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Author	Holledge, Masumi Maruo
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Integrin-Dependent Regulation of Proteoglycan Synthesis in Cultured Human Articular Chondrocytes Following Mechanical Stimulation

Masumi MARUO HOLLEDGE

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Declaration

I hereby declare that this thesis has been composed by myself and has neither been presented nor accepted in any previous application for a degree. All work presented was unless acknowledged, carried out by myself. All sources of information have been acknowledged by reference.

Masumi MARUO HOLLEDGE
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Publications associated with this thesis

Abstracts

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2. Integrin Dependent Up-Regulation of Proteoglycan Synthesis, in Cultured Normal Human Articular Chondrocytes Following Mechanical Stimulation, is Lost in Chondrocytes from Patients with Osteoarthritis and Rheumatoid arthritis (The British Society for Rheumatology Annual Meeting, 20-23 April 2004, Edinburgh, UK, and British Health Professionals in Rheumatology Spring Meeting, 21-23 April 2004, London, UK)

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Posters

1. Integrin Dependent Up-Regulation of Proteoglycan Synthesis, Following Mechanical Stimulation in Cultured Normal Human Articular Chondrocytes, is Lost in Patients with Osteoarthritis (Scottish mechano-transduction posters session, January 2003, Glasgow, UK)

2. Integrin Dependent Up-Regulation of Proteoglycan Synthesis, in Cultured Normal Human Articular Chondrocytes Following Mechanical Stimulation, is Lost in Chondrocytes from Patients with Osteoarthritis and Rheumatoid arthritis (Scottish mechano-transduction posters session, January 2005, Edinburgh, UK)

Abbreviations

AKA	above knee amputation
ADAM TS	a disintegrin and metalloproteinase with thrombospondin motifs
APS	ammonium persulphate
BSA	bovine serum albumin
C6S	chondroitin-6-sulphate
C4S	chondroitin-4-sulphate
C0S	chondroitin-0-sulphate
cAMP	cyclic adenosine monophosphate
CHAPS	3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate
CS	chondroitin sulfate
DMMB	dimethylmethylene blue
DNA	deoxyribo nucleic acid
DTT	dithyotheritol
ECM	extra-cellular matrix
EDTA	ethylene -diamine-tetra-acetic acid
FAK	focal adhesion kinase
FCS	foetal/fetal calf serum
GAG	glycosaminoglycan
GuHCl	guanidium hydrochloride
HABR	hyaluronic acid binding region
HCl	hydrochloride
Hg	mercury
Hz	hertz
IAP	integrin associated protein
IGF-1	insulin like growth factor 1
IgG	immunoglobulin G
IL	interleukin
IMDM	Isacove's modified Dulbecco's medium
kDa	kilo dalton
KS	keratan sulphate
MMP	matrix metalloproteinase
MPa	mega Pascal
mRNA	messenger ribonucleic acid
MS	mechanical stimulation
NaCl	sodium chloride
OA	osteoarthritic/osteoarthritis
PBS	phosphate buffered saline
PG	proteoglycan
PIP_2	phosphatidy linositol-4,5-bisphosphate
PKC	protein kinase C

PLC	phospho lipase C
PMSF	phenyl methyl sulfonyl fluoride
RA	rheumatoid arthritis
RGD	arginine-alanin-asparagines oligopeptides
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	N'N'N'N'-tetramethylethylenediamine
TGF	transforming growth factor
TKR	total knee replacement
TNF	tumour necrosis factor
Tris	Trizma® Base
Tween 20	polyoxyethelene 20 sorbitan monolaurate

Abstract

The loading of cartilage and chondrocyte cultures *in vitro* has demonstrated the importance of mechanical forces in regulating chondrocyte metabolism. The aim of my thesis was to investigate the effects of cyclical mechanical stimulation on proteoglycan (PG) metabolism in chondrocytes derived from patients with OA and RA, in comparison with that of chondrocytes from normal cartilage (Collins and McElligott Grade 0).

The chondrocytes were exposed to cyclical mechanical stimulation in an apparatus that functioned to produce a strain on the base of the culture dishes with attached cells in monolayer. Following 20 minute 4000μ strain cyclical mechanical stimulation at 0.33 Hz, the GAG synthesis of chondrocytes from normal cartilage (measured by the DMMB assay) increased significantly ($p=0.045$) compared to unstimulated controls. Increased GAG synthesis following mechanical stimulation was blocked in the presence of antibodies to α5 integrin, or αVβ5 integrin, or CD47. These experiments provided evidence that α5 integrin, αVβ5 integrin, and CD47 are involved in the signal transduction process that leads to accelerated PG synthesis, following cyclical deformation of chondrocytes from normal cartilage.

Cyclical mechanical stimulation applied to articular chondrocytes from patients with OA and RA in monolayer culture did not show any significant change in GAG synthesis, measured by the DMMB assay. Chondrocytes from OA grade III synthesized on average 65.0 per cent and 59.6 per cent less GAG (corrected by DNA) than OA grade I and II chondrocytes, which were statistically significant ($p=0.0038$ and $p=0.071$ respectively).

PGs extracted from medium by acetone precipitation were analysed by SDS-PAGE and Western blotting using anti-aggrecan G1 domain antibody and CS epitope monoclonal antibodies (3-B-3, 2-B-6 and 1-B-5) following deglycosylation. Electrophoretic patterns of PGs released from normal chondrocytes following mechanical stimulation showed that newly-synthesized aggrecan was not fragmented or degraded. The concentration of C4S disaccharide sulphation isomers with a molecular weight greater than 250 KDa was enhanced in normal chondrocytes compared to unstimulated controls. Epitopes recognised by 1-B-5 were expressed at low levels in both chondrocytes from normal and OA cartilage. There was no increase in 3-B-3 expression after 24 hours of chondrocyte stimulation. The intensity of the band with molecular weight 50 KDa small C4S-containing epitopes increased following an eight-times greater strain (32000μ) on chondrocytes from OA cartilage. OA grade III chondrocytes showed a lack of the synthesis of large molecular weight aggrecan core protein with a molecular weight of around 350 kDa. The apparent intensity of the large molecular weight 350 KDa band increased following the mechanical stimulation of OA grade I and II chondrocytes in the presence of anti-α5 integrin antibody.

Techniques for the preparation and maintenance of primary cultures of human articular chondrocytes in three-dimensional alginate culture were developed. Cyclical mechanical stimulation through compression of OA chondrocytes in three-dimensional alginate culture

(disk formation) resulted in a decrease in GAG synthesis. This decrease in GAG synthesis in the alginate-chondrocyte construct culture was seen after cyclical pressurization of 30 per cent at 0.33 Hz and 0.1 Hz. This decrease was blocked by the presence of either anti-β1 or anti-αVβ5 integrin antibodies. These experiments provided evidence for the first time that β1 and αVβ5 integrins are involved in the signal transduction process that leads to decreased PG synthesis following cyclical deformation of chondrocytes from OA cartilage.

Chapter One

Introduction

1.1 Articular cartilage

The prime function of cartilage is physical. Articular cartilage covers the heads of the long bones in synovial diarthroidial joints, providing an essentially smooth gliding articulating surface that is resistant to the considerable shear and compressive forces produced by normal joint movement (Muir 1995). Adult articular cartilage is avascular, aneural, and alymphatic. Cell nutrition is derived primarily from the synovial fluid (Barnett et al 1961).

1.1.1 Structure of articular cartilage

Articular cartilage consists of a matrix with sparsely distributed chondrocytes existing in isolation or in small clusters (chondrons). Articular cartilage matrix consists principally of 70 to 80 per cent water (Maroudas 1980), 10 to 30 per cent collagen (Kuettner et al 1991), and 3 to 10 per cent PGs (Poole 1997). The chondrocytes only occupy 1 to 10 per cent of the total cartilage volume (Meachim and Stockwell 1979, Hunziker et al 2002). The predominant hyaline cartilage component comprises of the following: (1) collagens (type II, VI, IX, XI, XII), (2) proteoglycans (aggrecan, biglycan, decorin, fibromodulin, lumican), (3) non-collagenous proteins (cartilage oligomeric matrix protein, tenascin, cartilage matrix protein, anchorin II, thrombospondin, chondrocalcin, fibronectin), (4) membrane proteins (syndecan, CD44, integrins) (Goldring 2000).

Cartilage varies in thickness, but in large human synovial joints such as knees, normal adult cartilage is 2 to 4 mm thick (Meachim and Stockwell 1979), on average 2.4 mm (Hunziker 2002). Cartilage thickness varies not only from joint to joint, but also from area to area within joints (Simon 1970, Kiviranta et al 1987a). Loading patterns contribute to these variations. Weight bearing and moderate exercise has been shown to increase articular cartilage thickness in animal studies (Kiviranta et al 1987b, 1988), whereas immobilisation or excessive exercise can decrease it (Kiviranta et al 1987b, Arokoski et al 1993, Palmoski et al 1979), demonstrating the importance of load to cartilage integrity. Investigations in humans have been impossible until very recently, since articular cartilage could not be quantified under *in vivo* conditions. However, the development of fat-suppressed gradient echo-magnetic resonance sequences, and three-dimensional digital post-processing techniques demonstrate that the thickness of the cartilage does not adapt to mechanical stimulation in humans (Eckstein et al 2002).

Articular cartilage is normally divided into four zones (superficial, intermediate or transitional, deep or radial, and calcified zones) parallel to the surface (Collins and McElligott 1960, Hunziker et al 2002). In human articular cartilage the superficial zone and the transitional zone each occupy about 10 per cent of the total cartilage thickness and the radial zone occupy about 80 per cent (Hunziker et al 2002). The matrix can be further subdivided into two regions: the pericellular (the territorial) and inter-territorial (Meachim and Stockwell 1979, Hunziker et al 2002). The pericellular matrix surrounds the chondrocyte plasma membrane. The unit of specialised pericellular matrix surrounding the chondrocyte or chondrocytes is termed the chondron. A chondron contains one or more cells, which can be isolated from the cartilage (Poole 1997). Chondrons appear to be resistant to mechanical, osmotic, and physicochemical changes induced by dynamic loading (Muir 1995). The pericellular matrix is very thin with a rim of approximately 5 to 12 μm in width, characterised by the absence of cross-banded fibrillar collagen, but it is very rich in PGs (Poole et al 1997, Mok et al 1994). A 'cargo-like' capsule with micro-pores connects the pericellular matrix to the inter-territorial matrix (Allen and Mao 2004). The inter-territorial and outermost matrix constitutes the largest domain. It is characterised by cross-banded collagen fibrils or fibres running in parallel and inter-spaced with PGs (Mok et al 1994). It has been shown by both *in vivo* and *in vitro* experiments that these two regions of matrix surrounding chondrocytes have different rate of turnover (Mok et al 1994).

1.1.2 Composition of articular cartilage

1.1.2.1 The chondrocytes

Chondrocytes are differentiated from mesenchymal cells during embryonic development and represent the sole cell type found in hyaline cartilage. Chondrocytes are discoidal or ovoid in shape with scalloped edges. Individual chondrocytes are located in their lacunae within the matrix, and each lacunae contains a single chondrocyte (Sandell and Aigner 2001). They contain extensive endoplasmic reticulum, mitochondria, lysosomes, and Golgi apparatus (Brighton et al 1984). These characteristics reflect the active role of chondrocytes in synthesis and maintenance of the cartilage matrix. The chondrocytes' primary function is to maintain the surrounding matrix which is turning over continuously. The cells detect changes in the composition of the surrounding matrix, by unknown mechanisms, in order to maintain the relative concentration of its components (Hauselmann et al 1994). Some chondrocytes have cilia that extend from cell into extra-cellular matrix and sense the mechanical environment of the cell (Wislman and Fletcher 1978). The chondrocytes in mature articular cartilage exhibit virtually no mitotic activity and a very low rate of matrix synthesis (Hedbom and Hauselmann 2002). There is no detectable cell division in healthy adult articular cartilage. However, cells appear to be divided anew in osteoarthritis lesions (Muir 1995).

Because chondrocytes are embedded in high concentrations of PGs, the extra-cellular ionic environment of the chondrocytes is very different from that of most other cells. Chondrocytes are surrounded by an extra-cellular fluid whose cation concentration is considerably higher than that in synovial fluid or standard tissue culture media, and whose anion concentration is lower. In addition, the osmolality of the interstitional fluid is high in order to maintain cartilage hydration under a high external load (Table 1.1)(Urban and Hall 1994).

	interstitional fluid		
	human hip surface zone	human hip deep zone	synovial fluid, Dulbecco's modified Eagle's medium (DMEM)
fixed charge density (meq/l H_2O)	100-180	150-240	0
$[Na^+]$ mM	200-240	220-300	130-150
$[K^+]$ mM	6-8	8-12	5
$[Ca^{2+}]$ mM	4-8	6-20	2
$[Cl^-]$ mM	80-100	60-90	150
pH	7-7.2	7.2-6.9	7.4
osmolality (mOsm)	350-400	350-470	280-300

Table 1.1 The composition of extra-cellular environment of articular chondrocytes (interstitional fluid)compared to the composition of synovial fluid and tissue culture media (Urban and Hall 1994)

The zonal difference in cartilage matrix structure is determined by embedded chondrocytes which produce the matrix. Studies of chondrocytes from these zones have demonstrated differences in cell density, cell proliferation, cell size, PG synthesis, collagen synthesis, cytoskeleton, and response to the pro-inflammatory cytokines (review by Aydelotte et al 1992, pig cartilage by Siczkowski and Watt 1990, bovine cartilage by Langelier et al 2000). In human articular cartilage, chondrocyte density is two to four fold higher in the superficial zone than in the deep zone (Hunziker et al 2002). Adult human humeral head cartilage contains about 25.000 cells per mm^3 in the superficial layer, and about 7000 cells per mm^3 in the deeper layers (Hunziker et al 2002). Another report says that adult human femoral head cartilage contains 10.000 cells per mm^3 (Venn and Maroudas 1977). The cellularity can vary as much as 10 fold or more according to species, age, degenerative change, zonal variation in the human articular cartilage within the joint, or variations dependent on the particular joint from which the cartilage has been obtained (Lipman 1989). Cells from the superficial layer have lower rates of synthesis of PGs and collagen when compared to cells in deeper layers in human and calf articular cartilage (Collins and McElligott 1960, Korver et al 1990). Turnover of PGs is

significantly higher in chondrocytes of the superficial layer. This may be due to the lower aggregation of PGs synthesized by these cells (Aydelotte et al 1988). The superficial layer has a higher water content and reduced GAG concentration (Meachim and Stockwell 1979). These metabolic differences are maintained by isolated chondrocytes from the various zones of articular cartilage in a variety of culture systems, demonstrating that they are an intrinsic property of the chondrocytes (Aydelotte et al 1988, Archer et al 1990). There are also structural differences between the PG produced by the different chondrocyte populations (Aydelotte et al 1992, Maroudas et al 1980). These zonal variations may be related to the difference in cell shapes of chondrocytes within the zones, or may result from differences in the chemical environment of each zone, or the changing physical environment, which is a function of depth from the surface.

Measurement of chondrocyte metabolism *in vitro* indicates much more rapid synthesis of matrix products than those seen *in vivo*, partially due to the increased availability of nutrients and oxygen *in vitro* (Benya 1990). Chondrocytes experience very low oxygen tension, about 1 kPa compared to 20 kPa in vitro (Holm et al 1981), as low as 1 per cent in deep layers compared with 24 per cent in normal atmosphere (Muir 1995) and metabolism is primarily by anaerobic glycolysis (Holm et al 1981, Brighton et al 1974). However, Brighton et al (1974) showed that *in vitro* articular cartilage, GAG metabolism has a broad tolerance to oxygen tension, only in 1 per cent and 90 per cent of oxygen depressed GAG synthesis. Nutrient and growth factor diffusion are limited *in vivo*, because of the small pore size in tissue (Holm et al 1981), whereas in cartilage explant culture, diffusion distances are reduced and the cartilage tissue swells considerably, due to disruption of the collagen meshwork. This results in a greater ease of diffusion and exposure of chondrocytes to higher nutrient and growth factor levels (Bayliss et al 1986). In isolated chondrocyte cell cultures, the cells are bathed in nutrient medium and have negligible surrounding matrix. As a consequence both rate and regulation of metabolism are considerably altered compared to chondrocytes *in vivo* (Benya 1990).

1.1.2.2 Extra-cellular matrix

Cartilage homeostasis is regulated by the interaction of chondrocytes with extra-cellular matrix. It is characteristic of extra-cellular matrix to have multiple compositions, including isoforms of the collagen (I-XXI), large and small PGs, structural glycoproteins (non-collagenous proteins), membrane proteins, and elastin (Haralson and Hassell 1995). Each element is involved in both of the supporting structure of matrix and the modulation of the biology of cells, in response to growth factors, cytokines, vitamins and hormones (Haralson and Hassell 1995). The chondrocytes are responsible for maintaining the biological composition of the extra-cellular matrix and in some manner respond to mechanical and biological signs to maintain homeostasis. This concept, in which cells produce an extra-cellular matrix and also are influenced by it, has been termed dynamic reciprocity (Scully 2001). The

extra-cellular matrix may be the regulatory apparatus of the cells and regulate extra-cellular matrix by itself.

1.1.2.3 Proteoglycan

Proteoglycan (PG) can be defined as 'any macromolecule that has a core protein containing at least one covalently bound glycosaminoglycan (GAG) chain' (Hascall and Kimura 1982). PGs function in a range of biological activities that includes: (1) assembly of ECM components, (2) modulation of cell growth, cell homeostasis, wound healing through interactions with various growth factors and other ligands, (3) regulation of cell adhesion, migration, and proliferation (Wight et al 1992). Some of the diverse actions of PGs are mediated by the core protein and others are mediated by the GAG chains. Their structural diversity and tissue distribution suggest a functional versatility (Kjellen and Lindahl 1991).

1.1.2.4 Glycosaminoglycans

A glycosaminoglycan (GAG) is a heteropolymer consisting of repeating disaccharide subunits containing hexosamine and hexuronic acid or hexose (Carney and Muir 1988). These are linear molecules which are highly charged due to the presence of carboxyl groups ($-COO^-$) and sulphate ester groups ($O-SO_3^-$) (Carney and Muir 1988). GAGs form a very heterogeneous population within cartilage, due to differences in their type and size, both of which are influenced by factors such as age, disease state, and joint loading (Hardingham and Bayliss 1990). The major GAGs found in cartilage PGs are chondroitin sulphate (CS), keratan sulphate (KS) and dermatan sulphate (DS). Table 1.2 shows structure of different GAG chains (Prydz and Dalen 2000).

The repeating unit of CS is glucuronic acid linked beta-(1,3) to N-Acetyl-galactosamine. Linear CS chains are polymers of this disaccharide unit joined by a beta-(1,4) linkage. The amine residues within the N-Acetyl-galactosamine can be sulphated in the -4 or -6 positions and these are referred to as chondroitin -4 or -6-sulphate (C4S or C6S). A GAG chain commonly exhibits regions of -6 sulphation followed by -4 sulphation, but un-sulphated (C0S) and disulphated residues are also seen (Carney and Muir 1988). The average CS chain consists of 25 to 30 disaccharides. C4S predominates in young cartilage and C6S in adult cartilage (Mourao et al 1988, Saamanen et al 1989). In OA cartilage, the proportion of C4S increases (Mankin and Lippiello 1971b) and following moderate exercise, the proportion of C6S increases (Saamanen et al 1989).

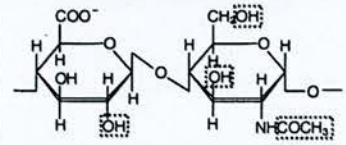
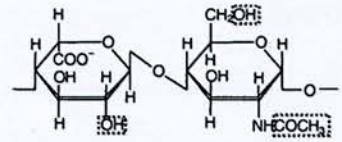
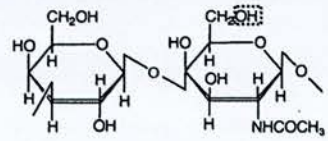
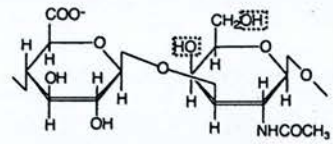
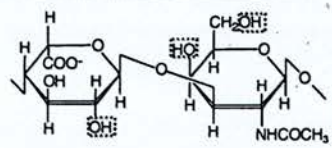
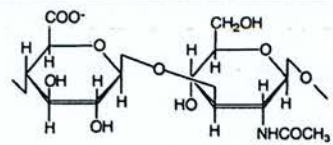
GAG	Hexuronic or Iduronic acid	Galactose	Hexosamine	Disaccharide composition
Heparan sulphate/ Heparin	D-glucuronic acid **(GlcA)** L-iduronic acid **(IdoA)**	-	D-glucosamine **(GlcNAc)**	 **GlcA β(1→4) GlcNAc α((1→4)**  **IdoA α(1→4) GlcNAc α(1→4)**
Keratan sulphate	-	Galactose **(Gal)**	D-glucosamine **(GlcNAc)**	 **Gal β(1→4) GlcNAc β(1→3)**
Chondroitin sulphate	D-glucuronic acid **(GlcA)**	-	D-galactosamine **(GalNAc)**	 **GlcA β(1→3) GalNAc β(1→4)**
Dermatan sulphate	D-glucuronic acid **(GlcA)** L-iduronic acid **(IdoA)**	-	D-galactosamine **(GalNAc)**	 **IdoA β(1→3) GalNAc β(1→4)**
Hyaluronic acid	D-glucuronic acid **(GlcA)**	-	D-glucosamine **(GlcNAc)**	 **GlcA β(1→3) GlcNAc β(1→4)**

Table 1.2 The structure of different glycosaminoglycans chains

The different sulphation positions in each GAG are marked by encircling (adapted from Prydz and Dalen 2000).

1.1.2.5 Chondroitin sulphate

The repeating unit of CS is glucuronic acid linked beta-(1,3) to N-Acetyl-galactosamine. Linear CS chains are polymers of this disaccharide unit joined by a beta-(1,4) linkage. The amine residues within the N-Acetyl-galactosamine can be sulphated in the -4 or -6 positions and these are referred to as chondroitin -4 or -6-sulphate (C4S or C6S). A GAG chain commonly exhibits regions of -6 sulphation followed by -4 sulphation, but un-sulphated (C0S) and disulphated residues are also seen (Carney and Muir 1988). The average CS chain consists of 25 to 30 disaccharides. C4S predominates in young cartilage and C6S in adult cartilage (Mourao et al 1988, Saamanen et al 1989). In OA cartilage, the proportion of C4S increases (Mankin and Lippiello 1971b) and following moderate exercise, the proportion of C6S increases (Saamanen et al 1989).

1.1.2.6 N- and O-linked oligosaccharide chains

In addition to the GAGs, N- and O-linked oligosaccharide are covalently bound to PG core protein. N-linked oligosaccharides have a complex triantennary mannose structure and form the majority of N-linked proteins (Carney and Muir 1988). These N-linked oligosaccharides are relatively abundant in cartilage PG, in particular, around the hyaluronic acid binding region, but their function is unknown. It has been suggested that an extra N-linked oligosaccharide, which exists in the B' components of the tandem repeat region of the G2 domain, may disrupt the folding of this region and prevent binding of the G2 domain to hyaluronic acid (Neame and Barry 1993). There are three distinct O-linked oligosaccharides distributed along the whole PG core protein. They have no known function but they may represent incomplete keratan sulphate II molecules since they have a similar structure to the linkage region of keratan sulphate II (Carney and Muir 1988). O-linked carbohydrate side chains may be important in fortifying the interaction between hyaluronic acid and the G1 domain (Hascall and Heinegard 1974) (Figure 1.1).

1.1.2.7 Aggrecan

1.1.2.7.1 The structure of aggrecan

Chondrocytes produce large PGs, in other words aggrecan, which contains both CS and KS, the phenotype-specific PG of cartilage. Aggrecan accounts for approximately 10 per cent of the dry weight (Ayad et al 1994) and about 80 per cent of total cartilage PG (Carney and Muir 1988). As many as 20 to 100 aggrecan molecules are formed by non-covalent bonding to

hyaluronic acid to produce aggregates with a molecular mass up to 5 x 10^8 Da (around 1-4 x 10^6) and up to 5 μm long, that are visible under the electron microscope (Rosenberg and Buckwalter 1986). The aggrecan molecule is made up of 87 per cent chondroitin sulphate (CS), 6 per cent keratan sulphate (KS) and 7 per cent core protein by weight, and has a molecular weight of about 2600 kDa (Ayad et al 1994). Aggrecan core protein contains three globular domains (G1, G2, and G3) and two extended regions, which form the inter-globular domain (IGD) region between G1 and G2, and the main GAG attachment region (Hardingham et al 1994) (Figure 1.1).

The G1 domain of aggrecan contains three protein motifs: an IgG fold, and two copies of a hyaluronan-binding motif (link module). In aggregates, the G1 domain of aggrecan binds to hyaluronan and this binding is stabilized by the link protein (Heinegard and Oldberg 1989).

The G2 domain is located towards the N-terminus of the molecule and shares some of the structural features of the G1-domain. However, it has no hyaluronan binding properties despite sharing considerable sequence homology with the G1 domain (Doege et al 1987). Although the exact function of it is still unknown, it appears to function by inhibiting aggrecan secretion (Watanabe et al 1997).

The G3 domain is located at the C-terminus of the molecule and contains regions with sequence similarity to C-type lectins, complement regulatory proteins (CRP) and members of the epidermal growth factor family (Baldwin et al 1989). The G3 domain may function to stabilise the molecule within the matrix, since it is homologous to a lectin specific to galactose and fucose. The G3 domain is thought to be associated mainly with immature, newly synthesised aggrecan (Dudhia et al 1996). This domain binds other matrix components that may permit essential cross-links during extra-cellular matrix formation development (Aspenberg et al 1997, 1999, Olin et al 2001). The CRP domain may also function by promoting GAG chain attachment to the core protein during aggrecan synthesis (Yang et al 2000). The G3 domain and CS-rich core protein are required for effective secretion of aggrecan (Kiani et al 2001).

It has been shown that both the G1 and G2 domains work in concert with each other inhibiting secretion of aggrecan, whilst the G3 domain and the CS core protein region promote secretion. Therefore, the G2 domain acts as a secretion retardant, ensuring that only fully glycosylated aggrecan monomer is secreted (Kiani et al 2001).

The binding of aggrecan to hyaluronan decreases with low pH, with no binding at pH 3.0. The G1 domain re-natures after exposure to many denaturing agents such as guanidium hydrochloride (GuHCl). The G1 domain also survives treatment with solvents such as ethanol

and acetone (Hardingham 1981). These characteristics are significant in extracting aggrecan from matrix.

1.1.2.7.2 Aggrecan core protein

Because the polysaccharide components (GAGs) are added to molecules post-translationally, it is necessary to analyse the core protein in order to better understand the relationships between the various PGs. The exceptional diversity of PGs is derived from both the number of different core proteins and from the poly-dispersity, produced by the large variety of post-translational modifications required to construct the final molecules. The molecular weight of full length aggrecan core protein in rat chondrosarcoma is deduced to be 220.952 kDa, using the data from its amino acid sequence of 2124 residues (Doege et al, 1987). Human aggrecan core protein is sizing 245 kDa (personal communication with prof Hardingham, Manchester). In addition to the full length of aggrecan core protein, there are a series of products generated by varying degrees of proteolytic truncation from the C-terminal end of core protein. These truncations result in the formation of distinct and electrophoretically separable species of aggrecan core, all of which lack the G3 globular domain and appear to be retained in the tissue through the hyaluronan-binding properties of the G1 domain (Sandy 2001).

1.1.2.7.3 The role of aggrecan

The role of aggrecan is often called 'space-filling' because when it aggregates, it occupies large solvent domains in interstitial spaces (Gallagher 1989). The GAG chains of the PGs are negatively charged due to carboxyl and sulphate groups (Carney and Muir 1988) and this creates a high fixed charge density. This high concentration of negative charges attracts an equally high concentration of counter ions and the resulting osmotic potential creates a powerful swelling force within the matrix (Carney and Muir 1988). The PGs are, however, prevented from swelling to their maximum hydrated volume by the collagen fibre network, which is kept under tension as a consequence. When cartilage is compressed, following joint loading, water is squeezed out of tissue, until the raised osmotic pressure is equal to the applied compression (Carney and Muir 1988).

1.1.2.7.4 Metabolism of aggrecan

1.1.2.7.4.1 Biosynthesis of aggrecan

In mature normal cartilage, there is a little collagen turnover, but considerable enzymatic breakdown and synthesis of PGs (Muir 1973). For functional PG aggregates to form,

hyaluronic acid and link protein must be synthesized simultaneously in order to allow extra-cellular assembly to take place (Poole 1986, Hascall 1981). PG synthesis occurs intra-cellularly in the endoplasmic reticulum and Golgi apparatus (Poole 1986, Lohmander and Kimura 1986, Alonso et al 1996) but synthesis of hyaluronic acid occurs on the cell membrane from where it is exposed directly (Prehm 1986).

Figure 1.2 outlines the general steps involved in PG synthesis. Synthesis of the protein and carbohydrate moieties of PG occur sequentially (Kimura et al 1981, Calabro and Hascall 1994), although the initial steps of GAG chain synthesis may occur while the core protein is still being synthesized (McQuillan et al 1986). The core protein is translated from mRNA by ribosomes on the rough endoplasmic reticulum (Upholt et al 1979), a process which has a half lifetime of approximately 60 minutes and represents 70 to 90 per cent of the time taken to secrete a completed PG molecule (Velasco et al 1988).

Following translocation to the trans-Golgi cisternae, more than 7000 post-translational modifications are performed in the 10 to 15 minutes remaining before secretion by exocytosis (Calabro and Hascall 1994, Fellini et al 1984). The bulk of these modifications, occurring in the Golgi apparatus, are concerned with the addition of approximately 200 sulphated GAGs per core protein and the addition of O- and N-linked oligosaccharides (Velasco et al 1988). N-linked and O-linked oligosaccharides are added to the PG precursor at an early stage, possibly con-translationally, in the endoplasmic reticulum (Lohmander and Kimura 1986). The N-linked oligosaccharides are added in the form of dolichol-bound high mannose primers, which are processed after the translocation of core protein to the Golgi apparatus (Lohmander and Kimura 1986). The O-linked oligosaccharide precursors are linked to protein core by galactosyl-transferases, and then extended by other transferases to be completed at about the same time as the CS chains (Thonar et al 1983). CS chains are added to the PG precursor at serine residues with neighbouring glycine residues. Polymerisation is initiated by the action of xylosyl-transferase, which functions to add xylose to the core protein in the endoplasmic reticulum and cis-Golgi (Sugumaran and Silbert 1991). Elongation of GAG chains by the addition of alternate glucuronic acid and N-Acetyl-galactosamine then takes place in the medial and trans-Golgi apparatus through the action of specific transferases (Sugumaran and Silbert 1991). Sulphation probably takes place in the trans-Golgi network during chain polymerisation and is mediated by sulpho-transferases (Lohmander and Kimura 1986, Godman and Lane 1964) which add sulphate from PAPS (Godman and Lane 1964, Nishimoto et al 1982). Terminal sulphation may stop chain polymerisation but little is known about chain length, which can vary significantly (Godman and Lane 1964, Telser et al 1966).

The high mannose O-linked oligosaccharide primers added to the core protein in the endoplasmic reticulum can be polymerised to either KS or O-linked oligosaccharides (Lohmander and Kimura 1986). The polymerisation of KS occurs simultaneously with that of

CS in the medial and trans-Golgi by the alternate addition of glucuronic acid and galactose (Velasco et al 1988). The proportion of KS in completed PGs is variable but the sum of O-linked oligosaccharides and KS is relatively constant, indicating their common origin (Lohmander and Kimura 1986). The mechanism that determines whether KS or oligosaccharide polymerisation occurs is unknown (Lohmander and Kimura 1986).

Hyaluronic acid synthesis is performed by hyaluronate synthetase which is found on the inner side of the cell membrane (Prehm 1986). It functions to link N-Acetyl-galactosamine and glucuronic acid sequentially, the product being extruded directly into the extra-cellular matrix. The incomplete hyaluronic acid is able to bind to other matrix components, such as link protein and aggrecan, whilst it is still enzyme-bound and this may serve to anchor a proportion of the pericellular matrix. The extra-cellular assembly, of PG aggregates on hyaluronic acid, takes place pericellularly and is dependent on a very specific interaction between the core protein linkage region and five disaccharides, which is stabilised by link protein (Kimura and Kuettner 1986). The bulk of the new hyaluronic acid, when released from its synthetase, diffuses away from the cell into the territorial matrix, but some of it will remain in the pericellular matrix bound to the chondrocyte surface hyaluronan receptor CD44 (Knudson 1993).

Once completed, the PG can have a large number of outcomes depending upon the cell type and function of the macromolecule. Mis-folded proteins and fractions of aggrecan precursor are targeted onto a smooth endoplasmic reticulum sub-compartment where it undergoes degradation (Alonso et al 1996), similar to other mis-folded proteins (Domowicz et al 2000). The time between GAG chain synthesis in the Golgi apparatus and the appearance of PG molecules is short (5 to 10 minutes). Half of the PGs synthesized by the cells spend less than 24 hours in the cell-associated matrix before moving into the inter-territorial matrix (Hassell et al 1986).

1.1.2.7.4.2 Catabolism of aggrecan

Aggrecan has been demonstrated to undergo proteolytic cleavage at a number of different sites within the molecule. The extra-cellular metabolism and turnover of aggrecan is markedly influenced by processing events which occur at both the N-terminal (G1 end) and C-terminal (G3 end) regions of the core protein. There are two pools representing the metabolically active and inactive turnover pools found both *in vivo* (Sandy et al 1989) and in cartilage explant studies (Sandy et al 1986) of mature cartilage. Molecules in the inactive pool may have an *in vivo* half-life of up to 300 days in mature animals (Maroudas 1980). Previous work, measuring both *in vivo* and *in vitro* turnover rates of PGs, showed dependence on age and on culture conditions. PGs from calf articular cartilage have longer half-lives than those of adult bovine

cartilage. PGs of steer articular cartilage, maintained in medium containing fetal calf serum, have longer half-lives than those from the same tissue maintained in medium alone (Campbell et al 1984).

Processing of the N-terminal (G1 domain side) during the biosynthesis and secretion of aggrecan is indicated by a low affinity of newly synthesized aggrecan molecules for hyaluronan (Sandy and Plaas 1989). Processing of the C-terminal region (G3 domain and CS-attachment region) in molecules apparently takes the form of a slowly progressive trimming in the inactive pool. Such C-terminal processing is suggested by analysis of extracted aggrecan molecules which have various sizes (Paulsson et al 1987, Stanescu 1990). These studies detected that only 30 per cent of molecules from mature bovine nasal cartilage have the G3 domain. Moreover, only about 50 per cent of the aggrecan molecules carried the G3 domain in rapidly growing immature rat chondrosarcoma cells, suggesting that a deficiency of G3 may be a common feature of aggrecan. As a consequence of C terminal trimming and a reduction in the length of the chondroitin sulphate attachment region, aggrecan molecules lacking a G3 domain are present at higher concentrations in mature tissues (Dudhia et al 1996).

A proportion of the molecules in the inactive pool will subsequently undergo proteolysis near the N-terminal to generate fragments which have lost the ability to bind hyaluronan. This population appear to be rapidly lost from the matrix (Sandy 1992). The catabolic process leading to the loss of PGs from matrix is thus mediated by cells (Campbell et al 1984).

Catabolism of aggrecan *in situ* was first found in the dissociation of the hyaluronic acid binding G1 domain (Roughley et al 1985, Tyler 1985, Ratcliffe et al 1986, Campbell and Handley 1987). An early observation by Sandy et al (1978) showed that aggrecan degradation in cartilage explants appeared to be initiated by proteolytic cleavage within or near N-terminal domains. Subsequently, Sandy et al (1991, 1992) discovered the existence of aggrecan fragments between the G1-G2 domain (Glu^{373}-Ala^{374}) called the NITEGE neo-epitopes. Flannery et al (1992) then identified others (Asp^{341}-Phe^{342}) called VDIPEN neo-epitopes. In 1997, Kuno et al succeeded in cloning an 'aggrecanase', an endogenous proteolytic enzyme of aggrecan. Abbaszade et al (1999) identified three aggrecanases (ADAMTS; a disintegrin and metalloproteinase with thrombospondin motifs gene family -1, 4, and 5), all of which release the NITEGE neo-epitopes. Products of aggrecan cleavage by MMPs at the VDIPEN site were demonstrated in normal and arthritic cartilage (Flannery et al 1992).

These cleavage events are likely to be of significant because they uncouple the G1 domain from the GAG-rich domains, which are then no longer anchored inside the cartilage, and hence might be lost (Sztrolovics et al 1997). It is now clearly established that aggrecanases are primarily responsible for the aggrecan catabolism in the early stages of aggrecan loss from

articular cartilage (Caterson et al 2000), which is induced by cytokines such as IL-1 and TNF stimulation (Arner et al 1998). When collagen catabolism is occurring, MMPs (MMP-1, 2, 3, 7, 8, 9, 10 and 11) appear to cleave the Asp^{341}-Phe^{342} bond as a later event in cartilage degeneration (van Meurs et al 1999a, 1999b, 1999c) As both types of aggrecan G1 fragments are found in normal cartilage, it is thought that MMPs and aggrecanases play a role in the turnover of human cartilage aggrecan. NITEGE and VDIPEN neo-epitopes are generated and enriched in the immediate pericellular matrix. The intense staining immediately surrounding the cells suggests that both aggrecanase enzyme and MMPs are probably generated and or activated by chondrocytes. The catabolism of the newly synthesized aggrecan molecule occurs almost exclusively in the cell-associated matrix (Hassell et al 1986). The amount of MMP-generated aggrecan G1-fragments in human joint cartilage increases up to about 20 to 30 years old of age, then remains constant with further increases in age (Mok et al 1994). When using immuno-histochemical staining technique for the VDIPEN and NITEGE neo-epitopes in human cartilage, staining intensity, in general, appears to increase with age (Hollander et al 1994). Moreover, aggrecan catabolism may differ with tissue origin and stimulatory agent (Sztrolovics et al 1997, 2002). Human articular cartilage shows little response to IL-1β in terms of GAG release, and there is no evidence for aggrecanase action. In contrast retinoic acid appears to stimulate aggrecanase activity with the release of GAG and production of the corresponding G1 region (Sztrolovics et al 2002). It has been noted that there may be some elevated proteolytic activities involved in the progress of cartilage distraction, in diseased joints such as OA and RA (see following OA and RA section).

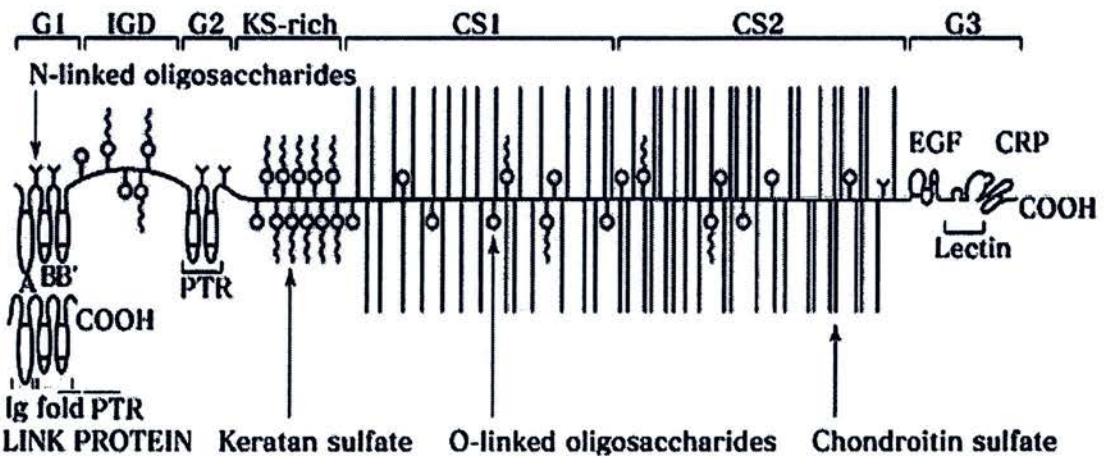


Figure 1.1 Schematic molecular models of aggregating PG aggrecan and link protein of cartilage

Aggrecan may contains as many as 100 chondroitin sulphate (CS) chains, up to 50 keratan sulphate (KS) chains, up to 50 O-linked, and 10 to 15 N-linked oligosaccharides chains (adapted from http://www.glycoforum.gr.jp/science/hyaluronan/HA05/HA05E.html#II).
PTR: proteoglycan tandem repeat

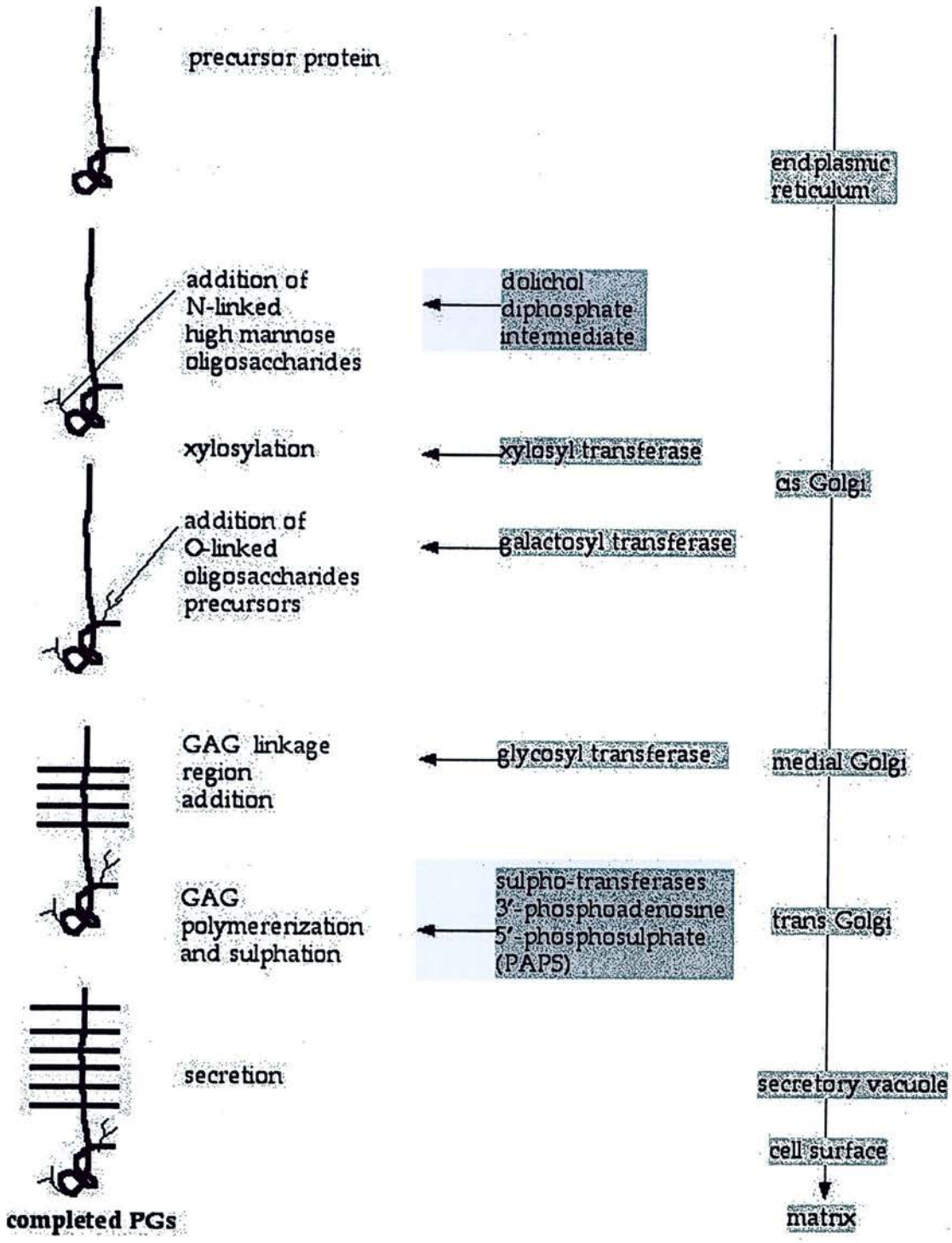


Figure 1.2 The general steps involved in PG synthesis

(adapted from Hassell et al 1986)

1.1.2.8 Small chondroitin sulphate and Dermatan sulphate proteoglycans

Biglycan, decorin, and fibromodulin together form a family of structurally related small PGs. In cartilage, biglycan is the predominant small PG. It contains two CS chains and dermatan sulphate. Decorin and biglycan constitute about two per cent of the total cartilage PGs (Gallagher 1989).

