TIMP-1), an inhibitor of interstitial collagenase, stromelysin and other metalloproteases. In addition, serine protease inhibitors PMSF, aprotinin, soybean trypsin inhibitor, benzamidine HCl and heparin as well as a serine/cysteine protease inhibitor, antipain, were used.

Confluent ovine articular chondrocytes were cultured under basal conditions for 48 hours, ^{125}I -IGFBP-5 added and the culture continued for another 24 hours in the presence or absence of protease inhibitors, IGFs or IL-1 α . At the end of the incubation, 50 μl aliquots of conditioned media were taken and the reaction stopped by the addition of 4x SDS buffer. Samples were analysed by SDS-PAGE, autoradiography (Fig. 5-4) and densitometry (Table 5-1).

As shown in Fig. 5-4, the intact ^{125}I -IGFBP-5 (29/31-kDa) underwent a limited proteolysis when incubated in chondrocyte culture in the absence of protease inhibitors, breaking down to 22- and 16-kDa degradation fragments. Proteolysis was

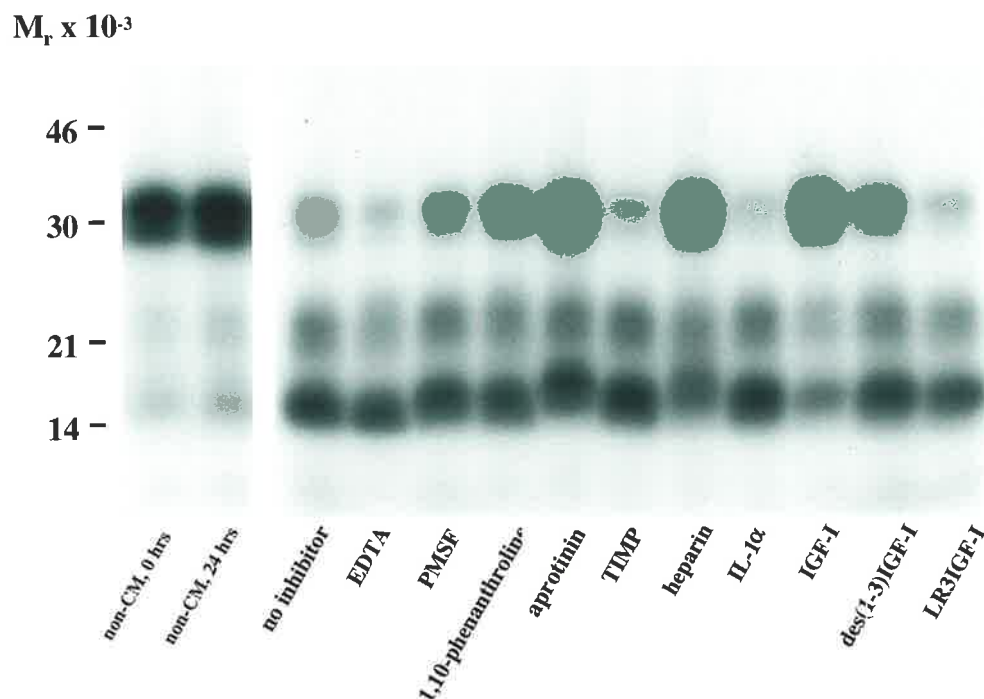


Fig. 5-4. Proteolytic degradation of ^{125}I -labelled rhIGFBP-5 in primary culture of ovine articular chondrocytes. ^{125}I -rhIGFBP-5 was incubated for 24 hours at 37°C with chondrocyte cell-layers that were previously cultured under the basal conditions. The incubation was performed in the presence or absence of various inhibitors, as indicated above. At the end of the incubation period, $50\ \mu\text{l}$ aliquots of each condition were taken and the reaction stopped by the addition of SDS buffer. The samples were analysed by SDS-PAGE and autoradiography. The first two lanes show ^{125}I -rhIGFBP-5 incubated in non-conditioned medium (control) for 0 and 24 hours. The positions of molecular weight markers are indicated on the left.

calculated by dividing the optical densities of the proteolytic fragments by the sum of the densities of the fragments and intact IGFBP-5. After adjusting for spontaneous proteolysis (proteolysis in conditioned medium in the absence of inhibitors minus proteolysis in non-conditioned medium), it has been estimated that 82% of the intact protein was proteolysed in ovine articular chondrocyte culture. The formula that was used to calculate the inhibition of proteolysis by particular inhibitor is shown in the legend of Table 5-1. While a serine protease inhibitor, aprotinin, effectively inhibited proteolysis of ^{125}I -rhIGFBP-5, inhibitors that chelate divalent cations, EDTA and 1,10-phenanthroline, did not affect the IGFBP-5 breakdown. It has to be pointed out that, as detected by light microscopy, EDTA and 1,10-phenanthroline were toxic on cells at the concentrations used. Nevertheless, the results obtained by using those two inhibitors are shown in Fig. 5-4 and Table 5-1. Similarly to the serine protease inhibitor, IGF-I and II and heparin in particular, partially inhibited the degradation of IGFBP-5. Although des(1-3)IGF-I and des(1-6)IGF-II also showed some activity in protecting IGFBP-5 at 100 ng/ml, they were less potent than the native IGFs whereas, at the same concentration, LR³IGF-I (100 ng/ml) did not affect the proteolysis. Similarly to LR³IGF-I, IL-1 α was ineffective in protecting IGFBP-5 from proteolytic degradation.

Table 5-1. Quantitative analysis of the inhibitory effects of various factors on ^{125}I -rhIGFBP-5 proteolysis during a 24-hour incubation with chondrocytes in culture. The autoradiographs, including that shown in Fig. 5-4, were analysed by laser densitometry and the inhibition of proteolysis calculated by the following formula: $(A-B) \times (100\%/A)$. A=proteolysis in conditioned medium without inhibitors, B=proteolysis in conditioned medium in the presence of indicated inhibitors. The results represent the means of two separate experiments.

Inhibitor (conc.)	Number of observations	Inhibition of proteolysis (%)
None	2	
EDTA, 5 mM	2	-2.8 ± 7.3
1,10-phenanthroline, 1 mM	2	10.2 ± 5.1
PMSF, 5 mM	2	3.8 ± 1.6
Aprotinin, 1 mg/ml	2	24.4 ± 5.2
Soybean trypsin inhibitor, 2 mg/ml	1	15
Benzamidine, HCl, 10 mM	1	11.8
TIMP, 20 $\mu\text{g/ml}$	1	-13.1
Heparin, 20 $\mu\text{g/ml}$	2	33.3 ± 1.8
IL-1alpha, 1000 u/ml	2	-6.8 ± 5.6
IGF-I, 100 ng/ml	2	37.2 ± 10.3
Des(1-3)IGF-I, 100 ng/ml	2	7.9 ± 0.5
LR3IGF-I, 100 ng/ml	2	-3.5 ± 7.6
IGF-II, 100 ng/ml	1	21.5
Des(1-6)IGF-II, 100 ng/ml	1	12.6

5.2.5. Proteolysis of ^{125}I -Labelled rhIGFBP-5 in a Cell-Free System in the Presence of Medium Conditioned by Ovine Articular Chondrocytes

From the previous results obtained by using unlabelled endogenous ovine IGFBPs and exogenous human IGFBP-5, it was expected that the IGFBP-5 proteolytic activity should be soluble in culture medium. In order to establish that, ^{125}I -IGFBP-5 was incubated in cell-free conditions with medium conditioned by ovine articular chondrocytes in the presence or absence of protease inhibitors. As in the experiments with the monolayers, inhibitors used included conventional inhibitors, heparin, IGFs and inflammatory cytokines.

Confluent ovine articular chondrocytes were cultured under basal conditions for 48 hours, conditioned medium collected and 20 μl aliquots incubated with ^{125}I -rhIGFBP-5 in the presence or absence of protease inhibitors, IGFs or cytokines for indicated periods of time. Reactions were stopped by the addition of 4x SDS buffer and samples analysed by SDS-PAGE, autoradiography (Fig. 5-5) and densitometry (Table 5-2).

As shown in Fig. 5-5, as well as in the chondrocyte culture, the intact ^{125}I -IGFBP-5 (29/31-kDa) underwent a time-dependant limited proteolysis when incubated with chondrocyte conditioned medium in a cell-free system, breaking down to 22- and 16-kDa degradation fragments. However, the proteolytic system in the

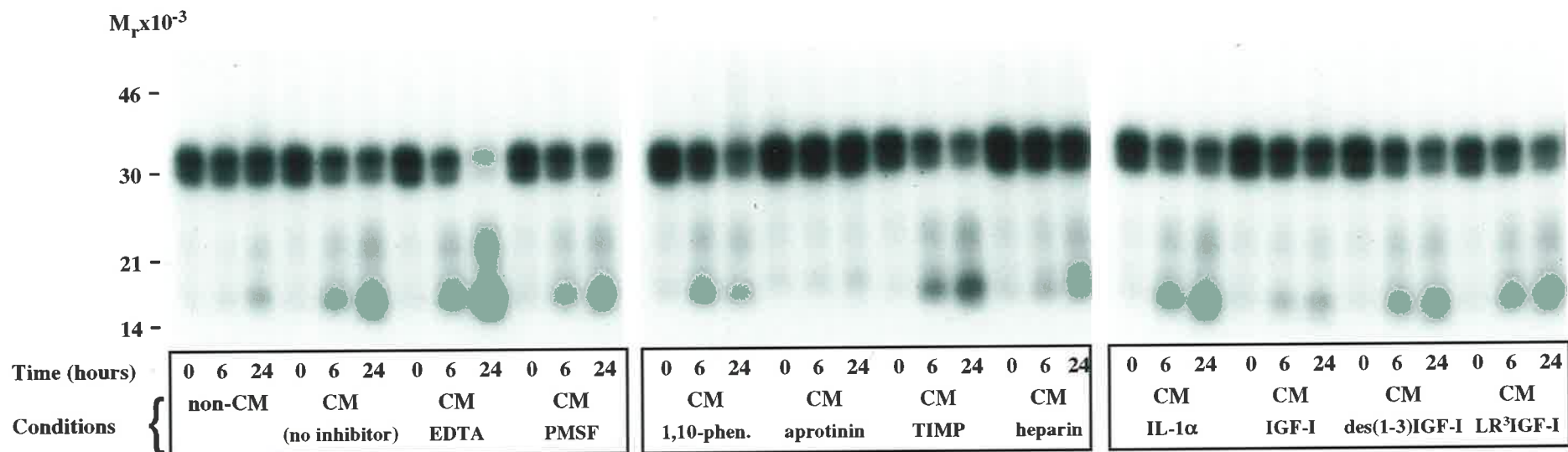


Fig. 5-5. Proteolytic degradation of ¹²⁵I-labelled rhIGFBP-5 in medium conditioned by ovine articular chondrocytes. ¹²⁵I-rhIGFBP-5 was incubated at 37°C for indicated periods of time in the cell-free system with medium conditioned by chondrocytes that were cultured under the basal conditions in the presence or absence of various inhibitors, as indicated above. The reaction was stopped by the addition of SDS buffer and the samples were analysed by SDS-PAGE and autoradiography. The positions of molecular weight markers are indicated on the left.

cell-free conditions was somewhat less efficient than the cell culture in degrading the IGFBP-5. In the absence of inhibitors only 47% of the protein was proteolysed in conditioned medium in cell-free conditions compared to 82% in cell culture. Aprotinin and antipain (results for antipain are shown in Table 5-2 only) were very potent in inhibiting proteolysis of ^{125}I -rhIGFBP-5 in the cell-free conditions, whereas EDTA and 1,10-phenanthroline were unable to significantly inhibit the IGFBP-5 breakdown. Heparin, IGF-I and II had a significant effect on IGFBP-5 stability, partially inhibiting its degradation. Des(1-3)IGF-I and des(1-6)IGF-II showed some marginal, non-significant activity in protecting IGFBP-5, whereas LR³IGF-I did not have any effect on the proteolysis. Likewise, the inflammatory cytokines, IL-1 α and TNF α , were ineffective in protecting IGFBP-5 from proteolytic degradation.

Table 5-2. Quantitative analysis of the inhibitory effects of various factors on ^{125}I -rhIGFBP-5 proteolysis during a 24-hour incubation in the cell-free conditions. The autoradiographs of samples incubated in the cell-free conditions, including those shown in Fig. 5-5, were analysed by laser densitometry and the inhibition of proteolysis calculated by the method given in the legend of Table 5-1. The results represent the means \pm SEM from up to four separate experiments. Level of significance was calculated by Student's t-test only if there were three or more observations. ND, not determined; NS, not significant ($p>0.05$).

Inhibitor (conc.)	Number of observations	Inhibition of proteolysis (%)	Significance
None	4		
EDTA, 5 mM	4	-20.9 ± 19.7	NS
1,10-phenanthroline, 1 mM	4	24.7 ± 10.8	NS
PMSF, 5 mM	4	17.3 ± 7.0	NS
Aprotinin, 1 mg/ml	4	64.9 ± 7.1	$p<0.005$
Soybean trypsin inhibitor, 2 mg/ml	2	-1.9 ± 23.8	ND
Benzamidine, HCl, 10 mM	2	1.6 ± 13.1	ND
Antipain, 4 mM	2	55.3 ± 0.2	ND
TIMP, 20 $\mu\text{g}/\text{ml}$	3	-4.9 ± 5.7	NS
Heparin, 20 $\mu\text{g}/\text{ml}$	4	28.6 ± 8.9	$p<0.05$
IL-1alpha, 1000 u/ml	4	6.0 ± 4.4	NS
TNFalpha, 50 ng/ml	2	2.1 ± 3.1	ND
IGF-I, 100 ng/ml	4	38.9 ± 8.0	$p<0.05$
Des(1-3)IGF-I, 100 ng/ml	4	9.9 ± 3.6	NS
LR3IGF-I, 100 ng/ml	4	-0.5 ± 4.7	NS
IGF-II, 100 ng/ml	2	38.3 ± 16.1	ND
Des(1-6)IGF-II, 100 ng/ml	2	18.6 ± 17.3	ND

5.3. Discussion

An IGFBP-5 proteolytic activity in ovine articular chondrocyte culture has been detected and partially characterised. In addition, the regulation of IGFBP-5 by IGFs and inflammatory cytokines at the post-translational level has further been defined.

When incubated in ovine articular chondrocyte culture, IGFBP-5 undergoes a time-dependant limited proteolysis breaking down to 22- and 16-kDa fragments. Moreover, the proteolytic activity appears to be soluble since substantial IGFBP-5 breakdown, resulting in the appearance of the two fragments, can also be seen in the medium conditioned by chondrocytes. However, a higher rate of IGFBP-5 proteolysis was observed in the presence of chondrocyte monolayers than in their absence, possibly due to a prolonged synthesis and interplay of the components of the IGFBP-5 proteolytic system (eg. enzyme, coenzyme, activators and inhibitors) in chondrocyte culture during the incubation period. It could also indicate a cell-membrane- or extracellular matrix-associative nature of the proteolytic activity or some other, less specific mechanism, by which the cells and/or the surrounding matrix increase the rate of IGFBP-5 proteolysis.

The protease inhibitor profile suggests that the enzymatic activity responsible for the IGFBP-5 cleavage belongs to a group of serine proteases. Serine protease inhibitors aprotinin, antipain and heparin were the most effective inhibitors of the IGFBP-5 proteolysis in the cell-free conditions, reducing the enzymatic activity by 65%, 55% and 29% respectively. Of the two non-specific (EDTA, 1,10-phenanthroline) and one specific (TIMP-1) metalloprotease inhibitors used, only 1,10-phenanthroline showed some potency in suppressing the proteolytic activity. This observation may imply that cation dependent proteases are not the principal enzymatic activity that is responsible for IGFBP-5 degradation, although their involvement cannot be ruled out completely. It is not unusual for certain cell types to produce more

than one enzyme, sometimes belonging to different classes of proteases, capable of proteolysing IGFBPs including IGFBP-5. Thus, murine osteoblasts produce metalloprotease-1 (MMP-1) and MMP-2 and a serine protease which all cleave IGFBP-5 (Thraikill *et al.*1995) while the all three MMPs capable of degrading IGFBP-3 have been detected in human dermal fibroblast culture (Fowlkes *et al.*1994a).

The inhibitor profile of the IGFBP-5 proteolytic activity in the presence of the chondrocyte monolayers was similar to that seen in the cell-free system, although aprotinin was not as potent in inhibiting the IGFBP-5 degradation as in the cell-free system. Furthermore, the proteolytic data obtained by using EDTA and 1,10-phenanthroline in chondrocyte culture should be interpreted cautiously as these two inhibitors had a toxic effect on the cell-layers as a consequence of their ability to sequester divalent cations and thus interfere with the cell adhesion. Nevertheless, it appears that the same protease was responsible for the IGFBP-5 degradation in the cell-free system as well as in the presence of the cell-layers.

The efficiency of heparin in inhibiting the chondrocyte-derived IGFBP-5 proteolytic activity, particularly in the presence of the cell-layers, is consistent with the previous reports of heparin's ability to inhibit degradation of IGFBPs including IGFBP-5 in cultures of various cell types (Arai *et al.*1994a; Parker *et al.*1995). Although heparin is a well known inhibitor of plasma serine proteases, especially in the combination with other serine protease inhibitors like antithrombin-III and α_1 -antichymotrypsin, its mechanism of action is not well understood. Nam *et al.* (1994) have shown that heparin binds to a human fibroblast-derived serine protease that cleaves IGFBP-5. As an additional illustration of the complexity of the IGFBP-5 proteolytic system, IGFBP-5 can interact directly with heparin and other GAGs (Arai *et al.*1994a; Arai *et al.*1996) as well as fibroblast extracellular matrix (Parker *et al.*1996) through the heparin binding site in the C-terminal domain of the molecule.

Therefore, it is possible that heparin interferes with the chondrocyte-derived IGFBP-5 proteolytic activity by binding to either the substrate or the enzyme. Either scenario probably involves a conformational change or hindrance of the active sites on the bound molecules. In addition, the involvement of the extracellular matrix either as a reservoir of serine protease inhibitors or as a source of IGFBP-5 interactive sites cannot be excluded, particularly in the light of a higher potency of heparin in the presence of the cell-layer. The fact that only a minimum amount of IGFBP-5 has been found associated with the chondrocyte extracellular matrix and cell-layer indicates a relatively low number of IGFBP-5 binding sites. However, it may also reflect a highly dynamic system in which the association of IGFBP-5 with the matrix could make the binding protein more susceptible to proteolysis and lead to its degradation.

Of the three chondrocyte-derived IGFbps which were purified by size-exclusion chromatography and incubated with chondrocyte conditioned medium, only IGFBP-5 showed instability while the levels of IGFBP-2 and the 24-kDa IGFBP remained steady. This finding suggests that the serine protease in ovine articular chondrocyte culture specifically cleaves IGFBP-5 whereas the other two endogenous binding proteins remain unaffected by its activity. Whether this protease has substrates other than IGFBP-5 remains, however, undetermined. Given the specificity of the protease and a high level of conservation among the IGFbps, one could expect that the mid-region of the IGFBP-5 molecule, which is the least conserved part of the molecule, would be a prime target for the catalytic activity of the enzyme. In this regard, all previous studies in which the N-terminal sequence analyses of the IGFBP-3 and -4 degradation fragments have been performed, report that the cleavage sites are positioned in the mid-regions of the binding protein molecules (Fowlkes *et al.* 1994a; Conover *et al.* 1995; Chernausk *et al.* 1995). Nevertheless, the position(s) of the cleavage site(s) inside the IGFBP-5 molecule remain(s) open to speculation until the sequences of the proteolytic fragments have been analysed.

Surprisingly, the proteolytic fragments could not be detected by immunoblotting, most likely as the result of a loss of the epitopes on the IGFBP-5 cleaved fragments. IGFBP-5 is highly conserved across species (eg. human and rat IGFBP-5 show 97% identity in the primary structure). Therefore, the IGFBP-5 molecule would not be expected to have a considerable number of sites with antigenic properties when used to immunise another species (in this study an anti-IGFBP-5 polyclonal serum raised in rabbit against human IGFBP-5 was used) which would result in a relatively small number of different antibodies in the anti-serum. It is likely that upon the cleavage of the IGFBP-5 molecule those few epitopes would be lost either because of their overlapping with the cleavage site(s) or through the conformational changes in the fragments which might take place as a result of the proteolysis. As a consequence, the anti-IGFBP-5 anti-serum would not be able to detect the IGFBP-5 fragments.