1.1.2.8.1 Decorin (PG-40, PG II, DS-PG)

PG II is a non-aggregating small proteoglycan in articular cartilage. It can also be found in many connective tissues (Jarvelainen et al 1991), such as human fetal membranes (Brennan et al 1984), sclera (Coster and Fransson 1981), human cervix (Uldbjerg et al 1983), bovine and human bone (Franzen and Heinegard 1984, Fisher et al 1987), bovine tendon (Vogel and Evanko 1987), rat, calf and pig skin (Miyamoto and Nagase 1980), and arterial wall (Kinsella and Wight 1990), although initially it was isolated from cartilage and bone (Rosenberg et al 1985, Fisher et al. 1983). Human fibroblast PG II was named decorin due to its appearance on the collagen network, decorating the collagen fibres at the 'd' and 'e' bands, as seen through the electron microscope (Scott and Orford 1981). Decorin is commonly found in the inter-territorial matrix, while biglycan is localized mainly pericellularly within the hyaline cartilage tissue (Miosge 1994).

From its core protein composition, decorin is further categorised as belonging in the leucine rich family, together with the other small proteoglycans; biglycan, fibromodulin, lumican, and PG-Lb. Electrophoretic separation indicates that decorin has an average molecular weight value of 98 kDa and a core protein of 38 to 47 kDa (depending on the system used), after chondroitinase ABC treatment (Glossl et al 1984, Roughley and White 1989). The GAG chain can be either CS or DS, with the latter containing variable quantities of iduronate and glucuronate, depending on the source (Sampaio et al 1988). For example, non-articular cartilage from immature pig larynges contain no DS. Human decorin contains DS chains and much shorter CS chains than the pig ones. The PG from human articular cartilage contains DS chains which are 50 per cent chondroitinase resistant (Sampaio et al 1988).

Although aggrecan is the most abundant species in articular cartilage in terms of mass, low molecular weight PGs are also present in similar molar concentrations (13.1 nM decorin compared to 13.3 nM aggrecan core protein), when the different molecular sizes of two PG classes are taken into account (Rosenberg et al 1985, Poole et al 1996). Ageing bovine articular cartilage contained more DS PG relative to fetal epiphyseal cartilage. There is a steady increase in the content of decorin in cartilage samples from 0 to 25 years old, and a subsequent decline in older tissue (Sampaio et al 1988).

Decorin's respective roles in the development and maintenance of cartilage are not known, but there are numerous studies of the interaction between decorin and collagen, because decorin is commonly found in the d and e bands of collagen fibrils, binding to triple helical collagen (Gallagher 1989). It has been proposed that decorin is a bidentate ligand attached to two parallel neighbouring collagen molecules in the fibril, helping to stabilize fibrils and orient fibrillogenesis (Scott 1996). It has been suggested that decorin participates in the regulation of the thickness of collagen fibres (Vogel et al 1984). Interaction occurs mainly via the leucine-rich repeats of the decorin core protein (Svensson et al 1995). *In vitro* studies of collagen fibrillogenesis have shown that the addition of DS-PG II results in the production of more uniform and thinner collagen fibrils (Vogel 1984, 1987). Decorin interacts with collagens I, II, III, and V (Bidanset et al 1992, Hedbom and Heinegard 1993, Whinna et al 1993), VI, XII, and XIV (Bidanset et al 1992, Burg et al 1996), IX (Hagg et al 1998), and may interact with type I and II collagen fibrils (Vogel et al 1984), and even type IV, which is localized in the pericellular matrix (Burrows et al 1994). These functions are not affected by the removal of the GAG chain, indicating that the function lies within the core glycoprotein.

In addition to collagen, decorin also binds to fibronectin. Interaction between decorin and the fibronectin cell binding domain is also via its core protein (Schmidt et al 1991). Bidanset et al (1992) found that this interaction only happens with fragmented fibronectin, not with intact fibronectin. When the binding occurs of decorin and fibronectin, it can alter cell adhesion and migration processes (Kinsella and Wight 1988, Schmidt et al 1987). For instance, fibronectin-mediated adhesion to fibroblasts is inhibited by decorin (Lewandowska et al 1987, Winnemoller et al 1991), possibly by attaching to specific regions adjacent to the fibronectin cell-binding site. Another study suggested that decorin impairs the ability of cells to form focal adhesions. Significantly fewer cells formed focal adhesions in the presence of decorin, as compared with untreated control cells (Bidanset et al 1992).

Furthermore, decorin's possible roles are reported in various ways: first, it has been demonstrated to be inhibitory for cell proliferation (Yamaguchi and Ruoslahti 1988), since its protein core has been shown to bind TGF-β (Yamaguchi et al 1990) and it neutralises the growth stimulating effect of TGF-β (Ruoslahti and Yamaguchi 1991). It may be important in the organogenesis (Scholzen et al 1994) and regulation of cell division and differentiation, the controlling of cytokine activity through binding to TGF-β (Yamaguchi et al 1990, Funderburgh et al 2001). Furthermore, there are possible interactions with PKC (Lam et al 2001), and thrombospondin (Iozzo 1999).

Interestingly, Cs-Szabo (1995, 1997), found that decorin increased both mRNA and protein levels in OA cartilage when compared to age-matched normal controls. Decorin is up-regulated in late stage OA (Bock et al 2001), and synthesis is increased in OA (Okimura et al 1997). Pearson and Sasse (1992) showed decorin mRNAs increased upon treatment of bovine

chondrocytes with retinoic acid, indicating that decorin could serve as a marker for phenotype change. Similarly, Dourado et al (1996) showed that the relative levels of the mRNAs of the small PGs increased in the hypertrophic phase of early OA that follows joint injury. Poole (1996), on the contrary, showed relating to OA cartilage, that there were no overall significant changes in the quantity, but that the distribution of decorin was changed. Some researchers have even reported the loss of decorin in human OA (Carroll et al 1989, Pettipher 1989, Witsch-Prehm 1992, van de Loo 1998).

Regarding decorin and mechanical stimulation in the articular cartilage, Korver et al (1992) have demonstrated a three-fold increase in synthesis of DS-PG in articular cartilage, following seven days of cyclic *in vitro* loading. Little et al (1996) showed that a high contact stress area produced more decorin than a lesser stress area. Similarly, 30 MPa hydrostatic pressure increased decorin mRNA (Lammi et al 1994).

1.2 Osteoarthritis

Damage to articular cartilage is a common problem affecting the joints of millions of people: 15 per cent of the world's adult population (Cole and Kuettner 2002, Lawrence et al 1998), 25 per cent by 2020 (Lohmander 2004). OA is the single largest cause of physical disability in the elderly (Hardingham and Bayliss 1990). OA is a clinico-pathologic consequence of many etiologic factors (Brandt et al 1986). Causative factors leading to the development of OA can be classed either as those that render cartilage abnormal, or as those that lead to abnormal loading of cartilage (Brandt et al 1986). As an example of abnormal loading of normal cartilage responsible for cartilage degeneration, Koshino et al (2003) showed that the correction of overloading of weight bearing resulted in successful regeneration of degenerated articular cartilage, using a high tibial valgus osteotomy treatment of medial compartmental OA of the knee, without autologous chondrocyte implantation. Thus, substantial loss of cartilage PG and simultaneous inhibition of GAG can be reversed (Page Thomas et al 1991), however, the poor healing ability of the cartilage makes OA a high priority health care problem.

1.2.1 Chondrocyte changes in OA

Many researchers have found that chondrocytes in OA cartilage show increased anabolic activity (Brand et al 1991, McElligott and Collins 1960, Ehrlich and Mankin 1980, Bollet 1967), however, a net loss of PG content is one of the hallmarks of all stages of OA cartilage degeneration (McElligott and Collins 1960, Mankin 1971, Aigner et al 1997). This leads to the

assumption that the balance between the anabolic processes (chondro-formation) and catabolic processes (chondro-resorption) of cartilage is disturbed in OA patients (Aigner et al 1997).

Normally in adult cartilage, chondrocytes are long-lived and seldom divide, but when the collagenous network of the local matrix is damaged, as occurs in OA, the capacity to replicate cells appears. Cell cloning is a typical feature of OA cartilage (Stockwell 1971). Why this should be, and how the response is initiated, is not understood. Cell proliferation and division in OA is initiated by growth factors (Malemud 1999). The increased proliferative activity of chondrocytes, which is found primarily in the upper zone of cartilage, might be due to an easier accessibility to the synovial fluid (Meachim and Collins 1962). Alternatively, damage in the collagen network, which is particularly found in the upper zone of OA cartilage, may contribute to the proliferation of OA chondrocytes. Chondrocyte proliferation results in a higher cellular content, and consequently an increased synthesis of the matrix, but unfortunately the fabric of the matrix is not restored to its former state (Muir 1995). It's inferior biomechanical properties lead to progressive deterioration of articular cartilage (Aigner and McKenna 2002).

It is also notable that various specific macromolecules in cartilage are intimately associated with each other and yet are synthesized by chondrocytes and degraded independently. This is difficult to explain (Muir 1995). Some authors have suggested that cell death is a central feature in OA cartilage degeneration (Vignon et al 1976), but chondro-necrosis or apoptosis can only be seen to a limited extent in early OA or aged human articular cartilage (Aigner and McKenna 2002). Because chondrocytes are the only source of matrix synthesis in articular cartilage, terminal differentiation of the articular chondrocytes must be responsible for the formation of OA cartilage. In other words, there is an inability of the cells to synthesize extra-cellular matrix components of their former state (Salter et al 1992).

Chondrocyte phenotypes are classically categorised in the main by sub-typing of the collagen expression (von der Mark 1986, Cancedda et al 1995). Mature chondrocytes express the typical cartilage collagen type II, IX, and XI as well as aggrecan and link protein (Vornehm et al 1996, Sandberg and Vuorio 1987, Muller et al 1997). When looking at *in situ* expression analysis of OA cartilage specimens, collagen type II and aggrecan (as a marker of differentiated, activated, and functional chondrocytes) were found mostly in the middle zones (Sandell and Aigner 2001). This indicates that chondrocytes start to re-express a chondro-progenitor phenotype in OA cartilage. Cells expressing type III collagen (a marker of dedifferentiated chondrocytes) were mainly found in the upper middle zone. Cells start to express type X collagen (as a reversion to a fetal phenotype), in the deepest zone of OA cartilage (Sandell and Aigner 2001). However, no specific markers have been established for the chondrocytes in the upper zones.

Chondrocyte changes in cartilage are reported by various authors. In contrast to normal chondrocytes, human OA chondrocytes were hypo-responsive to stimulation by insulin-like growth factor 1 (IGF-1), due to an alteration in the level of IGF binding proteins (IGFBP) (Tardif et al 1996). Trickey et al (2000) found significant differences in the mechanical properties of chondrocytes isolated from normal and OA cartilage. The increased elastic and viscous properties suggest that the mechanical environment of the chondrocyte may be altered in OA cartilage. Dozin et al (2002) have investigated the metabolic properties of human articular chondrocytes derived from young, aged and OA subjects, and their genetic adaptation to catabolic challenge (i.e. the inflammatory cytokines IL-1α and TNF-α). Results show that chondrocytes from aged subjects appear to be different from those of young patients, but were similar to OA chondrocytes, as indicated by cell morphology, cell proliferation rate and pattern of protein secretion (in particular stromelysin-1 and interstitial collagenase). Chondrocytes isolated from OA cartilage bound more TNF-α and IL-1β compared with chondrocytes isolated from morphologically normal cartilage from the same joint (Shlopov et al 2000). The volume and morphology of living *in situ* human chondrocytes increased with cartilage degeneration (Bush and Hall 2003). OA chondrocytes up-regulated the cell adhesion molecules such as CD44 (Ostergaard et al 1997b) and β1 integrin (Loeser et al 1995). Aberrant expression of α2, α4, and β2 integrins, which are not expressed in normal cartilage, has also been documented (Ostergaard et al 1998). Human OA chondrocytes express different percentages of α1 to 6 and αV chains, according to the severity of anatomical change and the phase of the cell cycle in different zones of OA cartilage (Lapadula et al 1997). Moreover, the lower prevalence of OA in ankle joints, rather than the knee, may in part be explained by differences, in metabolic and biochemical activities, between the chondrocytes in the different joints (Eger et al 2002, Cole and Kuettner 2002, Jubb 1984).

1.2.2 Extra-cellular matrix changes in OA

The expression of extra-cellular matrix molecules is modified in OA, and secondary changes in cell-matrix interactions are believed to influence the development and progression of the disease. A number of biochemical studies have demonstrated enhanced synthesis of extra-cellular matrix components in OA cartilage, such as fibronectin (Scott et al 1981, Burton-Wurster et al 1986, Brown and Jones 1990, Farquhar et al 1996), type VI collagen (McDevitt et al 1988), and tenascin (Sage and Bornstein 1991). The quantitatively minor matrix components may serve important roles in regulating the assembly of the matrix and its constituents (Burton-Wurster and Lust 1990). Fibronectin accumulation is one of the first changes to occur after injurious impact (at 10 days) in articular cartilage (Farquhar et al 1996). Fragments of fibronectin can induce expression of MMPs and matrix degradation (Homandberg et al 1992). Moreover, a fragment of link protein was found to stimulate PG and collagen synthesis in cartilage explant culture (Liu et al 2000). Studies have also reported the appearance of molecules in OA cartilage, such as collagen type I (Nimni and Deshmukh

1973), IIA (Aigner et al 1999) and III (Aigner et al 1993), which are rarely seen in normal articular cartilage.

Age is a major factor in occurrence of OA. The articular cartilage extra-cellular matrix undergoes extensive changes during ageing (DeGroot et al 1999). It is likely that aged cartilage chondrocytes experience different regulatory signals, in their extra-cellular matrix, than those of young tissue. Age-related changes in articular cartilage include an increase in decorin and biglycan (Kuettner et al 1992), hyaluronan acid concentration (Holmes et al 1988), changes in GAG composition (Bayliss et al 1978), and increase in non-enzymatic glycation (DeGroot et al 1999).

The main changes in the OA cartilage matrix are depletion of PGs and disruption of the collagen network (Chevalier 1993). In the OA cartilage, a decrease in chondroitin sulphate concentration was found according to the severity of the grade of OA, while the concentration of hydroxyproline (representing collagen) was virtually unchanged (Bollet et al 1963). PG and collagen degradation seems to occur separately. There may be tighter regulation of collagen degrading enzymes in the articular cartilage (Kozaci et al 1997). Moreover, the decrease in the quantity of organic matrix components can be seen in the areas of actual cartilage fibrillation and erosion (Anderson et al 1964), indicating that the degenerative joint disease OA is a focal process, and not due to a generalised matrix deficiency.

Cytokines are important as local mediators of inter-cellular communication in normal and diseased tissue (Chevalier 1993). Cytokines bind to specific receptors on target cells and induce intra-cellular signal transduction pathways (Chevalier 1993). For example, IL-1 induces signal transduction via the cytoplasmic domain of IL-1 receptor I, which in turn activates several signalling pathways, among which are the production of prostaglandins, NO synthesis, activation of protein tyrosine kinases, mitogen-activated protein kinases and calcium signalling (Tonon and D'Andrea 2000). There are many different cytokines including IL-1α, IL-1β, IL-2, 3, 4, 5, 6, 7, 10 (Flannery et al 2000, van Roon et al 1996, Lotz et al 1991, Nietfeld et al 1990, Moos et al 1999), tumour necrosis factor-α (TNF-α), and interferons (Goldring et al 1986, Verbruggen et al 1993) which can cause the destruction of cartilage. They are up-regulated in OA cartilage compared to normal cartilage, suggesting that they serve some regulatory (catabolic) function (Moos et al 1999).

Matrix metalloproteinases (MMPs) are involved in extra-cellular remodelling, and have a crucial role in progressive joint degradation (Streuli 1999). These proteinases are Zn^{2+} dependent endopeptidases. The MMPs are currently believed to number 16 and are generally subdivided into four main groups on the basis of their substrate specificity and internal homologies; namely collagenases, gelatinases, stromelysins, and membrane type

metalloproteinases (Lee et al 1998a). Enhanced levels of many MMPs including, MMP-3 (Wernicke et al 1996), MMP-7 (Ohta et al 1998), MMP-8 (Shlopov et al 1997), MMP-9 (Jin et al 2000) MMP-13 (Moldovan et al 1997), MMP-14 (Imai 1997) and aggrecanases (Lohmander et al 1993, Malfait 2002) have been reported to accompany increased matrix degradation in OA cartilage. In addition, MMP-generated and aggrecanase generated G1 neo-epitopes were detected in the more severely fibrillated superficial zone of the OA cartilage (Lark et al 1997). MMP-1, 8, and 13 are collagenase-1, 2, and 3 and serve as degraders of collagen. In particular MMP-13 (collagenase-3) degrades type II collagen (Shlopov et al 2000). MMP-1, 2, 3, 8, 9, 10, 13, and 14 all have the capacity to degrade aggrecan core protein between Asn^{341} and Phe^{342} (Lark et al 1997, Lee et al 1998) independently of aggrecanase activity (Hughes et al 1998). MMPs are responsible for the Asn^{341}-Phe^{342} cleavage, and they can cleave aggrecan core protein at different sites (Sandy et al 1991). Chondrocytes obtained from cartilage adjacent to OA lesions express higher levels of MMPs in comparison with chondrocytes from more normal looking cartilage located in the same joint (Shlopov et al 2000). Shlopov et al (2000) showed that up-regulated MMP-1 and MMP-13 synthesis can be seen in OA chondrocytes compared to normal ones, as a result of an increase in the number of the cytokine receptors per cell. They were able to create activated OA phenotype chondrocytes by incubating normal chondrocytes with TNF- α.

Collagen degradation is found to change in parallel with VDIPEN neo-epitope expression, from the middle to the late stage in arthritis models. VDIPEN neo-epitope and collagen degradation are stimulated by collagenase (Billinghurst et al 1997). It has been proposed that MMP-3 is one of the major degradative MMPs which activate collagenase to cause loss of cartilage in RA (Hasty et al 1990, Brinckerhoff et al 1991, Martel-Pelletier et al 1994). It has also reported that MMP-3 degrades a wide spectrum of matrix molecules such as cartilage link protein, collagen III, IX, X, fibronectin, and gelatine, and is particularly active against PG (Mehraban et al 1994) . However, Mudgett et al (1998) have shown that the exclusion of MMP-3 neither prevents nor reduces cartilage destruction in MMP-3 knockout mice. No single cytokine can stimulate all metabolic reactions observed in OA (Sandell and Aigner 2001). Both aggrecanase ADAM-TS4 and ADAM-TS5 mRNA exist in OA cartilage, and most important, a recent study shows that the aggrecanase inhibitors can block aggrecan degradation in OA cartilage *in vitro* (Malfait et al 2002). This means that inhibition of ADAM-TS4 and ADAM-TS5 is a potential target for the development of new cartilage-protective treatment (Stanton et al 2005).

Free radical nitric oxide (NO) has been implicated as a biological mediator in OA (Studer et al 1999). OA chondrocytes produce inducible enzyme nitric oxide synthase (iNOS) and NO spontaneously (Clancy et al 1998). The amount of NO produced by chondrocytes is greater than that produced by synoviocytes in OA joints (Melchiorri et al 1998). NO can inhibit PG synthesis *in vitro* (Studer et al 1999, Matsukawa et al 2004), and a selective inhibitor of iNOS can reduce the progression in experimental OA (Pelletier et al 1998). NO inhibits the

assembly of the intra-cellular activation of the focal adhesion complex, but has no effect on the extra-cellular aggregation of $\alpha 5\beta 1$ integrins (Clancy et al 1997). Within the joint, NO production causes a number of catabolic effects which are assumed to promote the degradation of articular cartilage, including the release of aggrecan from cartilage and down-regulation synthesis of PG and collagen II (Clancy et al 1998).

Better understanding of chondrocyte cell biology is required to develop methods to arrest the onset of deterioration in OA. The study of chondrocytes is made more difficult by the need to consider transient intra-cellular and extra-cellular physicochemical changes. When chondrocytes *in vivo* are subjected to mechanical stress, the immediate and also long-term activity of the cell is affected. The situation is further complicated by spatial heterogeneity of the extra-cellular matrix (Muir 1995).

1.3 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease with two major pathological components; synovial proliferation and cartilage and bone destruction. The available data suggest that the overall prevalence of diagnosed RA is approximately one percent of adult population. Rates for women are approximately 2.5 times higher than those for men (Lawrence et al 1998).

1.3.1 Pannus in RA

The invasion of cartilage by synovial pannus is a characteristic feature of rheumatoid arthritis. Even though there are numerous previous studies on rheumatoid synovium, there is still a controversy about the cell composition and nature of synovial pannus tissue in RA (Mitrovic 1985). The inflammatory processes that operate in rheumatoid synovial tissue (pannus), that can lead to cartilage degradation, are just beginning to be elucidated. Rheumatoid synovial fibroblasts are a major component of pannus tissue. The pannus tissue gliding over the cartilage may originate from the cartilage by itself. Under the influence of rheumatoid inflammation, specialized, differentiated cells tend to lose their phenotype, acquiring morphologic characteristics of fibroblasts, and destroying the matrix (Mitrovic 1985). The overall process may be explained in terms of fibrous metaplasia of hyaline cartilage. Hyperplastic synovioblasts synthesize and secrete MMP-3 continuously (Okada et al 1989), and MMP-3 degrades a wide spectrum of extra-cellular matrix macromolecules. Indeed, greater amounts of MMPs exist than their natural inhibitors (tissue inhibitors of metalloproteinases TIMP), and this implies joint damage in RA. Oncostatin M, which induces MMP and TIMP-3, is undetectable in the synovial fluid of OA, but is present in the fluid of RA patients, and its mRNA and protein levels were elevated in synovial cells (Li et al 2001). MMPs are considered the most influential

proteolytic enzymes in RA, and many MMPs and TIPMs are enriched in synovium and synovial fluid with RA (Sun and Yokota 2002).

1.3.2 Adhesion molecules in RA

Heterotypical interactions between chondrocytes and synovial cells are known to occur in RA. Synovial pannus formation is initiated with the recognition and adhesion of synovial cells to chondrocytes and to cartilage matrix, mediated by cadherins and integrins (Ishikawa et al 1994). A complex mechanism of inter-cellular calcium signalling between chondrocytes and synovial cells *in vitro* has been reported by D'Andrea et al (1998). The evidence is that these two cell types *in vitro* develop a cell network, which may result in an increased tissue sensitivity to changes in the extra-cellular environment (D'Andrea et al 1994).

Cell adhesion molecules are important in immune cell functions. They are receptors for soluble molecules, receptors for viruses, and ligands for other cell surface molecules (Haynes et al 1991). CD44 is up-regulated on many synovial cell types in patients with RA, and the level of CD44 present in synovial tissue is related to the degree of synovial inflammation (Haynes et al 1991, Takagi et al 2002). Integrin subunits of $\alpha 5$, $\alpha 6$, $\beta 1$, and $\beta 3$ are not up-regulated in RA synovial tissues (Johnson et al 1993), however, Wang et al (1997) showed rheumatoid synovial fibroblast invasion in cartilage was inhibited by antibodies to $\alpha 4$, $\alpha 5$, αV, and $\beta 1$ integrins. This indicates that integrin receptors have a role in the invasion of cartilage by rheumatoid synovial fibroblasts.

1.3.3 Chondrocyte changes in RA

There have been few studies of the role of chondrocytes in the process of cartilage destruction in RA. Cartilage destruction and synovial inflammation are separately controlled (van den Berg et al 1996). Fujii et al (1999) suggested that RA primarily involves articular cartilage and subchondral bone, and that the initiation of the rheumatoid process is possibly triggered by the autoimmune reaction involving type II collagen in the articular cartilage. T cells are required for immunity against type II collagen (Seki et al 1995) and autoimmune mouse is known to develop a spontaneous destructive arthropathy sharing many features with RA (Koopman et al 1988). Moreover, Zhang et al (1998) showed that link protein can produce a persistent, erosive, inflammatory polyarthritis in mice. Guerassimov et al (1999) described the arthritogenic capacity of link protein and G1 domain aggrecan in patients with RA. The patients also displayed proliferative responses to the G1 domain depletion of KS chains. Chondrocytes are responsible for alterations in the expression profiles of degenerative enzymes, such as MMP-9, as a part of the paracrine interactions of chondrocytes and macrophages (Dreier et al 2001)

Progressive degradation of type II collagen and aggrecan in cartilage is a major feature in both OA and RA joints. Previous research has explored the relationships between matrix synthesis, degradation and mechanical stimulation in chondrocytes from OA cartilage (Millward-Sadler et al 1999, 2000a, 2000b). However, very few studies have been undertaken of chondrocytes from RA cartilage. Normal cartilage is generally resistant to invasion by many kinds of cells and blood vessels. Pathological observations in 150 different RA knee joints by Woolley et al (1997) showed that, within the same specimen, individual cartilage erosion sites are spatially and temporally separated. This observation suggested that rheumatoid cartilage becomes deficient in its resistance to cellular invasion. Local as well as general factors may be involved. Murphy (2002) suggests that the marked regional variations in the production of MMPs in arthritic joints indicate that proteinase synthesis is dependent upon cellular events brought about by locally co-ordinated signals.

1.3.4 Inflammation in RA

Researchers have speculated that the inflammatory pathways in RA joints develop in two stages: the early stage of the disease is mainly mediated by T cells, while the late stage is mediated mainly by pro inflammatory cytokines. Anti-$CD4^+$ T cells treatment showed a trend towards efficacy (Veale et al 1999) but was less effective at the end stage of RA (Firestein and Zvaifler 1990). Recent advances in the treatment of RA, using anti TNF agents (Taylor et al 1999) and the IL-1 receptor antagonist, have suggested that the erosive event in a RA joint occurs at the late stage, dominated by cytokines and proteinases (van den Berg et al 2001). The most extensively studied cytokine in relation to cartilage breakdown is IL-1 (Dinarello et al 1996, 2000). Degradation of proteoglycan by IL-1 has been clearly demonstrated in in vivo studies (Page Thomas et al 1991). Chondrocytes in explant culture can be induced to degrade their surrounding proteoglycan with the application of compounds such as retinoic acid, IL-1 or TNF (Flannery et al 1999). IL-1 induces leukocyte infiltration and cartilage proteoglycan degradation in the synovial joint (Pettipher et al 1986).

Evidence of iNOS expression has been found in the rheumatoid joint. and patients with active RA have raised level of NO breakdown products in blood and urine (Ralston 1997). Recent studies have found that the inhibitory effect of IL-1 on matrix production by cultured chondrocytes is partially mediated by NO (Hickery et al 1998), and that NO activates MMPs in cultured chondrocytes (Murrell et al 1995). It has also been indicated that a NO production around the joint may contribute to the increased bone loss which is an early feature of RA (Gough et al 1994). In addition, iNOS activation may be partly responsible for the increased blood flow associated with joint inflammation in RA (Ralston 1997). Increased numbers of apoptotic synovial lining cells and chondrocytes in the tissue of samples from patients with RA can be seen with closely mirrored iNOS expression (van't Hof et al 2000). The inhibitors

of NO synthase suppress the development of disease in animal models of inflammatory arthritis (McCartney-Francis et al 1993).

1.3.5 Comparing RA with OA

Causative factors leading to the development of OA can be classed either as those that render cartilage abnormal or as those that lead to abnormal loading of cartilage (Brandt et al 1986). Thus mechanical stress of OA chondrocytes is probably more important in the development of OA, whereas RA chondrocytes are exposed to more inflammatory mediators, although the loss of PG is the first sign of pathology in the articular cartilage in both OA and RA joints (Joosten et al 1999). Lark et al (1997) found that similar aggrecan degradation mechanisms of MMPs and aggrecanases may be active in OA and RA, in spite of the contrasting pathological and clinical features of these two conditions. Obviously looking at the synovial tissue in RA joints is of primary importance, however, cartilage destruction in RA joints may proceed through pathways independent of the classic inflammatory ones in the synovial tissue. Looking at the initial site of cartilage or bone destruction in RA-like joints of mice, these appear in the absence of inflammatory cells (Gay et al. 1993). Cartilage and bone erosions occur extensively at sites of synovial attachment, it seems likely that a critical cell-matrix interaction occurs (Gay et al. 1993).

No study has been performed to test the effects of mechanical stimulation on human articular chondrocytes from RA cartilage. Thus it is important to compare the differences in the mechano-transduction pathway between OA and RA chondrocytes after mechanical stimulation. As mechanical loading of RA synovial cells has been shown to down-regulate cytokine MMP-13 genes (Sun and Yokota 2001), and the strain elevates the mRNA level of TIMP-1 and TIMP-2 (Sun and Yokota 2002), there is a possibility that mechanical stimulation effects cell-matrix interaction in RA joints. *In vitro* experiments using human synovial cell cultures also support a stimulus-dependent transcriptional regulation of MMPs and TIMPs (Sun and Yokota 2001).

1.4 Loading of articular cartilage

Changes in pressure on articular cartilage have been measured *in vivo* using a pressure-sensitive pseudo-femoral head (Hodge et al 1986). Within the joint cartilage compressive forces are transient but they can be very high, rising from one to two atmospheres to 100 atmospheres when the body is standing, and oscillate between 40 to 50 atmospheres when walking (Afoke et al 1987). Loading of articular cartilage *in vivo* results from weight bearing and joint movement, and generates a combination of tensile, compressive and shear stress on the material (Kempson et al 1973). Articular cartilage is mainly subjected to compressive loading,

under high hydrostatic pressure. Rises in hydrostatic pressure lead to a number of physico-chemical changes within the cartilage, such as loss of water, change of streaming potentials, and altered pH and osmolality (e.g. increased cation concentrations and decreased anion concentrations) (Urban and Hall 1994).

Loading can be mimicked *in vitro* either by mechanical compression of cartilage explants, or by exposing chondrocyte cultures to hydrostatic pressure or stretching. Numerous studies have examined the effects of loading on articular cartilage composition and metabolism, both *in vivo* and *in vitro*, as mechanical loading is essential to normal joint homeostasis. In animal models, immobilization of a limb, abnormally high joint forces, and ligamentous instability initiate degradation of cartilage and impair joint function (Palmoski et al 1979, Kiviranta et al 1987, McDevitt et al 1988, Pond and Nuki 1973).

1.4.1 Terminology used in mechanical stimulation

In physics, a force is a pulling, or attracting, or pushing effect that something has on something else. Stress is a measure of the force per unit of area, acting on a given surface. Tensile stress causes an increase in the length of a body and compressive stress causes a reduction in the length of a body. Shear stress results when the applied force lies on the plane of the surface. Strain is a measure of deformation per unit of length of a body and a ratio of the deformation to the initial length, see http://en.wikipedia.org/wiki/Stress_(physics).

The unit of stress is Newton (N)/m^2 or Pascal (Pa). 1 kPa is equal to 1000 N/m^2 or 7.5 mmHg.

1.4.2 The effect of loading articular cartilage and chondrocytes *in vitro*

Studies of loading *in vivo* have a number of drawbacks which have lead to the development of systems for cartilage loading *in vitro* (Sah et al 1991). Chondrocyte structure and function can be studied in cartilage explant or in isolated chondrocyte cell culture systems. *In vitro* systems allow real time measurement of physical parameters, such as cell shape and intracellular ion fluxes as well as metabolic labelling during loading (Lee and Bader 1995, Knight et al 1998, 2002, Guilak et al 1994). Loads of known magnitude and frequency can be applied to a defined area of cartilage *in vitro*, in a precisely maintained physico-chemical environment.

However, studies of *in vitro* loading place articular cartilage in a non-physiological environment, exposing the tissue to conditions never experienced *in vivo* in many aspects. For

example, when chondrocytes are isolated from the matrix, and placed in standard tissue culture medium, the extra-cellular osmolality and ionic environment are altered significantly. This process is known to influence cell volume (Urban and Hall 1994). In addition, the concentrations of cytokines and growth factors are altered in the presence of a high concentration of FCS in standard culture medium which could affect the chondrocytes metabolism.

Nevertheless, for investigating the mechanisms of cartilage matrix regulation, *in vitro* studies are the best method of controlling loading. Studies of loading conditions *in vitro* can be divided into three groups: first those looking at the mechanical compression of cartilage tissue explants or chondrocytes cultured in a three dimensional matrix (loading with cartilage or cell deformation), second those looking at the effects of hydrostatic pressure on cartilage explants or chondrocytes in a number of culture systems (compression with no deformation), and third those looking at the effects of stretch on chondrocyte cultures.

1.4.2.1 The effects of mechanical compression on cartilage explants and chondrocyte three-dimensional cultures

Articular cartilage can be easily maintained in explant form in a culture medium, so that the chondrocytes remain in contact with the native cartilage matrix. The use of cartilage explants has the advantage that chondrocytes are maintained in an environment similar to that experienced *in vivo* and cells retain a differentiated phenotype (Handley et al 1990). Explant cultures (*ex vivo*) can be maintained in a steady state for several weeks (Sah et al 1991). An articular cartilage explant culture system has been used to examine how newly synthesized molecules are incorporated into the existing cartilage matrix, and how they are turned over (Campbell et al 1984). The average half-life of PGs, in explants of articular cartilage from different sources, varies from 10 to 25 days (Campbell et al 1984), and this is shorter than that *in vivo* (Maroudas 1980a). Cartilage explants can be subjected to axial compression, hydrostatic pressure, or pure tissue shear. Explant culture is not, however, ideal for detailed studies of chondrocyte function since the cells' physical, chemical, and mechanical environment can not be controlled precisely. Another disadvantage of the explant culture system is that the newly synthesized aggrecan molecules cannot be recovered except using solvents, which cause the dissociation of the aggregation (Campbell et al 1984).

To overcome the disadvantages of other cultures, many researchers have seeded chondrocytes into three-dimensional matrices such as agarose or alginate. In three-dimensional cultures, the cells maintain their morphology and phenotype, and will rebuild their matrices with many of the characteristics of *in vivo* cells (Hauselmann et al 1994). Different groups have used agarose three-dimensional model systems (Buschmann et al 1995, Lee and Bader 1997, Toyoda et al

2003) as the physical properties of agarose are suited to mechanical loading. When chondrocytes are suspended in three-dimensional cultures, such as agarose and alginate, for three to four weeks, they form colonies of cells with 'halos' of matrix surrounding the cells, which is characteristic of chondrocytes (Kolettas et al 1995). These differentiated colonies re-express cartilage markers such as sulphated PG (aggrecan) and collagen type II, IX, and XI (Benya and Shaffer 1982, Bonaventure et al 1994). The culturing of chondrocytes in alginate offers an advantage over culturing the cells in agarose in examining the re-expression of the cartilage phenotype. Differentiated chondrocyte colonies from the alginate can be recovered at different time points and examined for their ability to produce normal biochemical and molecular cartilage markers (Hauselmann et al 1992).

In general, static compression of these cultures leads to reduced matrix PG synthesis over hours or days, but the degree of response is dependent on the amplitude of the applied load (Table 1.3 and 1.4). Burton-Wurster et al (1993) showed that 18-hour long single loading of up to 1.2 MPa resulted in the inhibition of PG and that depressed synthesis remained effective for several hours, while the cartilage explants regained their original weight 90 minutes after the removal of the load. Static compression of explants reduces protein synthesis (Burton-Wurster et al 1993, Kim et al 1994, Wong et al 1997), in particular of fibronectin (Burton-Wurster et al 1993), in contrast to fibronectin accumulation which is stimulated by cyclical impaction of explants (Farquhar et al 1996). Valhmu et al (1998) found that core protein mRNA was elevated after one to four hours of compression of bovine cartilage plugs and this then fell below control levels with longer periods of loading. Sulphate incorporation measured after one hour of compression demonstrated a similar increase. Earlier studies with canine cartilage plugs (Palmoski and Brandt 1984) and human ones (Schneiderman et al 1986) showed reduced $^{35}SO_4$ sulphate incorporation, following two hours and four hours of static compression respectively. Dynamic mechanical compression of chondrocyte and three-dimensional construct leads to increased matrix PG synthesis, although this varies with the frequency of the loading cycle (Table 1.4).

Recently Grodzinsky et al (2004) have shown that low amplitude dynamic compression on cartilage explants transiently up-regulated anabolic genes and then down-regulated them during a period of 24 hours, while catabolic genes, such as IL-1β, TNF-α and TGF-β, were increasingly up-regulated with time. This suggests that at early time points, there may be apparent differences between gene and protein expression. In addition, a single application of injurious compression of 60 per cent 20 MPa caused significant increases in MMP-3 and ADAM TS-5 mRNA expression over controls during periods of 6 to 12 hours, but less change to MMP-13 and other molecules (Grodzinsky et al 2004). Cultures in three-dimensional agarose gels responded to continuous compression by reducing PG (Buschmann et al 1995, Lee and Bader 1995) and protein synthesis (Buschmann et al 1995).

study (cartilage explants)	pressure (MPa)	compression amplitude (%)	loading time (hours)	compressed/ non-compressed PG synthesis
Jones et al (1982)	2.9		96	0.5
Palmoski and Brandt (1984)	0.006		2	0.3
	0.011		2	0.52
	0.06		2	0.35
Gray et al (1988)	0.1		12	1.1
	0.3		12	0.9
	0.5		12	0.8
	1.0		12	0.5
	1.5		12	0.3
	2.0		12	0.2
	2.5		12	0.1
	2.8		12	0.1
Sah et al (1989)	0.2		12	0.8
	0.5		12	0.3
Larsson et al (1991)	1.0		24	0.8
	1.0		48	0.5
	1.0		72	1
Kim et al (1994)		33	12	0.88
		50	12	0.9
		65	12	0.5
		77	12	0.48
		85	12	0.18
Burton-Wurster et al (1995)	0.025		18	0.53
	0.5		18	0.21
	1.2		18	0.18
Wong et al (1996)		40	23	0.7

Table 1.3 The effects of static mechanical load on cartilage explants

Results are expressed as the ratio compressed/non-compressed for total $^{35}SO_4$ incorporation

study (3D)	pressure (MPa)	compression (%)	cycle (Hz)	loading time (hours)	results	3D
Buschmann et al (1995)		25		16 (2 days culture)	1.00	agarose gel
		25		16 (41 days culture)	1.10	
		35		16 (2 days culture)	1.00	
		50		16 (2 days culture)	0.95	
		50		16 (41 days culture)	0.60	
		60		16 (41 days culture)	0.50	
Lee et al (1995)		15		48	0.9	
Ragan et al (2000)		60		22 (21 days culture)	0.75	alginate gel
		80		22 (21 days culture)	0.65	
		100		22 (21 days culture)	0.75	
Mizuno et al (2002)	2.8		0.015	120	1.4	collagen sponge
	2.8		0	120	1.3	
Lee et al (1997)		15	0	48	0.9	agarose gel
		15	0.3	48	0.85	
		15	1	48	1.4	
		15	3	48	1	
Demarteau et al (2003)		5	0.1	12 (3 days culture)	0.09 ~ 2.3	poly active foams
		5	0.1	12 (14 days culture)		
Davisson et al (2002)		10		24	0.75	non-woven, poly-glycolic acid composite
		30		24	0.95	
		50		24	0.4	
		10	0.001	24	1.5	
		10	0.1	24	1.25	
		50	0.001	24	1	
		50	0.1	24	2.8	
Toyoda et al (2003)	5			4	1.1	agarose gel
	5		1	4	0.95	
	5			20	1	
	5		1	20	0.9	

Table 1.4 The effects of mechanical compression of chondrocytes in three-dimensional cultures

Results are expressed as the ratio of compressed/non-compressed for total $^{35}SO_4$ incorporation.

Very few studies have investigated the effect of mechanical compression on cartilage collagen synthesis due to its low turnover rate (Muir 1973).

A number of experimental approaches have demonstrated that cartilage cells react to confined and unconfined compressive loading under *in vitro* testing conditions depending on time, magnitude, and frequency of loading (Gray et al 1988). Static compression inhibits GAG synthesis but repetitive loading of cartilage has produced variable responses depending on the range of forces applied, the technique for applying the forces, and the frequency of loading (Veldhuijzen et al 1987). Comparing static and dynamic compression, the tissue biosynthetic response depended on amplitude and frequency of loading (Sah et al 1989).

1.4.2.2 The effects of hydrostatic pressure on cartilage explants and chondrocytes in different culture systems

Articular cartilage is a unique tissue where hydrostatic pressure is a significant component of the mechanical loading environment (Ikenoue et al 2003). Cartilage, which is mainly subject to compressive loading, experiences particularly high hydrostatic pressures and alterations to its osmotic environment (Urban and Hall 1994). Hydrostatic pressure itself induces no fluid flow or tissue deformation, since water is virtually incompressible up to 50 MPa (Parkkinen et al 1994). Little is known how chondrocytes sense changes in hydrostatic pressure. Likely sites for sensors includes cell membranes because hydrostatic pressure spreads in the intra- and extra-cellular fluids equally. It is possible that there are several 'receptors' or 'monitors' activated at different levels of hydrostatic pressure including integrins. *In vivo* testing shows that chondrocytes within cartilage experience hydrostatic pressure levels of 0.1 to 20 MPa (Ikenoue et al 2003). Relatively few studies have examined the effects of hydrostatic pressure on articular cartilage or chondrocytes. However, the results for cyclic and static hydrostatic pressurisation are broadly in agreement with those seen following mechanical compression with deformation, which may imply that hydrostatic pressure is the key signal. Cyclical pressure stimulated PG synthesis in cartilage explants (Parkkinen et al 1994, Hall et al 1991, Lafeber et al 1992) and chondrocyte cultures (Parkkinen et al 1994, Kampen et al 1985), but the response was sensitive to the magnitude and frequency of the stimulus. Hall et al (1991) have found that the influence of hydrostatic pressure on incorporation rates of 35SO4 and [3H]proline into adult bovine articular cartilage slices *in vitro* depends on the pressure level and varied with the duration of the signal. Organ culture experiments of Klein-Nulend et al (1987) demonstrated that sites of proteoglycan production coincide with regions of pure hydrostatic pressure. Physiological levels of intermittent hydrostatic pressure enhanced mRNA signal levels for aggrecan and type II collagen when applied for four hours (Smith et al 1996). Up-regulation of aggrecan mRNA occurs when load-controlled intermittent hydrostatic pressure was applied (Ikenoue et al 2003). Changes in hydrostatic pressure are clearly important in regulating chondrocyte function.

1.4.2.3 The effects of stretch on chondrocyte cultures

Monolayer cultures are associated with some loss of chondrocyte phenotype over the culture period, and a result of the 'passage' (Aigner and McKenna 2002). Removal from the cartilage matrix and attachment to the tissue culture dishes results in changes of cell shapes and cell volumes, losses of cell-matrix interactions and alterations in the chemical and osmotic environments (Wong and Carter 2003). All of them may define the cells' phenotype *in vivo*.

In monolayer culture, chondrocytes undergo a gradual (a few weeks) dedifferentiation, characterised by a change in their spherical shape into a fibroblastic appearance (Benya et al 1978). At the same time, they replace the synthesis of cartilage specific collagen type II IX XI and aggrecan with molecules that are normally expressed by fibroblasts or pre-chondrocytes. These include collagen I, III and V (Benya et al 1978, von der Mark 1977) and dermatan sulphate containing PGs, small molecular weight PG and versican (Liu et al 1998). Culture systems such as agarose and alginate gel reduce the effects of chondrocyte isolation on phenotype by suspending the cells in an artificial matrix in which their shape is maintained and in which they do not form abnormal cell-substrate attachments. These methods are preferable to monolayer culture, but are not practical for techniques, such as those used in studies of cell stretching, that require cells to be immobilised on a substrate. Where it is essential to use a monolayer culture of chondrocytes, phenotypic changes can be kept to a minimum by using primary cultures which have been developed for only short periods of time (Thonar et al 1986). Recent studies show that the expression by chondrocytes of cartilage-specific molecules, such as type II collagen, remains in monolayer culture for a long time, despite other changes that indicate loss of phenotype (Kolettas et al 1995).

Chondrocytes can be grown on flexible membranes or cover-slips and then exposed to stretching by deformation of the culture substrate, which mimics the deformation of the cartilage substrate under load *in vivo*. This tension leads to direct deformation of the chondrocytes, similar to the distortion of the collagen network which occurs with a direct compressive load (Parkkinen et al 1994). Both intermittent stretching (Uchida et al 1988, de Witt et al 1984) and continuous stretching (Lee et al 1982) cause significant increases in sulphate incorporation, as well as increased type II collagen mRNA and PG core protein mRNA levels (Holmvall et al 1995). Fluid induced shear stress on chondrocytes, equivalent to a force of 1.6 Pa on the cell membrane, induces a two fold increase in $^{35}SO_4$ sulphate incorporation (Smith et al 1995) a 10 fold increase in IL-6 and TIMP-1 (Smith et al 1994), and a three to four fold increase in MMP-9 (Jin et al 2000). Exposure of fibro-chondrocytes to cyclic tensile strain in monolayer culture results in abrogation of the catabolic effects of IL-1β; a cyclic tensile strain of 6 per cent up-regulated PG synthesis and inhibited NO production (Xu et al 2000, Agarwal et al 2001). Interestingly, Matsukawa et al (2004) reported that PG synthesis was enhanced by cyclic tensile strain when chondrocytes were attached to a

fibronectin-coated surface. They also found that cyclic tensile strain decreased PG synthesis when chondrocytes were seeded on type I collagen coated surface. These data suggest that the response of chondrocytes to mechanical strain is influenced by the extra-cellular matrix.

1.5 Mechano-transduction

Many tissues use mechanical forces to control the homeostasis of their own extra cellular matrix through inducing cell shape change, cell differentiation, and cell growth (Chiquet et al 1999, Tagil et al 1999). The process by which mechanical loading influences the metabolism of chondrocytes and the synthesis of extra-cellular matrix is termed mechano-transduction. Cells transduce physical force induced signals into biochemical responses, which are critical for the adaptation of cells to mechanical loading (Ko et al 2001). This capability provides articular cartilage which can alter its structure and composition to meet the physical demands of the body. Mechanical stress (stimulation) selectively modulates the biosynthesis and retention of certain cartilage components. The synthesis of link proteins is much less affected by static loading than are aggrecan and small PGs (Kim et al 1996). Little et al (1997) have demonstrated that strenuous exercise in horses can lead to a disturbance in the metabolism of PGs by chondrocytes in articular cartilage regions subjected to high contact stress. These metabolic changes are not associated with widespread gross or histo-morphological evidence of cartilage degeneration.

1.5.1 Integrins as mechano-transducers

Control of chondrocyte function is central to the maintenance and integrity of the structure and organisation of cartilage. Cell and matrix interactions are important in the regulation of chondrocyte metabolism. Integrins are considered as molecules that are necessary for adhesive interactions between cells and the extra-cellular matrix, regulating cell adhesion, cell growth and cell motility. Integrins can also function as signal transducers that regulate gene expression and cellular growth. They are now particularly recognized as mechano-transducers (Shyy et al 1997).

1.5.2 Integrin mediated mechano-transduction

Integrins are families of cell-surface receptors that link the extra-cellular matrix to the cellular cytoskeleton, at places called focal adhesion sites (Hynes 1992, Schwartz 1995, Juliano 1993). Integrins are composed of α and β subunit heterodimers that consists of a large extra-cellular domain, a transmembrane region, and usually a short cytoplasmic domain. The extra-cellular domain binds either the extra-cellular matrix or the counter receptors on other cells. The

cytoplasmic domain forms links with cytoskeletal proteins and intra-cellular signalling molecules such as α-actinin and FAK (Hynes 1992, Lewis 1995).

One theory about the mechanism of integrin-mediated signalling is that integrins transmit signals by organising actin cytoskeleton (actin stress fibres) through intermediary molecules including α-actinin, talin, vinculin, paxillin, and tensin, thereby stabilizing cell adhesion and regulating cell shape, morphology, and motility (Ingber 1991, Juliano 1993). In accordance with this view, Wang et al (1993) showed that: (1) β1 integrin induced focal adhesion formation and support a force-dependent stiffening response; (2) an increase in the cytoskeletal stiffness requires an intact cytoskeleton. Their result suggests that mechanical stress is first received by integrins. Then, inter-linked actin micro-filaments transduce mechanical stress in concert with micro-tublins and intermediate filaments. Finally, this mechanism modulates gene expression. It is possible that intermediate filaments may transmit mechanical stress to the chromatin. Integrin-induced focal adhesions are clusters of integrins and associated proteins. Comparing stationary and migrating cells, focal adhesions are shown to move and be regulated by a clutch-like mechanism (Smilenov et al 1999).

Recently, integrins have been regarded as true receptors, capable of inducing biochemical signals within the cells that regulate gene expression and cellular growth. Upon clustering at focal adhesion sites, the integrins recruit the non-receptor kinases such as FAK and Src, cytoskeletal proteins, and signal transducing molecules such as Grb2, SOS, Ras, Raf, PLC-γ, and initiate the Ras and ERK signal transduction pathway (Parsons et al 1997, Juliano et al 1993, Clark et al 1995, Shyy et al 1997. Miyamoto et al 1995). The ability of tyrosine kinase inhibitors to inhibit the formation of focal adhesions suggests a role for tyrosine phosphorylation in the signalling pathways linked to integrin receptors (Burridge et al 1992). FAK appears to play a central role in integrin-mediated signal transduction. Integrin-mediated phosphorylation of FAK requires the cytoplasmic domain of the β integrin subunit (Schaller and Parsons 1994). It has been shown that FAK can bind to peptides from β1, β2 and β3 subunits. However, FAK cannot be co-immuno-precipitated with these subunits, suggesting that either the interaction is weak, or there is no direct association during the localization of FAK (Dedhar and Hannigan 1996). In these complexes, signalling proteins and their substrates are brought into close proximity, thereby facilitating signal transduction. The targeting of FAK to focal adhesions appears to involve multiple binding interactions. Integrins may induce the activation of FAK with the help of Src (Parsons et al 1997), which may lead to activation of the ERK pathway through Grb2-SOS-Ras or through activation of PLCγ (Shyy et al 1997). Integrins can also collaborate with growth factor receptors and their substrates to phosphorylate their receptor kinases, such as the serine-threonin kinase families (e.g. protein kinase C (PKC)) and mitogen activated protein (MAP) kinases, and to activate ERKs and JNKs upon ligand binding (Plopper et al 1995). Some inhibitors of PKC block cell attachment and spreading (Vuori and Ruoslahti 1993). PKC also seems to play a role in the formation of focal adhesions, and the association of PKC activation with FAK tyrosine

phosphorylation has been reported (Vuori and Ruoslahti 1993). Activation of MAP kinase kinase appears to lie downstream of increases in PKC activity, seen following mechanical stimulation (Watson and Krupinski 1994, Jalali et al 2001). Thus integrins may integrate a variety of different signalling pathways that are activated by both the extra-cellular matrix, and growth factors, to establish a well co-ordinated response. (Figure 1.3).

Integrins may integrate a variety of different signalling pathways that are activated by both the extra-cellular matrix and growth factors to establish a well co-ordinated response. Besides integrins, the extra-cellular matrix proteins, ligated to the integrins such as collagens, laminin, fibronectin, and vitronectin (Hynes 1992), also play a role in signal transduction. Furthermore, it was found that besides integrins, heparan sulphate proteoglycans are involved in the formation of focal adhesions and the actin stress fibres act co-operatively with integrins in generating signals in fibroblasts, plated on fibronectin (Delehedde et al 2001).

1.5.3 Ion channels in mechano-transduction

The first identified mechano-transducing activity in cells was a stretch-activated ion conductance (Watson and Krupinski 1994). Cytoskeletal elements are necessary for ion channel responses. The disruption of actin micro-filaments inhibits ion channel responses (Davies and Tripathi 1993). Almost all cells express stretch-activated ion channels (Gudi and Frangos 1994). This particular class of ion channels remains up to now partially unidentified, although it was the first identified cellular mechano-transducing function. Identification of downstream effects, regulated by these channels, is limited to correlative biological responses. Activation of mechano-sensitive ion channels has been proposed as a transduction mechanism between mechanical stress and cell. These stretch-activated channels allow passage of ions like Na^+, K^+ and Ca^{2+} (Ruknudin 1993). Direct Ca^{2+} influx through stretch-activated channels has been reported in cultured chick heart cells, that were stimulated by prodding with a pipette (Sigurdson 1992) Calcium ion is a ubiquitous intra-cellular second messenger involved in a large number of cellular processes. The findings suggest that mechanical perturbation leads to transient increases in intra-cellular Ca^{2+} in various cell types (Guilak et al 1994). Chondrocyte deformation, in the absence of matrix-related effects, initiates an intra-cellular biochemical signal, suggesting that the first signal is transduced through mechano-sensitive ion channels on the plasma membrane. However, the involvement of stretch-activated channels in the transduction of the mechanical stress stimulus into the nucleus is still controversial. Several studies failed to confirm that gadolinium inhibits the stretch-induced expression of 'immediate early genes' such as c-fos and protein synthesis (Ruwhof 2000). ('Immediate early genes' are activated very soon after the imposition of mechanical challenge, and they are involved in conveying signalling information and subsequently co-ordinating the adaptive response) (Watson and Krupinski 1994).

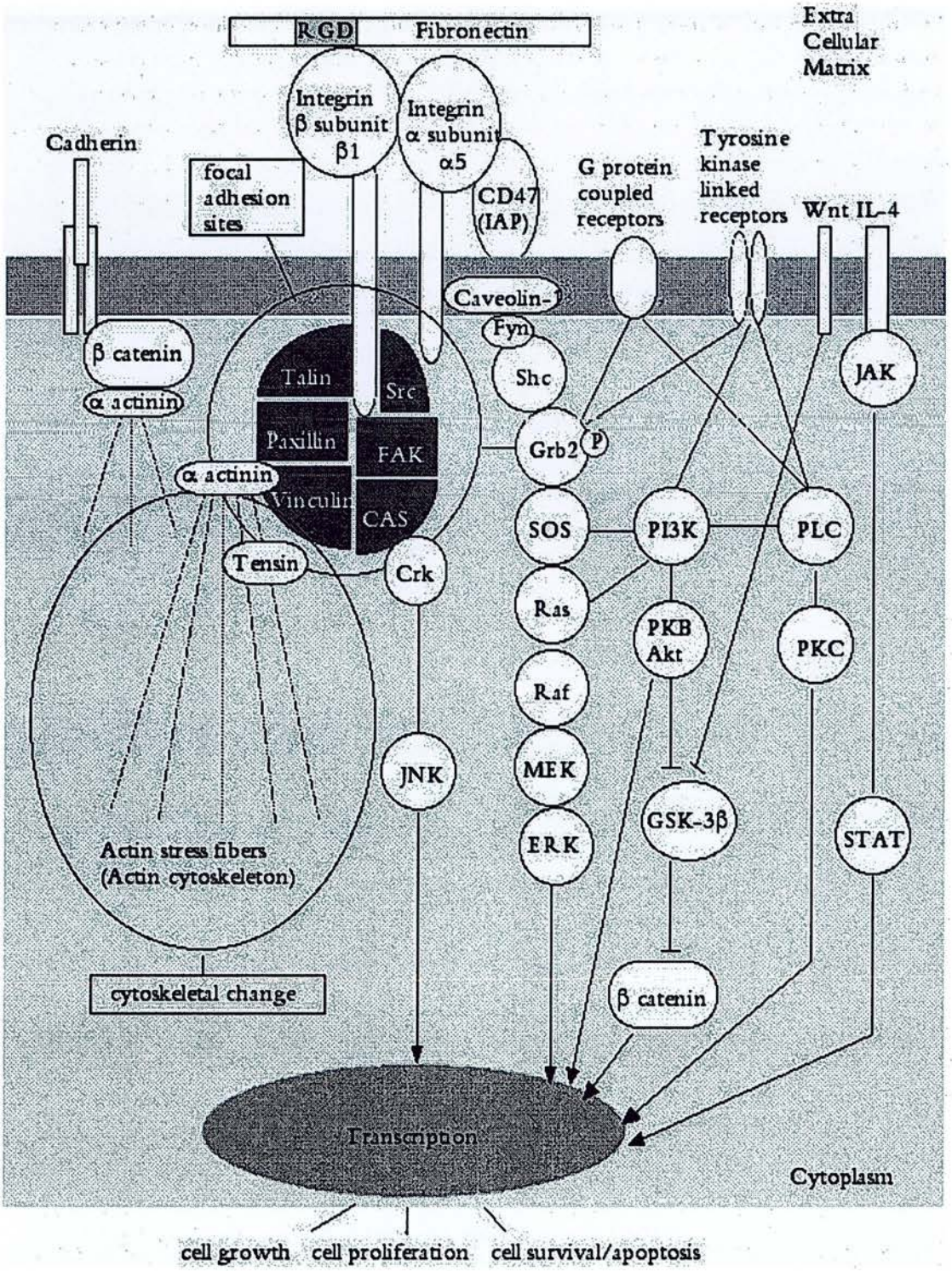


Figure 1.3 Diagram of possible mechano-transduction pathways

An important consequence of the activation of K^+ channels is depolarization of the cell, a phenomenon that has been confirmed using membrane potential sensitive dyes, as well as patch-clamping measurements (Nakache and Gaub 1988). The membrane hyperpolarization response that follows 0.33 Hz mechanical stimulation was shown to be inhibited by quinidine, indicating involvement of Ca^{2+} activated K^+ channels leading to the efflux of K^+ from the cell (Wright et al 1997). These are apamin-sensitive charybdotoxin and iberiotoxin-resistant small conductance Ca^{2+} activated K^+ channels (Wright et al 1992). The depolarization response does not involve activation of these ion channels, but rather it is the result of the activation of the tetrodotoxin-sensitive Na^+ channels (Wright et al 1992).

1.6 Integrins

Integrins are families of membrane-spanning glycoproteins that mediate cell adhesion to the extra-cellular matrix, the basement of membranes, other cells, and plasma proteins (Hynes 1987, Ruoslahti 1987). Integrins are also exist in the cartilage matrix (Shakibaei and Merker 1999). The prototype integrin is the fibronectin receptor originally purified from human placenta (Pytela et al 1986) and from human osteosarcoma cells (Pytela et al 1985). The name of the integrins came from their function of linking the matrix to the cytoskeleton (van der Flier and Sonnenberg 2001). The basic structure of the integrins is that of a heterodimeric complex of one 130 to 200 KDa α subunit, linked non-covalently to one 90 to 130 kDa subunit. Up to now, 19 α and 8 β subunits have been identified, making 25 different dimers (Table 1.5).

1.6.1 Structure of integrins

Integrins are comprised of one ligand binding globular, which is approximately 10 nm in diameter, and two extended tails (Springer 1997). Most integrins have a large extra-cellular domain and a much shorter cytoplasmic domain (van der Flier and Sonnenberg 2001) (Figure 1.4). The exception is the $\beta4$ subunit which has a long domain, and certain β subunits, such as $\beta1$, $\beta3$ and $\beta4$, that can undergo alternative splicing of the cytoplasmic domain. This may play a role in the regulation of integrin signalling (Giancotti 1999). For example, the long cytoplasmic tail of integrin $\beta4$ allows $\alpha6\beta4$ to recruit Shc, and the $\beta4$ tail is phosphorylated on tyrosine residues, binding to Shc directly (Giancotti 1999). There are also differences in signalling that arise from variations in the $\beta1$ cytoplasmic tail generated by alternative splicing (Fornaro and Languino 1997).

	β 1	2	3	4	5	6	7	8
α 1	■							
2	■★							
3	■							
4	■★						○	
5	■							
6	○			■★				
7	○							
8	○							
9	○							
10	○							
11	○							
D		○						
E							○	
L		○						
M		○						
V	■		■★★		■	○		○
X		○						
IIb			○					
IELb							○	

○= exist

■= expressed in articular cartilage (Salter et al 1992)

■★= neoexpression in OA cartilage (Ostergaard et al 1998)

■★★= over expressed in OA

Table 1.5 Integrin subunits

The α subunits are sub-divided into two groups based on structural differences. The first group is formed by α1, α2, αL, αM and αX. The members of the first group all contain an inserted domain (I-domain). This domain of about 180 amino acids is situated between the second and the third repeat (Loftus and Liddington 1997). The function of the I domain is probably to do with ligand binding when it is associated with an I-like domain in the β subunit region (Takagi et al 2002).

The second group is formed by α3, α5, α6, α7, α8, αIIb, αV and αIEL. Members of this group all share a post-translational cleavage of their precursors into heavy and light chains. The light chain is composed of the cytoplasmic domain, the transmembrane region, and a part of the extra-cellular domain (about 25 kDa), while the heavy chain contains the rest of the extra-cellular domain (about 120 kDa). All the RGD-reactive integrins are relatively closely related and belong to this group. They do not all contain I domains in the α subunit (Hynes 1992). A schematic diagram of α5β1 integrin is shown in Figure 1.4.

1.6.2 Function of integrins

The β cytoplasmic domains play a critical role in a variety of cellular processes including endocytosis (van Nhieu et al 1996), cross talk between integrins (Blystone et al 1995), assembly of fibronectin fibrils (Wu et al 1995), and cell motility (Pasqualini and Hemler 1994). The α subunit cytoplasmic domain is known to inhibit certain function of the β subunit (Burridge and Chrzanowska-Wodnicka 1996). It also influences cell motility (Bauer et al 1993). It is becoming clear that integrin signalling is determined by both α and β subunits.

Ligand binding of integrins is regulated by inside-out and outside-in signalling leading to the conversion of integrins from a low to a high affinity and avidity. Affinity modulation implies a structural change intrinsic to the integrins that results in a greater strength of ligand binding, and avidity modulation implies a change in the functional affinity of the interaction between integrins and ligands (Neri et al 1996). Integrins typically exhibit relatively low affinities for their ligands (dissociation constants K_D between 10^{-6} and 10^{-8} moles per litre) compared with the high affinities (K_D between 10^{-9} and 10^{-11}) of typical cell surface hormone receptors (Lodish et al 1995). Cells can rapidly change integrin function by altering the binding affinity of integrins for ligands (Schwartz et al 1995). Outside-in signalling is initiated by integrin ligation and clustering, and they regulate cell functions and behaviour in nearly every cell type. Ligation of integrins specifically blocks apoptosis indicating that integrin specific signals may be involved (Schwartz et al 1995, Pulai et al 2002), such as collagen and β1 integrin that reduce chondrocyte apoptosis in vitro (Cao et al 1999). Integrin-mediated signalling must also be co-ordinated with signals from other plasma membrane receptors, such as growth factors

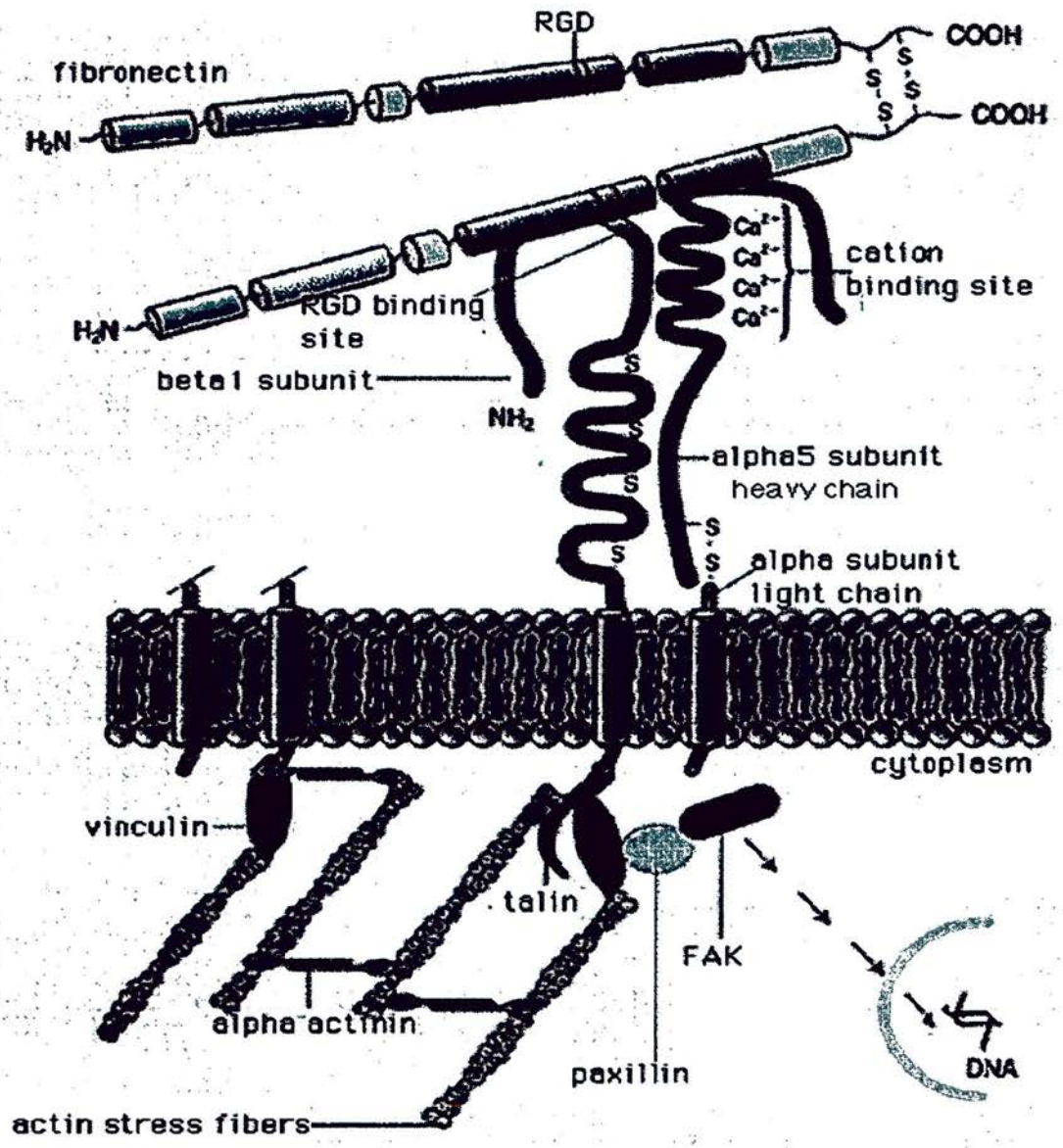


Figure 1.4 Schematic structure of alpha5beta1 integrin
(see http://cella.cn/book/10/01.htm)

receptors and G-protein linked receptors (Juliano 1993). Integrins can exist in various affinity states for their ligands (Hughes et al 1998). The α and β pairings specify the ligand binding abilities of the integrin heterodimers (Ruoslahti 1996).

1.6.3 Expression of integrins in human articular cartilage

A variety of integrins are expressed in human articular cartilage. The α5β1 integrin is prominent in adult human articular cartilage. This has been demonstrated by Salter et al (1992) by using immuno-histochemical staining. Expression of the α1β1 and α3β1 is variable and weaker than α5β1 (Salter et al 1992). Using a combination of immuno-fluorescence and immuno-precipitation, Woods et al (1994) showed that adult human articular chondrocytes express α1β1, α5β1 and αVβ5 integrins accompanied by a weaker expression of α3β1. Fetal chondrocytes and chondrosarcoma cells show higher levels of α3β1 and α6β1 integrins (Salter et al 1995, Holmvall et al 1995). With regard to OA cartilage, Loeser et al (1995) have shown that there is a particular increase in expression in α1β1 OA-like cartilage in monkey, and Ostergaard et al (1998) have demonstrated an increase in expression of α2β1 in human femoral head OA cartilage. In addition, only occasional α3, and no α2 or α4 at all, has been observed (Ostergaard et al 1998), and αV was seen in the superficial layer (Woods et al 1994) (Table 1.5).

1.6.4 Integrins and ligands

Integrins can adhere to an array of ligands. Common ligands are, for example, fibronectin and laminin, which are both part of the extra-cellular matrix or basal laminas. Both of the ligands mentioned above are recognized by multiple integrins. For adhesion to ligands, both integrin subunits are needed, as well as the presence of cations (Hynes 1992).

The α1β1 integrin is a receptor for collagen, and α5β1 receptor serves as the primary chondrocyte fibronectin receptor. They recognize fibronectin via an RGD sequence (Loeser et al 1995). The αVβ5 integrin is a receptor for vitronectin via an RGD sequence (Loeser et al 1995). Also, αVβ3 is a receptor for vitronectin in addition to thrombospondin, von Willebrands factor and fibrinogen (Hynes 1987). The β1 integrin attach to fibronectin, matrix Gla protein, and type II collagen, indicating that β1-containing integrins are the primary receptors in chondrocytes (Loeser 1994). A number of integrins mediate the binding of a cell to laminin. For example, α6β1, α7β1 and α3β1 have been shown to associate with proteins of the C-terminal globular domain of the laminin A1 chain (Nakahara et al 1996). It is likely that

the integrin and ligand interactions have not been fully described, and a potential exists for additional interactions to be discovered.

1.7 Cell membrane integrin-associated proteins

Integrins form multi-receptor complexes with other receptors on the same cell, with neighbouring cells and with molecules in extra-cellular matrix. These complexes recruit signalling molecules and result in focal complexes and focal adhesions. Integrins appear to co-operate with other cell surface receptors to influence a variety of signalling pathways. One of the receptors known to be associated with integrins is CD47 (Porter and Hogg 1998).

1.7.1 CD47 or integrin associated protein (IAP)

CD47 associates with integrins physically and functionally. Up to now, CD47 has been known to associate with the platelet fibronectin receptor αIIbβ3, the RGD receptor αVβ3, and the collagen receptor α2β1 integrins (Lindberg et al 1993, 1994, Gao et al 1996, Hermann et al 1999). In addition, recent studies have revealed that there is a physical association between CD47 and the α5 integrin in human articular chondrocytes (Orazizadeh 2004).

1.7.1.1 The structure of CD47

CD47, or integrin associated protein (IAP), was discovered originally as a plasma membrane molecule that co-purified with the integrin αVβ3 from leukocytes and placenta (Brown et al 1990). IAP is widely expressed in hematopoietic cells including erythrocytes, lymphocytes, platelets, monocytes, and neutrophils (Rosales et al 1992, Mawby et al 1994). Later, after its cDNA was cloned, antibodies that recognized IAP were shown to recognize CD47 (Lindberg et al 1994), demonstrating that the IAP and CD47 were the same molecules.

CD47 is a unique Ig superfamily member with an IgV (variable) extra-cellular domain, five membrane-spanning segments, and a short cytoplasmic tail with four alternatively spliced forms (Lindberg et al 1993, Reinhold et al 1995, Brown and Frazier 2001). The four isoforms (Form 1-4) of CD47 is shown in Figure 1.5.

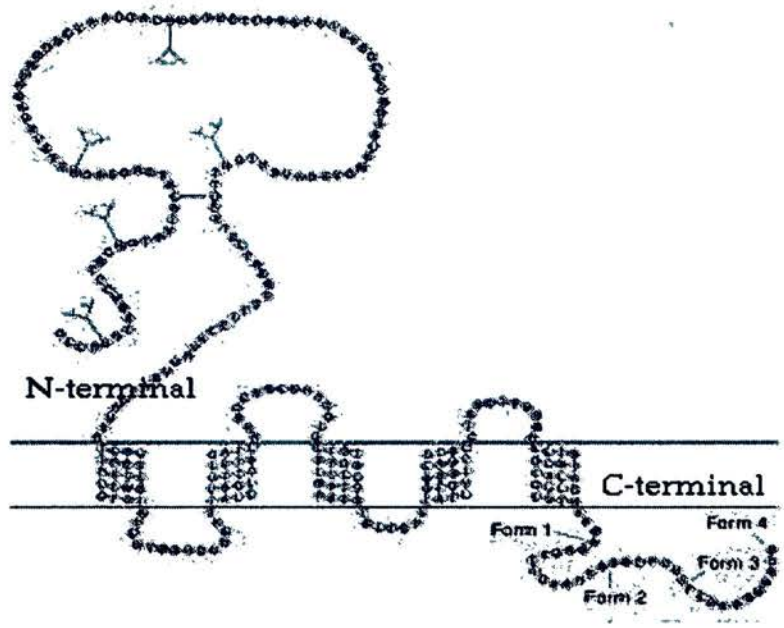


Figure 1.5 Structure model of CD47

The extra-cellular N-linked glycosylation sites and the Ig variable extra-cellular domain are followed by five transmembrane segments terminating in a cytoplasmic tail that is alternatively spliced (adapted from Brown and Frazier 2001).

CD47 is expressed at varying levels in a variety of cells and in almost all the tissues. However, the four alternatively spliced isoforms of CD47 mRNA have different tissue distributions. For example, the thymus and the spleen express predominantly Form 2 mRNA, while the brain expresses Form 4. The most abundant CD47 splices *in vivo* are Form 2 and 4 (Reinhold et al 1995). CD47 is strongly expressed on the plasma membrane of chondrocytes in all cartilage zones in normal and OA human articular cartilage (Orazizadeh 2004). The results of the analysis of the expression patterns of CD47 by western blotting suggest that the molecule may exist in more than one form in human articular cartilage (Orazizadeh 2004).

1.7.1.2 Functions of CD47

CD47 is reported to be involved in a number of cellular and molecular functions. Anti-CD47 monoclonal antibody B6H12 inhibits neutrophils trans-endotherial migration, chemotaxis to RGD-containing proteins, and adhesion to entactin (Cooper et al 1995). In addition, anti-CD47 antibody inhibits an increase in intra-cellular calcium concentration when endothelial cells are spread on vitronectin in an αV-dependent manner (Schwartz 1993). Anti-CD47 antibody can block integrin stimulated phagocytosis (Blystone et al 1995), and trans-epithelial

migration of polymorphonucleus (Parkos et al 1996). CD47 knockout mice have a severe host defence defect (Lindberg et al 1996). Salter et al (1997) have shown that anti-CD47 antibody (Bric 126) inhibited a hyperpolarization response in human bone cells following 0.33 Hz cyclical mechanical stimulation. Ticchioni (1997) have shown that signals via CD47 in T cells induce a tyrosine phosphorylation. Similarly, Orazizadeh (2004) showed that the tyrosine phosphorylation response of FAK following mechanical stimulation was absent in the presence of Bric 126 in human articular chondrocytes. However, very little study has been done to explore CD47 functions in human articular chondrocytes.

1.7.1.3 CD47 and integrin interactions

CD47 forms a physical complex with integrins. A blockage of CD47 with monoclonal antibodies shows an inhibition of certain levels of integrin signalling (Lindberg et al 1996). CD47 and integrin interactions initiate a signalling pathway to up-regulate integrin function (inside-out signalling) (Blystone et al 1995, Gao et al 1996). Moreover, once CD47 binds with its ligand thrombospondin, the affinity and/or avidity of αVβ3 integrin is increased. For example, the presence of thrombospondin or its agonist peptide causes tyrosine phosphorylation (Gao et al 1996, Frazier et al 1999, Green et al 1999). Another example is that the binding of CD47 to thrombospondin activated αIIbβ3 integrin in platelet, results in the spreading and aggregation of the platelet, and the enhanced tyrosine phosphorylation of focal adhesion kinase (Chung et al 1997). Fujimoto et al (2003) reported that CD47 and thrombospondin-1 binding interacted with αIIbβ3 integrin, changing integrin to a higher affinity state without the requirement of intra-cellular signalling.

Although the association of CD47 with certain integrins has been found to modulate their functions, some signal transduction through CD47 is integrin independent (Cooper et al 1995, Frazier et al 1999, Reinhold et al 1997, Liu et al 2001). Previous researchers have used a panel of function-blocking monoclonal antibodies to integrins, but they did not observe any significant effects on CD47-mediated regulation. There is a possibility that CD47 is involved in chondrocyte mechano-transduction in an integrin independent manner.

1.8 Aims of the Project

Previous studies have shown that human articular chondrocytes from normal, but not from osteoarthritic cartilage, respond to cyclical mechanical stimulation in an in vitro model system, with an integrin-dependent increase in aggrecan mRNA. This response was absent in OA chondrocytes (Millward-Sadler et al 2000a, 2000b). It remains unclear whether these changes at the mRNA level are associated with altered levels of proteoglycan synthesis and whether there was indeed differences in proteoglycan production by normal and OA chondrocytes in

response to mechanical stimulation. This has led to the hypothesis that chondrocytes from articular cartilage of normal or diseased (RA and OA) joints show differences in proteoglycan production following mechanical stimulation that may be influenced by signalling via integrins and the integrin-associated protein CD47.

Therefore specific aims of this study were

(1) to identify whether the relative increase in aggrecan mRNA following mechanical stimulation is translated into increased aggrecan production in normal chondrocytes,

(2) ascertain whether chondrocytes derived from OA and RA joints show an altered PG synthesis response (either no change or decrease) following mechanical stimulation,

(3) assess whether integrin receptors and the integrin associated protein CD47 are involved in any changes in responses to mechanical stimulation in OA, and RA chondrocytes.

Chapter Two

Materials and Methods

All chemicals used throughout this study were provided by Sigma-Aldrich Ltd. (Poole, Dorset, UK) unless otherwise stated. Anti-integrin antibodies used in this study are listed in Appendix.

2.1 Tissue sources and handling

All human articular tissues were obtained at the time of joint replacement surgery or above knee amputation due to peripheral vascular disease. Patients details are shown in each chapters. All tissue culture procedures were carried out in a lamina flow cabinet using aseptic techniques, utilising sterile, disposable plastic pipettes and pipette tips. Laboratory coat, gloves and over-sleeves were worn when handling biological tissue. If a given tissue was found to be infected, the joint was incinerated and the hood was fumigated.

2.2 Assessment of cartilage

The articular cartilage surface was assessed and graded macroscopically for the presence or absence of OA and the severity of OA using the grading system of McElligott and Collins (1960) (Appendix). Cartilage was also assessed microscopically following safranin O and Hematoxylin-and-eosin staining. Histological grading schemes were according to OARSI Osteoarthritis Cartilage Histopathology Grade Assessment (Pritzker et al 2005, Figure 2.1), modified Mankin scoring system. Two sets of full thickness pieces of cartilage were dissected from grade of each knee. One set was snap frozen in liquid nitrogen immediately and stored at –80°C for frozen sections. Another set was fixed in four per cent formalin for embedding in paraffin wax for paraffin sections. 4 μm sections were cut with a Bright cryostat. Sections were mounted on PLL coated glass slides and fixed with acetone (Fisher Scientific, Loughborough UK) for 20 minutes at -20°C. Sections were stained with hematoxylin blue (Fisons, USA) for 10 minutes, washed with water, stained for three minutes with fast green diluted 1:5000 in water, washed in 1 per cent acetic acid, then stained with 0.1 per cent safranin O for five minutes. Sections were then dehydrated in ascending grades of alcohol (64 per cent, 74 per cent, absolute), cleared in xylene (Genta Medical, UK) and mounted in pertex (Cellpath, UK).

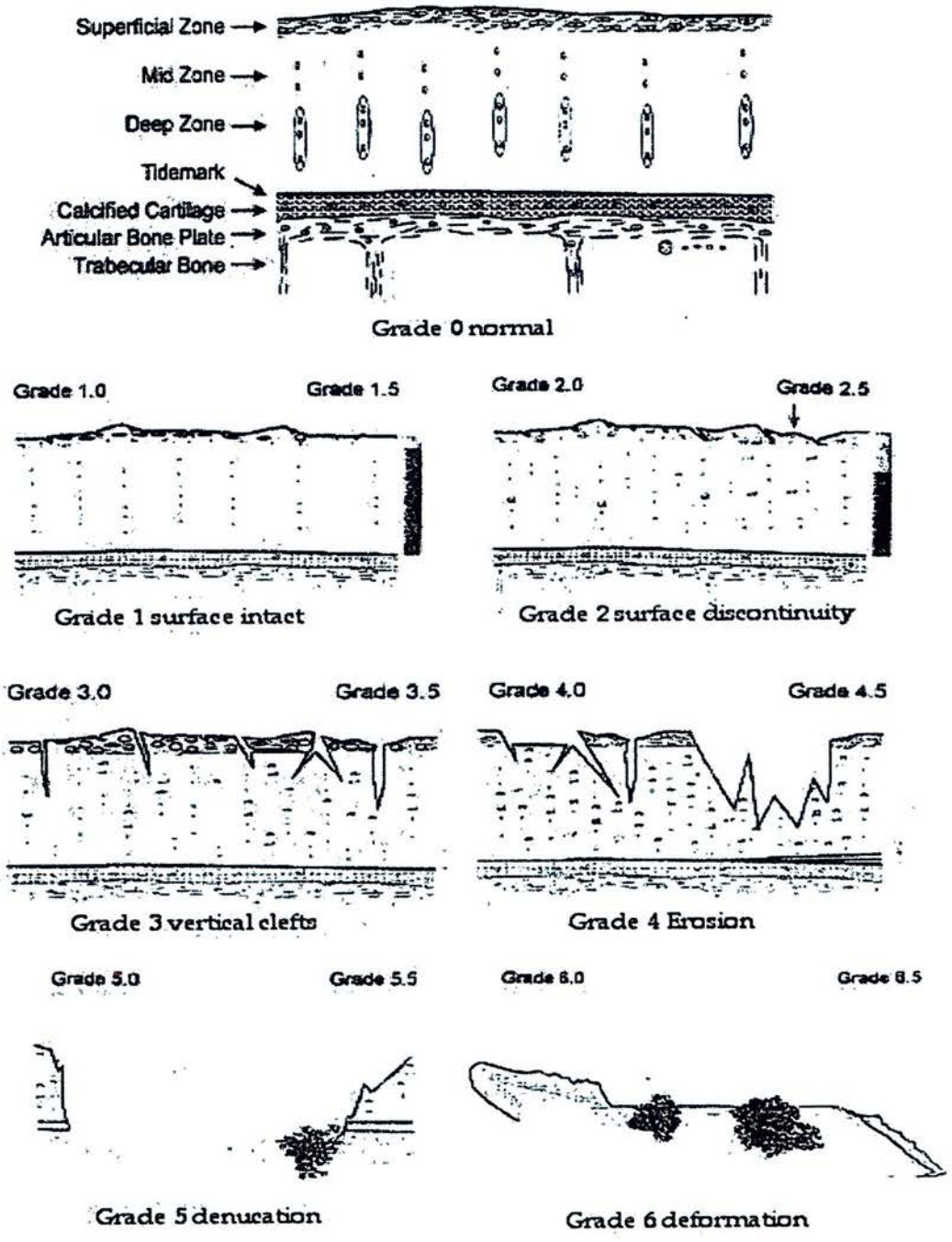


Figure 2.1 Histological grading schemes were according to OARSI Osteoarthritis Cartilage Histopathology Grade Assessment (Pritzker et al 2005)

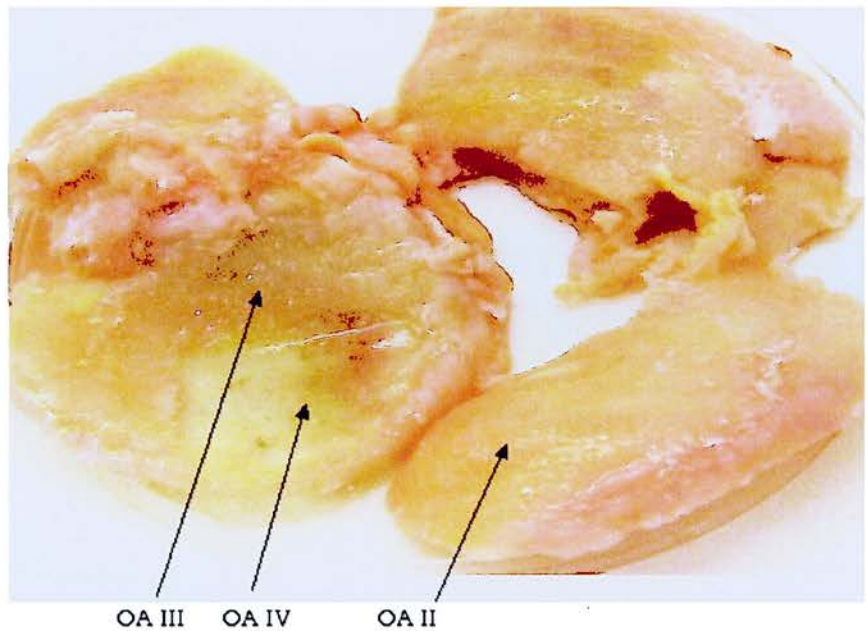


Plate 1A Typical OA knee joints, obtained at the time of joint replacement surgery

A knee joint obtained from 64 year old female (day book number DS069C) at the time of joint replacement surgery. Macroscopically graded OA articular cartilage was shown as numbers (I, II, III, IV). Area of softened OA grade II cartilage with destruction of superficial and deep layers is shown. On tibial plateau, can be seen OA grade III cartilage with extensive destruction of deep layer cartilage and next to exposure of bone, which is OA grade IV.

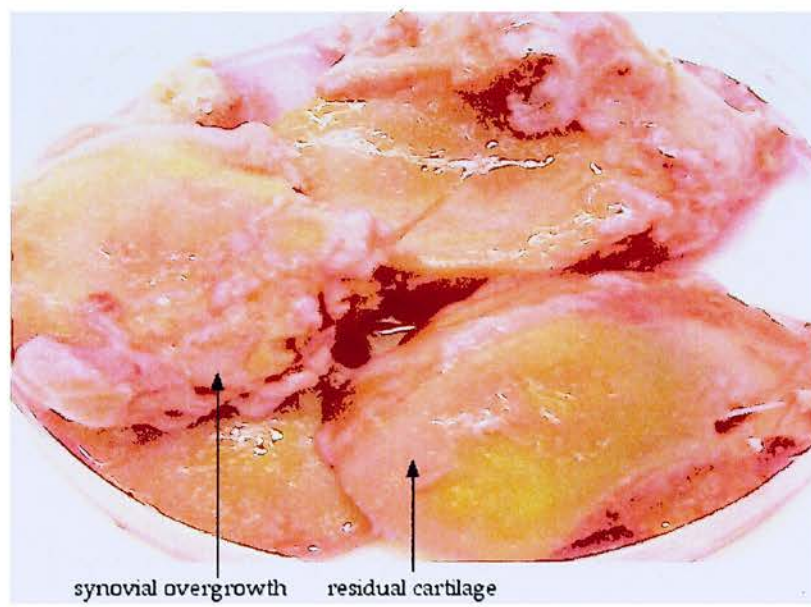


Plate 1B Typical RA knee joints, obtained at the time of joint replacement surgery.

A knee joint obtained from 55 year old female (day book number DS098C) at the time of joint replacement surgery. Thinned residual cartilage and synovial overgrowth are shown.

Plate 1

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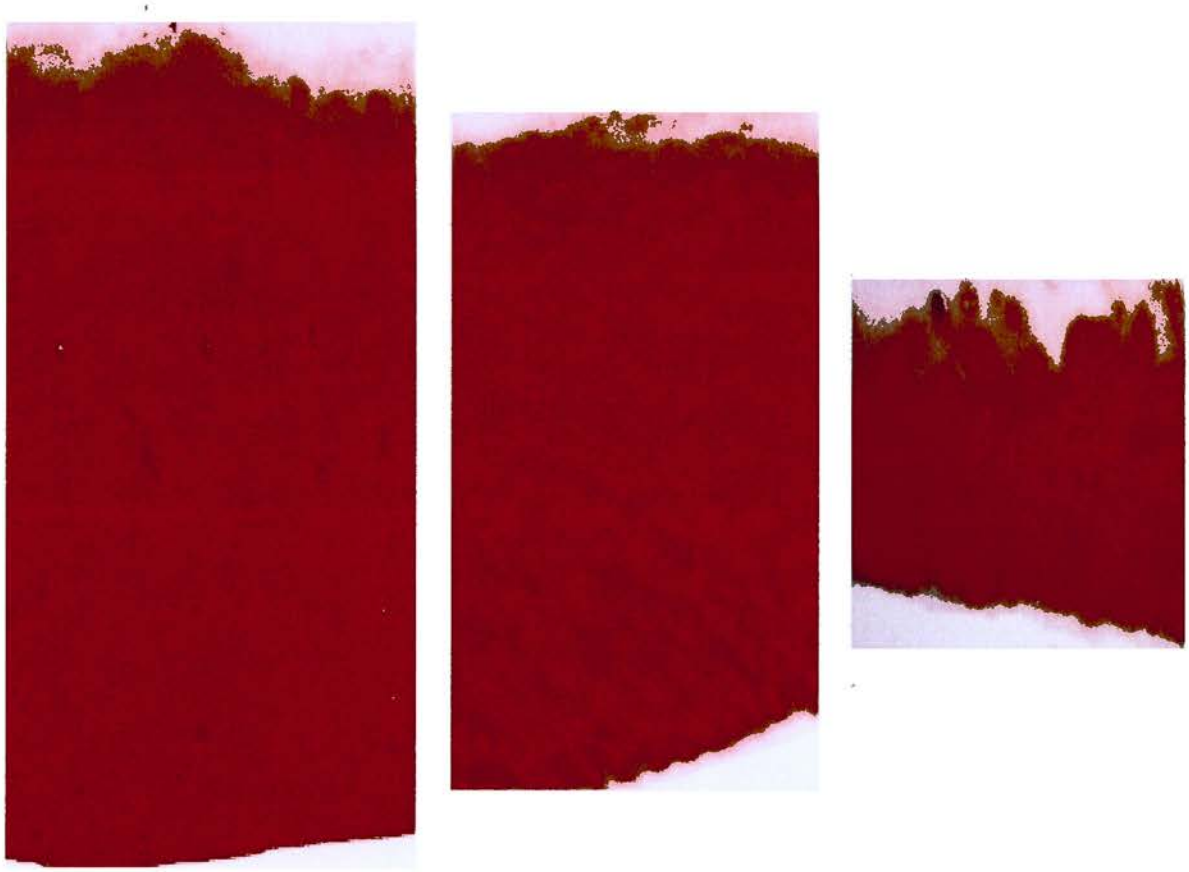


Plate 2 Section of cartilage

Plate 2A (left) Section of OA cartilage from knee joint of 80 year old female (day book number DS088C), stained with Safranin-O. Original magnification x10. OA grade 0-I cartilage exhibits strong staining of PG with safranin-O, no disruption of tissue structure with smooth articular surface and no cell clustering or cloning.