The 22- and 16-kDa IGFBP-5 degradation fragments could not be detected by Western ligand blotting (IGF-II used as a tracer) indicating a substantial loss of the affinity of the fragments for IGFs. It is well documented that the proteolytic fragments of IGFBPs, including IGFBP-5, have substantially reduced affinity for IGFs (see section *IGFBP protease* in Chapter 1). Although the physiological relevance of the IGFBP proteolysis is still unclear, it has been proposed by some authors that the fragmentation of certain IGFBPs like IGFBP-3 in prostate epithelial cell and ovarian granulosa cell cultures (Cohen *et al.* 1994a; Grimes and Hammond, 1994), IGFBP-4 in fibroblast and osteoblast cultures (Chernausek *et al.* 1995; Conover *et al.* 1995) and IGFBP-5 in osteoblast culture (Thraillkill *et al.* 1995; Andress *et al.* 1993) may lead to increased bioavailability of IGFs due to their subsequent release from the IGF-IGFBP complex and, consequently, higher bioactivity of the growth factors. The IGFBP-5 proteolytic activity in chondrocyte culture could certainly have the similar impact on the IGF activity. The IGF biological activity data

and results demonstrating the effect of IGFBP-5 are presented and discussed in more detail in the following chapter.

The ability of IGF-I and II to inhibit the activity of the ovine articular chondrocyte-derived IGFBP-5 protease is consistent with the previous findings showing that IGFBP-3 (Grimes and Hammond, 1994) and IGFBP-5 (Fielder *et al.* 1993a; Nam *et al.* 1994) can be protected by IGFs from proteolytic degradation. More recently, Matsumoto *et al.* (1996b) have demonstrated a partial inhibition of IGFBP-5 degradation by IGF-I and II in primary culture of rat articular chondrocytes. However, they incubated IGFBP-5 in medium conditioned by chondrocytes that were pre-treated with IGFs, whereas in this study, IGFs were added only at the start of the incubation of IGFBP-5 with either conditioned medium or complete cell culture.

The IGFs used showed different levels of efficiencies in inhibiting the IGFBP-5 proteolysis. They ranged from an inhibition of up to 39% and 38% in the cell-free system with IGF-I and IGF-II, respectively, to no inhibition at all when LR³IGF-I was used, while des(1-3)IGF-I and des(1-6)IGF-II showed an intermediate potency of no statistical significance. The potencies of the IGFs in inhibiting the activity of the IGFBP-5 protease correlate with their affinities for IGFBPs, indicating that IGF binding to IGFBP-5 is the mechanisms by which wild type IGFs protect IGFBP-5. Since the IGF binding regions are located in the N-terminal regions of IGFBP molecules and the protease expected to cleave in the mid-region of the IGFBP-5 molecule, a conformational change in the IGFBP-5 molecule upon IGF binding, that would result in a lower susceptibility of the IGFBP-5 molecule for the proteolysis, is a likely mechanism of the inhibition of the proteolysis although a simple blockage or hindrance of the cleavage site is not excluded. Since IGF-I shows only a marginal stimulation of the IGFBP-5 gene expression (Chapter 4), these results suggest that the protection of IGFBP-5 from proteolytic degradation is a principal mechanism by

which IGF-I increases the level of this binding protein in primary culture of ovine articular chondrocytes.

Contrary to the IGFs, IL-1 had no effect on the chondrocyte-derived IGFBP-5 proteolytic activity indicating that the stimulation of IGFBP-5 by this inflammatory cytokine is regulated primarily at the transcriptional level as shown in Chapter 4.

The nature of IGFBP-5 regulation by IGF-I and IL-1 in primary culture of ovine articular chondrocytes has by now become more evident. Apparently, there is a simultaneous, constitutive synthesis of IGFBP-5 and IGFBP-5 protease by chondrocytes in the basal conditions. IL-1 effectively induces the expression of IGFBP-5 while, on the other hand, IGF-I has only a marginal effect on the basal level of the IGFBP-5 synthesis but strongly inhibits its degradation. The growth factor and inflammatory cytokine act in a synergism to dramatically elevate the level of IGFBP-5, IL-1 by increasing the synthesis and IGF-I by protecting the newly synthesised binding protein. The effect of this co-ordinated upregulation of IGFBP-5 by IGFs and inflammatory cytokines on the metabolism of proteoglycans is investigated and discussed in the following chapter.

Chapter 6

Proteoglycan Synthesis in Chondrocyte Cultures in Response to IGFs and IL-1: the Effect of IGFBPs

6.1. Introduction

The opposite effects of the IGFs and inflammatory cytokines on cartilage metabolism, in particular in the metabolism of proteoglycans, are well documented (see Chapter 1). While IGF-I maintains the level of proteoglycans in cartilage matrix by increasing their synthesis, IL-1 exerts a catabolic effect on these cartilage-specific macromolecules. An increase in the rate of proteoglycan degradation and decreased proteoglycan synthesis are among the most prominent catabolic actions of this inflammatory cytokine in diseased articular cartilage. IL-1 can also induce cartilage non-responsiveness to IGF-I through a mechanism that remains unknown (Lazarus *et al.* 1993; Verschure *et al.* 1995).

The results from the previous chapters have shown that articular chondrocytes produce and secrete several IGFbps in primary culture. Furthermore, it has been demonstrated that IGF-I and IL-1 upregulate IGFbp-5 in ovine articular chondrocyte culture through different mechanisms which complement each other resulting in a substantially increased level of the binding protein when the cells are co-incubated with these two factors. These results, together with the findings of Olney *et al.* (1995) and Matsumoto *et al.* (1994), which demonstrate the ability of IL-1 and TNF α to increase the level of chondrocyte IGFbps, indicate that the inflammatory cytokines could interfere with the IGF bioactivity by affecting the level of IGFbps.

In order to assess the role of IGFbps in the regulation of cartilage metabolism, the effect of endogenous and exogenous IGFbps on the activities of IGF-I and IL-1 in chondrocyte culture was investigated.

6.2. Results

Stimulation of proteoglycan synthesis is a well recognised biological activity of IGFs in cartilage explants and chondrocyte cultures. Therefore, proteoglycan synthesis was determined indirectly by measuring the incorporation of [³⁵S]-sulphate into newly synthesised glycosaminoglycans in order to detect the level of bioactivity of the wild type IGFs and their structural analogues and establish the effect of IGFBPs and IL-1 on the activity of the growth factors.

6.2.1. Proteoglycan Synthesis in Primary Culture of Bovine Articular Chondrocytes in Response to IGF-I and des(1-3)IGF-I

The effect of the bovine articular chondrocyte endogenous IGFBPs on IGF bioactivity was investigated by exposing the monolayers to IGF-I or des(1-3)IGF-I and measuring the level of newly synthesised proteoglycans. Briefly, confluent chondrocytes were incubated with IGF-I or des(1-3)IGF-I for 48 hours, [³⁵S]-sulphate added in the final 24 hours and newly synthesised proteoglycans determined as described in Chapter 2.

As shown in Fig. 6-1, bovine articular chondrocytes responded to IGFs in a dose-dependant manner. Both growth factors stimulated [³⁵S]-sulphate incorporation to the same maximum level of approximately 3-fold over the control value. However, with the half-maximum effective dose of less than 2 ng/ml, des(1-3)IGF-I was 10-20-fold more potent than IGF-I. This clearly indicated the inhibitory effect of the 31/29-kDa IGFBP, secreted by bovine articular chondrocytes to culture medium, on the

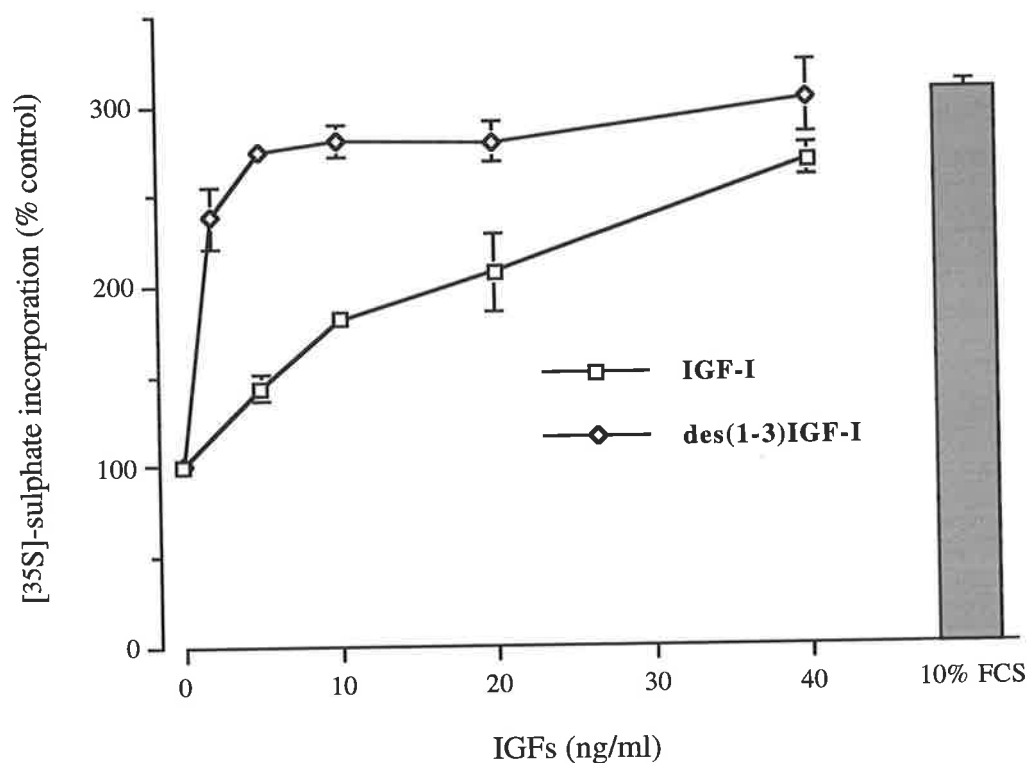


Fig. 6-1. Effects of IGFs on proteoglycan synthesis in bovine articular chondrocyte culture. Confluent chondrocytes were incubated with indicated concentrations of IGF-I or des(1-3)IGF-I or 10% FCS for 48 hours. [^{35}S]-Sulphate was added in the final 24 hours and its incorporation into newly synthesised proteoglycans determined. The results are expressed as percentage of the negative control (basal conditions) and represent the means \pm SEM pooled from three separate experiments, each performed in triplicate. The basal level value was 1150 ± 59 cpm. Results obtained with monolayers exposed to 10% FCS were used as a positive control.