Plate 2B (centre) Section of OA cartilage from knee joint of 78 year old male (day book number DS138C), stained with safranin-O. Original magnification x10. The OA section exhibits a reduction uptake of safranin-O, indicating a decrease in PGs, surface irregularities and clefts (OA grade II).

Plate 2C (right) Section of RA cartilage from knee joint of 43 year old female (day book number DS282C). Stained with safranin-O. Original magnification x40. The RA section exhibits reduced staining of safranin-O in the upper zone of cartilage indicating a decrease in PGs, surface irregularities and thinner than normal cartilage.

Plate 2

Microscopic grading showed a close relation to the macroscopical grading system of OA cartilage (Plate 1-2). When there is a discrepancy between macroscopic and microscopic grading, the result obtained was discussed in each chapter. Both the quantity of residual cartilage, and the quality, varied a great deal even in advanced stages of OA, so we included normal (grade 0) cartilage from OA joints in agreement with Maroudas and Venn (1980). A typical OA knee joint is shown at Plate 1A. In cases of RA, residual cartilage was collected without grading (Plate 1B) because the quantity of thinned residual cartilage was much less than from OA (Plate 2). The osteophytic region of the joints was not used in experiments, to prevent the use of neo-cartilage.

2.3 Chondrocyte cell culture

2.3.1 Monolayer culture

The cartilage was removed from the subchondral bone and cut into small pieces, which were placed in sterile tissue culture dishes containing antibiotics (antibiotic-antimycotic solution; 0.25 μg/ml amphotericin B (Gibco Paisley, UK), 100 U/ml penicillin and streptomycin (Gibco), 2 mM L-glutamine (Gibco) for 60 minutes. After removal of the antibiotic-antimycotic solution and washing with PBS without magnesium and calcium (Gibco), the cartilage pieces were incubated with 0.25 per cent trypsin (Gibco) at 37°C for 30 minutes followed by collagenase digestion (3 mg per ml, type H Sigma blend, clostridiopeptidase A) overnight at 37°C. The digested cartilage solution was poured through a 100 μm filter (Falcon, Becton Dickinson, UK), washed and centrifuged three times at 300 *g* for 10 minutes, and suspended in a IMDM with 10 per cent FCS (First Link Ltd, UK) containing the antibiotic-antimycotic solution. A sample of the cell suspension was used for cell counting and determination of the viability of the cells with trypan blue (0.4 per cent) then seeded into culture dishes (58 mm Nunc™, Denmark) at a cell density of 5×10^4 cells per ml of medium (25×10^4 cells per dish).

The cells were grown in monolayer culture in a IMDM with 10 per cent FCS in a humidified incubator which was maintained in an atmosphere of 95 per cent air and 5 per cent CO_2 at 37°C. Primary cells seeded at 5×10^4/ml grew up to 80 per cent confluence in a range of 6 to 13 days. The medium was changed every three to four days. At this point the cells spread polygonally, rather than in a fibroblastic or a spherical morphological pattern. The day before experiments were carried out (16 hours), the culture medium containing 10 per cent serum was replaced by medium with 0 per cent or 0.5 per cent serum. 0.5 per cent serum was added to medium in order to allow production of matrix (Lee et al 1995, and personal communication to Dr R. Al-Jamal, Edinburgh University).

2.3.2 Alginate culture

Chondrocytes were harvested from OA cartilage of mixed grades (OA grade I to III) using sequential digestion which is the same technique used as described in monolayer culture. For alginate culture the method of Ragan et al (1998) was followed with some modifications. 2 per cent alginate (Pronova Biomedical, Norway) solution was made by mixing 250 mg alginate powder with 12.5 ml of 0.5 M NaCl, heated to 50°C for two hours in advance in order to dissolve the mixture completely. Then the temperature of the mixture was brought back to 37°C.

2.3.2.1 Alginate bead culture

Isolated cells were mixed with the two per cent alginate solution at the cell concentration of $23x10^6$cells/ml alginate. This high concentration was chosen in order to have a approximately same numbers of cells in one alginate bead and one alginate disk. The alginate cell mixture was dropped into a 102 mM $CaCl_2$ solution through a 21 gauge needle to give a final cell density $25x10^4$cells/bead. The beads had a volume is 11 µl (0.0107 ± 0.0011). After 10 minutes incubated in $CaCl_2$ solution, beads were washed once with PBS, then maintained in culture medium (IMDM with 10 per cent FCS containing the antibiotic-antimycotic solution). The preparation of chondrocytes in alginate beads was performed as described by Guo et al (1989). The cells were maintained by changing the medium daily and incubated in a humidified atmosphere of five per cent CO_2 at 37°C.

2.3.2.2. Alginate disk culture

Isolated cells were mixed with a two per cent alginate solution at the density of 2×10^6cells/ ml alginate to give a final cell density of 30×10^4 cells per disk (the volume of 0.157 ml alginate per disk). This is chosen as a density similar to monolayer culture (25×10^4 cells per dish). Alginate-cell mixture was injected into an ethanol-sterilised, diffusion-permeable, custom-designed casting frame using a 21 gauge needle and a syringe (Plate 3A). The casting frame was made of two stainless steel rounds, 5 mm thick, with a central area of parallel mesh and separated by 2 mm spacers and membrane filters (5 µm pore size, Durapore Millipore). The mesh, filter paper, and frame were immersed in a solution of 102 mM $CaCl_2$, and left for 30 minutes to allow the alginate to gel. Disks (10 mm diameter, 2 mm height) were punched out from gelled alginate (Plate 3A). Each disk was transferred to a single well of a 12-well plate containing 2 ml medium. The medium was replaced everyday. Disks were removed for the mechanical stimulation and assayed on day three.

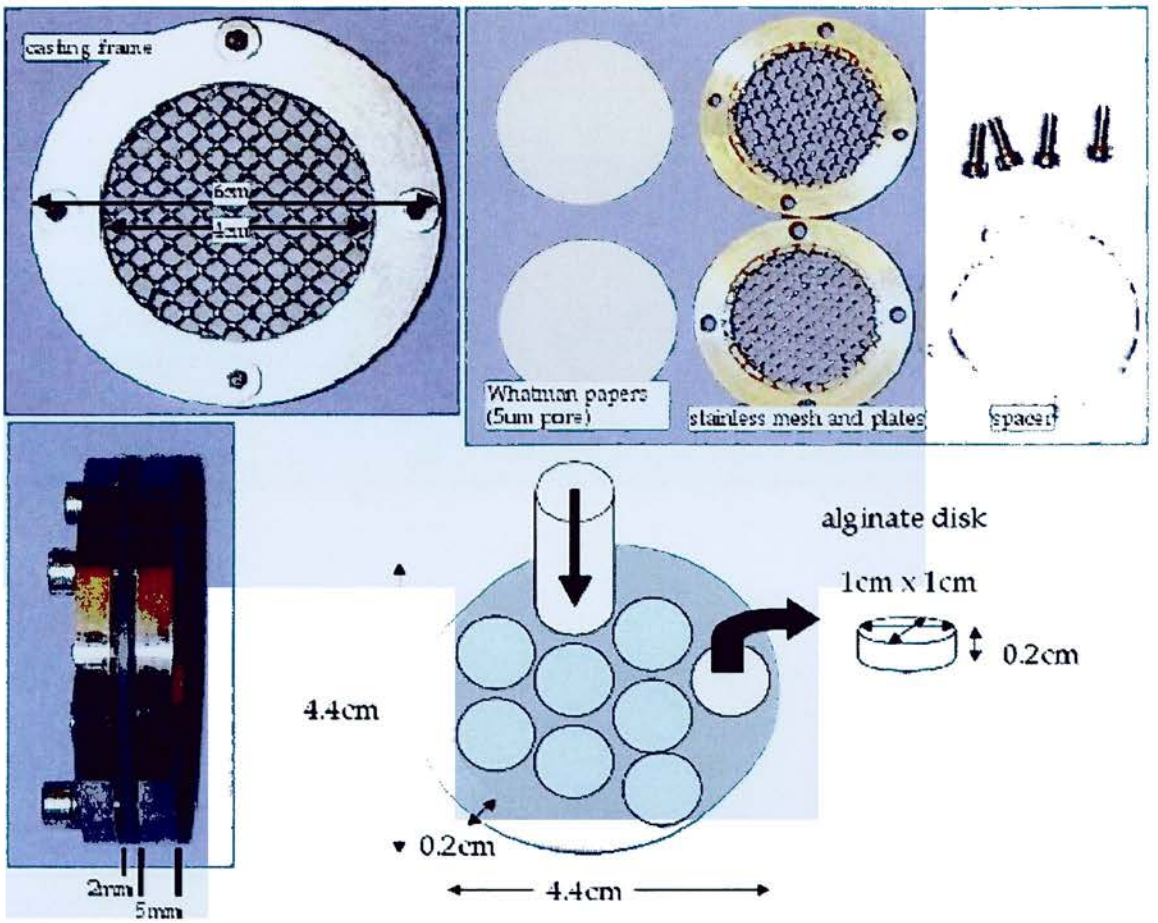


Plate 3A Custom-designed casting frame
Disks (1 cm x 1 cm diameter, 0.2 cm height) were punched out from gelled alginate.

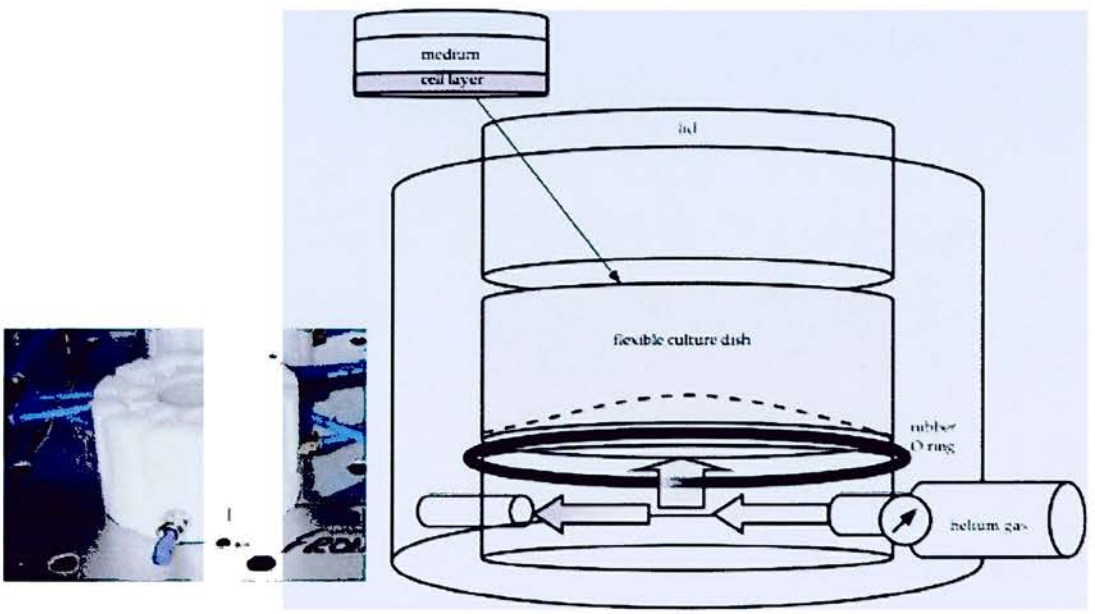
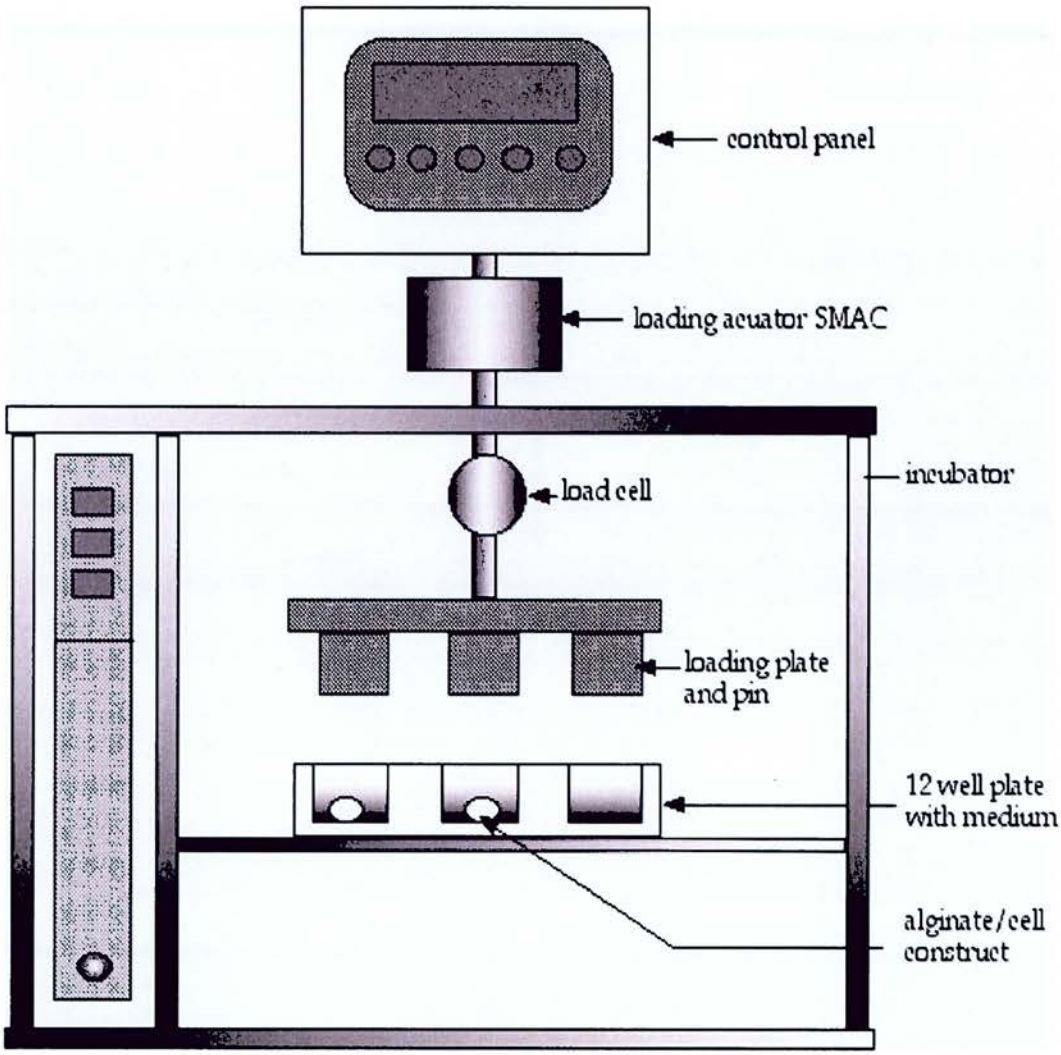
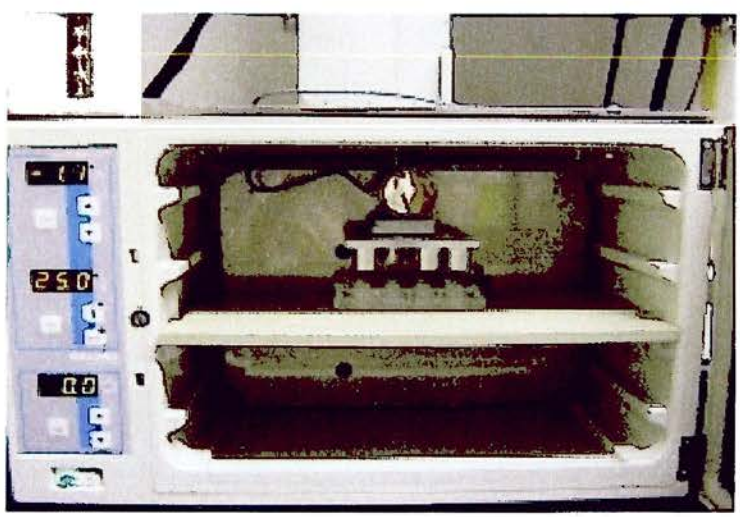


Plate 3B Monolayer culture pressure chamber
The cells in the culture dish are subjected to an increase in pressure and stretching as a result of the deformation of the base of the dish; set at 20 minutes, 0.33 Hz, 4000μ strain.

Plate 3

facing page 68





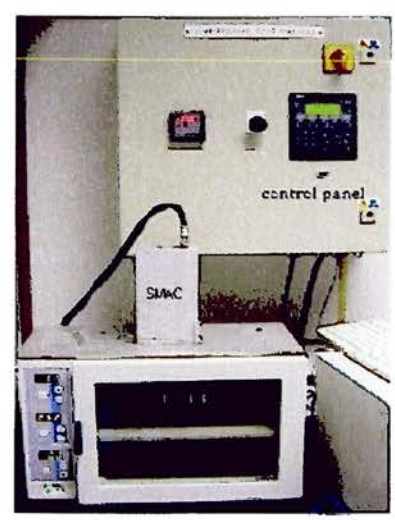


Plate 4 Three-dimensional culture pressure chamber
Schematic diagram of the apparatus used to apply compressive strain to alginate-chondrocyte construct.

Plate 4

facing page 69

2.4 Induction of cyclical mechanical stimulation

2.4.1 Mechanical stimulation of chondrocytes in monolayer culture

Cells grown in a monolayer culture in plastic tissue culture dishes were subjected to mechanical stimulation using an apparatus (Plate 4B) modified from that previously described by Wright et al (1996). The apparatus consists of (a) inlet and outlet ports to allow pressurization of the culture dishes with helium gas, (b) inlet and outlet valves fitted with an electrical timer to control the frequency and duration of pressure pulses, (c) four pressure chambers to insert the culture dishes (Plate 4B). It this system, the space above the dish is smaller than the space below the dish. When the pressure is applied to the system, the base of the dish becomes distorted because of the time constant for equilibration of pressure above and below the dish is different. The difference in pressure between above and below the dishes causes the base of the dish to flex, thereby stretching the plasma membranes of the adherent chondrocytes. This result in an electro-physiological response with chondrocytes isolated from normal cartilage showing hyperpolarization, those from OA depolarising. An electro-physiological response to mechanical stimulation is absent when non-deformable dishes are used. Helium gas at a range of frequencies of 0.1, 0.33, and 1Hz (Appendix I). Normal walking cycle is usually above 0.1Hz and less than 1Hz. The pressure at 30 mmHg to impart 4000μ strains (0.039 MPa). Hodge et al (1986) measured intra-articular pressures as fluctuating between 0.2 MPa and 4 MPa during the normal walking cycle. This cyclical pressurization technique is well documented to induce cell response (Wright et al 1992). Total duration of cyclical mechanical stimulation was 20 minutes followed by incubation at 37°C for 24 hours. Prior to mechanical stimulation, 1 ml of medium was removed from culture dishes (pre-medium) including control dishes without mechanical stimulation in order to assess baseline GAG synthesis. Following stimulation the remaining medium bathing the cells was removed and kept separately from the cell layer as post-medium and both sample pools were stored in –70°C until needed.

2.4.2 Mechanical stimulation of chondrocytes in alginate culture

Alginate beads and disks were placed into a specially designed compressive test apparatus (SMC Pneumatics (UK) Limited) (Plate 4). Loading acuator 'SMAC' is mounted vertically through the roof of the test chamber (tissue culture incubator) pointing downwards. There is a load cell mounted on the rod end of the 'SMAC' rod with a loading plate and pins on the other side of the load cell. It registers zero distances of materials, and cylinder movement allows compression and decompression of the material, moving the initial start set up zero distance to any distance of setting. Operating frequency range is from 0 to 3 Hz and

operating force is 2-200 N. The loading plate and pins were sterilised in 100 per cent methyl alcohol before each experiment.

Alginate beads or disks were cyclically (0.1 Hz, 0.33 Hz, and 1 Hz) compressed to 10 per cent, 30 per cent, and 50 per cent of original thickness. Control alginate-cell beads or disks were left uncompressed otherwise kept in the same conditions. After compression, the beads or disks were cultured for 24 hours and removed from culture for snap freezing

2.5 Extraction of PG

PG released in the medium was incubated with ice-cold three volumes of acetone in glass tubes at –20°C overnight. Then samples were centrifuged at 1800 rpm for 30 minutes at 4°C (MSE Mistral 2000R centrifuge, Sanyo, Leicester, UK).

PG in the cell layer was extracted by using GuHCl buffer; first, after the medium was aspirated, the cell layer was rinsed twice with ice-cold PBS (free of calcium and magnesium). Second, 2 ml of 4 M GuHCl extraction buffer containing protease inhibitors was added in the culture dish for 24 hours with agitation at 4°C (Hascall et al 1995) (Appendix). Third, GuHCl extracts were precipitated overnight with three volumes [v/v] ethanol at –20°C and precipitated again with ethanol overnight for the complete removal of GuHCl buffer. Fourth, all the samples were centrifuged at 1000 *g* for 30 minutes at 4°C.

Pellets were re-suspended in proteinase K buffer for GAG assay or in deglycosylation buffer for SDS-PAGE (Appendix).

2.6 Glycosaminoglycan assay using Dimethylmethylene blue (DMMB)

The two most common methods of assessing PG synthesis are the quantification of ^{35}S-sulphate incorporation into PG and the DMMB assay. The DMMB assay is a quantitative dye binding procedure. DMMB was initially used as a metachromatic histological stain (Taylor and Jeffree 1969). The metachromatic change in DMMB, which occurs when the cationic dye binds to sulphate groups, was adapted for the spectrophotometric determination of sulphated GAG (Humbel 1974). The assay excludes the binding of other commonly occurring polyanions such as hyaluronan, DNA and RNA by lowering the pH of the assay buffer and including NaCl in the buffer (Farndale et al 1986, Handley et al 1995). It was also found that the interference by other proteins could be eliminated by treating the sample with papain or

proteinase K (Farndale et al 1986). The sulphated GAG-DMMB complex shows a positive peak at 525 nm, and a negative peak at 590 nm. Hollander et al (1991) made use of this assay with a 96-well plate using a standard ELISA plate reader and were able to quantify sulphated GAG in large numbers of samples at the same time. The only the disadvantage of this assay is that the sulphated GAG-DMMB dye complex is unstable and tends to precipitate, it is therefore necessary to measure the absorption within 60 seconds.

2.6.1 DMMB assay with monolayer culture

Precipitated pellets from 1 ml pre-stimulation medium and 2 ml post-stimulation medium and 1 ml cell layer samples in GuHCl buffer were re-suspended in 100 µl of PBS containing proteinase K (1 mg/ml), followed by incubation at 60°C for 16 hours, then boiling for 15 minutes to denature enzymes. The DMMB assays were performed in 96-well plates using an ELISA plate reader (model MRX Dynatech Laboratories). 20 µl of sample was added to 250 µl of DMMB stock solution (Appendix) and the optical density (OD) was immediately measured at 490 nm. The mean OD of the blank wells was subtracted from all other wells to construct a standard curve and read off sample values. A standard solution of CS (chondroitin sulphate A, Sigma) was prepared at 100 µg/ml in distilled water and this stock was used to prepare a range of standard dilutions in individual plastic tubes. A standard curve was generated at the time of each assay using a solution of CS of a known concentration (Figure 2.2).

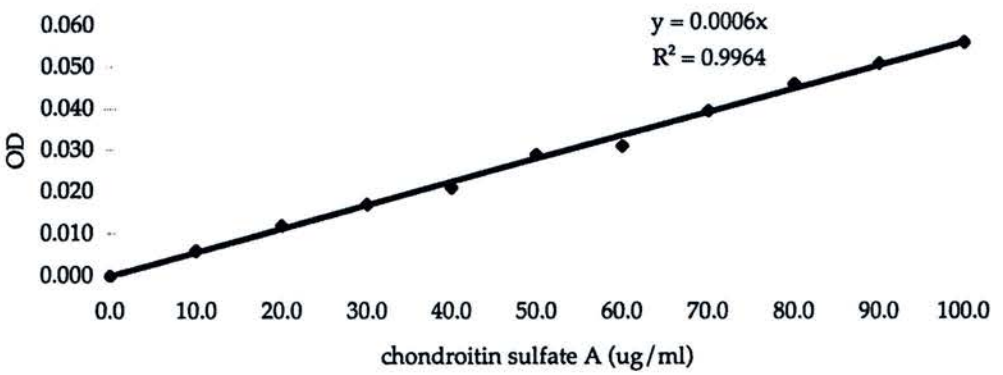


Figure 2.2 A typical Standard curve for using a known concentration of chondroitin sulphate A (0-100 µg/ml)

2.6.2 DMMB assay with alginate culture

Measurement of total sulphated GAG in cell culture digests containing alginate using DMMB is complicated by the fact that the carboxyl group of alginate binds with DMMB forming a complex which interferes with the spectrophotometric detection of sulphated GAG-DMMB. A sulphated group has a lower pH than that of the carboxyl group. Enobakhare et al (1996) have shown at a pH level of 1.5, only sulphated GAG will complex with DMMB.

Preparation of DMMB solution for alginate is as follows; 21 mg of DMMB was stirred with 5 ml of absolute ethanol and 2.0 g of sodium formate. The solution volume was then made up to 800 ml with distilled water. Concentrated formic acid was used to adjust the pH of the solution to 1.5. The volume of the solution was made up to 1 litre with distilled water.

The chondrocyte-alginate gels were disrupted and digested by addition of 148 µl of alginate digestion buffer (Appendix) per bead and 2 ml per disk of papain buffer (Appendix) containing 0.56 units/ml papain. The samples were incubated at 60°C for 24 hours. Alginate gel alone was used for the standard curve and the dilution of the samples. 40 µl of papain-digested sample was added to 250 µl of DMMB solution and the absorbance was determined immediately at 595 nm (Enobakhare et al 1996).

2.7 Determination of DNA content of cultures using a Hoechst DNA assay

Although equal numbers of chondrocytes were seeded, it was desirable to normalise metabolic indices by cell or DNA content. In order to assess cellular proliferation, the total content of DNA was quantified using the bisbenzimide fluorescent dye (Hoechst 33258); a method for determining DNA concentrations in aqueous buffers, that is not affected by contaminating protein or RNA (Kim et al 1988). One thing to note is that the DNA assay technique does not distinguish live and dead cells in culture.

10 µl of the proteinase K digested samples was mixed with 2 ml of Hoechst working solution (Appendix) and evaluated in a spectrofluorometer (LS50-B Luminescence Spectrometer Perkin Elmer) (Figure 2.3). The samples were measured at an emission wavelength of 458 nm and an excitation wavelength of 356 nm. Disposable polymethacrylate cuvettes (Elkay Ultra-Vn®) were purchased from Elkay products Inc, USA. A standard curve was generated at the time of each assay using a solution of calf thymus DNA (Sigma) of a known concentration (Figure 2.4).

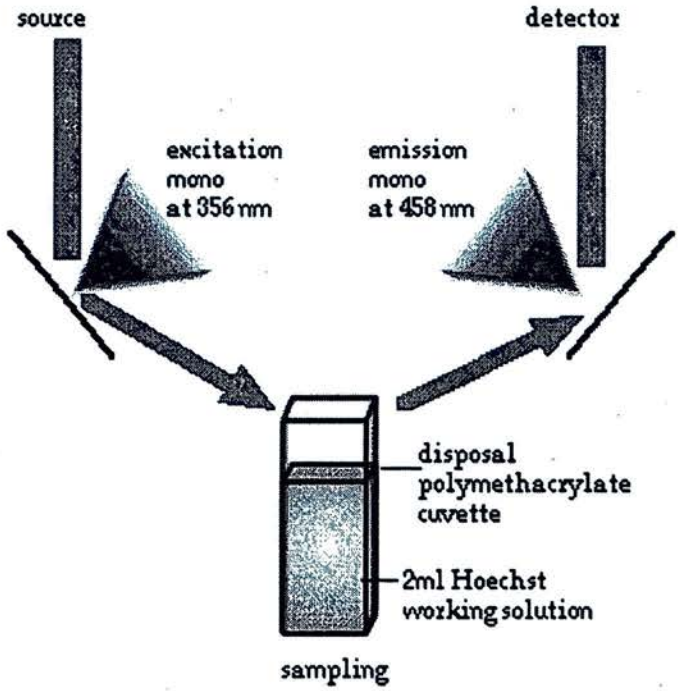


Figure 2.3 Fluorometer

Total DNA content with in the disks was measured by reaction of 10 µl of the digest with Hoechst 33258 dye solution and fluorometry as described in DNA assay in monolayer culture.

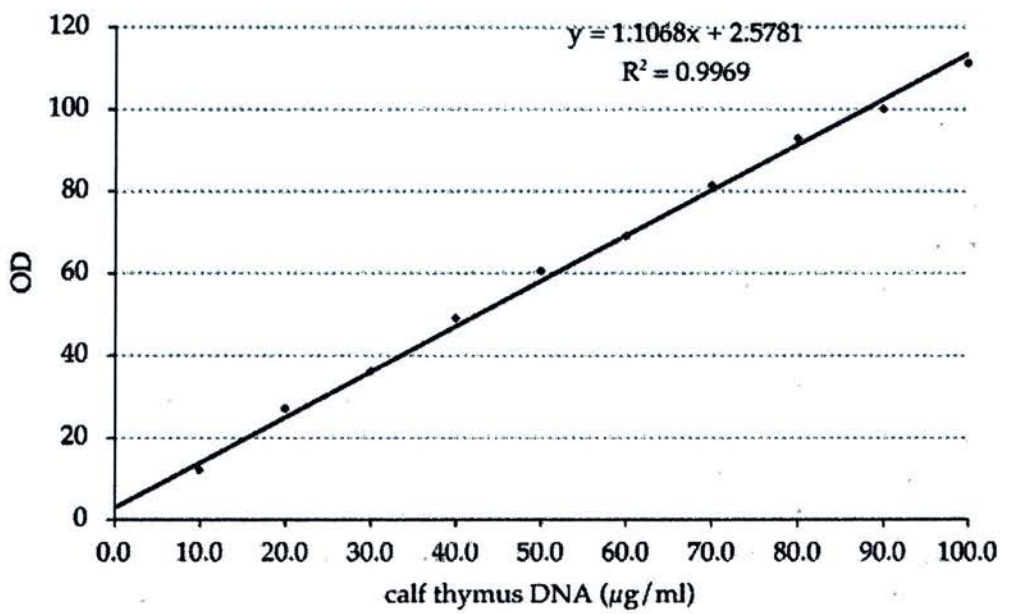


Figure 2.4 A typical standard curve for using a known concentration of calf thymus DNA (0-100 µg/ml)

2.8 Western blotting

2.8.1 Deglycosylation of PG (aggrecan and aggrecan catabolites)

This deglycosylation step is aimed at removing both CS and KS chains from the aggrecan core protein, generating CS and KS 'stabs' for 3-B-3, 2-B-6 and 1-B-5 antibody recognition. Furthermore the removal of both CS and KS is necessary to enable aggrecan metabolites to enter SDS-PAGE gels. The dried pellet samples after precipitation were incubated with 0.01 units/ml of keratanase II (Seikagaku) in distilled water at 37°C overnight, and subsequently with 0.1 units/ml chondroitinase ABC (Seikagaku) diluted in 0.1 M sodium acetate and 0.1 M Tris/HCl (pH 7.4) at 37°C for 4 hours (Appendix).

2.8.2 Analysis of PG core protein: assessing aggrecan and decorin core protein by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The method of SDS-PAGE in this study was modified from Laemmli (1970) using the continuous buffer system.

The composition of the gels and buffers used in the preparation is listed in Appendix I. Electrophoresis was performed using the electrophoresis system (Mini protean® 3 cell Bio-Rad lab Ltd). Glass gel plates (12 x 10, 12 x 9 cm), spacers, clamp assembly, casting stand and silicone gaskets were cleaned with 70 per cent industrial methyl alcohol. Glass plate sandwiches were assembled into the clamp and the assembly was transferred to the casting stand. A 10-well Teflon® comb was placed in between the plates and a mark was made below the edge of the teeth. The separating gel was prepared of appropriate acrylamide percentages for the resolution required (6 to 12 per cent polyacrylamide solution; see Appendix) and was poured between the glass plates and a layer of distilled water was added gently over the sitting gel to give a smooth surface. The gel was allowed to polymerise for 30 minutes at room temperature. The overlaid distilled water was drained off and the stacking gel was poured onto the surface of the separating gel. The comb was inserted, ensuring that no air bubbles were trapped. The gel was allowed to polymerise at room temperature for 15 minutes. After removing the comb, which leaves wells for samples, the gel was fitted into an electrophoresis tank with an electrode buffer. The samples in a total volume of 40 µl containing 1 to 10 µg GAG mixed with four times Laemmli sample buffer (Appendix) were then denatured by boiling for five minutes and pipetted into the wells. Electrophoresis was carried out at 75 volts for the first 30 minutes, then at 100 volts for two hours, until the samples reached the bottom of the separating gel. The gel was then removed from the apparatus.

PVDF membrane (Immobilon-P Millipore PVDF filter) was soaked in 100 per cent methanol for 15 seconds, then in distilled water for two minutes, and finally in transfer buffer (Appendix) for 15 minutes. Two pieces of 3 mm paper (Whatman®) were placed on top of the sponges on the cathode side of a transfer cassette. The gel was then placed on top of the paper and the PVDF membrane placed exactly above the gel. The remaining pieces of the paper were stacked on top of the membrane, followed by the second sponge. This transfer cassette was then closed, placed in the transfer tank (Bio-Rad Trans-Blot Cell), and run at 45 volts overnight at 4°C.

After transfer was completed, the PVDF membrane was incubated in blocking buffer (5 per cent [w/v] BSA in PBS-T (PBS contains 0.1 per cent Tween-20 (BDH)) overnight at 4°C. The membrane was washed in 25 ml PBS-T for 30 minutes with three changes at room temperature. Subsequently, the membrane was incubated with primary antibodies overnight at 4°C.

After further washing in PBS-T, a secondary antibody (alkinephosphatase-conjugated goat anti-rabbit IgG; Dako Cytomation, Denmark) diluted 1:2000 with the blocking buffer, or anti-sheep (horseradish peroxidase conjugated with anti-rabbit serum, Dako Cytomation, Denmark) diluted 1:1000 with the blocking buffer, was added to the membrane and incubated for 2 hours at room temperature with agitation.

The proteins were finally visualised by using chemiluminescence (ECL) or ECL plus detection kit (Amersham, UK) with the ECL detection kit, a mixture of 1 ml solution A and 1 ml of solution B were poured onto one membrane for one minute. For the ECL plus kit was used, a mixture of solution A: solution B was 1:40 was applied for five minutes. The solution was drained off and the membrane was wrapped in a translucent cover. Any bubbles were smoothed out. The film was exposed to a film as required depending on the signal and developed by a hyperprocessor (Amersham).

2.8.3 Preparation of culture media for SDS-PAGE and Western blotting with monoclonal antibodies to core protein epitopes (BC-3, BC-4, BC-13, BC-14) or chondroitin sulphate stubs (3-B-3, 2-B-6, 1-B-5)

The medium was incubated with three volumes of acetone in glass tubes at –20°C overnight and the GAG content of the samples was determined using the DMMB assay. Then the volume of sample required for deglycosylation was calculated (10 μg of GAG for a BC-3 blot, 20 μg of GAG for a BC-14 blot, 1 μg of GAG for a 3-B-3 blot, 2 μg of GAG for a 2-B-6 blot). After the deglycosylation procedure, two times sample buffer with 10 per cent mercaptoethanol was added. The composition of the buffers used in the preparation are listed in Appendix. Samples were electrophoresed on 4 to 12 per cent gradient gels (Invitrogen

EC6038) in SDS at 150V for one hour and 45 minutes. After electrophoresis the fractionated proteins were transferred electophoretically to nitrocellulose membranes 0.2 μm pore size (Protran BA Schleicher&Schuell BioScience, Germany, Perkin Elmer UK, NBA083G) at 100 V for one hour on ice. Then membranes were blocked with 5 per cent (w/v) BSA in TSA buffer, and immuno-blotted with dilutions of listed monoclonal antibodies (Appendix) in 1 per cent (w/v) BSA in TSA buffer (Appendix) overnight at room temperature. After three washes in TSA buffer, the nitrocellulose sheets were incubated with alkaline-phosphatase-conjugated rabbit anti-mouse Ig secondary antibody (Promega, S3721) 1:7500 in 1 per cent (w/v) BSA in TSA buffer at room temperature for one hour and immuno-positive bands visualised. The immuno-blots were incubated with NBT and BCIP (development substrate Promega catalogue #3771) in AP buffer for 5 to 15 minutes at room temperature to achieve optimum colour development (Hughes 1995).

2.9 Calculation and Statistical analysis

2.9.1 Calculation of the values of the results

The values of the results were calculated as follows;

$$\left[\mathrm{GAGsynth}\right]_{t2}^{t1} = \frac{\left[\mathrm{postGAG}\times 2\right]_{0}^{t2} - \left[\mathrm{preGAG}\times 3\right]_{0}^{t1} / 10}{\mathrm{DNA}}$$

$\left[\mathrm{GAGsynth}\right]_{t2}^{t1}$ (μg/μg/24 hours/dish): GAG synthesis (μg) per dish in 24 hours within the system between the time points of t1 (16 hours) and t2 (40 hours) and corrected by DNA (μg)

$\left[\mathrm{postGAG}\times 2\right]_{0}^{t2}$: GAG measured in post-medium at time point of t2 (40 hours) which amount is 2 ml out of total 3 ml

$\left[\mathrm{preGAG}\times 3\right]_{0}^{t1}$: GAG measured in pre-medium at time point of t1 (16 hours) which amount is 1 ml out of total 3 ml

As GAG in pre and post-medium samples were 10 times concentrated by acetone

precipitation, the values were divided by 10, and finally corrected by the value of DNA (μg) per dish, which contained $25x10^4$cells.

However, with personal communication with Dr Hughes (Cardiff University), it was not necessary to treat medium samples with proteinase K as GAG chains are released to medium. In addition, starving cells for 16 hours with serum-free medium prior to experiments probably inhibit cells synthesizing PG. The DMMB assay was modified for the experiments relating to section 2.8.3 as follows; serum starved cells for 30 minutes prior to experiments. Cells were then mechanically stimulated followed by 24 hours incubation. 40 μl medium out of the total 2 ml post-medium samples were used for DMMB assay.

The values of the results were calculated as follows;

$$[\text{GAGsynth}]_{t0}^{t1} = \frac{[\text{postGAG}]_{t0}^{t1} \div 25 \times 50}{\text{DNA}}$$

$[\text{GAGsynth}]_{t0}^{t1}$ (μg/μg/24 hours/dish): GAG synthesis (μg) per dish in 24 hours within the system between the time points of t1 (0 hours) and t2 (24 hours) and corrected by DNA (μg)

$[\text{postGAG}]_{t0}^{t1} \div 25 \times 50$: GAG synthesis (μg/ml) in 40 μl of medium was corrected to μg/ml, and then corrected to μg/dish/2 ml, and finally corrected by the value of DNA (μg) per dish, which contained $25x10^4$cells.

2.9.2 Statistical analysis

The mean and standard deviation (SD) were determined for each set of experiments. For statistical comparisons, when the F-ratio of the two variances reached significance, the non-parametric (unpaired two samples) Mann-Whitney-U test (two-tail) was used. When the ratio did not reach significance, the parametric Student's *t*-test was used. The Kruskal-Wallis-H-test and the Mann-Whitney-U test with Bonferroni correction post hoc test were used for multiple comparison tests.

Chapter Three

The Effects of Mechanical Stimulation on Proteoglycan Synthesis in Human Articular Chondrocytes in Monolayer Culture

Structural integrity of articular cartilage depends on (a) balance of matrix synthesis and degradation and (b) mechanical loading. Previous studies have shown that human articular chondrocytes from normal cartilage, but not from osteoarthritic cartilage, respond to cyclical mechanical stimulation with an increase in aggrecan mRNA. This response is absent in OA chondrocytes (Millward-Sadler et al 2000a, 2000b). The aim of this study is to examine the effects of mechanical stimulation on PG synthesis in chondrocytes. To test the hypothesis of that increased aggrecan mRNA is translated into increased aggrecan production in normal chondrocytes, a series of GAG assay was undertaken on monolayer cultured normal chondrocytes, which subjected to mechanical stimulation. In addition, a question of whether chondrocytes in OA and RA joints can respond to mechanical stimulation in the same way was tested and compared.

3.1 The influence of age, disease states, and culture period on baseline GAG synthesis

3.1.1 Aim

Samples obtained for this study include a variety of stages of OA and RA in addition to normal cartilage. The aim of this study in this section was to analyse the influence of age as well as disease states and culture period on GAG synthesis.

3.1.2 Materials and Methods

Human articular knee cartilage was obtained at the time of joint replacement surgery from patients with OA or RA, and from above knee amputation from patients with peripheral vascular disease. The articular cartilage surface was assessed macroscopically (McElligott and Collins 1960) and microscopically (OARSI) and graded into normal, OA (I-III) or RA. Normal chondrocytes were isolated from articular cartilage of one male (76 year old) and two females (66 and 73 year old). OA chondrocytes were isolated from the articular cartilage of four males with a mean age of 64.5 years (range 39-79), and six females with a mean age of 64.6 years (range 53-74). RA chondrocytes were isolated from the articular cartilage of three

females with a mean age of 57.3 years (range 39-74). Primary chondrocytes were seeded in monolayer at 5 x 10^4 cells/ml concentration. Chondrocytes were cultured to sub-confluent for an average of 8.1 days. The culture medium (IMDM) containing 10 per cent serum (FCS) was then replaced by serum free medium, and this was followed by 24 hours incubation at 37°C. PGs secreted into the medium were precipitated and the quantity of GAGs was measured by the DMMB assay. Briefly, precipitated pellets were re-suspended in 100 µl of PBS containing proteinase K (1 mg/ml), followed by incubation at 60°C for 16 hours, then boiling for 15 minutes. 20 µl of sample was added to 250 µl of DMMB stock solution (Appendix) and the optical density (OD) was immediately measured at 490 nm. The total content of DNA was quantified using the bisbenzimide fluorescent dye (Hoechst 33258).

3.1.3 Results

3.1.3.1 The influence of age on baseline GAG synthesis rates

There was no significant correlation between age of patients and the amount of GAG measured in medium. The mean age of patients was 64.6 years old (range 39-79) (Figure 3.1).

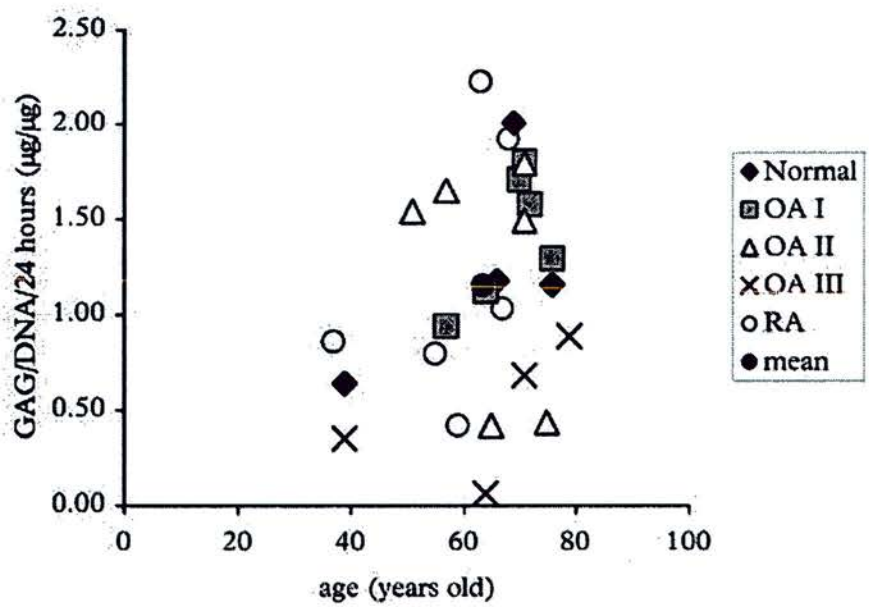


Figure 3.1 The influence of age on GAG synthesis

Baseline GAG synthesis in medium was assayed without mechanical stimulation (control group). The mean age was 64.6 years old. Correlation and coefficient analysis showed no correlation between age and the amount of GAG corrected for DNA (R^2=0.0816).

3.1.3.2 The influence of disease state on baseline GAG synthesis rates

GAG synthesis by chondrocytes from normal articular cartilage was 1.24 ± 0.56 (μg/μgDNA) (mean ± SD), and GAG synthesis by chondrocytes from OA and RA patients were 1.10 ± 0.58 and 1.20 ± 0.70 (μg/μgDNA) respectively. Chondrocytes from OA cartilage, graded from I to III, produced 1.40 ± 0.34 (μg/μgDNA) (OAI), 1.21 ± 0.62 (OAII), and 0.49 ± 0.36 (OAIII). OA grade III chondrocytes synthesized on average 65.0 per cent and 59.6 per cent less GAG (corrected by DNA) than OA grade I and II chondrocytes respectively, which were statistically significant p=0.0038 and p=0.071 respectively (Figure 3.2).

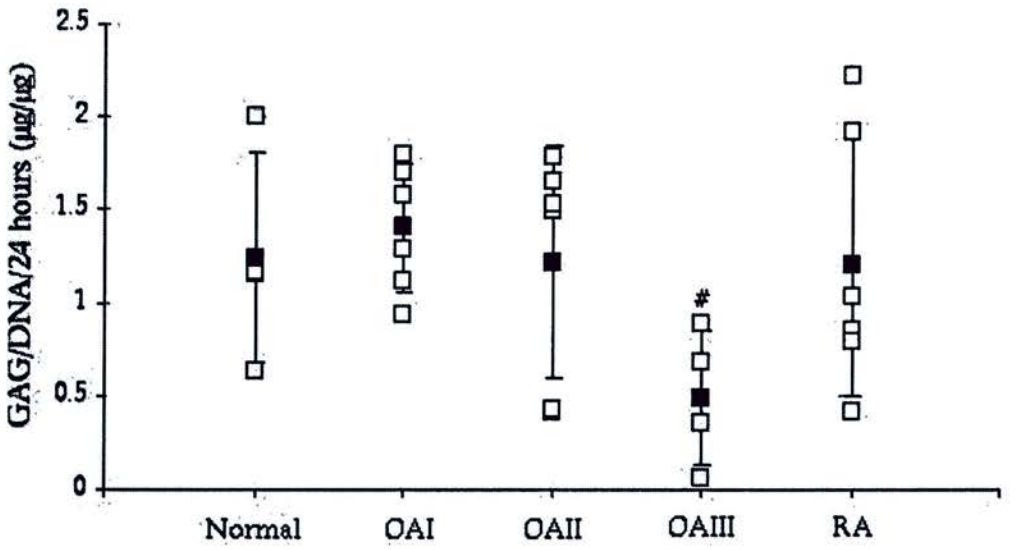


Figure 3.2 The influence of disease state on GAG synthesis

Baseline amounts of GAG synthesis in chondrocytes derived from articular cartilage graded as normal, OA (I-III), and RA were assayed for 24 hours without mechanical stimulation. Chondrocytes from OA grade III synthesized on average 65.0 per cent and 59.6 per cent less GAG (corrected by DNA) than OA grade I and II chondrocytes, which were statistically significant p=0.0038 and p=0.071 respectively. The vertical (Y) axis represents GAG/DNA (μg/μg). normal n=4, OAI n=6, OAII n=6, OAIII n=4, RA n=6.

3.1.3.3 The influence of the length of time in culture on PG synthesis rates

The amount of GAG was estimated at between six and 13 days after isolation and initiation of primary chondrocytes cultures. There was no correlation between the amount of GAG and the period cells were cultured before assay (Figure 3.3).

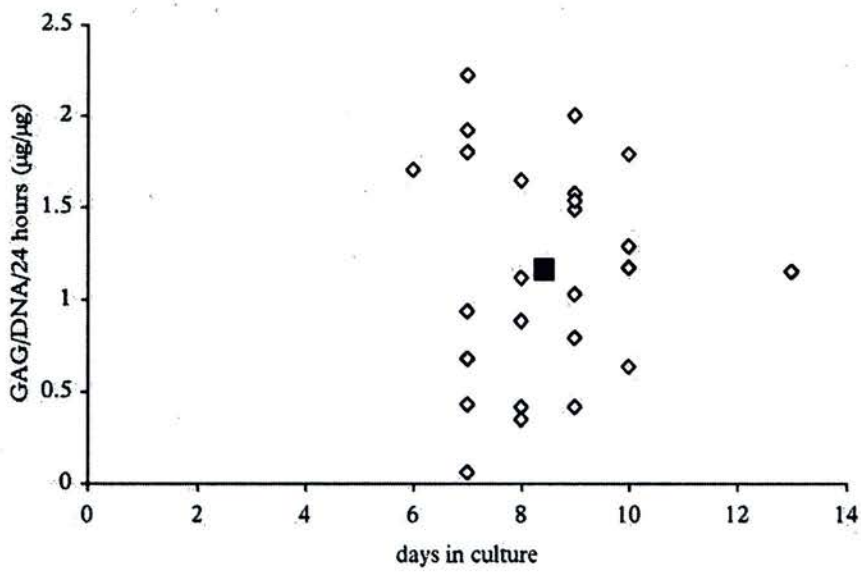


Figure 3.3 The influence of the number of days in culture on GAG synthesis

There was no correlation between the amount of GAG synthesis and the number of days in culture in human articular chondrocytes from normal, OA, or RA. The average culture period was 8.4 days (filled symbol). Correlation and coefficient analysis showed no correlation between age and the amount of GAG corrected by DNA (R^2=0.0802).

3.1.4 Discussion

3.1.4.1 The influence of age on baseline GAG synthesis rates

The amount of GAG released into medium from unstimulated cultured chondrocytes was independent of age in this study (Figure 3.1). PG synthesis rates have been previously reported to increase with age (McElligott and Collins 1960), although a recent report contradicted this, indicating that there was an age-related decrease in the PG synthesis of human articular chondrocytes (DeGroot et al 1999). One can speculate that there may be alterations in cartilage cell morphology and mechanical properties with ageing, as the development of osteoarthritis is closely associated with age. Dozin et al (2002) investigated some metabolic mediators (MMPs, IL-8, IL-6, nitric oxide) of human articular chondrocytes derived from young, old and osteoarthritic subjects. The mediator activity of chondrocytes from normal aged subjects appeared to be different from that of normal young patients, but similar to those of osteoarthritic chondrocytes. Differences in chondrocyte response in young and aged are also reported by Loeser et al (2000), who showed a significant age-related decline in chondrocyte response to IGF-1.

3.1.4.2 The influence of disease state on baseline GAG synthesis rates

My results have shown no significant difference in PG synthesis between normal and OA (grade I and II), or normal and RA chondrocytes, except only the end stage OA chondrocytes (OA grade III) decreased PG synthesis on average 60.45 per cent compared to normal chondrocytes, and also showed a statistically significant decrease in GAG synthesis compared to OA grade I and II chondrocytes. This may be due to the following reasons.

First, the pathological cartilage changes in knee OA are often focal and heterogeneous. It is technically difficult to divide cartilage samples with macroscopic gradings from 0 to IV from OA cartilage. It is possible that samples obtained remained still heterogeneous. In addition, cartilage was not isolated separately from the superficial, middle and deep layers. When undertaking comparative studies of normal and osteoarthritic cartilage, Schneiderman et al (1986) warned that cartilage from all areas of a normal joint should not be included because the superior surface of the cartilage in an osteoarthritic joint has usually been worn away. PG synthesis rates have been shown to differ in different cartilage zones (Lafeber et al 1992a, 1992b). They demonstrated that PG synthesis rates in chondrocytes located in the superficial layer of normal cartilage were higher than those from cartilage derived from OA after being cultured for four days.

Second, there are technical drawbacks to the use of chondrocytes in monolayer cell cultures. We cultured chondrocytes on average for eight days up to 12 days. It was demonstrated that disease-related increases in synthesis in cartilage explants were lost after three to four days in culture. During culture, a transition from higher to lower PG synthesis rates was observed in OA cartilage when compared with normal cartilage (Lafeber et al 1992b, Venn et al 1995). This would suggest that it is unlikely that isolated chondrocytes, cultured for varying periods up to 12 days, would maintain any disease or age-related metabolic differences. Chondrocytes in monolayer culture have a completely different cell morphology; chondrocytes in monolayer culture are polygonal, whereas chondrocytes in cartilage are spherical. The different shape of chondrocytes in monolayer culture is known to influence their metabolism. In addition, we used a relatively low cell density ($5x10^4$ cells/ml) compared to that used by Bulstra et al (1989) who used a high-density culture ($2x10^5$ cells/ml) to maintain the characteristic phenotype of the chondrocytes *in vitro*.

However, the cells used in this study were in primary culture within 12 days of isolation, and expressed keratan sulphate, one of the marker of differentiated chondrocyte function (Jobanputra et al 1992). Chondrocyte phenotypic expression of aggrecan and type II collagen may remain unchanged for one to two weeks, although a marker dedifferentiation type I collagen mRNA was detectable in monolayer culture after four days (Zaucke et al 2001).

Loeser et al (1995) discussed about chondrocyte dedifferentiation in monolayer culture; cells retained a chondrocyte phenotype after 7 to 10 days of culture. In addition, they did not note type I collagen expression until day 14 of culture (Loeser 1993). Pruzanski et al (1995) showed that OA human articular chondrocytes synthesize secretory non-pancreatic phospholipase A_2 ($sPLA_2$), a known inducer and promoter of the inflammatory process in the joints, up to a maximum of 60 days in monolayer culture. This activity was the same as in alginate beads three-dimensional cultures. The exact time course of the dedifferentiation of human chondrocytes in monolayer culture, especially of the osteoarthritic phenotype, is still largely unknown.

Third, we need to take into account the fact that our samples are almost all from knee joints, obtained at the time of joint replacement surgery. These are likely to be of advanced (end-stage) OA, although cartilage obtained was usually macroscopically and microscopically a mixture of early and late stages of OA. Samples of early stage osteoarthritic chondrocytes are not easy to obtain at the time of joint replacement surgery. The early stage of osteoarthritic cartilage tends to be found on the non-weight bearing lateral femoral condyle side, whereas the advanced OA cartilage is often seen on the weight bearing medial tibial plateau. In this study, grading OA cartilage is liable to sampling according to topographic differences, comparing weight-bearing region and non-weight-bearing regions, within the joint. The cartilage areas subjected to high contact stresses such as medial tibial side were found to contain more PG but incorporated less $^{35}SO_4$ per μgDNA than low stress area (such as femoral side) (Little et al 1997). This topographically dependent chondrocyte biosynthetic expression was observed *in vitro* culture (Little et al 1997). Ideally we need to take normal and osteoarthritic grade I to III cartilage samples from the same distinct joint regions for comparison.

One thing to note is that the DNA assays do not distinguish live from dead cells in culture. The decreased PG synthesis in OA grade III chondrocytes could be due to higher cell apoptosis rates for advanced OA chondrocytes compared to normal or other grades (Todd Allen et al 2004). All the chondrocytes showed greater than 95 per cent viability as measured by trypan blue exclusion when the cells were seeded. In order to eliminate cell death during the culture, one would need to test cell viability trypan blue assay on the seventh or eighth culture day.

The amounts of GAG, synthesised by unstimulated chondrocytes derived from RA knee joints, were also not significantly different from normal chondrocytes. This is surprising because the cartilage in knee joints obtained by surgery from patients with RA is usually thinner than even OA grade III cartilage (Figure 1.4). Chondrocytes in residual cartilage in RA joints may be functioning more than chondrocytes in OA grade III cartilage. Woolley and Tetlow (1997) observed the hyperplastic and hypertrophic outgrowth of chondrocytes from

cartilage in a minority of RA specimens. In addition, histological studies suggest that both erosive and reparative processes were occurring at different sites within the same specimen (Woolley and Tetlow 1997).

PG synthesis rates have been previously reported to increase in OA (Brand et al 1991). In particular, enhanced PG production in OA joints has been repeatedly demonstrated (McElligott and Collins 1960, Ehrlich and Mankin 1980, Bollet 1967). Increased sulphate incorporation with PG's is up to twice that of normal cartilage. This increase was found to be proportional to the severity of the disease and it is highly significant in moderate OA with a sharp fall off in severe OA (Mankin et al 1971). Although most of the studies which have examined the relationship between PG synthesis and disease have used cartilage explants, isolated human articular chondrocytes derived from OA cartilage have also shown that the rate of $^{35}SO_4$ uptake increased with the severity of the disease. Only chondrocytes isolated from the severest grades of OA showed a depressed synthesis (Bulstra et al 1989, Teshima et al 1983).

The increases in PG synthesis in human OA knee cartilage were reported from the middle and the deep zones. Cells in the superficial zone show increased proliferation and decreased PG synthesis, compared with chondrocytes from the deep layer (Schneiderman et al 1986, Lafeber et al 1992a, 1992b, Aigner and Dudhia 1997). Proliferation of chondrocytes in OA superficial cartilage zone in early OA is often seen in clusters suggesting the abnormal development of cells (Hulth et al 1972, Telhag 1972).

The activity of PG synthesis in OA chondrocytes is still controversial. It may be the same or lower than normal. Some studies have found it unchanged or even decreased in OA cartilage. Byers et al (1977) concluded that sulphate incorporation did not increase in cartilage from osteoarthritic hip joints, as compared with normal cartilage on a total tissue basis. Mackenzie et al (1977) also showed that comparable rates of sulphated GAG synthesis exist in osteoarthritic and normal cartilage from subjects in the same age range. Maroudas and Venn (1980) suggested that when dry weight basis analysis is used there is no difference between the sulphate uptake of OA cartilage as compared with normal, throughout its depth (Maroudas and Evans 1974). Bollet and Nance (1966) did not find increased sulphate uptake, but only increased turnover of chondroitin sulphate in the osteoarthritic regions.

Absolute PG synthesis varied widely in control samples of unstimulated cells. This may have been the result of number of factors such as the length of time in culture, the length of time after the operation before cartilage removal, age, disease state, as well as the possible influence of other unrelated diseases. This variability makes data analysis difficult, especially when number of samples (n) is unavoidably small due to limited tissue availability.

3.1.4.3 The influence of the length of time in culture on PG synthesis rates

There was no correlation between GAG synthesis rates and the number of days in culture of human articular chondrocytes, whether from normal, OA, or RA. The range of PG synthesis assayed in chondrocytes from separate human cartilage samples, after being in culture for the same number of days, was very wide, especially in the case of longer culture periods. PG synthesis in cells from one individual need to be followed over a 0 to 14 day period in culture.

3.2 The effects of mechanical stimulation on GAG synthesis

Human articular chondrocytes from normal, but not from OA chondrocytes, respond to cyclical mechanical stimulation with an increase in aggrecan mRNA (Millward-Sadler 2000a, 2000b). The aim of this study was to examine the effects of mechanical stimulation on GAG production by chondrocytes from normal, OA, and RA articular cartilage.

3.2.1 The effects of mechanical stimulation on GAG synthesis in human articular chondrocytes derived from normal cartilage

3.2.1.1 Aim

The aim of the study in this section was to test the hypothesis of that increased aggrecan mRNA is translated into increased aggrecan production in normal chondrocytes in monolayer, which subjected to mechanical stimulation.

3.2.1.2 Materials and Methods

Human articular knee cartilage was obtained at the time of joint replacement surgery from patients with OA (three cases), and from above knee amputation from a patient with peripheral vascular disease (one case) (Appendix), from one male (76 year old) and two females (66 and 73 year old). The articular cartilage surface was assessed macroscopically (McElligott and Collins 1960)(Appendix) and microscopically (OARSI) and graded as normal. To confirm whether chondrocytes in osteoarthritic cartilage are normal cells or phenotypically different from normal, cells were distinguished using the hyperpolarization response. Since OA cells showed a depolarization response to the same mechanical stimulation, hyperpolarized cells were considered as normal. Primary chondrocytes were seeded in monolayer at 5 x 10^4 cells/ml concentration. Chondrocytes were cultured to sub-confluent for an average of 10.5 days. The culture medium (IMDM) containing 10 per cent serum (FCS)

was then replaced by serum free medium, and cells were subjected to short-time (20 minutes) cyclic (0.33 Hz) mechanical stimulation of 4000μ strain. This was followed by 24 hours incubation at 37°C. PGs secreted into the medium were precipitated and the quantity of GAGs was measured by the DMMB assay. The total content of DNA was quantified using the bisbenzimide fluorescent dye (Hoechst 33258).

3.2.1.3 Results

Cultured primary chondrocytes isolated from normal cartilage, when exposed to mechanical stimulation, showed a significant increase by 113 per cent (*p=0.045) in GAG synthesis compared to unstimulated controls (Figure 3.4).

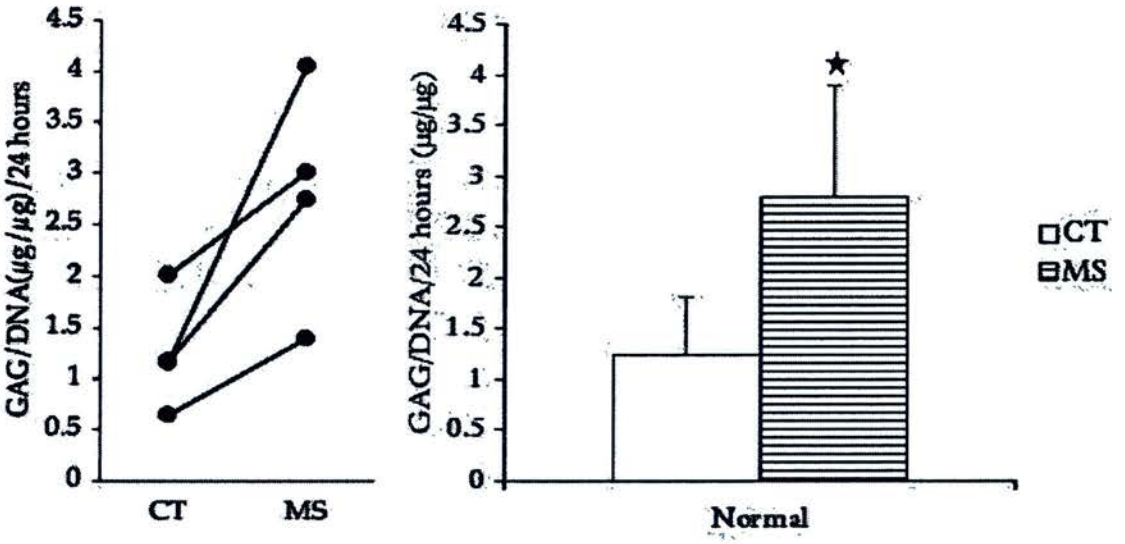


Figure 3.4 The effects of mechanical stimulation on GAG synthesis in human articular chondrocytes derived from normal cartilage

Cells subjected to mechanical stimulation of 4000μ strain at 0.33 Hz for 20 minutes, followed by 24 hours incubation at 37°C. There were significant increases in GAG release into medium following mechanical stimulation (mean ± SD: 1.24 ± 0.57 μgGAG/μgDNA compared with non-stimulated controls: 2.80 ± 1.1 μgGAG/μgDNA) (n=4) (*p=0.045)(Right). Mechanical stimulation increased GAG synthesis consistently compared to paired controls (CT: chondrocytes from same cartilage sample without mechanical stimulation)(Left).

3.2.1.4 Discussion

The results in this study demonstrate that mechanical stimulation modifies the metabolism of adult human articular chondrocytes derived from normal cartilage in monolayer culture. GAG production from normal stimulated human chondrocytes increased significantly compared to paired unstimulated chondrocytes. This means that human articular chondrocytes, derived from normal cartilage, responded to mechanical stimulation and as a result GAG synthesis was stimulated. Smith et al (1995) showed a two-fold increase in GAG synthesis measured by $^{35}SO_4$ incorporation rates using human articular chondrocytes derived from non-arthritic joints cultured in high-density monolayer with fluid induced stress. Lammi et al (1994) also showed a frequency-dependent stimulation of $^{35}SO_4$ sulphate incorporation of PG by cyclic pressures (0.5 Hz).

Studies have previously demonstrated that chondrocytes extracted from the normal articular cartilage of human knee joints show a reproducible membrane hyperpolarization response, protein tyrosine phosphorylation, an increase in relative levels of aggrecan mRNA and a decrease in relative levels of MMP-3 mRNA due to 0.33 Hz mechanical stimulation (Salter et al 2002). These cellular events occur very rapidly after stimulation e.g. tyrosine phosphorylation in one minute, and an elevation of aggrecan mRNA within one hour. We postulated that anabolic protein synthesis would follow mechanical signalling and mRNA production. This was confirmed by the observation that after 24 hours of mechanical stimulation, GAG synthesis was markedly elevated. Increased PG synthesis following mechanical stimulation was previously shown by increases in $^{35}SO_4$ incorporation (Bavington et al 1996). Changes in GAG synthesis may occur as a result of mechanical stimulation with a maximum response at a frequency of 0.33 Hz.

The system employed in this study, cyclic gas pressurization, causes changes in μ strain on the base of the culture dish. Mechanical stimulation of 4000μ strain (0.039 MPa, 30 mmHg) results in deformation of approximately 0.4 per cent of the base of the dish. This is associated with a plasma membrane deformation of cells because chondrocytes are attached to the base of the dish (Wright et al 1996). De Witt et al (1984) and Lee et al (1982) have shown increased PG synthesis of 110 per cent and 40 per cent in cultured chondrocytes stimulated by cyclical stretch with a cell deformation of 10 per cent and 5.5 per cent respectively. The level of stimulation used in this study is far less than theirs. In addition, contact pressures in cartilage *in vivo* can be up to approximately 20 MPa. When walking the pressure cycle is between 3 to 4 MPa (Urban 1994) with average compression amplitudes of over 13 per cent (Wright et al 1996). *In vitro*, chondrocytes produce a cartilaginous matrix under cyclic hydrostatic stress of approximately 3 MPa, and *in vivo*, chondrocytes produce a cartilaginous matrix under a compressive hydrostatic stress of 2 MPa (Tagil et al 1999). However chondrocytes can respond to strains as low as 0.006 MPa compressive and tensile stimuli (Wright et al 1996). Isolated

chondrocytes may sense stimuli differently without their extra-cellular matrix and substrate stiffness may influence that. To seed cells on a flexible substrate and apply defined cyclic strain to it can be easily controlled, but the actual forces acting on individual cells are difficult to measure because the substrate is much stiffer than the cells (Chiquet et al 1999). We have not made an exact measurement of the strain transmitted to the adherent cells, but when chondrocytes attached to pyrex glass petri dishes, whose degree of deformation is much less than that of plastic culture dishes, the cell response to pressure disappeared (Wright et al 1996).

Mechanical stimulation influences cells to produce PG in different steps in different ways. The translation of PG core protein takes approximately 120 minutes and post-translational steps, including GAG chain elongation and sulphation, take a further 15 minutes in chondrosarcoma cell line (Fellini et al 1984). Mechanical stimulation may act to stimulate PG synthesis in the short term by provoking translational or post-translational steps, or increasing gene expression. We have examined aggrecan mRNA expression in relation to 0.33 Hz of mechanical stimulation of human normal articular chondrocytes by semi-quantitative RT-PCR and showed that aggrecan mRNA levels increased within one hour of stimulation, with a return to baseline level within 24 hours (Millward-Sadler et al 2000a, 2000b). In these experiments, the stimulation period was 20 minutes, a relatively short observation period compared to those reported previously, e.g. one and a half hours (Parkkinen et al 1992), six hours (Bavington et al 1996), and eight hours (Liu et al 2001). However, a short application of the load can alter chondrocyte activity for many hours and acute events can initiate long-term responses. For example, five minutes at a very low pressure, applied every 27 hours, stimulates an increase in sulphate incorporation into mandibular chondrocytes by 40-60 per cent (Urban 1994).

3.2.2 The effects of mechanical stimulation on GAG synthesis in human articular chondrocytes derived from cartilage of patients with OA

3.2.2.1 Aim

The aim of this study in this section was to examine the effects of mechanical stimulation on GAG production by chondrocytes from OA articular cartilage.

3.2.2.2 Materials and Methods

Human articular knee cartilage was obtained at the time of joint replacement surgery from patients with OA. The articular cartilage surface was assessed macroscopically (McElligott and Collins 1960) and microscopically (OARSI) and graded into OA I to III. OA chondrocytes were isolated from the articular cartilage of four males with a mean age of 64.5 years (range

39-79), and six females with a mean age of 64.6 years (range 53-74). Primary chondrocytes were seeded in monolayer at 5 x 10^4 cells/ml concentration. Chondrocytes were cultured to sub-confluent for an average of 8.0 days. The culture medium (IMDM) containing 10 per cent serum (FCS) was then replaced by serum free medium, and cells were subjected to short-time (20 minutes) cyclic (0.1 Hz, 0.33 Hz, 1 Hz) mechanical stimulation of 4000μ strain or 32000μ strain. This was followed by 24 hours incubation at 37°C. PGs secreted into the medium were precipitated and the quantity of GAGs was measured by the DMMB assay. The total content of DNA was quantified using the bisbenzimide fluorescent dye (Hoechst 33258).

3.2.2.3 Results

OA chondrocytes culture in monolayer exposed to cyclical mechanical stimulation of 4000μ strain at 0.33 Hz for 20 minutes followed by 24 hours incubation at 37°C showed no significant changes in GAG synthesis when compared to paired controls of all grades of OA (Figure 3.5). OA grade III chondrocytes synthesized significantly less GAG (#$p<0.05$) than OA grade I or II chondrocytes.

OA chondrocytes were also subjected to mechanical stimulation at different frequencies and to greater strain. Two different frequencies of mechanical stimulation regime and one of much higher pressure induced strain (eight times more) were chosen; namely 4000μ strain at 0.1 Hz, 4000μ strain at 1 Hz, and 32000μ strain at 0.33 Hz. The amount of GAG detected in medium was not different at these frequencies or higher strain (Figure 3.6).

3.2.2.4 Discussion

Articular chondrocytes derived from the cartilage of patients with OA, exposed to mechanical stimulation, showed no significant change in GAG synthesis compared to paired controls. Cells retain their *in vivo* abnormal metabolic characteristics, or at least they do not respond to mechanical stimulation. As a result, GAG synthesis did not change following stimulation. There were changes in expression of surface receptors for ECM molecules in OA cartilage (Ostergaard et al 1998) and there are a lot of evidence of altered cellular responses to mechanical stimulation in chondrocytes derived from osteoarthritic cartilage, such as differences in membrane electro-physiological response, gene expression of aggrecan and MMP-3, and cytokine autocrine and paracrine signalling (Millward-Sadler and Salter 2004). In particular, there was up-regulation of aggrecan mRNA levels and down-regulation of MMP-3 in normal chondrocytes following mechanical stimulation. This was dependent on release of IL-4. In contrast OA chondrocytes released IL-1β and there was no change in

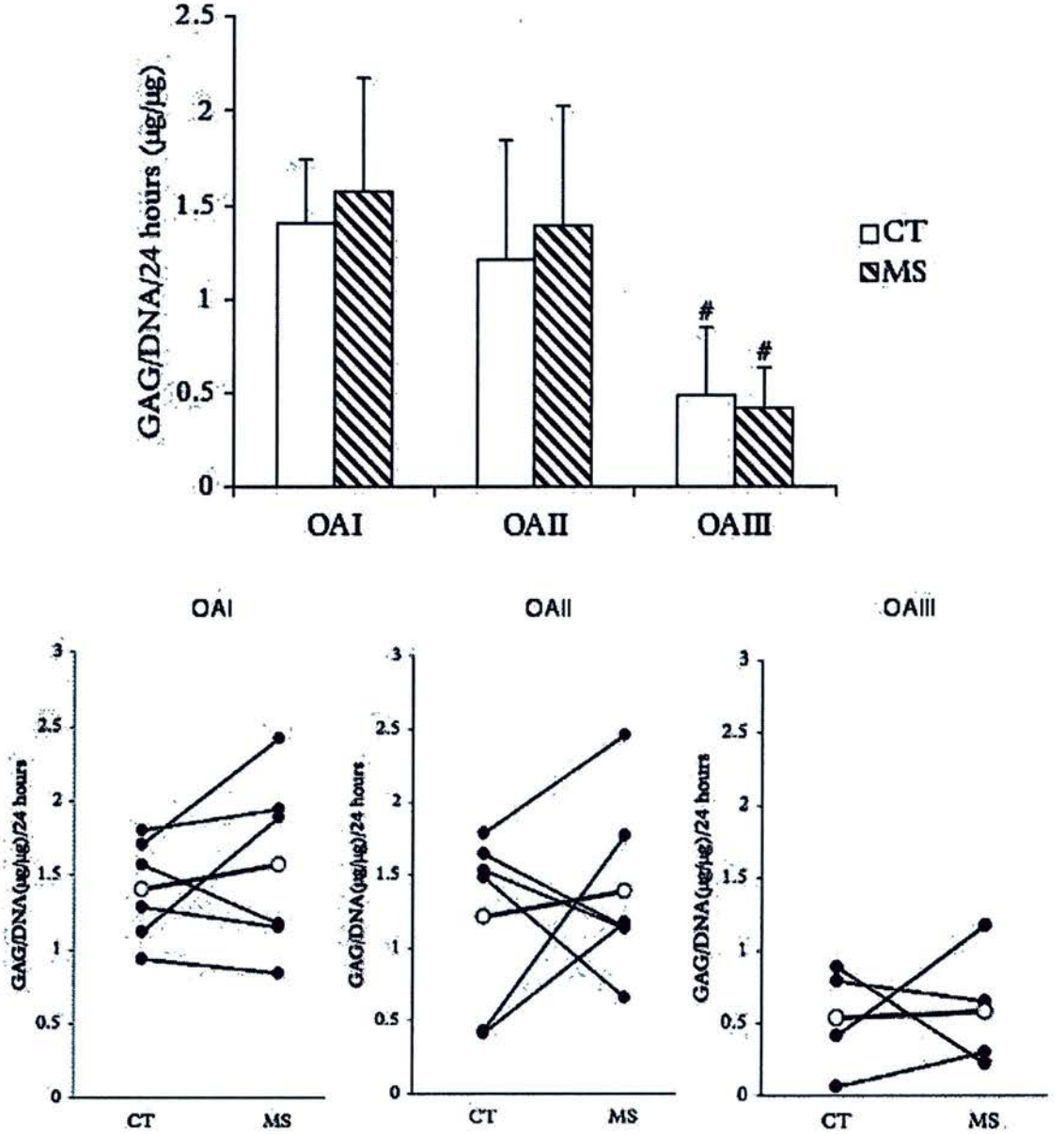


Figure 3.5 The effects of mechanical stimulation on GAG synthesis in human articular chondrocytes derived from cartilage of patients with OA

OA chondrocytes culture in monolayer exposed to cyclical mechanical stimulation of 4000µ strain at 0.33 Hz for 20 minutes followed by 24 hours incubation at 37°C showed no significant changes in GAG synthesis when compared to paired controls of all grades of OA. OA grade I n=6, OA grade II n=6, OA grade III n=4. Mechanical stimulation did not show consistent effects on chondrocytes compared to paired controls (same cartilage sample number without mechanical stimulation).

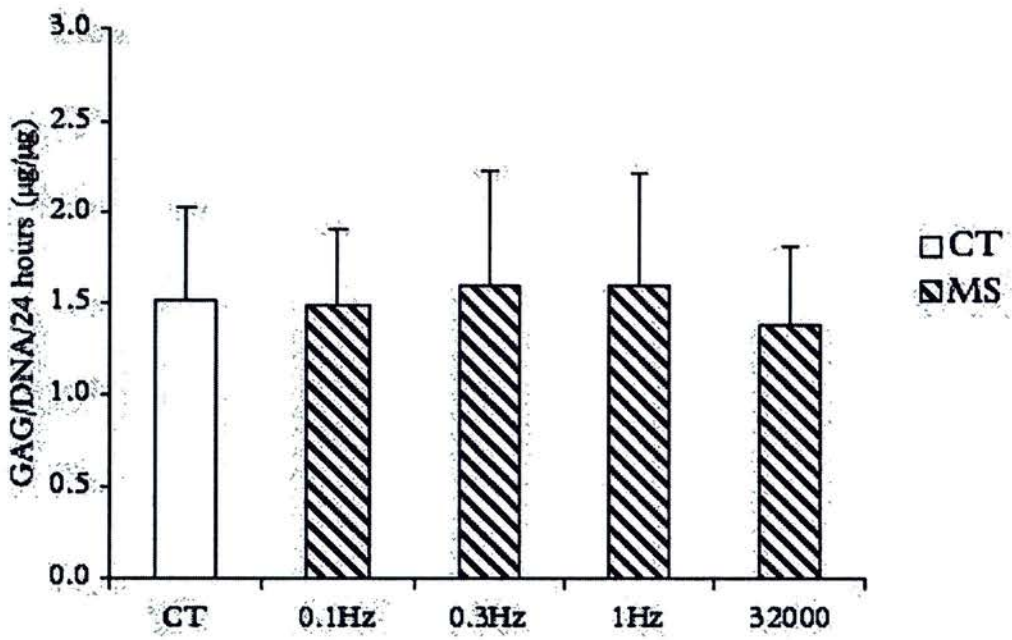


Figure 3.6 The effects of different mechanical stimulation on GAG synthesis in human articular chondrocytes derived from the cartilage of patients with OA

OA chondrocytes (mixed grade I-III) were subjected to different regimes of mechanical stimulation; 0.33 Hz at 32000µ strain (32000), 0.1 Hz at 4000µ strain (0.1 Hz), and 1 Hz at 4000µ strain (1 Hz) in addition to 0.33 Hz at 4000µ strain (0.3 Hz) followed by 24-hour incubation. The amount of GAG detected in medium was not different at these frequencies or higher strain when compared to controls (not stimulated, CT) (n=3).

aggrecan or MMP-3 mRNA in response to mechanical strain (Millward-Sadler et al 2000a, 2000b).

Because chondrocytes isolated from cartilage from patients with OA did not respond to 0.33 Hz of 4000µ strain cyclical mechanical stimulation, although this induced increased GAG synthesis in normal chondrocytes, I have done some additional experiments. Chondrocytes from OA cartilage were stimulated with eight times more strain (32000µ strain), and the same 4000µ strain with different frequencies of 0.1 Hz and 1 Hz. Again the results were that GAG synthesis in OA chondrocytes was not altered. As a previous report has shown (Wright et al 1992), cell response to different mechanical stimuli is variable and the dose response curve of osteoarthritic chondrocytes needs to be systematically measured. Cyclic loading can stimulate synthesis, but the response varies with loading frequency. At frequencies approaching those of the normal walking cycle (more than 0.1 Hz) synthesis rates were stimulated by around 30 to 50 per cent, while at low frequencies synthesis was inhibited (Urban 1994, Kim et al 1994). Different frequencies stimulate PG synthesis in cartilage, at directly compressed or uncompressed sites, within the same tissue (Parkkinen et al 1992). A frequency of 1 Hz tended

to increase the bone ingrowth, whereas 0.17 Hz caused a marked decrease in bone formation and induced cartilage production (Tagil et al 1999). In addition, it is reported that the zones and sites of articular cartilage differ in their response to joint loading *in vivo*. The superficial parts of the cartilage were sensitive to inhibition following excess pressure.

The altered response to mechanical stimulation may be an example of phenotypic difference between normal and OA chondrocytes. One hypothesis of the pathogenic event in OA could be that it is related to these changes in chondrocytes biosynthesis activity. An altered response of PG synthesis to mechanical loading can be seen from the early stage OA and beyond. By altering the distribution of mechanical loading across the joint by menisectomy, a disturbance in the metabolism of cartilage PG was provoked after six months (Little et al 1996). Teshima et al (1983) found abnormally high $^{35}SO_4$ incorporation in isolated osteoarthritic chondrocytes compared to normal. Little et al (1997) suggest that the changes in chondrocytes activities are environmentally induced rather than genotypic by comparing the same joint regions of neonatal sheep that had not borne weight, and skeletally mature.

Much osteoarthritis research has been directed to exploring whether OA is due largely to a failure of anabolic activity of OA chondrocytes, or due mainly to over-activation of cartilage catabolism by biochemical factors (Aigner 2004). However, in recent years there has been more research into the molecular and genetic phenotypes of chondrocytes that determine the homeostasis of extra-cellular matrix (Aigner 2004). Chondrocytes in OA cartilage showed significant phenotypic differences in matrix production, including aggrecan, from those in normal cartilage in an *in situ* analysis on the single cell level (Aigner and Dudhia 1997). In this report, the authors suggested three steps in the OA cartilage destruction process; (1) an increase of collagen type II and aggrecan synthesis, (2) modulation of the chondrocyte phenotype with the expression of atypical gene products such as collagen type III, (3) suppression of aggrecan core protein and collagen type II mRNA expression (Figure 3.7).

As the results have shown, anabolic activity in chondrocytes is low in our specimens and they did not response to mechanical stimulation. The results are consistent with the hypothesis that chondrocytes may change their phenotype. Thus there is a possibility that mechanical stimulation may work only to damage the collagen network, promoting its destruction and thereby further loss of PG.

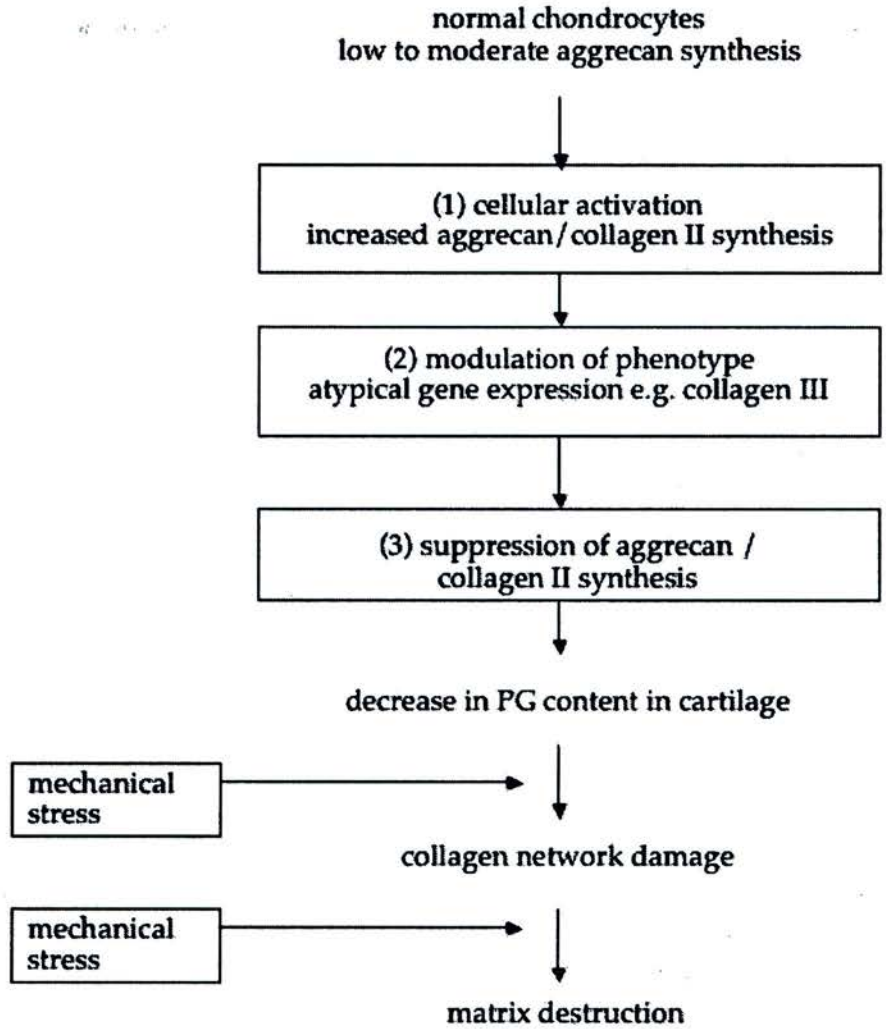


Figure 3.7 schematic representation of the three steps of cellular events in OA cartilage degeneration (hypothesis)

(1) cellular activation of chondrocytes, (2) modulation of the cellular phenotype, (3) suppression of anabolic activity. Adapted from Aigner and Dudhia (1997).

Further reports suggest that chondrocytes isolated from normal and OA cartilage behave differently. Tardif et al (1996) and Loeser et al (2000) demonstrated that, in contrast to normal chondrocytes, human osteoarthritic chondrocytes were hypo-responsive to stimulation by IGF-1. Trickey et al (2000) showed significant differences in cell mechanical (visco-elastic) properties in chondrocytes isolated from macroscopically normal and osteoarthritic human cartilage. The results in our study support the idea that there are fundamental differences between the expression of genes and molecular phenotypes of normal and OA chondrocytes that determine the homeostasis of cartilage matrix. In future research, it will be important to

further characterise the 'osteoarthritic' cellular phenotype, and to find additional markers to monitor the cellular processes.

3.2.3 The effects of mechanical stimulation on GAG synthesis in human articular chondrocytes derived from cartilage of patients with RA

3.2.3.1 Aim

The aim of this study in this section was to examine the effects of mechanical stimulation on GAG production by chondrocytes from RA articular cartilage.

3.2.3.2 Materials and Methods

Human articular knee cartilage was obtained at the time of joint replacement surgery from patients with RA of one male (37 years old), and six females with a mean age of 62.4 years (range 55-68). Primary chondrocytes were seeded in monolayer at 5 x 10^4 cells/ml concentration. Chondrocytes were cultured to sub-confluent for an average of 8.2 days. The culture medium (IMDM) containing 10 per cent serum (FCS) was then replaced by serum free medium, and cells were subjected to short-time (20 minutes) cyclic (0.33 Hz) mechanical stimulation of 4000μ strain. This was followed by 24 hours incubation at 37°C. PGs secreted into the medium were precipitated and the quantity of GAGs was measured by the DMMB assay. The total content of DNA was quantified using the bisbenzimide fluorescent dye (Hoechst 33258).

3.2.3.3 Results

Chondrocytes derived from the cartilage of patients with RA were subjected to cyclical mechanical stimulation of 4000μ strain at 0.33 Hz for 20 minutes followed by 24 hours incubation at 37°C. The result showed no significant change in GAG secretion in the medium compared to paired controls (Figure 3.8).

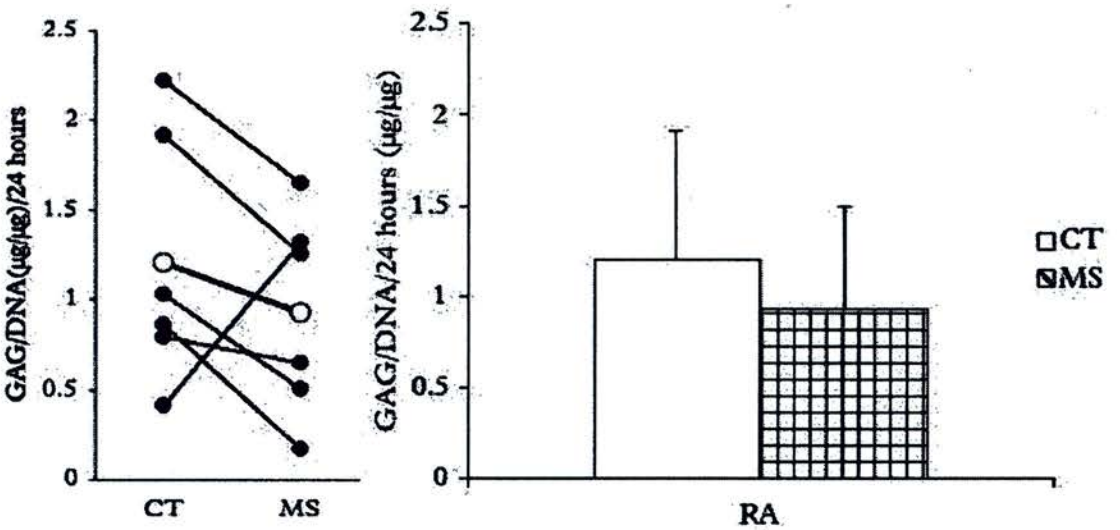


Figure 3.8 The effects of mechanical stimulation on GAG synthesis in human articular chondrocytes derived from cartilage of patients with RA

Chondrocytes derived from the cartilage of patients with RA were subjected to cyclical mechanical stimulation of 4000µ strain at 0.33 Hz for 20 minutes followed by 24 hours incubation at 37°C. The result showed no significant change in GAG excretion in the medium compared to paired controls (not stimulated) (n=6).