IGF-I bioactivity. In addition, the level of proteoglycan synthesis stimulation obtained in chondrocyte cultures that were incubated with medium containing 10% FCS (positive control) was similar to the levels of maximum stimulation observed when IGFs were used.

6.2.2. Proteoglycan Synthesis in Primary Culture of Ovine Articular Chondrocytes in Response to IGFs and Insulin

Ovine articular chondrocytes were exposed to the wild type IGFs, their structural analogues or insulin and the levels of newly synthesised proteoglycans were measured in order to evaluate the effect of the endogenous IGFBPs on IGF bioactivity. Briefly, confluent chondrocytes were incubated with IGF-I, des(1-3)IGF-I, LR³IGF-I, IGF-II, des(1-6)IGF-II or insulin for 48 hours, [³⁵S]-sulphate added in the final 24 hours and newly synthesised proteoglycans determined as described in Chapter 2.

Ovine articular chondrocytes responded to IGFs in a dose-dependant manner (Figs. 6-2 and 6-3). As shown in Fig. 6-2, half-maximum effective doses of IGF-I, des(1-3)IGF-I and LR³IGF-I were 10, 1 and 1 ng/ml, respectively. All three growth factors increased the proteoglycan synthesis to a similar maximum level of 2.5-3 fold over the control value. The IGF-II, des(1-6)IGF-II and insulin results are presented separately in Fig. 6-3. The N-terminus truncated analogue of IGF-II was only slightly more potent in enhancing proteoglycan synthesis than IGF-II and its potency appeared to match that of IGF-I. These results implied that the locally produced IGFBPs diminished the actions of IGF-I, IGF-II and even des(1-6)IGF-II in ovine

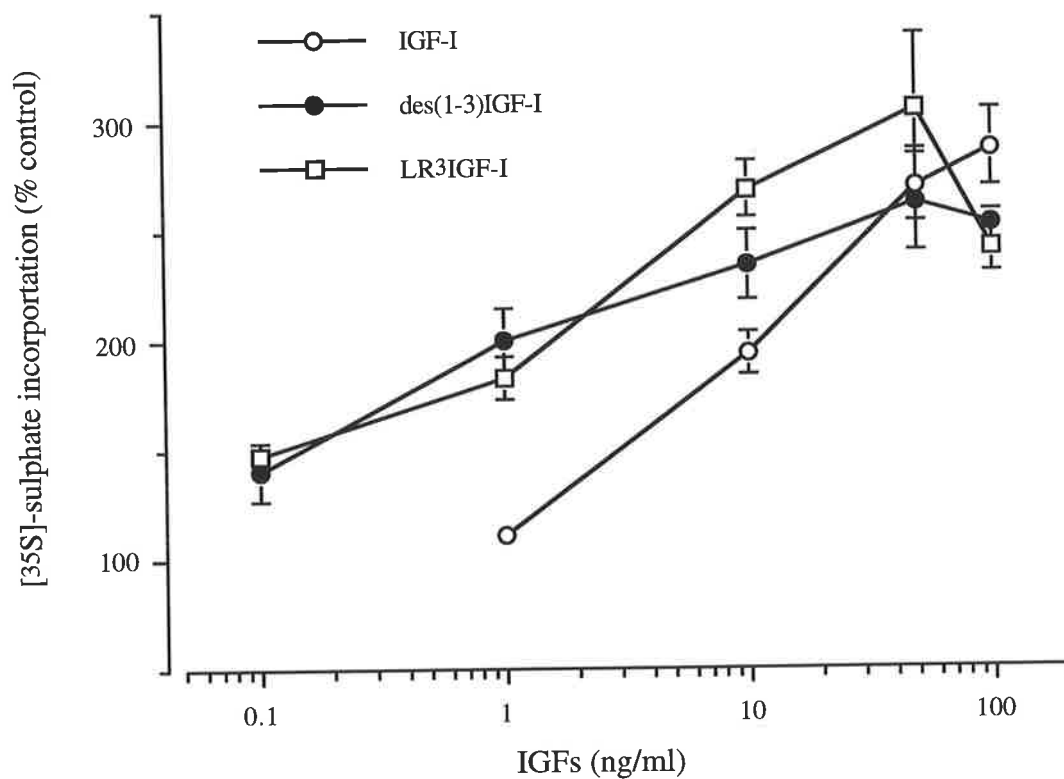


Fig. 6-2. Effects of IGF-I and its structural analogues on proteoglycan synthesis in ovine articular chondrocyte culture. Confluent chondrocytes were incubated with indicated concentrations of the growth factors for 48 hours. [^{35}S]-Sulphate was added in the final 24 hours and its incorporation into newly synthesised proteoglycans determined. The results are expressed as percentage of control and represent the means \pm SEM pooled from five separate experiments, each performed in triplicate.

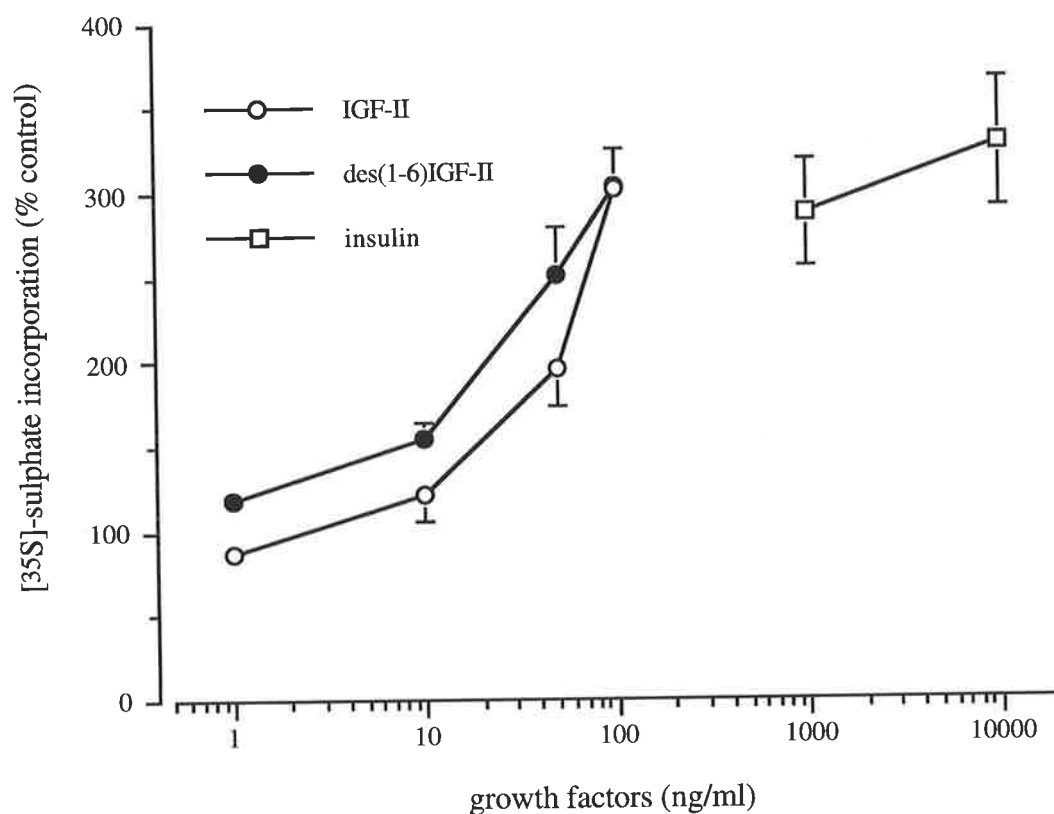


Fig. 6-3. Effects of IGF-II, des(1-6)IGF-II and insulin on proteoglycan synthesis in ovine articular chondrocyte culture. Confluent chondrocytes were incubated with indicated concentrations of the growth factors for 48 hours. [^{35}S]-Sulphate was added in the final 24 hours and its incorporation into newly synthesised proteoglycans determined. The results are expressed as percentage of control and represent the means \pm SEM from a single experiment, performed in triplicate.

articular chondrocyte cultures whereas the activities of des(1-3)IGF-I and LR³IGF-I remained unaffected. At supraphysiological concentrations, insulin was able to stimulate the maximum level of proteoglycan synthesis seen with other growth factors, thus indicating that the action was mediated through the IGF-I receptor.

6.2.3. The Effect of Exogenous IGFBPs on IGF-stimulated Proteoglycan Synthesis in Primary Culture of Ovine Articular Chondrocytes

Since the negative effect of the endogenous IGFBPs on the IGF bioactivity in chondrocyte cultures has been indirectly demonstrated by using IGF analogues, a further step was to establish the role of the individual IGFBPs. For that purpose, recombinant bovine IGFBP-2 (rbIGFBP-2) and rhIGFBP-5, that are structural and functional counterparts of the IGFBPs detected and identified in medium conditioned by ovine articular chondrocytes, were used.

To determine the effects of recombinant IGFBP-2 and -5 on IGF-stimulated proteoglycan synthesis, chondrocytes were incubated with increasing concentrations of IGFs for 48 hours in the presence or absence of rIGFBPs. [³⁵S]-Sulphate was added in the final 24 hours and newly synthesised proteoglycans determined as described in Chapter 2.