3.2.3.4 Discussion

The results showed that chondrocytes, isolated from the articular cartilage of patients with RA exposed to mechanical stimulation, did not demonstrate any significant change in GAG synthesis, compared to paired controls. Little is known as to the effect of mechanical stimulation in chondrocytes in cartilage obtained from patients with RA, or of the role of mechanical stimulation in the regulation of pro-inflammatory cytokines. No studies have been made to test the effect of mechanical loading and unloading on human articular chondrocytes from RA cartilage. Recently mechanical loading of RA synovial cells has been shown to down regulate MMP-13 genes (Type II collagen proteinase) while unloading is followed by up regulation (Sun and Yokota 2001). Xu et al (2000) have suggested the possibility of treating arthritic joints by cyclic tensile strain, which acts as an antagonist of IL-1β actions *in vitro*. The x-rays of hands of patients with rheumatoid arthritis showed greater joint destruction in the dominant hand. Owsianik et al (1980) recorded this difference using a radiological assessment. The small hand joints are not weight bearing. It is likely that daily use leads to joint destruction in RA joints. Our results suggest impaired anabolic response to mechanical stimulation in RA joints.

There were no statistical differences in GAG production between chondrocytes from OA and RA after mechanical stimulation. This suggests that differences between these pathological joint conditions are not related to the ability of chondrocytes to produce PG in response to mechanical stimulation. Pulsatelli et al (1999) showed that chondrocytes isolated from articular cartilage in patients with RA did not produce more chemokines, such as IL-8, than OA or post-traumatic patients. However, cells from diseased tissue in RA may have different sensitivities to IL-1β and TNF-α. We have found no differences between chondrocytes isolated from RA and OA joints except that OA III chondrocytes produce significantly less GAG. However, chondrocytes from RA joints have clearly lost the ability to respond to mechanical stimulation in our system. We can only speculate that the abnormal environment surrounding chondrocytes in RA joints may alter the chondrocyte phenotype, probably in a similar way to OA chondrocytes in terms of the response to mechanical stimulation.

3.2.4 The effects of mechanical stimulation on cell-associated GAG synthesis in human articular chondrocytes derived from normal, OA, and RA cartilage

3.2.4.1 Aim

Half of the PGs synthesized by the cells spend less than 24 hours in the cell-associated matrix before moving into the inter-territorial matrix (Hassell et al 1986). Biosynthesis and Catabolism of PG is not well understood. The aim in this study in this section was to analyse the effects of mechanical stimulation on GAG synthesis which retained in cell-associated matrix.

3.2.4.2 Materials and Methods

Human articular knee cartilage was obtained at the time of joint replacement surgery from patients with OA or RA, and from above knee amputation from patients with peripheral vascular disease. The articular cartilage surface was assessed macroscopically (McElligott and Collins 1960) and microscopically (OARSI) and graded into normal, OA (grade I to III) or RA. Normal chondrocytes were isolated from articular cartilage of two male (76 and 39 years old) and one females (69 years old). OA chondrocytes were isolated from the articular cartilage of four males with a mean age of 65.7 years (range 57-71), and two females (57 and 76 years old). RA chondrocytes were isolated from the articular cartilage of three females with a mean age of 51.6 years (range 37-63). Chondrocytes derived from normal cartilage, OA cartilage and RA cartilage were subjected to cyclical mechanical stimulation of 4000μ strain at 0.33 Hz for 20 minutes followed by 24 hours incubation at 37°C. The cell-associated GAG was extracted from the cells in monolayer culture using guanidium hydrochloride (GuHCl) extraction buffer. GuHCl extracts were precipitated overnight with three volumes [v/v]

ethanol at –20°C and precipitated again with ethanol overnight for the complete removal of GuHCl buffer. Fourth, all the samples were centrifuged at 1000 *g* for 30 minutes at 4°C. Pellets were re-suspended in proteinase K buffer for DMMB assay.

3.2.4.3 Results

The result showed no significant change in cell-associated GAG synthesis after mechanical stimulation compared to paired unstimulated controls (Figure 3.9).

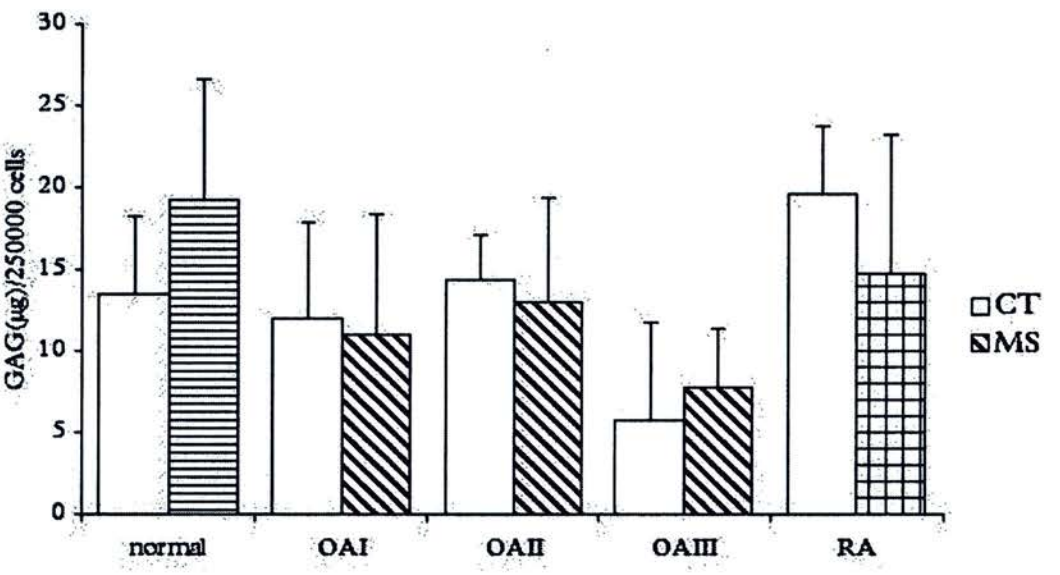


Figure 3.9 Effect of mechanical stimulation on the cell-associated GAG synthesis

Mechanical stimulation (MS) did not alter cell-associated GAG synthesis compared to paired controls (CT). Values are given as the mean ± SD. (normal n=3, OAI n=2, OAII n=2, OAIII n=2, RA n=3)

3.2.4.4 Discussion

The results show no significant change in cell-associated GAG after mechanical stimulation, compared to paired unstimulated controls. Smith et al (1995) have shown that the cell-associated GAG accounted to only 5 per cent of the total of newly synthesized GAG present in the medium fraction in human articular chondrocyte monolayer cultures. Fluid shear stress did not alter the distribution of GAG in the medium fraction, or the cell-associated fraction, measured by sulphate incorporation. In our system, the total amount of GAG in the cell-associated matrix was around 60 to 70 per cent of the total amount of GAG released in

medium (Fig 3.9). This is because the chondrocytes were cultured on average for eight days and the cell-associated matrix accumulated during this pre-experimental period. This accumulation may mask the effects of mechanical stimulation, and it may not be appropriate to use the cell-associated fraction when comparing the amount of GAG before and after mechanical stimulation. It did not , however, demonstrated that mechanical stimulation did not alter the distribution of GAG from the cell-associated fraction to the medium fraction.

3.3 Summary

The chondrocytes were exposed to cyclical mechanical stimulation in an apparatus that functioned to produce a strain on the base of the culture dishes with attached cells in monolayer. Following 20 minute 4000μ strain cyclical mechanical stimulation at 0.33 Hz, the GAG synthesis of chondrocytes from normal cartilage (measured by the DMMB assay, corrected by DNA) increased significantly compared to unstimulated controls.

Cyclical mechanical stimulation applied to articular chondrocytes from patients with OA and RA in monolayer culture did not show any significant change in GAG synthesis, measured by the DMMB assay. Chondrocytes from OA grade III synthesized on average 65.0 per cent and 59.6 per cent less GAG (corrected by DNA) than OA grade I and II chondrocytes, which were statistically significant ($p=0.0038$ and $p=0.071$ respectively). Chondrocytes from OA cartilage were stimulated with eight times more strain (32000μ strain), and the same 4000μ strain with different frequencies of 0.1 Hz and 1 Hz. Again the results were that GAG synthesis in OA chondrocytes was not altered.

Cyclical mechanical stimulation did not alter the amount of GAG in cell-associated matrix in chondrocyte monolayer culture from normal, OA and RA cartilage.

Chapter Four

The Role of α5 Integrin, αVβ5 Integrin and Integrin Associated Protein CD47 in Responses to Mechanical Stimulation in Human Articular Chondrocytes in Monolayer Culture

Cell-matrix interactions mediated by integrins are important in the regulation of chondrocyte function and development. A series of experiments have been undertaken to establish whether α5 integrins, αVβ5 integrins and the integrin associated protein CD47 are involved in chondrocyte mechano-transduction following cyclical mechanical stimulation of 0.33 Hz (two seconds on, one second off), 4000μ strain. These integrins were chosen because (1) studies have shown that α5 integrin, αVβ5 integrin, and CD47 are expressed in cartilage (Salter et al 1992, Orazizadeh 2004), (2) to test α5 integrin involvement in mechano-transduction pathway as a part of α5β1 complex, (3) to test CD47 involvement in α5β1 integrin mediated chondrocyte mechano-transduction as indicated by a study of Orazizadeh 2004, (3) to compare classical mechano-transduction mediator α5β1 (e.g. fibronectin receptor) with other integrin such as αVβ5 (e.g. vitronectin receptor).

4.1 The effects of anti-integrin antibodies on GAG synthesis in response to cyclical mechanical stimulation in human articular chondrocytes derived from normal cartilage

4.1.1 Aim

The aim of the study in this section was to test the hypothesis of that increased GAG synthesis is mediated by α5β1 integrin in normal chondrocytes, which subjected to mechanical stimulation. Moreover, the involvement of αVβ5 integrin and CD47 in chondrocyte mechano-transduction was tested.

4.1.2 Materials and Methods

The articular cartilage surface was assessed and graded macroscopically for the presence or absence of OA and the severity of OA using the OA grading system of McElligott and Collins (1960). Cartilage was also assessed microscopically following safranin O and Hematoxylin-

and-eosin staining. Histological grading was undertaken using the OARSI Osteoarthritis Cartilage Histopathology Grade Assessment (OARSI).

Human articular knee cartilage of normal appearance was obtained at the time of joint replacement surgery from patients with OA (one 76 year old male and two females of 66 and 73 year old), and from above knee amputation from a patient with peripheral vascular disease 76 year old male). Primary chondrocytes were studied in non-confluent monolayer culture. Cells were seeded at $25x10^4$ into dishes and cultured on average for 10.5 days. Cells were maintained in serum free medium for 16 hours prior to mechanical stimulation at 0.33 Hz 4000μ strain for 20 minutes, and this was followed by 24 hours incubation at 37°C. Experiments were repeated using single dishes of human articular chondrocytes derived from at least four adult donors.

Function blocking monoclonal antibodies to α5 integrin (P1D6 MAB1956, Chemicon International), αVβ5 integrin (P1F6 MAB1961, Chemicon International) and CD47 (Bric 126, IBGRL Research) at a concentration of 1 μg/ml, were incubated with chondrocytes cultured in monolayer at 37 °C for 30 minutes, prior to 0.33 Hz 4000μ strain mechanical stimulation for 20 minutes. GAG synthesis was measured after 24 hours incubation, using the methods for assay of DMMB and DNA in described in Chapter Two. Details of results were calculated as in Chapter Two. Details of results of individual DMMB and DNA assays and calculated values are shown in tables (Appendix).

4.1.3 Results

Microscopic grading (OARSI) showed a close relation to the macroscopic grading of OA cartilage (Appendix). Cartilage graded as macroscopically normal was confirmed as microscopically normal cartilage except in cells from two cases (14013 and DS088C) showed very early OA (grading 1, stage 1, score 1). Cells from these two cases were, however, categorised as normal chondrocytes as their electro-physiological responses following 0.33 Hz cyclical mechanical stimulation was one of hyperpolarization.

In normal chondrocytes there were significant increases in GAG synthesis following mechanical stimulation at 0.33 Hz, 4000μ strain (mean ± SD: 2.63 ± 0.84 μgGAG/μgDNA/ 24 hours compared with non-stimulated controls: 1.40 ± 0.89 (†P=0.046) in the presence of non-specific control antibody of mouse IgG (Figure 4.1). However, in the presence of antibodies to α5 integrin, αVβ5 integrin, and CD47, the accelerated GAG synthesis response was blocked completely, showing no difference between control chondrocytes and mechanically-stimulated chondrocytes (Figure 4.1). Incubation with mouse IgG and

antibodies to, α5 integrin, αVβ5 integrin, and CD47 alone without mechanical stimulation did not influence GAG synthesis (mean ± SD: 1.22 ± 0.76, range 0.379 - 2.98 μgGAG/ μgDNA/24 hours).

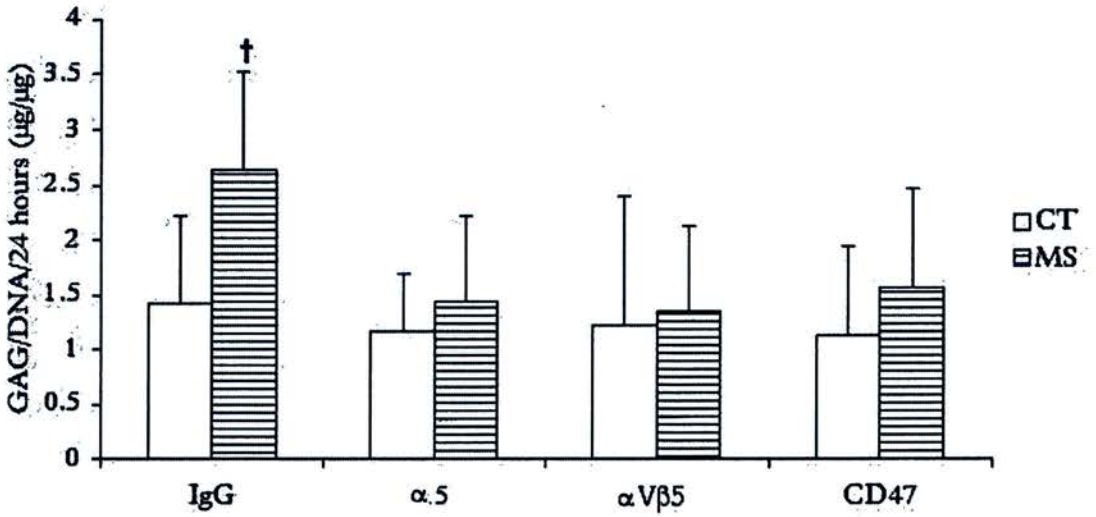


Figure 4.1 The effects of anti-integrin antibodies on GAG synthesis in response to cyclical mechanical stimulation in normal chondrocytes.

Normal chondrocytes were exposed to cyclical mechanical stimulation (0.33 Hz, 4000μ strain for 20 minutes at 37 °C) followed by 24 hours incubation in the presence or absence of anti-integrin antibodies. The GAG synthesis of chondrocytes from normal cartilage increased significantly compared to unstimulated controls in the presence of control antibody mouse IgG (†p=0.046). The increased GAG synthesis following mechanical stimulation was blocked in the presence of antibodies to α5 integrin, or αVβ5 integrin, or CD47. The white bars (CT) are paired controls without mechanical stimulation. Values are given as means ± SD (n=4).

4.1.4 Discussion

In these experiments only one antibody concentration (1 μg/ml) was tested, selected on the basis of experiments investigating the electro-physiological response of chondrocytes to mechanical stimulation.

4.1.4.1 The role of α5 integrin in mechano-transduction

Following 20 minute 4000μ strain cyclical mechanical stimulation at 0.33 Hz, the GAG synthesis of chondrocytes from normal cartilage increased significantly compared to

unstimulated controls in the presence of control antibody mouse IgG. Increased GAG synthesis following mechanical stimulation was blocked in the presence of anti-α5 integrin antibody (P1D6). A reduction in GAG synthesis, in response to mechanical stimulation in the presence of P1D6, is consistent with previous results which showed that P1D6 inhibited the hyperpolarization response (Millward-Sadler et al 2000a), and is consistent with the hypothesis that α5β1 integrin, the fibronectin receptor, is the predominant integrin dimer in cartilage (Salter et al 1992) mediating the mechano-transduction.

The anti-a5 integrin antibody P1D6 is a monoclonal Ig isotype subclass IgG_3 antibody that recognises α subunit of the α5β1 complex (Wayner et al 1988). P1D6 is regarded as a function blocking antibody as P1D6 inhibited human cell adhesion to fibronectin, but not collagen or laminin, following reaction with α5β1 integrin (Wayner et al 1988). Recently it has been reported that P1D6 blocks the recognition by α5β1 of the PHSRN (pro-his-ser-arg-asn) synergy sequence in fibronectin, by blocking the synergy recognition site in the I domain of α5 subunit (Takagi et al 2002, Burrows et al 1999) (Figure 4.2). Binding of P1D6 to α5β1 does not block the recognition of RGD, and is not affected by anti-β1 monoclonal antibodies (Burrows et al 1999). The anti- α integrin antibody P1D6 blocks the up-regulation of GAG synthesis in response to mechanical stimulation in normal chondrocytes by blocking cell - fibronectin interaction through α5 subunit.

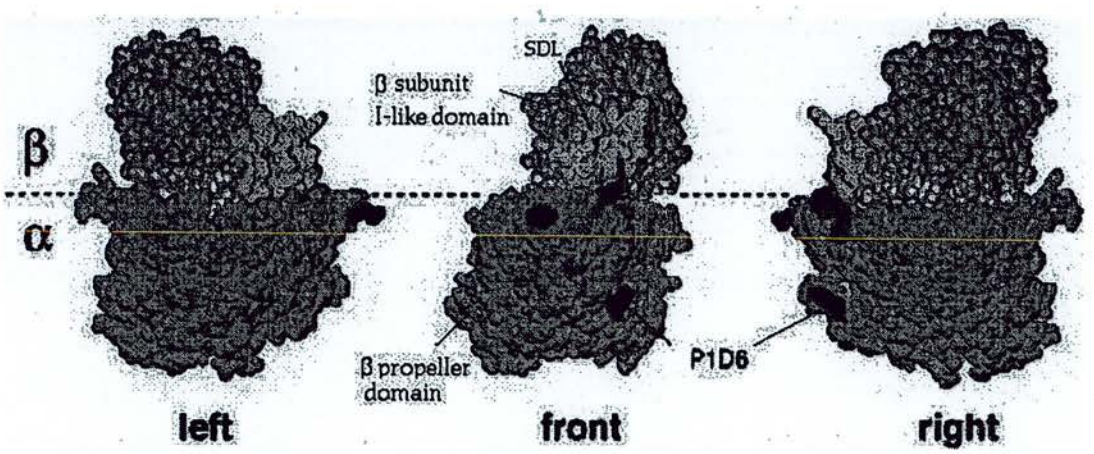


Figure 4.2 Location of the epitopes for function blocking monoclonal antibody P1D6

Integrins contain two non-covalently associated glycoprotein alpha and beta subunits with extra-cellular domains. Both subunits have a 'head' region. The head piece contains a beta-propeller domain in the alpha subunit and I-like domain in the beta subunit. These two domains are correctly folded only when they are associated. Beta subunit's I-like domain and alpha subunit's β-propeller domain is shown in a space filling model. The monoclonal antibody P1D6 epitope is shown. The species specific residues recognized by P1D6 have been precisely mapped in

the beta-propeller domain on alpha subunit. SDL: specificity determining loop. Adapted from Takagi et al (2002).

Although the available evidence points to both α and β subunits being required for a complete response, there are several reports that have specifically focused on the role of the α5 subunit. The β1 subunit is shared by other receptors for laminin and collagen, but the α chain is unique to the fibronectin receptor. The only integrin in which the α5 subunit is known to occur is α5β1 (Otey et al 1990, Yang et al 1993)(Table 1.3). The α chain of integrins was thought to confer the specificity for the recognition of different ligands, whereas the common β1 chain was thought to be involved in signal transduction (Groux et al 1989, Yamada et al 1991). Recently, however, it has been found that both α and β chains contribute to both ligand and binding events (Kornberg et al 1991, Schwartz et al 1991, Werb et al 1989). Cao et al (1998) have demonstrated that the α5 integrin subunit mediates cell adhesion, spreading and signalling on fibronectin, by modulating the function of the cytoplasmic domain of β1 subunit. The binding of α5 integrin to its ligand fibronectin activates a signalling pathway (outside-in signalling) in muscle cells, which involves FAK and PKC. This activates integrins and leads to cell adhesion (inside-out signalling) (Disatnik and Rando 1999). It has previously been shown that α5-deficient embryonic cells survive poorly (Yang et al 1993) and undergo apoptotic cell death on a substrate of fibronectin (Taverna et al 1998). The α5 integrin subunit acted as a mechano-receptor in human umbilical vein endothelial cells (Yano et al 1997) suggesting that α5 is vital for the mechano-transduction pathway. Yano et al have demonstrated that the α5 subunit recognized cyclic strain through the cytoplasmic domain of the β1 subunit. It has also been shown that mechanical loading induces an increase in the amount of α5 integrin mRNA in chondrosarcoma cells but not β1 or αV integrin mRNA (Holmvall et al 1995). As demonstrated by this study, cyclic mechanical stimulation of 0.33 Hz 4000μ strain increases GAG synthesis. The α5 subunit is involved in these processes, although the mechanisms by which mechanical signals are transformed into cellular response are not well understood. There is a binding region to ligands such as fibronectin on the α5 subunit, and ligand occupancy is required to respond to mechanical stimulation. The cytoplasmic domain of the β1 subunit has a critical role for tyrosine phosphorylation (Cao et al 1998), and for recruiting other focal adhesion molecules such as talin and α-actinin (Horowitz et al 1986, Otey et al 1990). If either of the integrin subunits is functionally blocked, one could hypothesise that the increase in PG synthesis following mechanical stimulation would be blocked. In support of this, Scully et al (2001) have shown that blocking the β1 integrin subunit with RGD peptide and anti-β1 antibodies (4-B-4) reduced PG synthesis in bovine chondrocytes in alginate bead culture. They suggested that type II collagen and β1 integrin interaction modulated the effect of TGF-β on chondrocytes.

One of the roles of α5 integrin as a part of α5β1 integrin is to mediate of cell attachment to fibronectin and intracellular signalling activation (outside-in signalling). This is followed by

inside-out signalling activating other molecules including integrins themselves ultimately leading to cellular responses, such as an increase in PG (GAG) synthesis.

4.1.4.2 The role of αVβ5 integrin in mechano-transduction

Increased GAG synthesis following mechanical stimulation was blocked in the presence of anti- αVβ5 integrin antibody (P1F6). This was an unexpected result, as previous reports have shown that anti- αV integrin antibodies (VNR-147; Wright et al 1997), anti- αVβ3 integrin antibodies (23C6; Salter et al 2002) and anti- αVβ5 integrin antibodies (P1F6; Millward-Sadler et al 1999) did not affect hyperpolarization responses in normal chondrocytes. In human bone cells, P1F6 reduced the hyperpolarization response at 0.104 Hz mechanical stimulation but not 0.33 Hz. This was the only indication that there may be a role for αVβ5 integrin in mechano-transduction in these cells. However, the membrane hyperpolarization response in chondrocytes does not always relate with anabolic responses. For example, inhibition of the hyperpolarization response by blocking stretch-activated ion channels did not influence the up-regulation of aggrecan mRNA in normal chondrocytes following mechanical stimulation (Millward-Sadler et al 2000). The effect of anti-αVβ5 integrin antibodies on GAG synthesis, in response to mechanical stimulation, is potentially interesting.

The anti-αVβ5 integrin antibody P1F6 is a monoclonal Ig isotype subclass IgG_1 antibody that recognises the αVβ5 complex. P1F6 is reported as function blocking antibody as P1F6 inhibited human cell adhesion to vitronectin (Weinacker et al 1994) and does not cross react with any other αV containing integrins. However, P1F6 is not well documented in terms of the inhibitory effect of human cell adhesion to fibronectin. Giuffrida et al (2004) have reported that the pre-treatment with P1F6 did not block the migration of epithelial cell line cells on fibronectin.

Human articular chondrocytes express αV integrin as a part of αVβ5 integrin dimer much less strongly than α5β1 integrin (Woods et al 1994). Precise identification of the functional activity of αVβ5 integrin has been difficult because of the simultaneous expression of multiple integrin β subunits on cells as part of other αV-containing heterodimers αVβ1 (Bodary and McLean 1990, Vogel et al 1990), αVβ3 (Pytela et al 1985, Cheresh et al 1989b), αVβ5 (Sonnenberg et al 1988, Cheresh et al 1989a), αVβ6 (Sheppard et al 1990), and αVβ8 (Moyle et al 1991). Both αVβ3 and αVβ5 integrins are notably abundant in cartilage (Chapter One Table 1.3). Pasqualini et al (1993) have generated a panel of novel monoclonal antibodies recognising the β5 subunit directly, showing that the β5 subunit can play a role in cell adhesion to both vitronectin and fibronectin. This implies that αVβ5 integrin may have a role in signalling on vitronectin or fibronectin in a similar way to α5β1 integrin. In addition,

αVβ5 integrin may have a role in signalling on fibronectin and vitronectin indirectly through other extra-cellular matrix proteins, such as βig-h3, which has recently been confirmed as an αVβ5 ligand (Kim et al 2002). On the other hand, some of the reports showed that αVβ3 integrin, but not αVβ5 integrin, was able to localise in focal adhesions, and mediate cell spreading and migration on vitronectin and fibronectin as a substrate (Leavesley et al 1992, Wayner et al 1991). The cytoplasmic tail of the β5 subunit is structurally distinct from that of the β1 subunit, although β1 and β5 cDNA sequences are the most closely related integrin β subunits (Ramaswamy and Hemler 1990, Mclean et al 1990, Suzuki et al 1990). Both α5 and αV integrins bind to fibronectin and are expressed on the same cell surface, although they may be responsible for different intracellular signalling pathways. It has recently reported that fibronectin- α5β1 interaction regulates angiogenesis, and αVβ3 seems participate in the same pathways. However, the pathways are distinct from those involving fibronectin and αVβ5 integrin (Giuffrida et al 2004).

Integrin ligand interactions are diverse. Indeed, there are nine different integrin combinations (αVβ5, αVβ1, αVβ3, αIIbβ3, αVβ6, α3β1, α4β1, α4β7, α5β1). All of them can function as a fibronectin receptor (Pasqualini et al 1993). Despite this apparent redundancy, genetic studies imply that many integrins have unique functions (Hynes 1996). However, post-ligand-binding events are still not fully understood. It appears that β5 integrin activity can be stimulated into a more fully functional state (Pasqualini et al 1993). In this regard, β5 integrin may adhere to fibronectin with different affinities, compared to β1 integrin.

Although a specific role for the αVβ5 integrin dimmer has not been clarified, the αV subunit has been shown to mediate increases in intracellular Ca^{2+} (Yamada and Miyamoto 1995). For example, binding αVβ5 integrin to its ligand osteopontin affects changes in intracellular Ca^{2+} levels (Denhardt and Noda 1998). Moreover, Schwartz and Denninghoff (1994) have shown that αV integrin, but not α5β1 integrin, mediates the rise in Ca^{2+} during the spreading of endothelial cells, although substrate binding was predominantly mediated by α5β1 integrin. They suggested that the different binding activities of integrins may reflect differences in regulatory functions, so that adhesion and signalling processes may be mediated by different integrins.

Mechanical deformation of many cell types results in an increase in intracellular Ca^{2+}, such as fibroblasts (Grierson et al 1995), bone cells (Hung et al 1995), osteoclasts (Xia et al 1995, Wiltink et al 1995), smooth muscle cells (Ohata et al 1995), lung epithelial cells (Wirtz et al 1990), endothelial cells (Sigurdson et al 1993, Naruse et al 1993), a hepatocyte cell line (Nebe et al 1995), and articular chondrocytes (Yellowley et al 1997). Intracellular Ca^{2+} can be a modulator of the cytoskeleton (Schwartz 1993b, Wu et al 2000), probably via a pathway that

involves PIP_2, the levels of which are controlled by Rho (Burridge et al 1996). Mechano-transduction in normal chondrocytes is, a calcium-dependent pathway (Wright et al 1996). It has been shown that a number of ion channels are involved in the mechano-transduction pathway, including gadolinium sensitive stretch-activated ion channels, L-type Ca^{2+}-activated K^+ channels (Wright et al 1996). Somatostatin and cadmium chloride, which block L-type calcium channels, inhibit the chondrocyte hyperpolarization response. EGTA, which chelates extra-cellular Ca^{2+}, reduces the response, and thapsigargin, which raises intracellular Ca^{2+}, causes the further hyperpolarization after mechanical stimulation (Wright et al 1996). Stretch-activated ion channels have been implicated in chondrocyte mechano-transduction by use of the stretch-activated ion channel blocker, gadolinium. These stretch-activated channels allow the passage of ions like Na^+, K^+ and Ca^{2+}(Ruknudin 1993). Direct Ca^{2+} influx through stretch-activated channels was reported in cultured chick heart cells that were mechanically stimulated (Sigurdson 1992), and Ca^{2+} influx through stretch-activated ion channels has been studied by others (Wiltink et al 1995, Ohata et al 1995, Wirtz et al 1990, Naruse et al 1993).

The influx of Ca^{2+} through stretch-activated ion channels may be one of the primary events to occur in response to mechanical stimulation. The influx of calcium stretch-activated ion channels may activate the chondrocyte mechano-transduction pathway directly. Alternatively, stretch-activated ion channels may interact with other integrins, forming an integrated signalling complex. Since there are several ion channels as well as integrin-mediated calcium waves, the elevation of intracellular calcium may be controlled by a number of ion channels and integrins. Moreover, different ion channels may play distinct roles in converting the same mechanical signal into different cellular responses. For example stretch-activated channels are involved in cell proliferation, whereas calcium channels mediate cell differentiation responses (Wu et al 2000). It is tempting to speculate that $\alpha V\beta 5$ integrin has a role in integrin-regulated calcium entry in response to cyclical mechanical strain as αV integrin is likely to influence Ca^{2+} influx. Increases in intracellular Ca^{2+} concentration regulate many mitogenic signals and pathways (Burridge et al 1996). The mechanism by which integrins elevate intracellular Ca^{2+} has not yet been elucidated. This mechanism may explain how mechano-transduction, which eventually leads to increased PG synthesis in normal chondrocytes, is blocked by anti-$\alpha V\beta 5$ integrin antibodies (Figure 4.3).

4.1.4.3 The role of CD47 in mechano-transduction

Following 20 minute 4000μ strain cyclical mechanical stimulation at 0.33 Hz, the GAG synthesis of chondrocytes from normal cartilage increased significantly compared to unstimulated controls in the presence of control antibody mouse IgG. Increased GAG synthesis following mechanical stimulation was blocked in the presence of anti-CD47 antibody (Bric 126). CD47, or integrin associated protein (IAP), is a transmembrane

glycoprotein of the Ig super-family, it can modulate a range of cellular functions. Bric 126, an antibody to CD47, was found to inhibit the electro-physiological response in human bone cells (Salter et al 1997) and in normal chondrocytes (Orazizadeh 2004) following 0.33Hz 4000μ strain mechanical stimulation, but had no effect on the depolarization response induced by 0.104 Hz mechanical stimulation (Salter et al 1997). The inhibition of accelerated GAG synthesis in response to 0.33Hz 4000μ strain cyclical mechanical stimulation in the presence of Bric 126 is consistent with the mechano-transduction mechanism previously proposed.

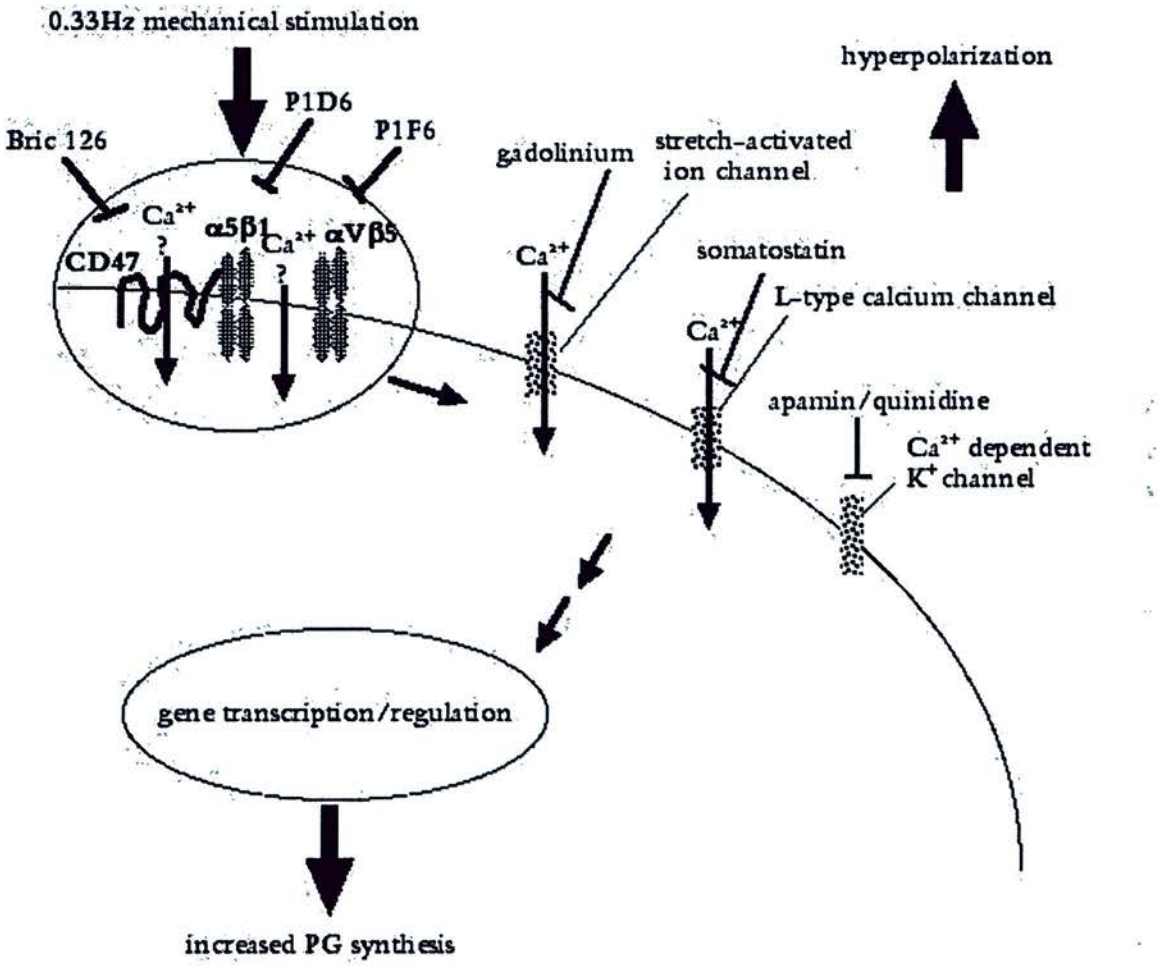


Figure 4.3

The model suggested by this study is one of mechano-transduction pathways, which are calcium-dependent, and activated by 0.33Hz 4000μ strain mechanical stimulation in normal articular chondrocytes

CD47 appears to be involved in integrin-mediated signal transduction (Brown 1990, Zhou 1993, Blystone 1995) following cell adhesion to vitronectin or fibronectin (Lindberg et al

1993, 1994, 1996), cell spreading in response to vitronectin (Gao et al 1996b), and the inward calcium currents induced in endothelial cells (Schwartz et al 1993a 1993b) and fibroblasts (Tsao and Mousa 1995). Recent data show that CD47 can associate with, and modulate, integrins of the β1 (Wang et al 1998), β2 (Van Strijp et al 1993, Ishibashi et al 1994) and β3 (Gao 1996a, 1996b, Chung et al 1997) families. Blystone et al (1995) have reported that CD47 has no effect on α5β1 integrin interaction with fibronectin in the absence of αVβ3 integrin. This implies that CD47 may modulate α5β1 integrin function indirectly through the αVβ3 integrin and CD47 signalling complex. There is a physical association between CD47 and α5 integrin in human articular chondrocytes (Orazizadeh 2004). Since integrins, including α5β1, have no intrinsic enzymatic activity of their own, they must interact with other proteins to be activated (Damsky and Werb 1992). In addition, fibronectin-mediated adhesion to α5β1 integrin needs a higher affinity state in the receptor to be activated (Arroyo et al 1992, 1993, Faull et al 1993, O'Toole et al 1994, Sanchez-Mateos et al 1993, Blystone et al 1995). Integrin-related receptors such as CD47 may have a role in modulating their affinity states as part of a cross-talk between integrins. CD47 is a strong candidate for a role in modulating the function of α5β1 integrin in chondrocyte mechano-transduction. The results obtained here support the idea that CD47 modulates α5β1 integrin-mediated signal transduction.

The anti-CD47 antibody Bric 126 is a monoclonal Ig isotype subclass Ig2b antibody that recognises the IgG domain of CD47 on cell membranes (Avent et al 1988). Bric 126 is considered to be a function blocking or function modifying antibody (Shahein et al 2002), as Bric 126 inhibited the binding of pig-CD47 expressing cells to fibronectin and thrombospondin (Shahein et al 2002). In addition, another monoclonal anti-CD47 antibody, B6H12, specifically interrupts CD47 and thrombospondin (Ticchioni et al 1997). Shahein et al has speculated that Bric 126 and B6H12 probably recognize functionally important epitopes of CD47. In addition, B6H12 has been shown to inhibit ligand binding to αIIbβ3 on several cell lines and to block the increase in intracellular Ca^{2+} (Schwartz et al 1993a). Some other antibodies such as 2D3, a non-function blocking antibody that bind to CD47, failed to block platelet adhesion to thrombospondin (Ticchioni et al 1997). Because there is no evidence that fibronectin is a ligand for CD47, the effect of Bric 126 is likely to a consequence of CD47-mediated enhancement of α5β1 integrin interaction with fibronectin. In this way, the anti-CD47 antibody Bric 126 blocks the elevation of GAG synthesis in response to mechanical stimulation in normal chondrocytes. Interestingly, there is a similar mechano-sensitive regulatory pathway comprising integrin, CD47 and thrombospondin in fibroblasts and in endothelial cells (Graf et al 2003).

Although the association of CD47 with certain integrins was usually found to modulate their function, some signal transduction through CD47 is integrin-independent (Reinhold et al 1997). CD47 seems to be expressed at higher levels than integrins. For example, CD47 is present on lymphocytes, where little αVβ3 integrin is expressed, and on erythrocytes, where

no integrin is expressed (Brown et al 1990). Similarly, direct association of CD47 with β1, or β2, or β3 integrins in neutrophils could not be detected by a co-immuno-precipitation (Liu et al 2001). In addition, same authors have observed that blocking β1 or β3 integrins has no effect on CD47-mediated regulation of neutrophil trans-epithelial migration. It is likely that the integrin CD47 complex regulates mechano-transduction in different cell types in response to different mechanical stimuli.

As already mentioned, several ion channels and several integrins control the elevation of intracellular calcium (Figure 4.3). Intracellular Ca^{2+} influx is controlled by adhesion to the extra-cellular matrix through integrin receptors via integrin-associated, voltage-independent, receptor-activated membrane calcium channels in endothelial cells (Schwartz 1993b). This requires CD47 (Schwartz 1993a). This means that CD47 is likely to be an integrin-associated, transmembrane, voltage-independent calcium channel. Inhibition of the hyperpolarization response in normal chondrocytes to a 0.33 Hz 4000μ strain cyclical strain, with the anti-CD47 antibody Bric 126, supports a role for integrin-regulated calcium entry, although it is difficult to determine which integrins and ion channels are involved, as they function as complex in regulating mechano-transduction.

The results obtained in the present study demonstrate that the mechano-transduction pathway leading to increased GAG synthesis in normal chondrocytes is dependent on integrin and extra-cellular matrix interactions. The precise role of integrins and CD47 in the response to cyclical mechanical stimulation leading to PG synthesis remains to be elucidated, but they do appear to be implicated in the mechano-transduction pathway.

4.2 The effects of anti-integrin antibodies on GAG synthesis in response to cyclical mechanical stimulation in human articular chondrocytes derived from OA cartilage

4.2.1 Aim

Many of the effects of extra-cellular molecules on cellular processes are mediated through signal transduction via the integrins. In understanding the degradation and repair processes that are operating in the cartilage extra-cellular matrix in OA joints, the interaction of extra-cellular matrix molecules with chondrocytes, which is modulated by integrins, may be important. A parallel set of experiments to chondrocytes from normal cartilage was carried out with chondrocytes from OA cartilage (grade I to III), to investigate whether there was a role for α5 integrin, αVβ5 integrin and CD47 in the response to 0.33 Hz 4000μ strain mechanical stimulation.

4.2.2 Materials and Methods

Human articular knee cartilage was obtained at the time of joint replacement surgery from patients with OA. The articular cartilage surface was assessed macroscopically (McElligott and Collins 1960) and microscopically (OARSI) and graded into OA I to III. OA chondrocytes were isolated from the articular cartilage of four males with a mean age of 64.5 years (range 39-79), and six females with a mean age of 64.6 years (range 53-74). Primary chondrocytes were seeded in monolayer at 5 x 10^4 cells/ml concentration. Chondrocytes were cultured to sub-confluent for an average of 8.0 days. The culture medium (IMDM) containing 10 per cent serum (FCS) was then replaced by serum free medium and left 16 hours Function blocking monoclonal antibodies to α5 integrin (P1D6), αVβ5 integrin (P1F6) and CD47 (Bric 126) at a concentration of 1 μg/ml, were incubated with chondrocytes cultured in monolayer at 37 °C for 30 minutes, prior to 0.33 Hz 4000μ strain mechanical stimulation for 20 minutes. This was followed by 24 hour incubation at 37°C. PGs secreted into the medium were precipitated and the quantity of GAGs was measured by the DMMB assay. The total content of DNA was quantified using the bisbenzimide fluorescent dye.

4.2.3 Results

Microscopic grading (OARSI) showed a close correlation with the macroscopic grading of OA cartilage (Appendix). Cartilage graded as macroscopically OA grade was confirmed as microscopically OA cartilage. Chondrocytes from these cases after 0.33 Hz cyclical mechanical stimulation depolarized. OA grade I and II cartilage were not clearly divisible, for example, OA grade II DS037C and 14078 scored similar to OA grade I cartilage (grading 2 stage 1 and score 2).

In OA chondrocytes there were no significant increases in GAG synthesis following mechanical stimulation at 0.33Hz, 4000μ strain (mean ± SD: 1.40 ± 047, 1.03 ± 0.56, 0.37 ± 0.24 μgGAG/μgDNA/24 hours compared with non-stimulated controls: 0.99 ± 0.47, 0.85 ± 0.43, 0.30 ± 0.27 in the presence of non-specific mouse IgG control antibody in OA grade I, II, and III respectively (Figure 4.4, 4.5, 4,6). OA grade III chondrocytes synthesized less GAG compared to OA grade I and II ($p=0.031$ I vs. III, $p=0.056$ II vs. III) in the presence of non-specific mouse IgG control antibody of and this was comparable with the result obtained without antibodies. In the presence of antibodies to α5 integrin, αVβ5 integrin, and CD47, GAG synthesis was not different in control chondrocytes and mechanically-stimulated chondrocytes in all grades of OA. Incubation with mouse IgG and antibodies to α5 integrin, αVβ5 integrin, and CD47 without mechanical stimulation (mean ± SD: 1.22 ± 0.76, range 0.379 - 2.98 μgGAG/μgDNA/24 hours) did not affect GAG production in OA chondrocytes.

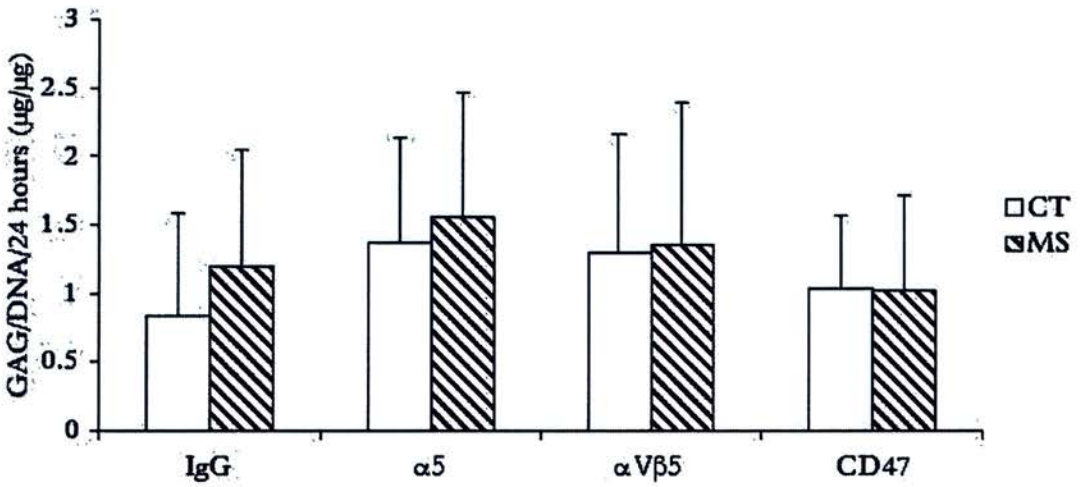


Figure 4.4 The effects of anti-integrin antibodies on GAG synthesis in response to cyclical mechanical stimulation in OA grade I chondrocytes

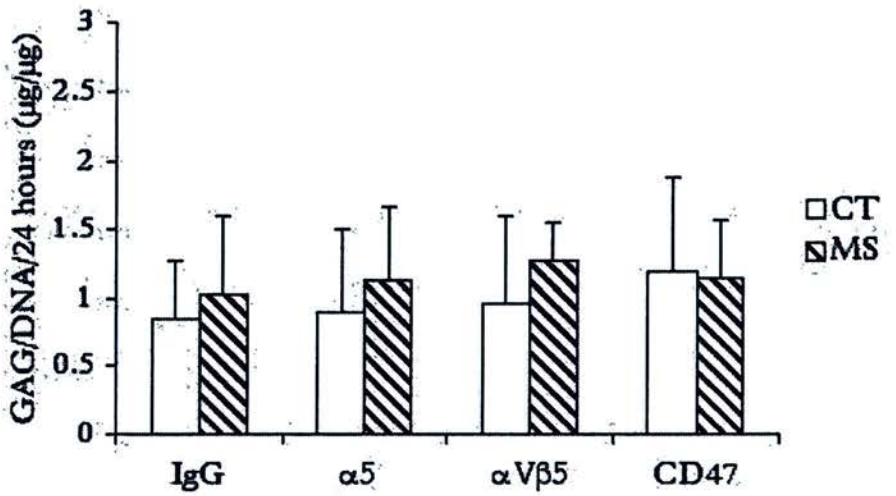


Figure 4.5 The effects of anti-integrin antibodies on GAG synthesis in response to cyclical mechanical stimulation in OA grade II chondrocytes

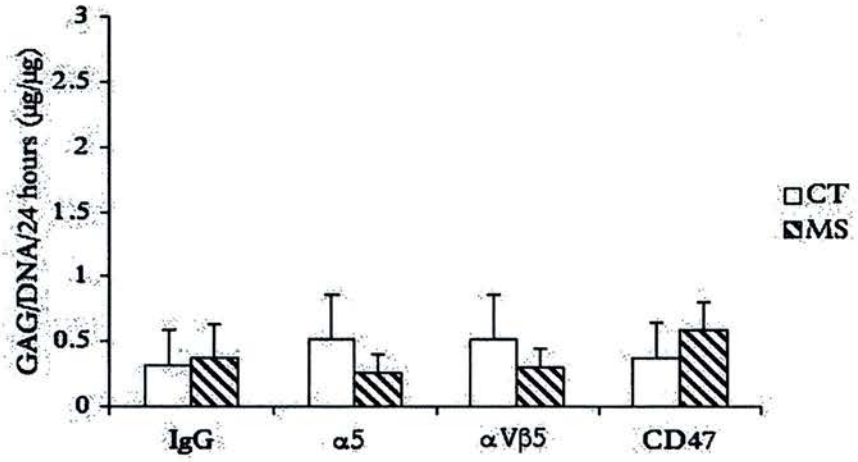


Figure 4.6 The effects of anti-integrin antibodies on GAG synthesis in response to cyclical mechanical stimulation in OA grade III chondrocytes

Cyclical mechanical stimulation at 0.33 Hz 4000μ strain did not modulate GAG synthesis in OA chondrocytes in GAG synthesis in response to mechanical stimulation in the presence of anti-integrin α5, αVβ5, and CD47 antibodies (Figure 4.4, 4.5, 4.6). The white bars (CT) are paired controls without mechanical stimulation (MS). Values are given as means ± SD (n=6).

4.2.4 Discussion

As shown in Chapter Three, the signal following 0.33 Hz 4000μ strain mechanical stimulation was mediated by integrins including α5β1 and αVβ5 integrins, and CD47 and resulted in increased PG synthesis in normal chondrocytes. However, the same mechanical stimulation did not affect PG synthesis in OA chondrocytes in the presence or absence of function blocking anti-integrin antibodies. This may be for the following reasons.

Firstly, integrins in OA chondrocytes may not recognize mechanical stimuli in the same way as normal chondrocytes because their extra-cellular matrix composition, including their integrin ligands, may have been changed during the OA process. It has been shown that extra-cellular matrix proteins such as fibronectin, collagen, and other integrin ligands can alter chondrocyte functions (outside-in signalling) (Kaplan et al 1982) by modulating extra-cellular protease activity (Attur et al 2000, Pacifici et al 1991, 1992) and catabolic cytokines by inside-out signalling (Homandberg 1999). In particular the capability of fibronectin and fibronectin fragments, as components of the cartilage extra-cellular matrix, to influence cartilage homeostasis have been studied extensively (Homandberg 1999). Osteoarthritis-affected cartilage exhibits enhanced expression of fibronectin mRNA (Rencic et al 1995). Fibronectin synthesis is increased in OA cultured chondrocytes, in cartilage explants (Burton-Wurster et al 1990, Brown et al 1990, Lust et al 1987, Wurster and Lust 1984), and *in vivo* OA models

(Burton-Wurster et al 1986). Fibronectin is produced locally and accumulated within the joint (MacLeod et al 1996). The highest rate of synthesis was in the deep zone of the cartilage in normal human articular cartilage, but the greatest increase of fibronectin fragments was detectable near the surface (Burton-Wurster et al 1986, Jones et al 1987, Rees et al 1987) or close to eburnated areas (Chevalier et al 1992). Enhanced fibronectin synthesis is partly influenced by biomechanical forces. Intermittent cyclic loading altered the synthesis and release of fibronectin significantly in cartilage explants (Steinmeyer et al 1997) and fibronectin synthesis increased in canine articular cartilage explant cultures after cyclic impact (Farquhar et al 1996). Compressive loading and unloading has also been shown to affect fibronectin synthesis (Burton-Wurster et al 1993). It would be interesting to see if OA chondrocytes produced more fibronectin in response to mechanical stimulation as normal chondrocytes. This could explain the increases in fibronectin found in abnormal cartilage in OA joints.

Incubation of fragments of fibronectin with cartilage explants enhances loss of cartilage matrix PG. This is mimicked by stimulatory antibodies to $\alpha5\beta1$ integrin and blocked by RGD peptides (Homandberg 1992). Interestingly, the fibronectin fragments do not cause collagen degradation or cell death (Xie et al 1993), but PG loss (Homandberg 1994). If the fibronectin fragments are removed from cartilage cultures, the PG synthesis rates increase to 140 per cent of control values (Homandberg 1992). Intact fibronectin or fragmented fibronectin can induce the release of IL-1β (Pacifici et al 1992), IL-6, GM-CSF, stromelysin-1, and MMPs, particularly MMP-3 (Yonezawa et al 1996, Homandberg et al 1999, Bewsey et al 1996). Arner and Tortorella (1995) have reported that RGD peptides can increase MMP production in bovine chondrocytes in response to IL-1. Ligation of $\alpha5\beta1$ with agonists similar to fibronectin (anti-$\alpha5\beta1$ antibodies; JBS5) up-regulated the production of inflammatory mediators (nitric oxide, prostaglandin E2, IL-6, IL-8) via autocrine IL-1β production. By contrast the $\alpha V\beta3$ complex with a specific function-blocking monoclonal antibody (LM609) which mimics the functions of osteopontin decreased the production of inflammatory mediators in OA cartilage (Attur et al 2000). They showed that normal human cartilage is equally susceptible to $\alpha5\beta1$ integrin-mediated induction of inflammatory mediators. It appears that low concentrations of fibronectin fragments enhance anabolic activities in normal cartilage homeostasis, whereas increased accumulation or synthesis of fibronectin and its fragments may interact with $\alpha5\beta1$ integrin and induce the production of inflammatory mediators. However, the available data do not rule out the involvement of other receptors other than $\alpha5\beta1$ integrin; both $\alpha5\beta1$ and $\alpha4\beta1$ receptors are required for fibronectin fragment mediated MMP gene induction in fibroblasts (Huhtala et al 1995). Similarly, Pacifici et al (1992) reported that the occupancy of both $\alpha5\beta1$ and $\alpha2\beta1$ integrins by ligands was required in order to induce a maximal IL-1 release response from human blood mononuclear cells. In addition to changes in fibronectin, changes in extra-cellular matrix composition, such as the changes in PG content (Brandt et al 1991, Cs-Szabo et al 1995, 1997), disruption of the collagen II network (Brandt et al 1986), or increases in a variety of glycoproteins such as fibromodulin, decorin and biglycan at the mRNA level (Dourado et al 1996) and on the protein level (Cs-Szabo et al 1995, 1997, Liu et

al 2003), and up-regulation of collagen type IV expression (McDevitt et al 1988), have been reported. As a result of these changes in the extra-cellular matrix, synthetic activity and production of proteinases by chondrocytes may be significantly altered.

Secondly, even though some integrins in OA chondrocytes, most notably $\alpha5\beta1$ integrin, may recognize the same mechanical stimuli, downstream signalling events may be significantly different. As a result, the mechano-transduction pathways in OA chondrocytes may be changed from those operating in normal chondrocytes. Previous reports from our laboratory have shown differences, at multiple stages of cellular response, in OA chondrocytes compared to normal ones (Salter et al 2002, Millward-Sadler and Salter 2004). For example, instead of the membrane hyperpolarization response in normal chondrocytes, OA chondrocytes show a depolarization response. There is no increase in the level of aggrecan mRNA and there is an IL-1β cytokine loop in mechanical signal transduction in OA chondrocytes and an IL-4 autocrine loop in normal chondrocytes. Furthermore, the actin cytoskeleton is required for integrin-dependent mechano-transduction in normal chondrocytes but not in OA.

Thirdly, the integrins expressed in OA chondrocytes and the distribution of integrins in OA cartilage may be different from normal. Ostergaard et al (1998) compared the distribution of integrins in normal and OA cartilage and found neo-expression of $\alpha2$, $\alpha4$, and $\beta2$ integrin subunits in OA cartilage. The $\alpha5$ integrin subunit was seen in all cases with similar distribution, but the αV integrin subunit was greater in the superficial layer (Woods et al 1994, Ostergaard et al 1998), and was zone specific (Wong et al 1997). Chondrocytes from OA affected cartilage express more of the $\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$, and αV subunits than $\alpha4$ and $\alpha6$ (Lapadula et al 1998). There was no topographic variation in the expression of CD47, in either normal or OA articular cartilage. Different grades of OA do not show any modification of the strong expression of CD47 in all zones of articular cartilage (Orazizadeh 2004). IL-1 has been shown to up-regulate $\alpha5$, $\alpha2$, and αV integrin subunit mRNA levels in osteosarcoma cells (Milam et al 1991) and IL-1 stimulation increased the expression of $\beta1$ and αV integrin subunits on the chondrocyte surface in primary rabbit articular chondrocytes (Arner and Tortorella 1995). Changing the number of integrins expressed on the cell surface may alter cell response to mechanical stimulation and other stimuli.

Fourthly, increased catabolic responses in OA cartilage may be more prominent than decreased or unchanged anabolic responses. Integrins may be more involved in catabolic responses in OA chondrocytes than anabolic responses. Integrin function may be altered in response to abnormal stimulation. Reports have demonstrated a super-induction of pro-inflammatory mediators in OA cartilage compared to normal cartilage, which include nitric oxide (Amin et al 1995, 1998, Melchiorri et al 1998), PGE2, cyclo-oxygenase-2 (Amin et al 1997), IL-1β, IL-6, and IL-8 (Attur et al 1998). These inflammatory mediators are up-regulated and spontaneously released from OA-affected cartilage (Amin et al 1997, Attur et al 2000). The up-

regulation of these cytokines and mediators exerts detrimental effects on chondrocyte function (Moos et al 1999, Pelletier et a 1998). As catabolic responses, such as the release of GAG from cartilage explants following fibronectin fragment stimulation in chondrocytes, has been shown to be blocked by synthetic peptide analogues of the RGD sequence (Homandberg et al 1994a) and by anti-sense oligonucleotides of the α5 subunit (Homandberg et al 2002), there is a possibility that chondrocyte proteolytic activities could be obstructed by function blocking anti-integrin antibodies.

4.3 The effects of anti-integrin antibodies on GAG synthesis in response to cyclical mechanical stimulation in human articular chondrocytes derived from RA cartilage

4.3.1 Aim

Cyclical mechanical stimulation applied to articular chondrocytes from RA cartilage did not show any significant change in GAG synthesis. The aim of this study in this section was to examine the effects of anti-integrin antibodies on GAG production in response to cyclical mechanical stimulation in chondrocytes from RA articular cartilage.

4.3.2 Materials and Methods

Human articular knee cartilage was obtained at the time of joint replacement surgery from patients with OA. The articular cartilage surface was assessed macroscopically (McElligott and Collins 1960) and microscopically (OARSI) and graded into OA I to III. OA chondrocytes were isolated from the articular cartilage of from patients with RA of one male (37 years old), and six females with a mean age of 62.4 years (range 55-68). Primary chondrocytes were seeded in monolayer at 5 x 10^4 cells/ml concentration. Chondrocytes were cultured to sub-confluent for an average of 8.2 days. The culture medium (IMDM) containing 10 per cent serum (FCS) was then replaced by serum free medium and left 16 hours Function blocking monoclonal antibodies to α5 integrin (P1D6), αVβ5 integrin (P1F6) and CD47 (Bric 126) at a concentration of 1 μg/ml, were incubated with chondrocytes cultured in monolayer at 37 °C for 30 minutes, prior to 0.33 Hz 4000μ strain mechanical stimulation for 20 minutes. This was followed by 24 hours incubation at 37°C. PGs secreted into the medium were precipitated and the quantity of GAGs was measured by the DMMB assay. The total content of DNA was quantified using the bisbenzimide fluorescent dye (Hoechst 33258).

4.3.3 Results

Microscopic assessment of RA synovial tissue showed synovial inflammation was very variable as judged by the following morphological characteristics: (1) hyperplasia of synovial lining cell layer, (2) activation of resident cells in synovial stroma (3) inflammatory infiltration. Two cases (DS190C and DS140C) showed little inflammation, two cases (DS098C and DS105C) showed moderate inflammation and two cases (DS157C and DS052C) showed strong inflammation in synovial tissue (Appendix).

Cyclical mechanical stimulation at 0.33Hz 4000μ strain did not modulate GAG synthesis in response to mechanical stimulation in RA chondrocytes in the presence of anti-integrin α5, αVβ5, and CD47 antibodies. None of the results from the paired samples were significantly different from each other (Figure 4.7). The response of RA chondrocytes to mechanical stimulation was partially modulated by anti-α5 integrin antibody, but not by αVβ5 integrin or CD47. GAG synthesis increased from a mean ± SD of 1.36 ± 0.78 to 1.56 ± 0.91 in human RA chondrocytes exposed to 20 minutes cyclical (0.33 Hz, 4000μ strain) mechanical stimulation at 37°C in the presence of anti-α5 integrin antibody, but this was not statistically significant (n=6). In two samples which showed inflammatory synovial tissue, GAG synthesis increased from a mean ± SD of 1.45 ± 0.23 to 2.62 ± 0.24, when exposed to mechanical stimulation in the presence of anti-α5 integrin antibody.

4.3.4 Discussion

This study reports that RA chondrocytes have lost their capacity to respond to cyclical mechanical stimulation in the presence and absence of anti-α5 or αVβ5 integrin antibodies or anti-CD47 antibody. The role of chondrocytes in the course of cartilage destruction in RA is not well understood. There are a few studies which have demonstrated differences between RA chondrocytes and normal or OA chondrocytes, in cartilage. One of the differences is the change of expression of integrins in diseased cartilage (chondrocytes). Looking at RA tissue sections, Jobanputra et al (1996) reported that chondrocytes in all zones of RA cartilage were markedly stained with α5 with no focal differences. In addition, they showed that inflammatory synovial fluid from RA patients could lead to increased α5 integrin expression in normal human articular chondrocytes in monolayer culture. Takagi et al (2001) have shown that the proportion of CD44-positive chondrocytes in RA is significantly higher than that in OA, using immuno-histochemical techniques.

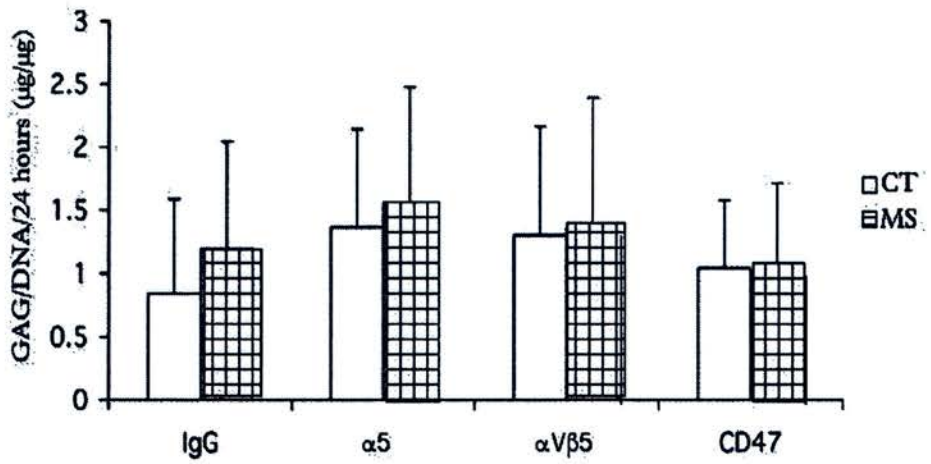


Figure 4.7 The effects of anti-integrin antibodies on GAG synthesis in response to cyclical mechanical stimulation in RA chondrocytes.

Cyclical mechanical stimulation at 4000µ strain (0.33 Hz) did not modulate GAG synthesis in RA chondrocytes in response to mechanical stimulation in the presence of anti-integrin α5, αVβ5, and CD47 antibodies. None of the results from the paired samples were significantly different from each other. The white bars (CT) are paired controls without mechanical stimulation. Values are given as means ± SD (n=6).

Their data also showed that different CD44 isoforms are expressed in RA chondrocytes compared with normal ones, indicating a phenotypic change in RA chondrocytes. In this study, in the presence of the anti-α5 integrin antibody, GAG synthesis increased by 92 per cent. The α5 subunit may be involved in the cellular response to mechanical stimulation in RA cartilage. Signalling through integrin receptors is an important stimulus for cell activity. In some cells, integrins are known to regulate MMP expression. In rabbit synovial fibroblasts there is co-operative signalling by α5β1 and α4β1 integrins, regulating MMP gene expression (Huhtala et al 1995). Engagement of the α2β1 receptor in dermal fibroblasts increases collagenase gene expression (Riikonen et al 1995). In contrast, α5 engagement inhibits RA fibroblast-like synoviocytes collagenase expression (Sarkissian et al 1999). However few studies have been done on the role of integrins in RA chondrocytes compared with integrins in synovial tissue, and no previous studies have been done on whether matrix synthesis is modulated by integrins in chondrocytes from patients with RA.

Rheumatoid synovial fibroblasts are a major component of pannus tissue. The invasion of cartilage by synovial pannus is a characteristic feature of rheumatoid arthritis. Turning to RA synovial tissue, there are numerous reports of changes in extra-cellular matrix composition, such as increased fibronectin (Vartio et al 1981, Dutu et al 1986, Clemmensen et al 1983, Vartio et al 1981, Elices et al 1994), vitronectin (Nikkari et al 1995), and tanascin (Cutolo et al

1992). Similarly, α3, α4, α5, α6, β1, and β5 integrin expression are increased compared to OA or normal cells in rheumatoid hyperplasia (Johnson et al 1993, Baeten et al 2000, Nikkari et al 1995, Nikkari et al 1993, Ishikawa et al 1994, Rinaldi et al 1994), but αV expression remains controversial (Nikkari et al 1995). It is not known whether these changes in synovial tissue can also be seen in RA cartilage tissue. Certainly in both kinds of tissue, integrins act as adhesion molecules between chondrocytes and rheumatoid synovial fibroblasts. Blocking of α4, α5, αV, and β1 integrins could lead to an inhibition of rheumatoid synovial fibroblast invasion into cartilage (Wang et al 1997). Normal synoviocytes do not adhere to the cartilage surface, whereas in RA synovial fibroblasts adhere directly to the cartilage surface (Ishikawa et al 1991). It may be important to see whether the invasion of the cartilage by synovial pannus is due to changes in the articular cartilage as well as changes in the synovium.

It is possible that in RA chondrocytes are functionally altered by many cytokines. RA is a chronic inflammatory disease and increased levels of cytokines are a major feature (Sugiura et al 2002). One cytokine, TGF-β, has been reported to have an important role in unresolved inflammation, immune suppression, and angiogenesis (Blobe et al 2000). TGF-β is highly expressed in joints in RA and considered to be a regulator of anti-inflammation in RA (Fava et al 1989, Taketazu et al 1994, Lafyatis et al 1989). Cytokines including TGF-β (Ignotz et al 1986, Heino et al 1989), IL-1β (Santala and Heino 1991), TNF-α (Santala and Heino 1991), and interferon-γ (Defilippi et al 1991) can selectively regulate integrin and adhesion molecule expression. For example, TNF-α and IL-1β can down-regulate α6 subunit expression in endothelial cells (Defilippi et al 1992). TGF-β increases β1 subunit mRNA and protein levels (Heino et al 1989), causes higher fibronectin binding capacity and elevated adhesion of cells. It is interesting to note that in two joint diseases with different etiological backgrounds, OA and RA, the pattern of integrin expression is different (Jobanputra et al 1996, Nikkari et al 1995, Lapadula et al 1998, Ostergaard et al 1998, Baeten et al 2000). Adhesion receptors of the integrin family appear to have a critical role for cell phenotype and function.

The use of surgical specimens from patients with rheumatoid disease may create a bias with regard to disease duration and activity. In addition to disease duration and disease activity, drugs may also be a confounding factor. Numerous previous studies have shown the effect of anti-rheumatic drugs on synovial histology (Tak et al 1995, Veale et al 1999, Youssef et al 1997, Smeets et al 2000, Tak et al 1996, Taylor et al 1999). However, Baeten et al (2000) has shown that synovial histology is dependent on local disease activity, but not on disease duration in clinically manifest RA.

4.4 Summary

The results reported here indicate that integrin-mediated adhesions to extra-cellular matrix proteins are involved in the signal transduction pathway that leads to accelerated PG synthesis following mechanical stimulation. The blocking effect of monoclonal antibodies to α5, αVβ5 and CD47 suggests that the occupancy of all these receptors by ligands is required in order for 0.33 Hz 4000μ strain cyclical mechanical stimulation to induce an increased GAG synthesis response from human normal articular chondrocytes. There must be a complex interactive role for specific receptors in the regulation of PG synthesis. The normal up-regulation of PG synthesis that follows cyclical mechanical stimulation of normal chondrocytes is lost in OA and RA chondrocytes.

Chapter Five

The Effects of Mechanical Stimulation on Aggrecan Expression in Human Articular Chondrocytes in Monolayer Culture (Western Blot Analysis)

The identification of the structure, biosynthesis and catabolism of proteoglycans, which are controlled during cellular responses, has initiated a new and important area of research (Hardingham and Fosang 1992, Caterson et al 1990). The current study has shown that 0.33Hz 4000μ strain cyclical mechanical stimulation of normal chondrocytes in monolayer cell culture increases GAG synthesis around two-fold (Chapter Three), and this is blocked by anti-α5 integrin, anti-αVβ1 integrin and anti-CD47 antibodies (Chapter Four). The same mechanical stimulation does not alter GAG synthesis in OA or RA chondrocytes (Chapter Three).

It can be postulated that the increased synthesis of GAG in normal chondrocytes is related to a change of anabolic activity in chondrocytes. There have been few studies of how mechanical stimuli influence aggrecan synthesis, especially metabolic analysis of aggrecan species. The aim of the work reported in this chapter was to see whether the increases in GAG synthesis, released from normal chondrocytes, were associated with changes in aggrecan synthesis and to explore which aggrecan species were involved.

GAG synthesis following mechanical stimulation does not increase in OA and RA chondrocytes as in does in normal chondrocytes. It is important to determine whether this is due to decreased anabolic activity or increased proteolytic activity. It is especially important to examine the effects of mechanical stimulation as these may influence the metabolism of diseased (OA and RA) chondrocytes differently in comparison with normal chondrocytes.

The aim of the work reported in this chapter was to analyse aggrecan populations (species) synthesized by mechanically-stimulated, monolayer-cultured chondrocytes derived from articular cartilage graded as normal, OA and RA.

5.1 Development of protocols for Western blotting

Preliminary experiments were performed to identify cultural conditions for applying antibodies to Western blotting. Antibodies which recognize aggrecan (PG), anti-aggrecan G1

domain antibody (anti-HABR) and monoclonal antibodies to aggrecan chondroitin sulphate epitopes (3-B-3, 2-B-6, 1-B-5), were assessed separately.

5.1.1 Development of protocols for Western blotting with an anti-aggrecan G1 domain antibody (anti-HABR)

5.1.1.1 Aim

The constituents of the cell culture medium can inhibit or stimulate matrix production by chondrocytes. The concentration of serum (FCS) is especially important (Lee et al 1995). The aim of this section is to maximise detection of a range of aggrecan species in different cultural conditions (0.1 per cent, 0.5 per cent, and 0 per cent serum).

5.1.1.2 Materials and Methods

Human articular knee cartilage was obtained at the time of joint replacement surgery from patients with OA and RA or from above knee amputations in patients with peripheral vascular disease. The articular cartilage surface was assessed macroscopically (McElligott and Collins 1960) and microscopically (OARSI) and graded into normal, OA (grade I to III) and RA.

Two samples of OA cartilage (sample day book number 14038 and 14044) and one samples of RA cartilage (DS359C) were used. As a control, proteoglycans were initially eluted from cartilage with a GuHCl buffer. Cartilage from same donor was subsequently digested and chondrocytes were seeded into monolayer culture. After an average of 7.1 days, when the cells were sub-confluent, the medium were replaced by 0 per cent (serum free), 0.1 per cent and 0.5 per cent FCS containing IMDM. Then 1 ml of medium per dish was collected and analysed after 24 hours incubation. 1 ml of the undigested control, digested samples, and 1 ml of medium with 0 per cent, 0.1 per cent, 0.5 per cent serum were precipitated and run in a 7.5 per cent SDS-PAGE, under reducing conditions following deglycosylation.

5.1.1.3 Results

Seven bands (350, 250, 160, 110, 90, 65, and 50 kDa) were detected in the control samples which were extracted with buffer alone from OA cartilage (sample day book number 14038) (Figure 5.1 lane 6). Cultured chondrocytes, which were incubated with 0 per cent (serum-free) medium for 24 hours, showed a single band with molecular weight of ~65 kDa (lane 2).

When cells were incubated with 0.5 per cent serum medium for 24 hours, they showed a high molecular weight band of ~350 kDa in addition to ~65 kDa band (lane 1, black arrow). The medium controls showed no bands for serum free (0 per cent) medium (lane 3), but there was a band with molecular weight of ~65 kDa in 0.1 per cent (lane 5) and in 0.5 per cent (lane 4) serum containing medium. The same experiments were repeated with RA cartilage and chondrocytes (day book number DS359C) and the same result was obtained (Figure 5.2).

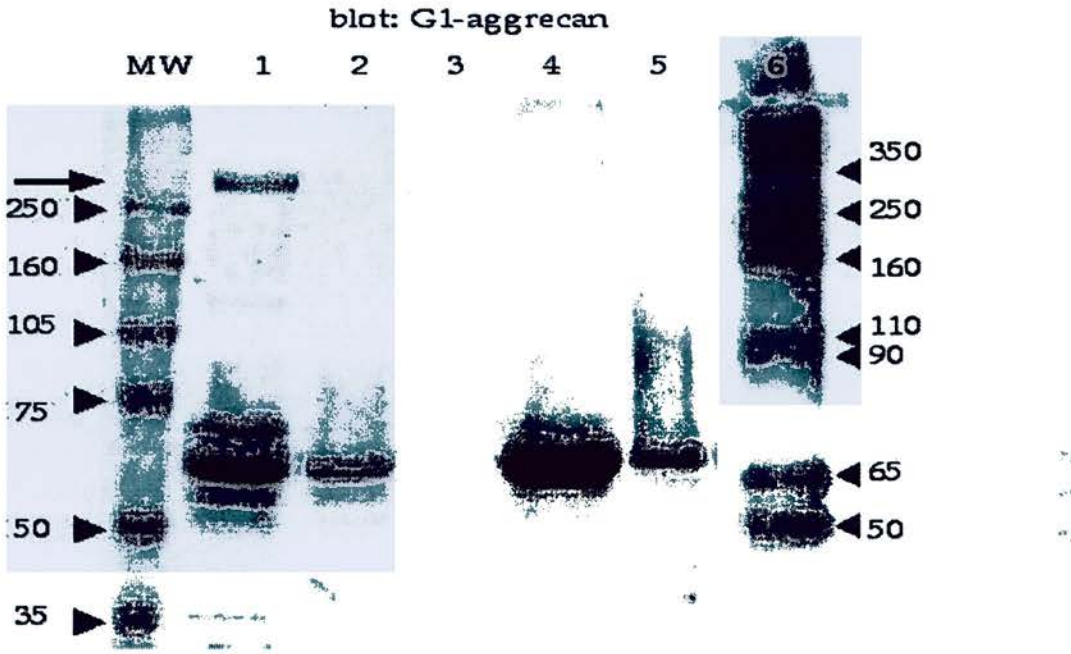


Figure 5.1 Western blotting using anti-aggrecan G1 domain antibody in OA samples and controls

PGs extracted from 1 ml of medium obtained from cultured chondrocytes incubated for 24 hours were run in a 7.5 per cent SDS-PAGE under reducing conditions following deglycosylation. G1 domain containing aggrecan core protein was detected by probing blots with polyclonal anti-aggrecan G1 domain antibody. The molecular weight (MW) marker in kilodaltons (kDa) is shown on the left, and the seven species of the G1 domain aggrecan in eluted articular cartilage are shown in the right.

Lane 1: 1 ml of medium (0.5 per cent serum) obtained from monolayer-cultured OA (day book number; 14038 OAI) chondrocytes,
Lane 2: 1 ml of medium (serum-free) obtained from monolayer-cultured OA (14038 OAI) chondrocytes,
Lane 3: 1 ml of serum-free medium (negative control),
Lane 4: 1 ml of 0.5 per cent serum containing medium (negative control),
Lane 5: 1 ml of 0.1 per cent serum containing medium (negative control),
Lane 6: 1 μg GAG extracted directly from OA cartilage (14038 OAI) (positive control)

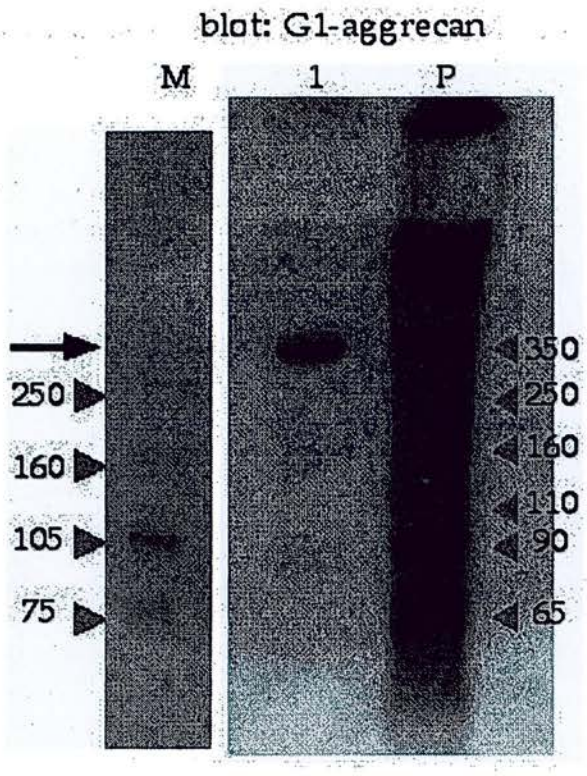


Figure 5.2 Western blotting using the anti-aggrecan G1 domain antibody in an RA sample

The large molecular weight band of ~350 kDa is a full-length aggrecan core protein corresponding with the highest band of positive control.

Lane 1: 1 ml of medium in the presence of 0.5 per cent serum obtained from monolayer-cultured RA (day book number; DS359C) chondrocytes,
P: 1 μg per lane GAG containing extract from RA cartilage (DS359C),
M: molecular weight (MW) in kilodaltons (kDa) (marker)

5.1.1.4 Discussion

Cultured OA and RA chondrocytes, which were incubated with 0.5 per cent serum medium for 24 hours, showed a high molecular weight band with molecular weight of around 350 kDa in addition to a band with molecular weight of around 65 kDa. Cultured OA and RA chondrocytes, which were incubated with 0 per cent serum medium for 24 hours, showed only a weak band with molecular weight of around 65 kDa. The controls, which were extracted with buffer alone from OA cartilage, showed seven bands including 350 kDa band. This ~350 kDa band is a full-length aggrecan core protein corresponding with the large molecular weight band synthesized from cultured OA and RA chondrocytes (Figure 5.1, 5.2). The lower band with molecular weight of ~65 kDa includes albumin, as 0.5 per cent and 0.1 per cent serum (FCS) containing medium without culturing chondrocytes showed the same

molecular weight band, and 0.5 per cent serum containing medium showed stronger band than 0.1 per cent ones.

Chondrocytes in serum free-medium will stop synthesizing matrix (Lee et al 1995). Usually 0.5 per cent serum is added to cell culture medium in order to allow the production of matrix at the same time as serum does not interfere with analysis (Lee et al 1995, and personal communication from Dr R. Al-Jamal, Edinburgh University). Thus subsequent experiments using anti-aggrecan G1 domain antibody were performed using 0.5 per cent serum containing medium. Any band of ~65 kDa was regarded as a non-specific albumin band.

A rabbit polyclonal antibody, which recognises the anti-hyaluronic acid binding region (HABR) which reacts with the G1 domain of the core protein of aggrecan has been used by other researchers (Poole et al 1991), and a range of species of core protein of aggrecan released into medium or synovial fluid from explants or knee joints have been reported (Sandy et al 1992, Ilic et al 1992). However, no previous studies have examined the range of G1-domain-containing species of core protein of aggrecan released into medium from monolayer-cultured chondrocytes using Western blotting.

Aggrecan with extreme poly-dispersity, in size and composition, in cartilage has been found extensively. The heterogeneity of aggrecan in cartilage is attributed to the size and number of CS and KS chains, to the pattern of sulphation of the GAG, and to proteolytic activity on the core protein. The variations occurring in the size of the core protein of aggrecan is an important aspect for characterising aggrecan. The molecular weight of the full length of aggrecan core protein in rat chondrosarcoma is deduced to be 220.952 kDa using the data from its amino acid sequence of 2124 residues (Doege et al 1987). Other methods, depending on the one used, have given sizes ranging from 180 kDa to 450 kDa (Hassell et al, 1986).

Despite the lack of precise structural data, the full length of peptides (un-degraded product) including G1 domain has been reported as 350KDa molecular weight, after deglycosylation with a method using Western blot. In addition, there are at least six smaller G1 containing fragments in human articular cartilage of 250, 160, 110, 90, 65, and 50 kDa (Sandy et al 1992, Ilic et al 1992) (Figure 5.3, 5.4).

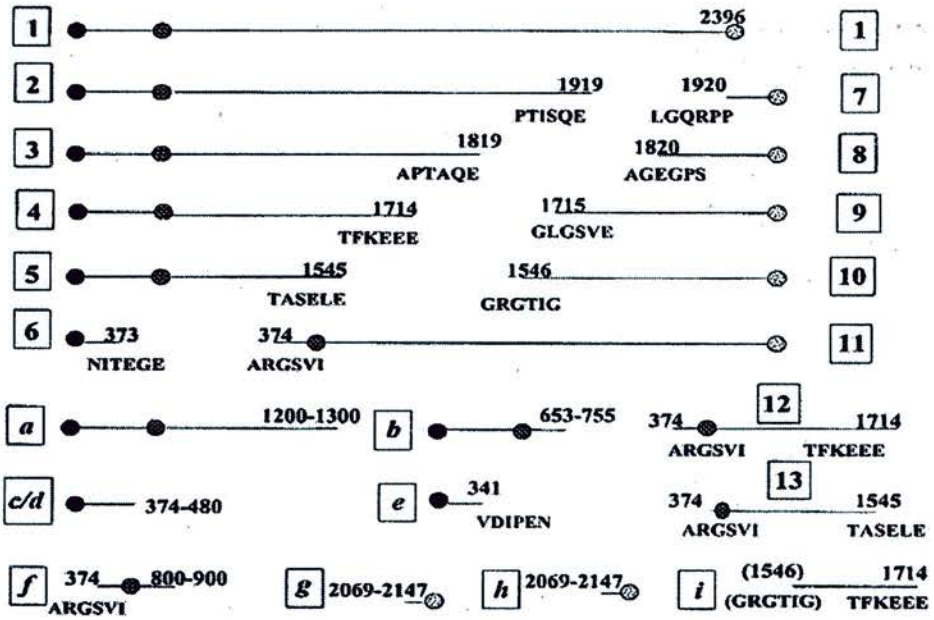


Figure 5.3 Schematic diagram of the aggrecan core species

The figure illustrates the structure of the human aggrecan peptides.
Filled circle: G1 domain, checked circle: G2 domain, stippled circle: G3 domain (Adapted from Sandy et al 2001).

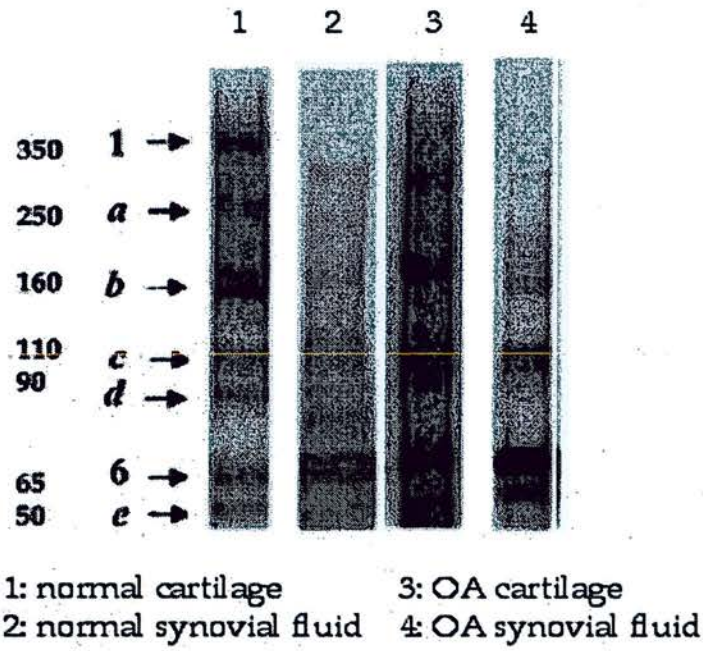


Figure 5.4 Western blots with anti-aggrecan G1 domain antibody showing the major aggrecan core species in human articular cartilage and synovial fluids

There are at least seven major core species in human cartilage of different ages. The species labelled 1 - 6, a - e are shown in Figure 5.3 (adapted from Sandy et al 2001).

5.1.2 Development of protocols for Western blot with monoclonal antibodies to aggrecan chondroitin sulphate epitopes

5.1.2.1 Aim

The aim of this section was to maximise detection of a range of aggrecan species under different cultural conditions (0.5 per cent and 0 per cent serum) with appropriate controls.

5.1.2.2 Materials and Methods

Two samples of OA cartilage (sample day book number 14075 and 14085) were used. As a control, proteoglycans were directly extracted from cartilage with GuHCl buffer. Using the same cartilage, which was subsequently digested, chondrocytes were seeded into a monolayer culture. When cells were sub-confluent (average culture period 7.1 days), the medium (IMDM with 10 per cent FCS) was changed to 0 per cent (serum free) or 0.5 per cent FCS containing IMDM, and 1 ml of medium per dish was collected and analysed after 24 hours incubation. PGs were extracted from 1 ml medium from cultured OA chondrocytes incubated for 24 hours, and from 1 ml of medium controls containing 0 per cent or 0.5 per cent serum. Then the samples were run in a 4 to 12 per cent gradient SDS-PAGE under a reducing condition following deglycosylation.

5.1.2.3 Results

Large molecular weight aggrecan of more than 250 kDa was detected in chondrocyte cultures with 0.5 per cent serum containing medium (Figure 5.6, lane 1,2) and in serum free medium (Figure 5.6, lane 3, 5, 6). However, 0.5 per cent serum interfered with immuno-staining (lane 1, 2) especially with molecular weight of ~300 kDa. The control with 0.5 per cent medium only showed thick non-specific albumin bands with molecular weight of ~65 kDa (lane 4). OAII chondrocytes (day book number 14085) released a band with molecular weight of ~50 kDa in both 0.5 per cent serum containing medium (lane 1, 2) and serum-free medium (lane 3), but OAI chondrocytes (day book number 14075) did not show the same band (lane 5, 6) with molecular weight of ~50 kDa. The whole cartilage control (lane P) showed large molecular weight aggrecan metabolites of more than 250 kDa, as well as the lower molecular weight band with molecular weight of ~50 kDa.

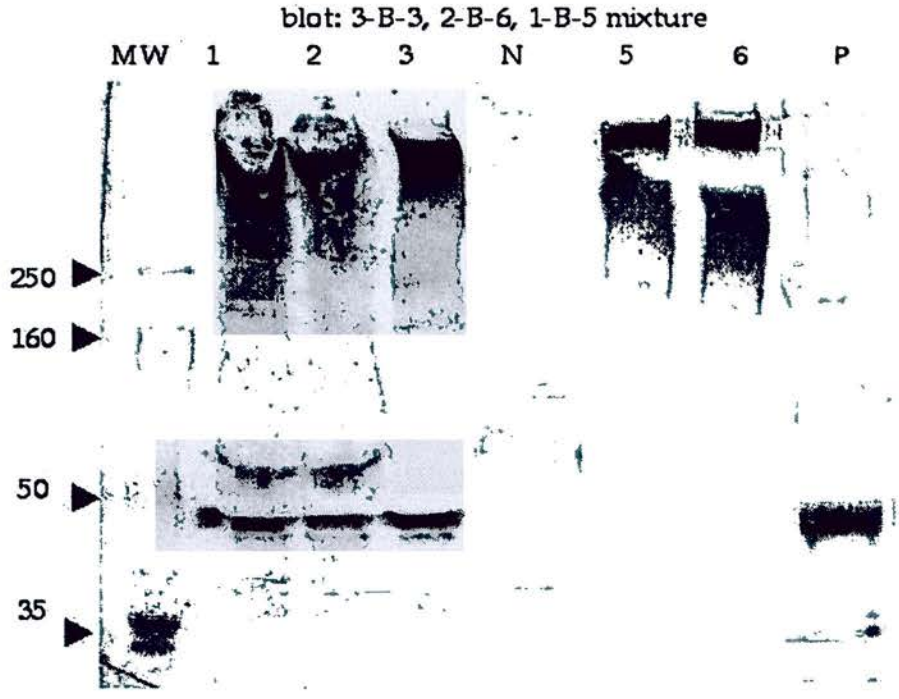


Figure 5.6 Western blots from OA samples and controls using monoclonal epitope antibodies

PGs extracted from 1 ml obtained from cultured chondrocytes incubated for 24 hours were run in a 4-12 per cent SDS-PAGE gradient gel under a reducing conditions. CS stubs were detected by probing blots with a monoclonal antibody mixture (3-B-3, 2-B-6, 1-B-5). Molecular weights (MW) in kilodaltons (kDa) are indicated on the left.