The addition of 1000 ng/ml rbIGFBP-2 to culture medium effectively inhibited IGF-I-induced proteoglycan synthesis in ovine articular chondrocyte culture. As shown in Fig. 6-4, the activity of 10 ng/ml and 50 ng/ml IGF-I was significantly

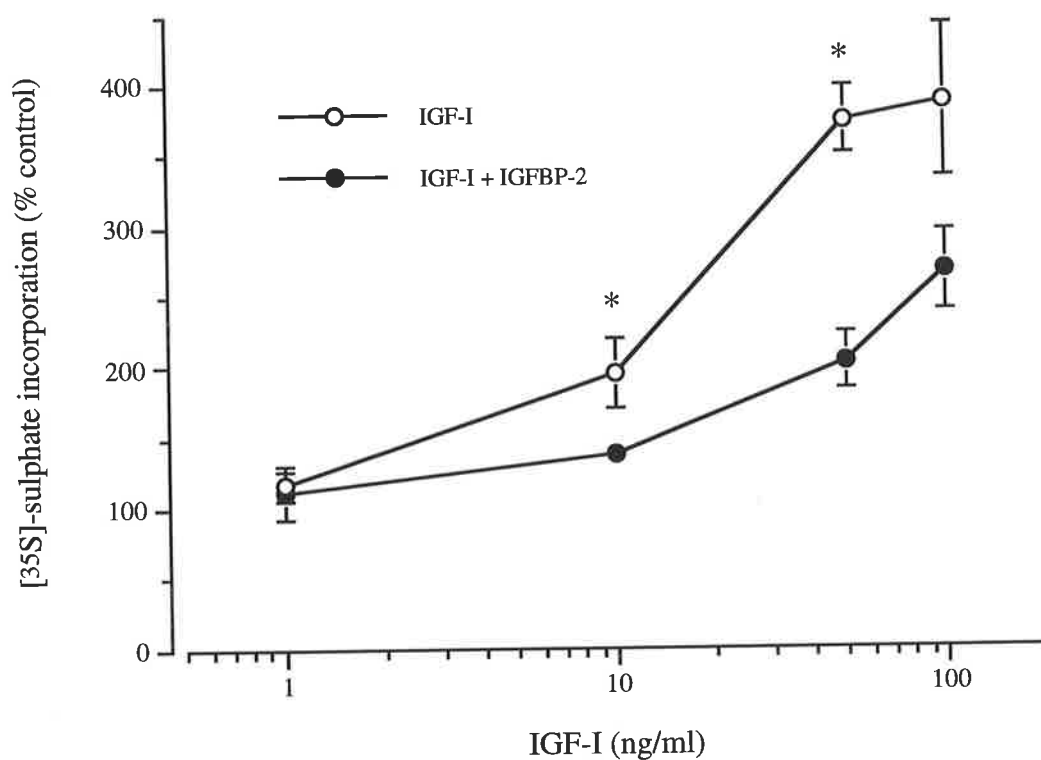


Fig. 6-4. The effect of rbIGFBP-2 on IGF-I-induced proteoglycan synthesis. Confluent chondrocytes were incubated with indicated doses of IGF-I for 48 hours in the presence or absence of 1000 ng/ml rbIGFBP-2. [^{35}S]-Sulphate was added in the final 24 hours and its incorporation into newly synthesised proteoglycans was determined. Results are expressed as the percentages of control values representing the means \pm SEM from a single experiment, performed in triplicate. *, $P < 0.05$, compared to corresponding cultures treated with IGF-I only (Student's t-test).

reduced by 60% and 65%, respectively. The ability of IGFBP-2 to inhibit the action of IGF-I was less evident at 100 ng/ml IGF-I, probably reflecting the increasing IGF-I:IGFBP-2 molar ratio and saturation of IGF-I binding sites on the IGFBP-2 molecule. IGFBP-2 did not affect the basal level of proteoglycan production.

Since IL-1 α increased the IGFBP-5 level in ovine articular chondrocyte culture, particularly when co-incubated with IGF-I (as shown in Chapter 3), it was postulated that increased levels of this binding protein could affect the IGF-I bioactivity. As shown in Fig. 6-5, similarly to rbIGFBP-2, the addition of rhIGFBP-5 also reduced the stimulatory effect of IGF-I on proteoglycan synthesis, although with somewhat lower potency than IGFBP-2. Thus, the activity of IGF-I at 10 and 50 ng/ml was suppressed by 50% and 30%, respectively. In contrast, the co-incubation of rhIGFBP-5 with the same concentrations of des(1-3)IGF-I or LR³IGF-I did not result in any significant change in the activities of the analogues (Fig. 6-6). The basal production of proteoglycans remained unaffected in the presence of IGFBP-5.

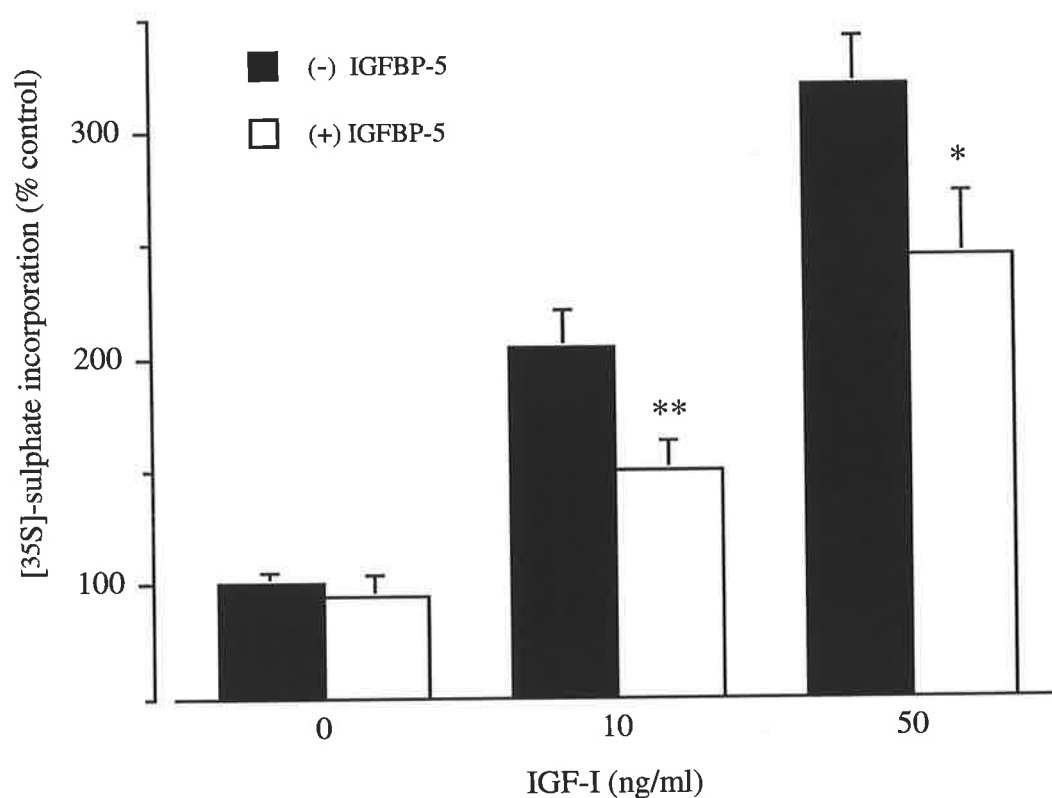


Fig. 6-5. The effect of rhIGFBP-5 on IGF-I-induced proteoglycan synthesis. Confluent chondrocytes were incubated with indicated doses of IGF-I for 48 hours in the presence or absence of rhIGFBP-5. Five hundred ng/ml of IGFBP-5 was added at the beginning of the 48-hour incubation period and additional 500 ng/ml was added 1 hour prior to the addition of [35 S]-sulphate. [35 S]-Sulphate was added in the final 24 hours and its incorporation into newly synthesised proteoglycans was determined. Results are expressed as the percentages of control values with columns representing the means and positive SEM from five separate experiments, each performed in triplicate. *, $P < 0.05$, **, $P < 0.005$, compared to corresponding cultures treated with IGF-I only (Student's t-test).

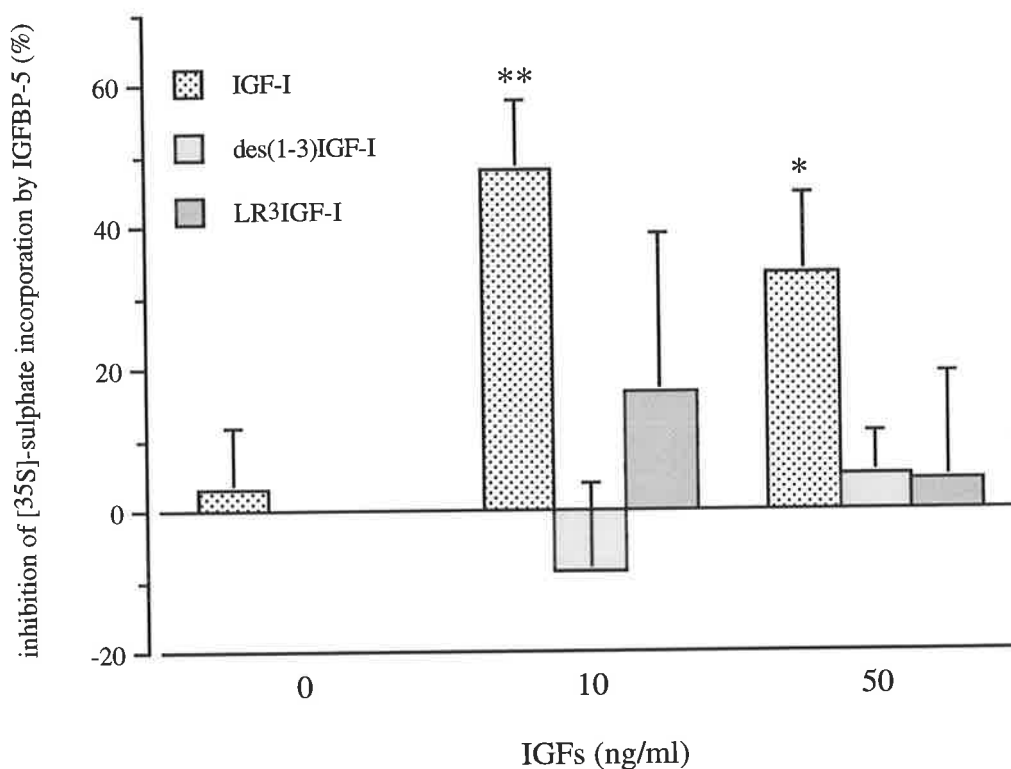


Fig. 6-6. The inhibition of IGF-stimulated proteoglycan synthesis by rhIGFBP-5. Confluent chondrocytes were incubated with 10 or 50 ng/ml of IGF-I, des(1-3)IGF-I or LR³IGF-I for 48 hours in the presence or absence of rhIGFBP-5. Five hundred ng/ml of IGFBP-5 was added at the beginning of the 48-hour incubation period and additional 500 ng/ml was added 1 hour prior to the addition of [³⁵S]-sulphate. [³⁵S]-Sulphate was added in the final 24 hours and its incorporation into newly synthesised proteoglycans was determined. Results are expressed as the percentages of inhibition of IGF-stimulated proteoglycan synthesis by rhIGFBP-5. Columns represent the means and positive SEM from a single experiment performed in triplicate. *, P<0.05, **, P<0.005, compared to corresponding cultures treated with IGFs only (Student's t-test).

6.2.4. The Effect of IL-1 on IGF-I-Stimulated Proteoglycan Synthesis in Ovine Articular Chondrocyte Culture: the Role of the Endogenous IGFBPs

The previous results of this study have revealed that locally produced and exogenously added IGFBPs, including IGFBP-5, inhibit the IGF-I-induced proteoglycan synthesis. Intriguingly, IL-1 α and TNF α have been shown to increase the level of IGFBP-5 in chondrocyte culture, in particular when co-incubated with IGF-I, pointing to a possibility that this cytokine-driven upregulation of IGFBP-5 could affect the IGF activity.

Therefore, in order to examine whether locally produced IGFBPs mediate the negative effect of the inflammatory cytokines on proteoglycan synthesis, confluent chondrocytes were incubated with IGF-I, des(1-3)IGF-I or LR³IGF-I for 48 hours in the presence or absence of IL-1 α . [³⁵S]-Sulphate was added in the final 24 hours and newly synthesised proteoglycans determined as described in Chapter 2. Neither basal nor des(1-3)IGF-I and LR³IGF-I-stimulated proteoglycan productions were affected by the cytokine, whereas IGF-I-stimulated proteoglycan synthesis was diminished by 45% (Fig. 6-7), clearly indicating that the negative effect of IL-1 α was mediated by locally produced IGFBPs, in particular IGFBP-5.

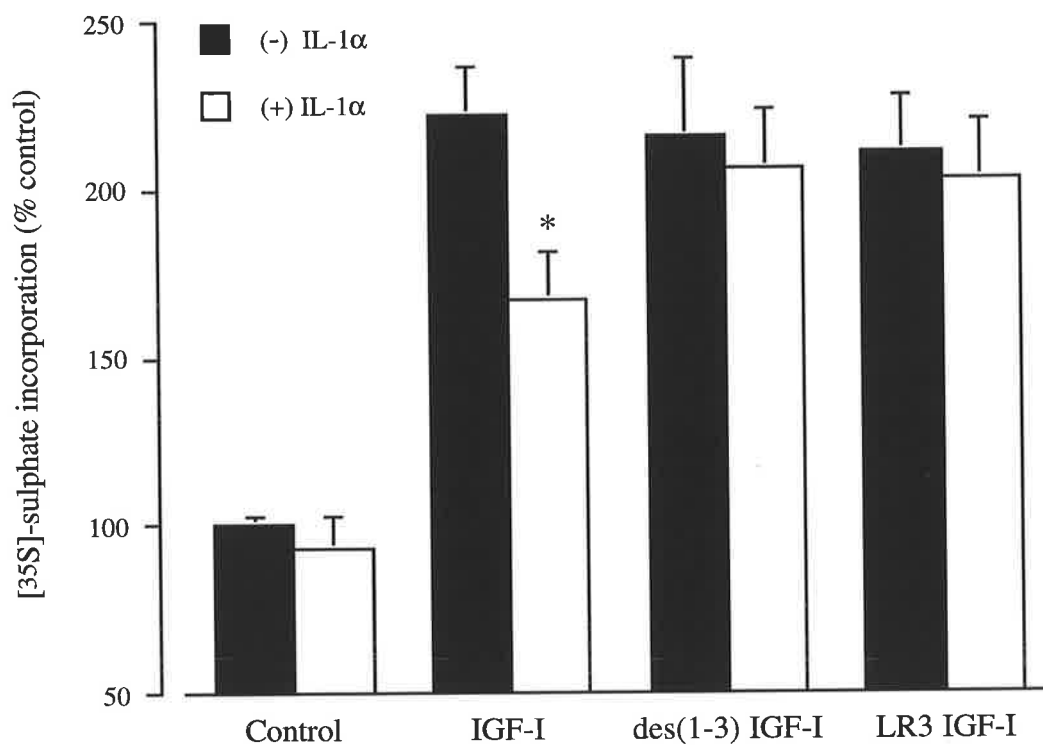


Fig. 6-7. The effect of IL-1 on the proteoglycan synthesis. Chondrocytes were incubated with 50 ng/ml IGF-I, des(1-3)IGF-I or LR³IGF-I for 48 hours in the presence or absence of 1000 u/ml IL-1 α . [³⁵S]-Sulphate was added in the final 24 hours and its incorporation into newly synthesised proteoglycans determined. Columns represent the means with positive SEM from three separate experiments, each performed in triplicate. *, P<0.05, compared with IGF-I treated cultures (Student's t-test).

6.3. Discussion

The previous results of this study pointed to the possibility that inflammatory cytokines could affect IGF bioactivity in ovine articular chondrocyte culture through the increased levels of IGFBP-5. Therefore, proteoglycan synthesis was measured and the effect of IGFBPs on the activities of IGF-I and IL-1 investigated.

IGF-I has been shown to be ten times less potent than its structural analogues with reduced affinity for IGFBPs, des(1-3)IGF-I and LR³IGF-I, in stimulating proteoglycan synthesis in ovine articular chondrocyte culture. This clearly demonstrates that locally produced IGFBPs negatively affect the activity of IGF-I, probably by sequestering the growth factor, thus reducing the concentration of "free" IGF-I available to interact with the cell receptors.

The activities of IGF-II and des(1-6)IGF-II were lower than those of IGF-I and des(1-3)IGF-I, respectively. Given the generally higher affinity of IGF-II than IGF-I for IGFBPs and the higher potency of IGF-II and des(1-6)IGF-II in increasing the level of IGFBP-5 in ovine articular chondrocyte culture, it seems likely that the lower potency of the IGF-II factors in stimulation of proteoglycan synthesis was a result of the stronger sequestering effect of the locally produced IGFBPs. The fact that insulin, which also binds to and acts through the IGF-I receptor at supraphysiological concentrations, could reach the maximum level of proteoglycan synthesis stimulation, suggests that the activity of IGFs was signalled through the IGF-I receptor. Since IGF-II has somewhat lower affinity than IGF-I for the IGF-I receptor, the involvement of the IGF-I receptor in the mediation of the IGF signals is obviously another factor which could contribute to the relatively low potencies of IGF-II and des(1-6)IGF-II. In addition, the IGF-II receptor, which binds IGF-II but do not transduce its signal (Morgan *et al.* 1987; Kiess *et al.* 1987b), and whose expression in

chondrocytes has been documented (Tardif *et al.* 1996), could be another factor that sequesters the growth factor thus further diminishing its bioactivity.

The negative effect of endogenous IGFBPs has also been demonstrated in bovine articular chondrocyte culture. Similar to ovine articular chondrocytes, des(1-3)IGF-I was 10-20 times more potent in stimulating proteoglycan synthesis than IGF-I.

By demonstrating the negative effect of the endogenous IGFBPs in chondrocyte cultures, these results complement previous reports of the inhibitory effect of exogenous IGFBPs on IGF activity in cartilage explants (Tesch *et al.* 1992; Schiltz *et al.* 1993). Moreover, the IGF-I bioactivity data obtained in this study suggest that there is a negative feedback mechanism by which chondrocytes modulate the activity of this growth factor. An increase in the concentration of IGF-I leads to elevated amounts of IGFBP-5 in chondrocyte culture. That, in turn, may lead to more IGF-I being sequestered by the binding protein, resulting in an efficient system which "buffers" the increase in the concentration of IGF-I.

Two of the three IGFBPs appearing in ovine articular chondrocyte culture were tested individually for their ability to affect the IGF-I bioactivity. Both exogenously added IGFBPs, rbIGFBP-2 and rhIGFBP-5, additionally suppressed the IGF-I activity in ovine articular chondrocyte culture. Thus, ovine chondrocytes appear to have a mechanism, that functions at two levels, by which they negatively control the activity of IGF-I. The first one is represented by IGFBP-2 which is constitutively synthesised and secreted by ovine articular chondrocytes and not affected by the presence of IGF-I, while the second level of the IGF-I activity modulation depends on IGFBP-5 whose presence is increased by IGF-I. The second level of the IGF-I activity suppression may be an additional way by which chondrocytes protect themselves from any sudden surge in IGF concentration in their immediate environment *in vivo*. A sudden increase in the concentration of IGF-I could result in the IGF-I receptor downregulation similar to that seen in bovine articular chondrocytes

(Watanabe *et al.*1985) and fibroblasts (Conover and Powell, 1991). As a result, in the long term, desensitised cells would be less responsive to the IGF signal. Thus, IGFBPs may provide more balanced conditions for chondrocytes with regard to IGF supply.

Others have demonstrated the ability of some IGFBPs to act independently of the IGF system. Thus, IGFBP-1 stimulates migration of ovary cells (Jones *et al.*1993b), IGFBP-3 inhibits growth of fibroblasts (Cohen *et al.*1993) and breast cancer cells (Oh *et al.*1993a), while the C-terminus truncated IGFBP-5 enhances mitogenesis of osteoblasts (Andress and Birnbaum, 1992). Neither of the two binding proteins tested in this study showed any activity in basal conditions, in the absence of IGF-I, indicating their inability to affect proteoglycan synthesis in the IGF-independent manner. However, the possibility that the IGFBPs may independently affect some other aspect of the cartilage biology cannot be excluded. In addition, IGF-I in complex with IGFBPs might be more protected in the protease-rich environment of articular cartilage, concentrated near the site of action or simply "stabilised" similarly to the IGF-I stabilisation by IGFBP-1 (Hober *et al.*1994).

The significance of the IGFBP-5 proteolysis in regards to the IGF bioactivity remains unclear. Some authors have proposed that the IGFBP-5 proteolysis could be a mechanism by which IGFs are released from the complex with the binding protein thus becoming more available to interact with the receptors (Andress and Birnbaum, 1992; Andress *et al.*1993; Jones *et al.*1993a). The potentiation of the IGF activity also requires the association of IGFBP-5 with cell monolayers or extracellular matrix. Since IGFBP-5 only marginally associated with ovine articular chondrocyte monolayers and extracellular matrix and because the presence of IGF-I protected the binding protein from the proteolytic degradation, it seems unlikely that IGFBP-5 degradation has this effect on IGF activity in chondrocyte culture under the given experimental conditions. Even if IGF-I was displaced from the complex with IGFBP-

5, there would be sufficient amounts of IGFBP-2 and the 24-kDa IGFBP to sequester newly released IGF-I preventing it from interacting with the receptor. However, in the long term, IGF-I-IGFBP-5 complex may serve as the IGF reservoir near the site of action, from which the growth factor is slowly released as a result of IGFBP-5 proteolysis.

IL-1, which induced IGFBP-5 expression and, in the presence of IGF-I, caused a 33-fold increase in IGFBP-5 protein level in ovine articular chondrocyte culture, diminished IGF-I-stimulated [³⁵S]-sulphate incorporation into newly synthesised proteoglycans by 45% whereas the activities of des(1-3)IGF-I and LR³IGF-I remained unaffected. This demonstrates the negative effect of the endogenous IGFBPs and strongly suggests that the suppressive effect of IL-1 α on IGF-I-induced proteoglycan production was mediated through increased levels of IGFBP-5.

Joosten *et al.* (1991) have demonstrated that murine arthritic cartilage is non-responsive to IGF-I, resulting in decreased proteoglycan synthesis, in spite of the presence of functionally unaltered IGF receptors on the chondrocytes. The studies of Dore *et al.* (1994) and Tardif *et al.* (1996) that followed, reported increased IGFBP production by human OA chondrocytes accompanied by their non-responsiveness to IGF-I stimulation. These findings, corroborated with recent reports of elevated IGFBP levels in synovial fluids of OA and RA patients (Matsumoto *et al.* 1996a; Fernihough *et al.* 1996; Kanety *et al.* 1996), point to a possible involvement of IGFBPs in the pathophysiological processes in articular cartilage. The results obtained in this study have revealed the capability of IGFBPs, in particular IGFBP-2 and -5, to suppress the activity of IGF-I in chondrocyte culture, by modulating its availability locally, at the site of action. Moreover, it has been demonstrated that IL-1 suppresses the activity of IGF-I but not the activities of IGFs that do not bind to IGFBPs, strongly suggesting the involvement of IGFBPs in mediating the effect of the inflammatory cytokine.

In conclusion, the IGFBP-mediated decrease in proteoglycan synthesis could be a relevant *in vivo* mechanism by which IL-1 α exerts its catabolic effect and disturbs the balance between the synthesis and degradation of cartilage matrix macromolecules in pathological conditions.

Chapter 7

General Discussion

An *in vitro* model system, utilising primary cultures of chondrocytes of a non-human origin, was used throughout the study. Given the analogy of cartilage structure and function that exists across the mammalian species and a high level of conservation among the factors used, it can be assumed that the results obtained in such way can be used to interpret events that take place in humans and are a sound basis for any subsequent investigation *in vivo*.

The direct interference of IL-1 with the IGF-I-driven proteoglycan synthesis in chondrocyte cell culture, as demonstrated in this study, is a unique example of the interplay of anabolic and catabolic factors and is yet another illustration of the complexity of mechanisms that regulate cartilage matrix metabolism. Furthermore, the involvement of IGFBPs in this interplay shows how important these factors are in the maintenance of cartilage matrix *in vivo*, particularly in pathological states.

The function of articular cartilage, which is best reflected in its ability to sustain compressive and shear forces, can be impaired if the strict structural requirements are not met. A balance between the synthesis and degradation of cartilage-specific proteoglycans is one of the crucial determinants of the structural integrity of this highly specialised connective tissue. Although the ability to increase proteoglycan synthesis in cartilage is a well known feature of IGF-I, little is still known about the role of the local modulators of the IGF activity, IGFBPs, in the regulation of cartilage matrix metabolism.

The negative effect of chondrocyte IGFBPs on the IGF-I-stimulated proteoglycan synthesis, demonstrated in this study, implies that it could be the mechanism that protects articular cartilage from overexposure to the growth factor. The basal level of protection is provided by IGFBPs whose presence is not IGF-dependent. Thus, IGFBP-2 is there to sequester the growth factor and diminish its availability to the receptors. In addition, any sudden increase in the concentration of IGF-I near the site

of action is swiftly buffered with IGFBP-5. This binding protein, which appears to be constitutively produced and immediately proteolysed in the absence of IGF-I, binds to the growth factor and becomes more resistant to proteolysis. Consequently, the rise in the concentration of IGF-I is accompanied by the increase in intact IGFBP-5 concentration, resulting in keeping the concentration of "free" IGF-I at the low level.

By the same token, the sequestration of IGF by IGFBPs could be a mechanism that provides more balanced exposure of chondrocytes to IGFs in the long term. The association of IGF-I with IGFBPs could result in a stabilisation of the IGF-I molecule, similar to that seen when IGFBP-1 is incubated with IGF-I (Hober *et al.* 1994), and/or attachment and localisation of the growth factor to cartilage extracellular matrix or cell surface via IGFBP-5 (Jones *et al.* 1993a; Parker *et al.* 1996). Both mechanisms would lead to a prolonged half-life of the growth factor in cartilage environment. In addition, a gradual release of IGF-I from the IGF-IGFBP complexes through the diminished but not completely inhibited proteolysis of IGFBP-5, which would generate IGFBP-5 fragments with reduced affinity for IGF-I, could allow the growth factor to exert its activity over a prolonged period of time. That would provide a balanced signal for stimulation of proteoglycan synthesis without requiring excessive amounts of the stimulating factor. This hypothetical mechanism could explain how a relatively low level of total IGF-I (2-10 ng/g tissue) and "free" IGF-I (below 1 ng/g tissue) detected in articular cartilage (Schneiderman *et al.* 1995) would still be sufficient to affect proteoglycan synthesis. Furthermore, the proteoglycan synthesis data I obtained using IGF-I and its structural analogues with reduced affinity for IGFBPs, des(1-3)IGF-I and LR³IGF-I, show that 10 ng/ml of IGF-I is equipotent with 1 ng/ml of the IGFBP-non binding IGFs. In respect to the IGF activity, the IGF-I analogues are equivalent to and could be viewed as "free", IGFBP-non bound, IGF-I. Even at concentrations as low as 0.1 ng/ml, the IGF-I analogues were very effective in increasing the proteoglycan synthesis reaching 25%

of the maximum level of stimulation. These findings indicate that the level of total and "free" IGF-I found in articular cartilage is adequate to efficiently stimulate proteoglycan synthesis and challenges the view of Schneiderman *et al.* (1995) that IGF-I is not present in sufficient amount to be able to affect proteoglycan metabolism.

There is no detectable expression of IGF-I mRNA in normal cartilage of various developmental stages (Shinar *et al.* 1993; Middleton *et al.* 1996; Wang *et al.* 1995b) and no evidence of IGF-I production in cartilage explants *in vitro* (Luyten *et al.* 1988; Schneiderman *et al.* 1995). The lack of IGF-I production by articular chondrocytes has indirectly been confirmed in this study by demonstrating the ability of IGFBPs to inhibit proteoglycan synthesis induced by exogenously added IGF-I but failing to affect it in the absence of IGF-I. Therefore, IGF-I found in articular cartilage is most likely to be of the endocrine origin, synthesised in liver and transported by the circulation and synovial fluid to the site of action. On the other hand, IGF-II appears to be produced locally, at least in early developmental stages (Shinar *et al.* 1993; Wang *et al.* 1995b), and may also play an autocrine role in mature cartilage. As demonstrated in this study, chondrocyte IGFBPs have the capacity to deal with changes in the levels of both IGFs, in particular IGF-II. Thus, IGF-II is more potent than IGF-I in increasing the level of IGFBP-5. In turn, the higher level of IGFBP-5, together with other factors involved, probably contributes to the lower bioactivity of IGF-II as compared to IGF-I.

The identity of the chondrocyte IGFBP-5 protease remains unknown since the initial attempts to purify the enzymatic activity were unsuccessful and detection of the N-terminal sequences of the IGFBP-5 degradation fragments was not feasible because of limited amounts of the rhIGFBP. A more thorough investigation of these issues need to be carried out in the future. The protease inhibitor profile indicates that it could be one of the serine proteases found in cartilage (Mankin and Brandt, 1989). Although of the three IGFBPs present in chondrocyte culture, it cleaves only IGFBP-

5, it is not likely to be specifically designed to degrade this binding protein. Many IGFBP proteolytic activities discovered so far have been found to be identical to previously described proteases that cleave other substrates. Thus, IGFBP-5 can be cleaved by matrix metalloprotease 1 and 2 (Thraillkill *et al.*1995), as well as by serine protease PSA and NGF γ -subunit (Rajah *et al.*1996). The physiological role of the IGFBP-5 proteolytic activity remains unclear. It could serve to eliminate excessive amounts of intact IGFBP-5 from cartilage in the absence of IGFs but also, as mentioned earlier, to slowly release IGF from the IGF-IGFBP complex. The ability of the wild type IGFs to partially inhibit the activity of the protease and thus increase the level of IGFBP-5 indicates that there is a very efficient negative feed-back mechanism which affects the IGF activity in cartilage and takes place at the post-translational rather than transcriptional level.

A possibility that chondrocyte IGFBPs exert IGF-independent actions in cartilage cannot be excluded. Thus, IGFBP-2 which, like IGFBP-1, possesses the RGD sequence could interact with integrin receptors and be involved in cell adhesion and migration, similar to IGFBP-1 which regulates migration of ovary cells (Jones *et al.*1993b). Likewise, IGFBP-5 has been shown to affect the activity of IGF-I in osteoblasts and at the same time exerts its activity independently of the growth factor (Andress and Birnbaum, 1992). However, any independent action of IGFBPs in cartilage remains open to speculation and, at this stage, there is no evidence to support that concept.

The results obtained with IL-1 and TNF α in this study indicate that they affect the IGF system in cartilage by increasing the level of IGFBP-5 and thus disturb a delicate balance which exists between IGFs and IGFBPs. The relevance of this finding is emphasised by the fact that these inflammatory cytokines, whose levels are elevated in joints affected by RA and advanced OA, are regarded as the principal catabolic factors that contribute to cartilage destruction (Holt *et al.*1992). The stimulation of IGFBP-5

mRNA and protein level by IL-1, as seen in this study, could account for the increased expression of this binding protein by human OA chondrocytes (Olney *et al.*1996). Moreover, the cytokine-upregulated IGFBP-5 could contribute to the IGFBP increase detected in synovial fluid of OA and RA joints (Matsumoto *et al.*1996a; Fernihough *et al.*1996; Kanety *et al.*1996). The stimulation of IGFBP-5 by inflammatory cytokines could have major repercussions on the IGF activity in diseased cartilage. As demonstrated in this study, the suppressive effect of IL-1 on the IGF-I-stimulated proteoglycan synthesis is mediated *in vitro* by increased levels of chondrocyte-derived IGFBP-5. It could be a relevant *in vivo* mechanism by which IL-1 inhibits proteoglycan synthesis in pathological conditions of cartilage. Although the rates of syntheses of cartilage-specific macromolecules, including proteoglycans, are increased in the initial phase of OA, the advanced stage is characterised by diminished synthesis and increased breakdown of proteoglycans (Mankin *et al.*1981; Ryu *et al.*1984). It is in the later stages of degenerative arthritis that the presence of inflammatory cytokines becomes more substantial and their negative effects on metabolism of proteoglycans more prominent. Thus, the IGF-mediated attempts to repair cartilage damage by offsetting increased proteoglycan degradation with increased synthesis may be hindered by IL-1 which interferes with IGF-stimulated proteoglycan synthesis through increased levels of IGFBP-5. This mechanism of IGFBP-mediated suppression of proteoglycan synthesis would contribute to proteoglycan displacement from cartilage matrix and subsequent cartilage erosion and loss of function. A similar mechanism of proteoglycan synthesis suppression is likely to exist in RA as well, especially given the role of IL-1 and TNF α in the aetiology of this inflammatory disease.

The ability of des(1-3)IGF-I and, in particular, LR³IGF-I to avoid the inhibitory effect of chondrocyte-derived IGFbps and fully exert their activities in the presence of IL-1, indicates that these IGF-I analogues have a potential to be used as therapeutic

agents in the treatment of diseased cartilage in order to restore the rate of proteoglycan synthesis. However, in spite of exceptional folding properties of LR³IGF-I for example (Milner *et al.*1995), a delivery of the analogues to the site of action could be quite a challenge because of the presence of various proteolytic activities in the arthritic joint. In addition, the stimulation of proteoglycan synthesis *per se* would not solve the problem of undersized and non-aggregating proteoglycans caused by the cytokine-mediated increase in the activities of matrix metalloproteases. A recent advance in the treatment of OA by using the IL-1 receptor antagonist in an animal model system (Caron *et al.*1996) and the studies showing clinical improvements in RA patients treated with an antibody against TNF α (Elliott *et al.*1993) and soluble TNF receptor fusion proteins (Moreland *et al.*1996; Baumgartner *et al.*1996) demonstrate that the catabolic and inflammatory effects of the cytokines can be blocked to a certain extent. Therefore, a use of the IGFs in a combination with the agents that block the activity of the cytokines would be a more comprehensive approach in restoring the metabolism of functionally correct proteoglycans in diseased cartilage. Furthermore, a novel gene therapy approach in the treatment of OA and RA utilising the intraarticular transfer of genetically modified synoviocytes containing the IL-1 receptor antagonist gene (Makarov *et al.*1996; Otani *et al.*1996; Pelletier *et al.*1997), demonstrates the feasibility and therapeutic value of targeted delivery of specific "anti-arthritic" genes to the articular joint. Thus, particularly in the light of the recent promising studies of the *ex vivo* gene transfer to chondrocytes (Kang *et al.*1997), a similar strategy may also be used to deliver the genes of chondroprotective agents such as IGFs to sites of cartilage loss.

The other approach could involve the use of IGFBP neutralising antibodies in order to diminish the effect of IGFBPs. Antibodies directed against IGFBPs, including IGFBP-5, are becoming more readily available and it is likely that in the near future genetically engineered human monoclonal antibodies that block IGF-binding site(s) on

IGFBP molecules will be constructed. The neutralisation of the negative effect of IGFBPs on the IGF action would allow IGFs to fully exert its activity in cartilage. However, it is hard to predict whether the other aspects of IGF biology in cartilage, such as the half-life and diffusion rate, would also be affected by those antibodies. Also, regardless of the increased permeability of arthritic cartilage it is not clear whether the size of the antibodies would allow them to efficiently penetrate and diffuse through cartilage. Anyhow, in spite of all the obstacles, the potential use of IGFBP neutralising antibodies remains a fruitful avenue to pursue to improve the current therapies available for the treatment of diseases of cartilage.

In conclusion, the suppression of the IGF-I-stimulated proteoglycan synthesis by IL-1 is mediated by increased levels of IGFBP-5. Therefore, chondrocyte-derived IGFBP-5 could play a critical role in the regulation of cartilage matrix degradation in inflammatory and degenerative arthritides.

Appendix

Buffers, Solutions and Gels

Buffers and solutions for Western analysis

Acrylamide stock solution

40.0% (w/v) acrylamide

1.5% (w/v) bisacrylamide

Stacking gel buffer

0.48 M Tris HCl

0.03 M Tris

pH 6.8

Separating gel buffer

1.27 M Tris

0.24 M Tris HCl

pH 8.8

Electrophoresis buffer

0.025 M Tris

0.192 M glycine

0.1% (w/v) SDS

pH 8.3 - 8.4

Western saline

0.01 M Tris
0.15 M NaCl
pH 7.4

Gel recipes:

Stacking gel

Stacking gel buffer	4.0 ml
Acrylamide	1.2 ml
10% (w/v) SDS	0.16 ml
H ₂ O	10.4 ml
10% (w/v) APS	160 μ l
TEMED	16 μ l

Separating gel

Separating gel buffer	9.4 ml
Acrylamide	17.8 ml
10% (w/v) SDS	0.375 ml
H ₂ O	15.8 ml
10% (w/v) APS	187 μ l
TEMED	9.7 μ l

Electrophoretic transfer buffers:

Anode solution 1

0.3 M Tris
20% (v/v) methanol
pH 10.4

Anode solution 2

0.025 M Tris
20% (v/v) methanol
pH 10.4

Cathode solution

4 mM 6-amino-n-hexanoic acid
20% (v/v) methanol

Buffers and solutions for Northern analysisTBE buffer

50 mM Tris
42 mM boric acid
1 mM EDTA
pH 8.4 - 8.5

Agarose-formaldehyde gel-running buffer (5x)

0.1 M MOPS (pH 7.0)
40 mM sodium acetate
8 mM EDTA (pH 8.0)

Agarose-formaldehyde gel-loading buffer

50% glycerol
1 mM EDTA (pH 8.0)
0.25% bromophenol blue
0.25% xylene cyanol FF

Transblot buffer (10x)

100 mM Tris
50 mM sodium acetate
5 mM EDTA

Gel recipes:

Agarose gel

1% agarose in TBE buffer

Agarose-formaldehyde gel

1% agarose
1/5 vol 5x running buffer
2.2 M formaldehyde

Buffers for size-exclusion chromatography

FPLC running buffer

1 M acetic acid

0.15 M NaCl

RIA buffer

30 mM NaH₂PO₄

0.2% protamine sulphate

10 mM EDTA

0.2% NaN₃

0.05% Tween-20

pH 7.5

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ERRATA

- Page 5, line 6 Insert after “(Mankin and Brandt, 1989).”: “Type XI collagen has been shown to form covalent linkages between type II collagen fibres thus stabilising the type II collagen network in articular cartilage and defining the size of the pore within the collagen network (Diab *et al.* 1996).”
- Page 5, line 9 “(Gay *et al.* 1981; Burgeson *et al.* 1982).” should read “(Gay *et al.* 1981; Burgeson *et al.* 1982; Petit *et al.* 1993).”
- Page 34, line 5 “Sertoli cell culture” should read “Sertoli cell cultures”
- Page 53, line 5 “by Milli-Q” should read “by a Milli-Q”
- Page 53, line 20 “replaced with the medium” should read “replaced with medium”
- Page 54, line 17 ”washed in 1% Triton X-100 (v/v)” should read ”washed in 1% Triton X-100 (v/v) made up in Western saline”
- Page 60, line 10 “¹²⁵I-IGFBP-5 (10000-30000 cpm)” should read “¹²⁵I-IGFBP-5 (1 µl, 10000-30000 cpm)”
- Page 60, line 12 “(DMEM with 20 mM Hepes, 0.01% BSA)” should read “(DMEM with 20 mM Hepes, 0.01% BSA, pH 7.4)”
- Page 60, line 24 “Approximately 250,000 cpm of ¹²⁵I-IGFBP-5” should read “Approximately 5 µl of ¹²⁵I-IGFBP-5 (250,000 cpm)”
- Page 61, line 15 “Proteoglycans were precipitated” should read “Glycosaminoglycans were precipitated”
- Page 104, line 2 “spontaneous proteolysis” should read “spontaneous degradation”
- Page 147 The pH of the transblot buffer is 7.8.

New references for the Bibliography section:

Diab, M., Wu, J. J., and Eyre, D. R.(1996). Collagen type IX from human cartilage: a structural profile of intermolecular cross-linking sites. *Biochem. J.* **314**, 327-332.

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