Lane 1 and 2: 1 ml of medium (0.5 per cent serum) obtained from monolayer-cultured OA (day book number; 14085 OAII) chondrocytes
Lane 3: 1 ml medium (0 per cent serum) obtained from monolayer-cultured OA (14085 OAII) chondrocytes
Lane 4: 1 ml of medium (0.5 per cent serum) (negative control)
Lane 5 and 6: 1 ml of medium (0 per cent serum) obtained from monolayer-cultured OA (14075 OAI) chondrocyte
Lane 7: positive control (14085 OAII cartilage extraction)

5.1.2.4 Discussion

Large molecular weight aggrecan of more than 250 kDa was detected in chondrocyte cultures with 0.5 per cent serum containing medium and in serum free medium. The whole cartilage control also showed large molecular weight aggrecan metabolites of more than 250 kDa. However, 0.5 per cent serum interfered with immuno-staining. The epitope monoclonal antibodies were able to detect metabolites in small amounts, and also when using manufactured 4 to 12 per cent gradient gel gave clearer results. Subsequent experiments using monoclonal antibodies were performed with serum-free medium. Monoclonal antibodies 3-

B-3, 2-B-6, and 1-B-5 were used to detect aggrecan products generated by chondroitinase ABC and keratanase I and II. Aggrecan fragments were detected by these antibodies which recognize many sites along the core protein of the aggrecan. These monoclonal antibodies 3-B-3, 2-B-6, and 1-B-5 recognize specific epitopes 6, 4, and 0-sulphated unsaturated disaccharides of CS respectively (Caterson et al 1985) (Figure 5.5).

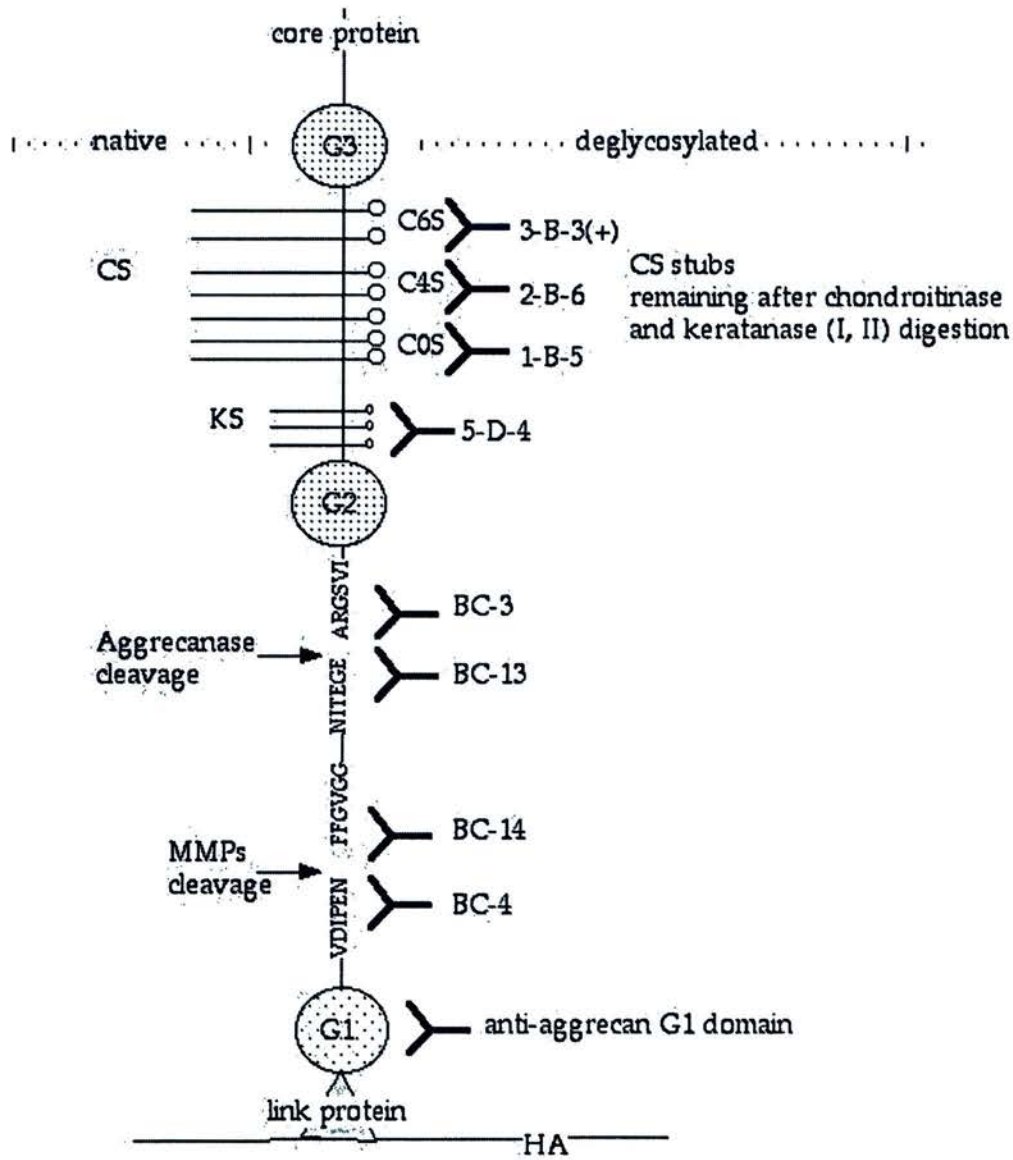


Figure 5.5 A PG monomer and the binding sited of the monoclonal and polyclonal antibodies used in this study

This schematic diagram shows the epitopes and catabolic neo-epitopes in aggrecan as recognized by the monoclonal antibodies and anti-aggrecan G1 domain antibody. To the right of the core protein there are CS and KS oligosaccharide stubs produced by chondroitinase and keratanase (I, II). (Adapted from Caterson et al 2000).

It has been shown that after electrophoretic separation, aggrecan from fetal cartilage migrates as a single narrow band, whereas aggrecan from adult cartilage migrates in more diffused broad band or multiple closely spaced bands (Sweet et al 1977, Rizkalla et al 1992). As all samples used in this study are of adult cartilage, aggrecan migrated as diffuse broad bands is comparable with previous results. An example of the analyses of PG metabolites with monoclonal antibodies 3-B-3, 2-B-6, and 1-B-5 by other researchers is shown in Figure 5.7 (Rees et al 2000). They have examined the catabolism of aggrecan in tendon explant cultures from compressed and tensional regions. A broad range of 3-B-3 positive high-molecular-mass PGs (more than 100 kDa) were present in the medium. Analyses of tendon PG metabolites in medium with the monoclonal antibody 2-B-6 showed little positive immuno-staining for the high-molecular-mass PG species. Detection of tendon PG metabolites using monoclonal antibody 1-B-5 showed positive immuno-reactivity with high-molecular-mass (more than 100 kDa) metabolites in medium obtained from tendon explants.

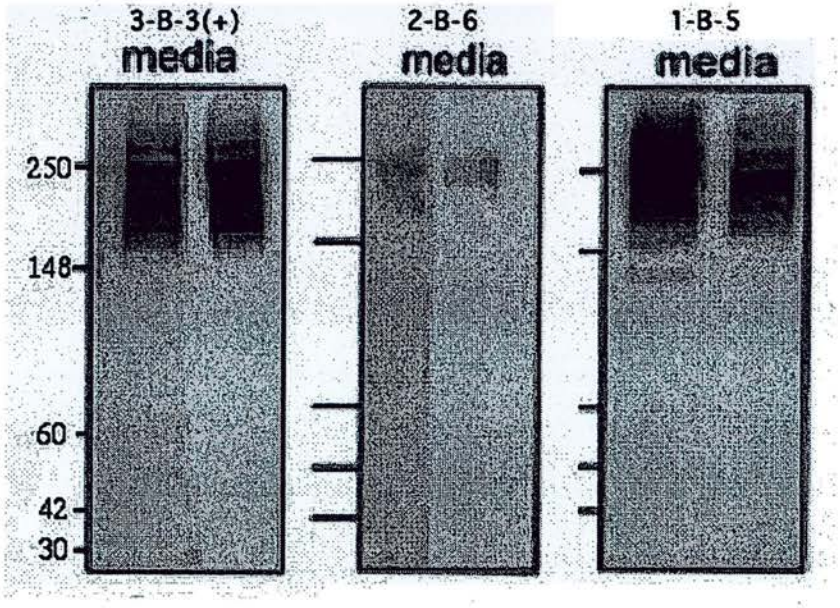


Figure 5.7 PG metabolites released into culture media of tendon explants detected by monoclonal antibodies of 3-B-3, 2-B-6, and 1-B-5

Examples of a broad range of 3-B-3, 2-B-6, and 1-B-5 positive high-molecular-mass PGs (more than 100 kDa) were present in media. (Adapted from Rees et al 2000).

5.2 The influence of disease states on baseline expression of aggrecan species

5.2.1 Aim

In this study, a significant decrease in GAG synthesis was only seen in advanced stage OA chondrocytes (OA grade III), unlike normal, OA grade I, and OA grade II chondrocytes (Chapter Three). The precise mechanisms causing the changes in PG that take place during the OA process are still unknown. The changes may involve anabolic or catabolic modification of newly synthesized PGs. To gain further insight, PGs, synthesized in OA and RA chondrocytes, were compare with those from normal chondrocytes in monolayer culture.

5.2.2 Materials and Methods

Human articular knee cartilage was obtained at the time of joint replacement surgery from patients with OA or RA, and from above knee amputation from patients with peripheral vascular disease. The articular cartilage surface was assessed macroscopically (McElligott and Collins 1960) and microscopically (OARSI) and graded into normal, OA (I to III) or RA. Chondrocytes were isolated from the normal articular cartilage of one male (76 year old) and two females (66 and 73 year old). OA chondrocytes were isolated from the articular cartilage of four males with a mean age of 64.5 years (range 39 - 79), and six females with a mean age of 64.6 years (range 53 - 74). RA chondrocytes were isolated from the articular cartilage of three females with a mean age of 57.3 years (range 39 - 74). Primary chondrocytes were seeded in monolayer at 5 x 10^4 cells/ml concentration. Chondrocytes were cultured to sub-confluent for an average of 8.1 days. The culture medium containing 10 per cent serum (FCS) was then replaced by serum free medium (IMDM) or medium with 0.5 per cent serum, and this was followed by 24 hours incubation at 37°C. PGs secreted into the medium were precipitated and the quantity of GAGs was measured by the DMMB assay. Equal amounts of GAG were loaded onto SDS-PAGE under a reducing conditions (1 to 2 μg GAG per lane). Experiments were repeated using single dishes of human articular chondrocytes derived from at least three adult donors (Appendix).

5.2.3 Results

5.2.3.1 Western blotting with anti-G1 domain aggrecan antibody

Aggrecan core species of molecular weight 350 kDa, which were detected in other grades of OA, RA and normal chondrocytes, were absent in the culture medium cultured from OA grade III chondrocytes (Figure 5.8). The results from OA grade II chondrocytes varied

significantly. For example, day book number 14087 showed aggrecan core species of molecular weight 350 kDa, although the quantity appeared to be less than normal or OA grade I chondrocyte cultures. Day book number 14035 showed a less intense 350 kDa band, and day book number DS130C did not show a 350 kDa band. Normal, OA grade I, and RA chondrocytes synthesized and released 350 kDa molecular weight core protein species into the culture medium (Figure 5.8, see arrow). The macroscopic and OARSI microscopic gradings were closely related. The observation of decreased full-length aggrecan core protein species in OA grade II chondrocytes was more noticeable when the OARSI score was lower (Appendix). Day book number 14087 OAII sample scored 1, whereas day book number 14085 scored 16. The former showed a clear band with molecular weight of 350 kDa but the latter did not exhibit the same band, as was the case in OA grade III chondrocytes.

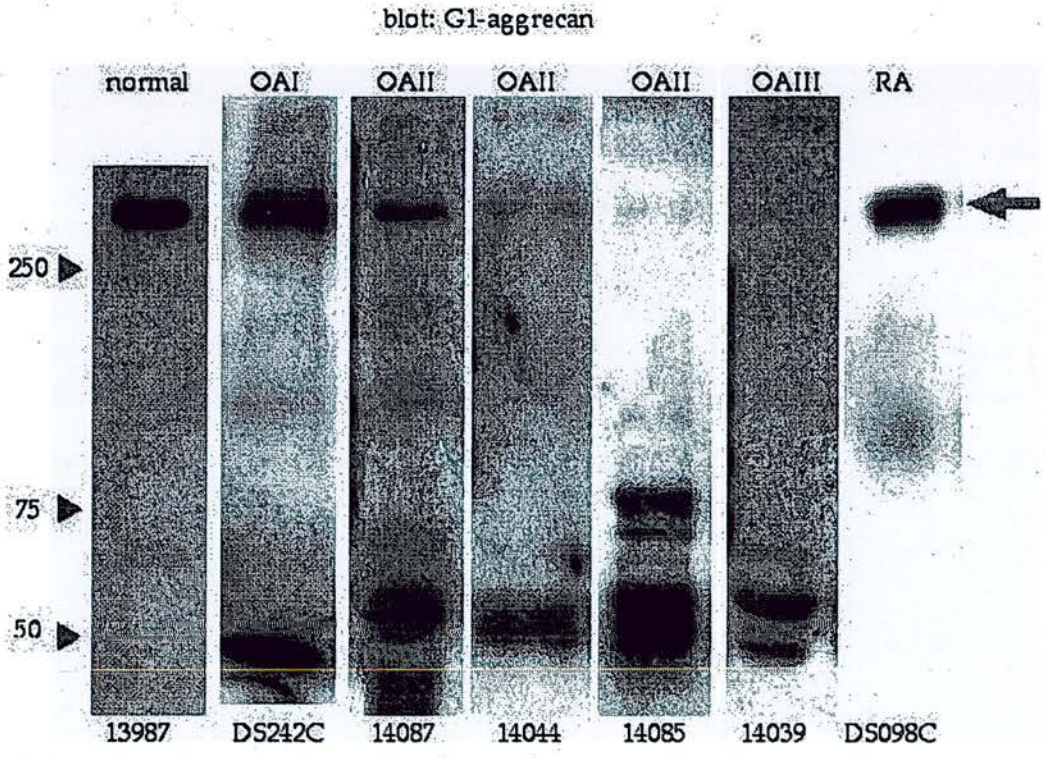


Figure 5.8 Western blots showing baseline aggrecan species expression in OA, RA and normal control samples

Un-degraded full-length aggrecan species (see arrow) were present in normal, OA grade I, and RA samples. However, high molecular weight PGs was not detected in OA grade III and in some OA grade II samples.

5.2.3.2 Results of Western blotting with monoclonal anti-aggrecan epitope antibodies

The fragments released into medium from chondrocytes were compared using the monoclonal epitope antibodies 3-B-3, 2-B-6, and 1-B-5. The C6S, C4S, and C0S levels were lower in OA grade III samples than in other extracts. Some of the OA grade II samples contained more C4S epitope than normal or RA samples.

A broad range of 3-B-3 positive high-molecular-mass PGs (more than 250 kDa) were present in the medium cultured from normal, OAI, OAII, and RA chondrocytes. However there were lesser amounts of 3-B-3 positive high-molecular-mass PGs and smaller fragments with molecular weight of ~250, ~75, and ~50 kDa from OA grade III chondrocytes (Figure 5.9).

Analyses of PGs in the medium with monoclonal antibody 2-B-6 showed less positive immuno-staining for high-molecular-mass PG species, especially in the culture medium cultured from RA chondrocytes (Figure 5.10). Strong 2-B-6 positive high-molecular-mass PGs staining could be seen in OA grade II chondrocytes. In addition to a broad range of 2-B-6 positive high-molecular-mass PGs (more than 250 kDa), there was a band with molecular weight of ~50 kDa in the medium cultured from normal, OAI, OAII, and RA chondrocytes, but not from OA grade III (see arrow).

Detection of PGs using monoclonal antibody 1-B-5 showed positive immuno-reactivity with high-molecular-mass (i.e. more than 250 kDa) species in medium but the immuno-reactivity of the detected bands was decreased in OA grade I to III (Figure 5.11).

5.2.4 Discussion

The results in this section showed that only aggrecan core species, immuno-reactive with G1 aggrecan antibody released from normal, OA grade I, and RA chondrocytes, was 350 kDa in size. The release of the 350 kDa band from OA grade II chondrocytes varied significantly, and was absent in the culture medium cultured from OA grade III chondrocytes (Figure 5.8).

The results also showed that a broad range of 3-B-3 positive high-molecular-mass PGs were present in the medium cultured from normal, OAI, OAII, and RA chondrocytes. However there were lesser amounts of 3-B-3 positive high-molecular-mass PGs from OA grade III chondrocytes (Figure 5.9). Strong 2-B-6 positive high-molecular-mass PGs staining could be seen in OA grade II chondrocytes. In addition, there was a band with molecular weight of

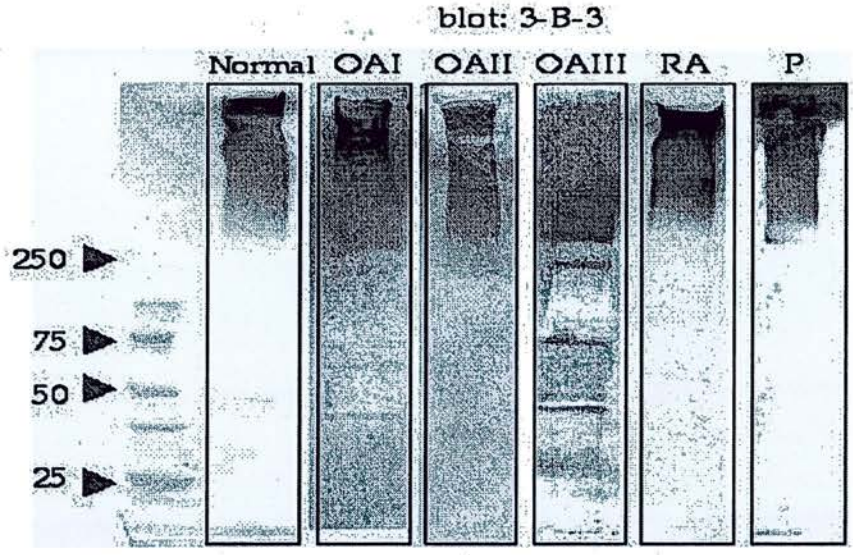


Figure 5.9 Western blots showing baseline PGs in medium from cultured chondrocytes and controls using the 3-B-3 epitope antibody

3-B-3 positive high molecular mass PGs were present in normal, OA grade I, II, and RA samples as well as from samples directly eluted from cartilage). However, this expression was lost in OA grade III, and smaller bands appeared with molecular weight of ~250, ~75, and ~50 kDa.

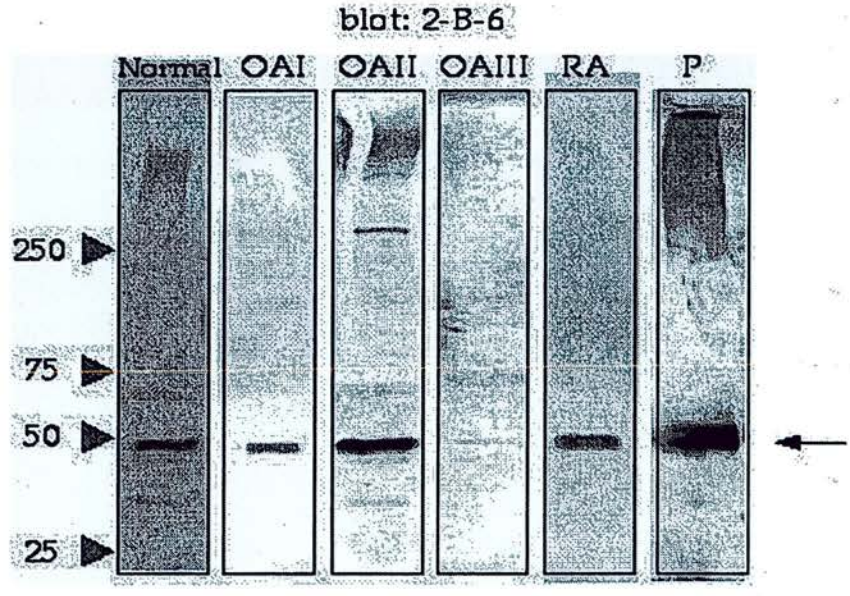


Figure 5.10 Western blots showing baseline PGs in medium from cultured chondrocytes and controls using the 2-B-6 epitope antibody

Analyses of PGs in medium with the monoclonal antibody 2-B-6 showed less immuno-staining for high-molecular-mass PG species, especially in the medium from RA chondrocyte cultures. Strong 2-B-6 positive high-molecular-mass PG staining could be seen in medium from OA grade II chondrocyte cultures. In addition to a broad range of 2-B-6 positive high-molecular-mass PGs (more than 250 kDa), there was a band with molecular weight of ~50 kDa in the medium cultured from normal, OAI, OAII, and RA chondrocytes, but not in OA grade III (see arrow). **P**: positive control (extraction from cartilage)

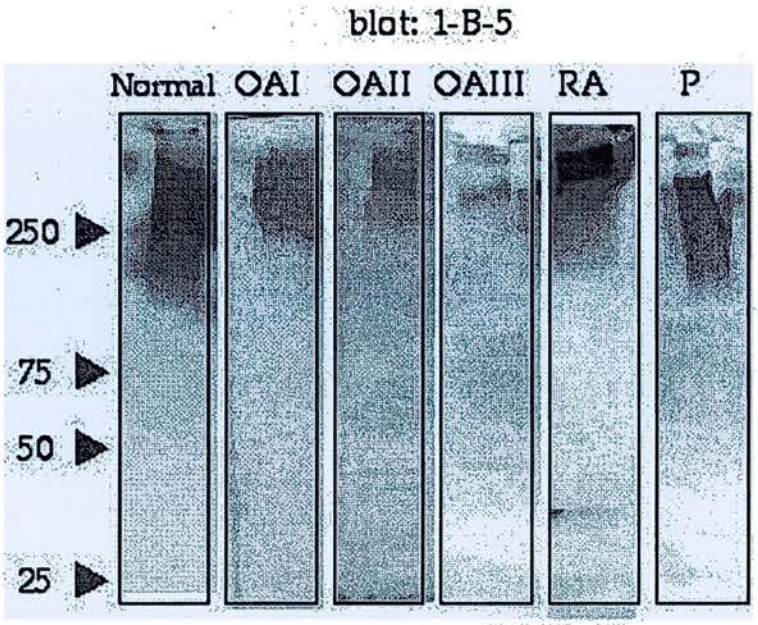


Figure 5.11 Western blots showing baseline PGs in medium from cultured chondrocytes and controls using the 1-B-5 epitope antibody

1-B-5 positive high molecular mass PGs were less apparent in OA chondrocytes.
P: positive control (extraction from cartilage)

around 50 kDa in the medium cultured from normal, OAI, OAII, and RA chondrocytes, but not from OA grade III chondrocytes. Detection of PGs using monoclonal antibody 1-B-5 decreased in OA grade I to III (Figure 5.11).

Chondrocytes were isolated from articular cartilage and cultured chondrocytes in monolayer. The medium was harvested after 24 hours when the cells remained sub-confluent. The PGs released directly into the culture medium were mostly newly synthesized PGs.

Core protein heterogeneity in aggrecan from normal human articular cartilage has been reported and identified as a consequence of aggrecan turnover in normal cartilage (Vilim et al 1993). Although the presence of many of these species in cartilage has been known for many years, the extent to which they reflect anabolic and catabolic processes remains uncertain.

One of the first changes observed in OA cartilage is a decrease in its PG content (Mankin and Lippiello 1971), but rates of PG synthesis observed in OA chondrocytes are still controversial. In many studies, PG synthesis rates increased in OA cartilage (Brand et al 1991, McElligott

and Collins 1960, Ehrlich and Mankin 1980, Bollet 1967). In other studies, they were unchanged or even decreased (Byers et al 1977, Mackenzie et al 1977).

During the biosynthesis of aggrecan, there is a marked variation in the capacity of newly synthesized molecules to bind hyaluronan (Sandy 1992). The pool of aggrecan synthesized over a 24-hour period and released into the medium of post-confluent primary cultures exhibits a low affinity for hyaluronan (Sandy and Plaas 1989). This population appears to have a half-life of about 24 hours *in vivo* before undergoing assembly into link stabilized aggregates (Sandy and Plaas 1989). The delay in acquisition of high affinity binding may be necessary to allow newly secreted molecules to diffuse to PG-deficient regions of matrix distant from the cells. Once synthesized aggrecan is aggregated, and this population may have an *in vivo* half-life of up to 300 days in mature animals (Sandy 1992), before undergoing proteolytic trimming at the C-terminal and N-terminal (Figure 5.12). Flannery et al (1992) reported that there may be loss of the G3 region soon after synthesis of aggrecan. Experiments with calf chondrocytes in culture showed that there was a rapid removal of G3 from newly synthesized aggrecan.

The only aggrecan core species, immuno-reactive with G1 aggrecan antibody to be released from normal chondrocytes, has a molecular weight greater than 250 kDa. It has been reported that 30 to 50 per cent of the total aggrecan core protein exists in full-length in cartilage (Sandy et al 2001). Therefore, newly synthesized aggrecan is more likely to be intact (un-degraded product). In normal cartilage, primary cleavages occur in the chondroitin sulphate-rich region and cleavage in the inter-globular domain is slow and secondary. Studies of the aggrecanase mediated degradative pathway in rat chondrosarcoma cells showed that only species present before IL-1β addition was full length aggrecan. There was a lag period, after IL-1β addition, of 20 hours before any catabolism occurred, and the first products, which appeared between 20 and 45 hours, were peptides 4, 5, 7, 8, 10 (Figure 5.13). These products were those expected from cleavage within the chondroitin sulphate-attachment region but without inter-globular domain cleavage. A second phase of catabolism began at about 60 hours with the appearance of peptides 12, 13, and 6. These are terminal products to be expected following inter-globular domain cleavage (Sandy et al 2000) (Figure 5.13).

It has been reported that aggrecan G1 fragments increase in cartilage with age as well as with the degree of histological damage in OA (Hollander et al 1994). However, few studies have been done on the biosynthetic activity of cultured chondrocytes. In this study, OA chondrocytes lost the ability to synthesize full-length intact aggrecan species when extracted from chondrocyte with higher degrees of histological damage. This was especially noticeable in OA grade III chondrocytes, measured by the G1 domain containing aggrecan species. The results obtained here are similar to those of Malemud et al (1995), which showed that the profiles of chondrocytes from aged-matched non-arthritic cartilage and chondrocytes from osteoarthritic cartilage were virtually identical except for the almost complete absence of

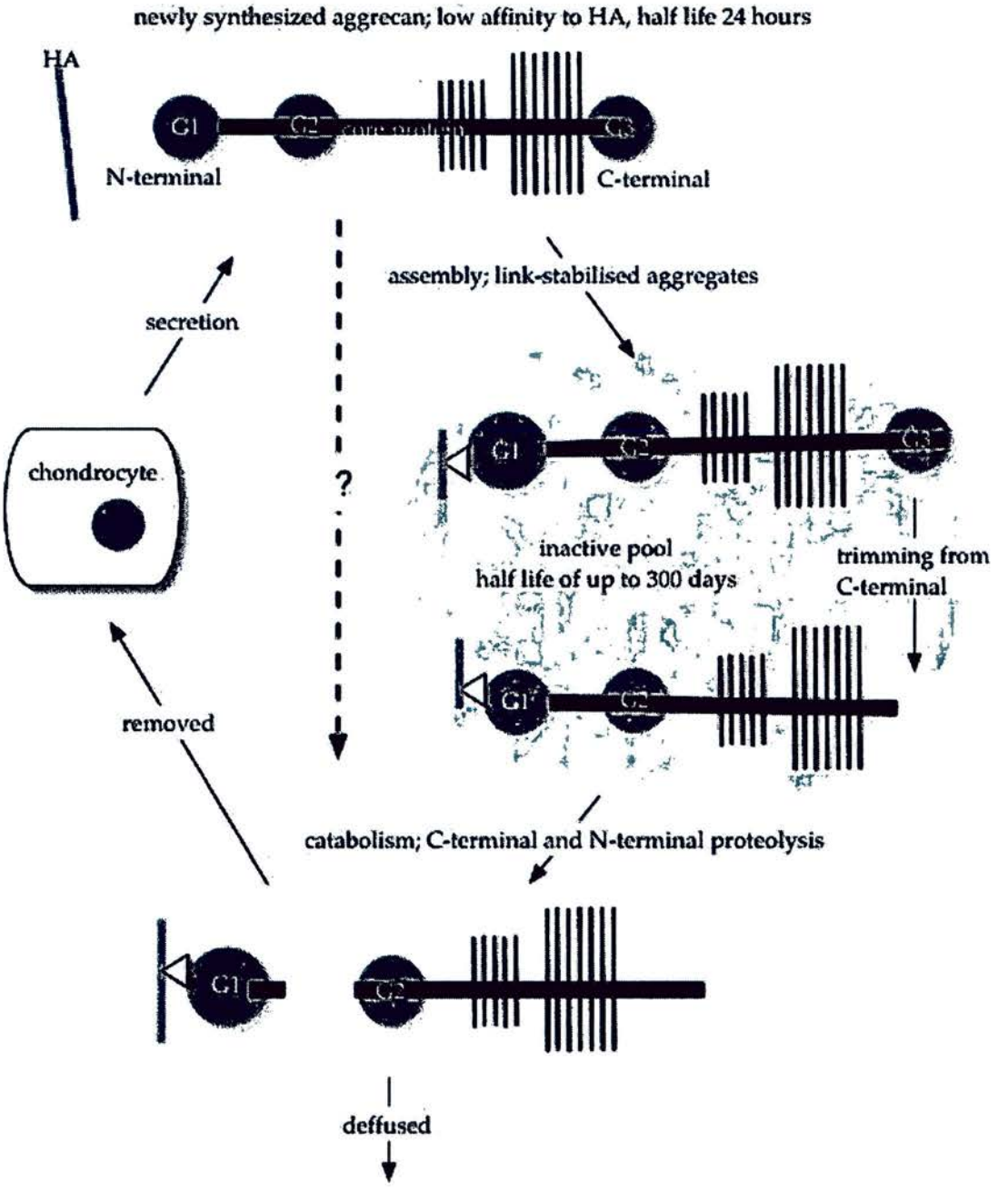


Figure 5.12 Extra-cellular metabolism of aggrecan

Aggrecan monomers are secreted by chondrocytes and enter a transient pool with a low affinity of binding for hyaluronan. The majority of this pool undergoes assembly into link-stabilised aggregates and acquires a high binding affinity for hyaluronan. The molecules then undergo proteolytic trimming at the C-terminal and N-terminal (adopted from Sandy 1992).

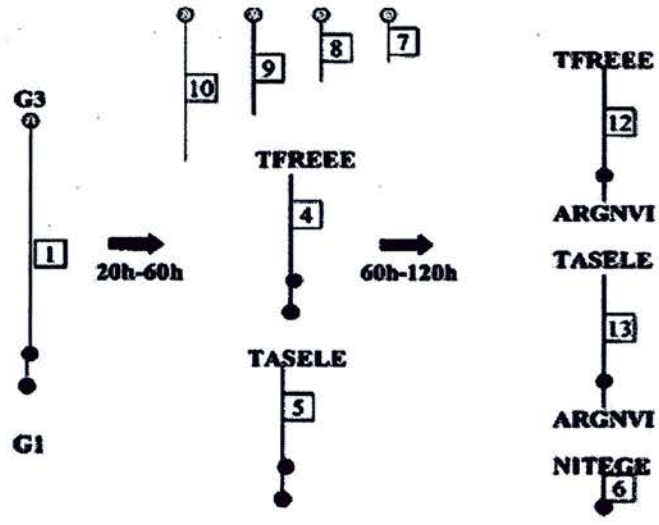


Figure 5.13 A model of the kinetics of the aggrecanase-mediated degradative pathway (adopted from Sandy et al 2000).
Peptides numbers refer to those shown in Figure 5.3.

some of the large (~520 kDa) molecular mass core proteins of PGs in the chondrocytes derived from osteoarthritic cartilage. Total GAG production from OA grade III chondrocytes was much smaller, on average 65.0 per cent and 59.6 per cent less GAG (corrected by DNA), than for OA grade I and II chondrocytes (Chapter Three). It is possible that newly synthesized PGs from OA grade III chondrocytes degraded more rapidly, or that OA grade III chondrocytes synthesized PGs more slowly than normal chondrocytes. The former theory is supported by Sandy et al (1984). They provided evidence that newly synthesized PGs in cartilage, from operated joints of experimental OA, are more rapidly degraded in the tissue than PGs in the control cartilage.

My results have demonstrated that RA chondrocytes synthesized large (~350 kDa) G1 containing molecular mass core proteins of aggrecan, which were virtually identical to those synthesized by normal or early stage of OA cartilage chondrocytes. However, diminished C4S reactivity in the high molecular weight protein from the RA samples suggests that RA chondrocytes synthesize different aggrecan species from normal chondrocytes in culture. Shiozawa et al (1992) found that rheumatoid synovium can spread easily over the eroded cartilage surface (hyaluronidase treated) *in vitro*. Synovial tissue extension was inhibited when the cartilage surface was covered by C4S. This result suggests that PGs in cartilage can inhibit rheumatoid synovial extension over the cartilage as pannus, and that this inhibitory effect is lost in RA. The results obtained with 2-B-6 in RA samples may be the result of degradative processes in RA chondrocytes. CS-Szabo et al (1995) have shown that PGs from RA cartilage were degraded and contained fewer PG populations than either normal or OA samples.

3-B-3 and 1-B-5 immuno-reactive proteins with lower electrophoretic mobility were not detected. High molecular weight proteins (around 300 to 400 kDa) were predominantly released from normal, early OA, and RA chondrocytes (Figure 5.9, 5.11). The strong immuno-reactivity with 3-B-3 and 1-B-5 confirms that the populations of aggrecan contain C6S and C0S disaccharide isomers. OA grade II samples contained more high molecular weight (around 300 to 400 kDa) C4S-containing epitopes than for normal or RA samples, and this is consistent with previous quantitative analysis of epitope contents in cartilage (CS-Szabo et al 1995). The band sizing just under 50 kDa when stained with 2-B-6 (see arrow) is most likely to be the small proteoglycan decorin, as the core protein of decorin is 38 to 47 kDa after chondroitinase ABC treatment (Roughley 1989, Liu 2003), and decorin contains CS isomers (personal communication with Dr Hughes, Cardiff University), especially, a greater amount of C4S than C6S of the N-Acetyl-galactosamine residues, in contrast with aggrecan, which always show more C6S (Melching and Roughley 1989).

The concentration of high molecular mass chondroitin-6, 4, 0-sulphate positive epitopes decreased in OA grade III chondrocytes compared to normal chondrocytes, in this study. C6S containing epitopes produced by OA grade III chondrocytes were fragmented and the number with molecular weight sizes below 250 kDa increased. It has been proposed that altered anabolic activity in OA is related to a change in the differentiated state of the chondrocytes (Aigner and Dudhia 1997). Fragments with these epitopes may be expressed as part of the chondrocyte response to the disease state. Changes in the concentration of chondroitin sulphate in human OA cartilage have been demonstrated in many reports, although changes in the structure of PGs are still controversial. The length of the CS chains has been reported to be shortened (Vasan 1980, Bollet et al 1966) or unchanged (Brocklehurst et al 1984, Rizkalla et al 1992), while the sizes of the PG monomers can vary (Brocklehurst et al 1984, Inerot et al 1978). An increase in the concentration of C4S in OA cartilage has been shown by Cs-Szabo et al (1995). The epitopes recognized by 3-B-3 (-) (monoclonal antibody to native terminal CS disaccharide epitope) were shown to be absent or only weakly expressed in normal cartilage, but expressed highly in pathological OA specimens (Slater et al 1995). Chondroitin sulphate chains from arthritic cartilage have significantly altered sulphation of the terminal residues. In normal cartilage around 60 per cent of terminal GalNAc4S was 4.6-disulphated, but this was reduced to around 30 per cent in OA cartilage (Plaas et al 1998). In experimental canine OA, induced by sectioning the cruciate ligaments in the stifle joint, newly synthesized PG from cartilage explants had longer CS chains but were smaller in size (Carney et al 1985). A summary of some of these investigations is listed on Table 5.1.

endogenous	normal	OA (arthritic)	tissue	references
size		smaller PG, shorter CS chain	human cartilage	Vasan 1980
		decreased CS size shorter CS chain	human cartilage	Bollet et al 1966
		unchanged molecular size	human cartilage	Brocklehurst et al 1984, Roughley et al 1981
		unchanged CS chain larger molecular size in advanced OA	human cartilage	Rizkalla et al 1992
		smaller PG monomer	dog cartilage	Inerot et al 1978
sulphation	disulphated	mono-sulphated	human cartilage	Plaas et al 1998
composition	C6S	C4S	human cartilage	Cs-Szabo et al 1995
		increased 3-B-3 (-) reactivity	human cartilage	Slater et al 1995
		carbohydrate deficient (smaller CS rich region)	dog cartilage	Inerot et al 1978
Newly synthesized	normal	OA (arthritic)	tissue	reference
size		Larger (PG monomer)	Human cartilage explant	Martel-Pelletier et al 1987
		Less PG core proteins	human cartilage explant / monolayer	Malemud et al 1995
	loading unchanged		bovine sesamoid bones	Korver et al 1992
		ACL transection longer CS chains smaller PG	cartilage explant	Carney et al 1985
aggregating property		slow aggregation	human cartilage explant	Martel-Pelletier et al 1987
	loading unchanged		bovine sesamoid bones	Korver et al 1992
sulphation	ACL rupture increased monosulphated		human OA cartilage	Plaas et al 1998
composition	exercise increased C6S		Beagle dog cartilage	Saamanen et al 1989

Table 5.1 Studies of the PG CS epitopes in normal and OA cartilage

5.3 The effects of mechanical stimulation on aggrecan core species released from cultured chondrocytes

5.3.1 Aim

The aim of the experiments described in this section was to investigate possible changes in aggrecan species and the level of epitope expression following exposure of cultured chondrocytes to cyclic mechanical stimulation. In addition, a series of experiments were also undertaken to establish whether α5 integrins, αVβ5 integrins or the integrin associated protein (IAP) CD47 were involved in those processes, following cyclical mechanical stimulation.

5.3.2 Materials and Methods

Human articular knee cartilage was obtained at the time of joint replacement surgery from patients with OA and RA and from above knee amputation in patients with peripheral vascular disease. The articular cartilage surface was assessed macroscopically (McElligott and Collins 1960) and microscopically (OARSI) and graded as normal, OA grade I to III or RA. Chondrocytes were isolated from normal articular cartilage of one male (76 year old) and two females (66 and 73 year old). OA chondrocytes were isolated from the OA articular cartilage of four males with a mean age of 64.5 years (range 39-79), and six females with a mean age of 64.6 years (range 53-74). RA chondrocytes were isolated from the articular cartilage of three females with a mean age of 57.3 years (range 39-74). Primary chondrocytes were seeded in monolayer at 5 x 10^4 cells/ml concentration. Cells were cultured to near confluent for an average of 8.1 days. On the day before experiments were carried out, the culture medium containing 10 per cent serum (FCS) was replaced by serum free medium or medium with 0.5 per cent serum for 16 hours.

Cells were exposed to function-blocking monoclonal antibodies to α5 integrin (P1D6 MAB1956, Chemicon International), αVβ5 integrin (P1F6 MAB1961, Chemicon International) or CD47 (Bric 126, IBGRL Research) at a concentration of 1 μg/ml, in monolayer culture at 37 °C for 30 minutes. Cells were then mechanically stimulated at 0.33 Hz 4000μ strain or 0.33 Hz 32000μ strain for 20 minutes in the presence or absence of antibodies, and this was followed by 24 hours incubation at 37°C. PGs in the obtained medium were precipitated and the quantity of GAGs was measured by DMMB assay. Identical amounts of medium (1 ml medium per lane)or GAG (1 to 2 μg GAG per lane) were loaded onto SDS-PAGE under reducing conditions. Experiments were repeated using single dishes of

human articular chondrocytes derived from at least four adult donors. The cultured medium was collected and analysed (Appendix).

5.3.3 Results

Results were obtained from cultured chondrocytes derived from normal, OA and RA cartilage.

5.3.3.1 Results obtained from normal chondrocytes

Identical amounts of medium (1 ml medium per lane) were loaded onto SDS-PAGE under reducing conditions. The anti-aggrecan G1 domain antibody (anti-HABR) was used on 7.5 per cent gel to examine the extent of aggrecan production from monolayer cells in 24 hours after mechanical stimulation. Aggrecan peptides with a molecular weight of 350 kDa were detected after 24 hours as well as smaller molecular weight bands, in both controls (CT: without mechanical stimulation) and MS (mechanically stimulated at 0.33 Hz 4000μ strain) samples. The density of these bands increased following mechanical stimulation (see arrow).

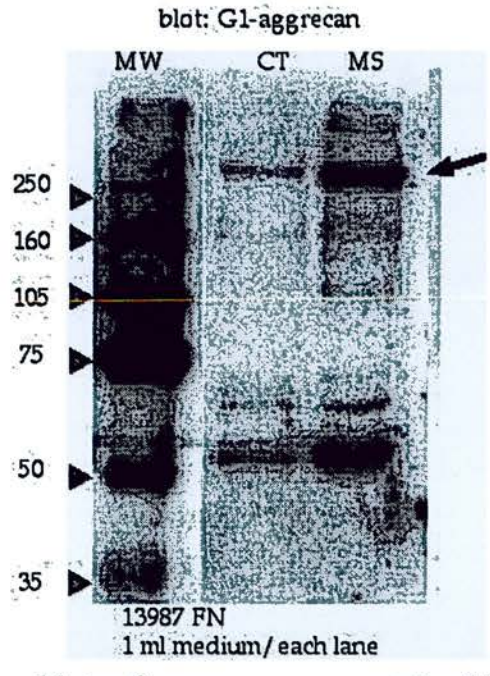


Figure 5.14 Western blots of aggrecan core proteins following 0.33 Hz cyclical mechanical stimulation of normal chondrocytes

Normal chondrocytes (day book number 13987) were subjected to 0.33Hz 4000μ strain mechanical stimulation for 20 minutes. The same amount of medium samples (1 ml) from resting chondrocytes (CT) and stimulated

chondrocytes (MS) cultured in medium with 0.5 per cent FCS were collected and precipitated. Extracts were deglycosylated and blotted with anti-G1 aggrecan antibody 1:400. The stimulated (MS) chondrocytes released large molecular weight core protein (~350 kDa see arrow) and some smaller bands (~160, 110,55 kDa) more intensively than control samples.

CT: resting chondrocytes (without mechanical stimulation)
MS: mechanically stimulated

In order to compare the abundance of different peptides 24 hours after mechanical stimulation, the same amount of GAG was loaded (1μg per lane) instead of the same amount of medium. The only peptide detected after 24 hours was a full-length peptide with a molecular weight of 350 kDa, in both controls without mechanical stimulation and MS (mechanically stimulated) samples (Figure 5.15).

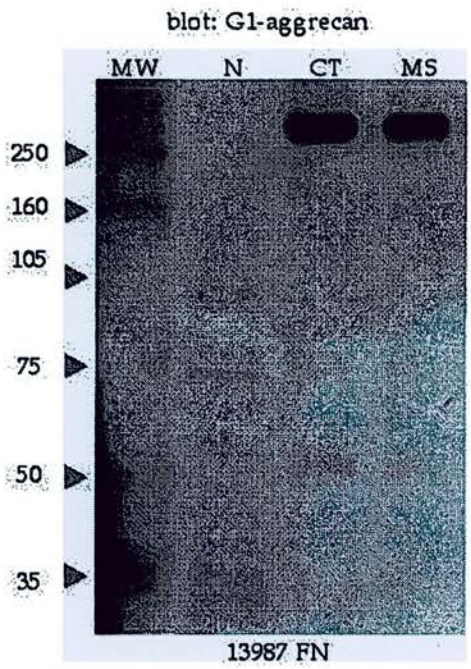


Figure 5.15 Western blots of aggrecan core proteins following 0.33 Hz cyclical mechanical stimulation of normal chondrocytes

Normal chondrocytes (day book number 13987) were subjected to 0.33Hz 4000μ strain mechanical stimulation for 20 minutes. Medium samples from resting chondrocytes (CT) and stimulated chondrocytes (MS) cultured in medium with 0.5 per cent FCS were collected and precipitated. Extracts were deglycosylated and 1 μg GAG per lane was blotted with anti-G1 aggrecan antibody 1:400. Resting (CT) and stimulated (MS) chondrocytes released only large molecular weight core protein (~350 kDa) with no noticeable difference between the two. The blot shown is representative of a series of experiments including three different donors of normal cartilage.

N: negative control (0.5 per cent serum containing medium)
CT: resting chondrocytes (without mechanical stimulation)
MS: mechanically stimulated

In the presence of anti-integrin antibodies to α5, αVβ5, and CD47, which have been shown to block chondrocyte mechano-transduction, the only peptide detected was a full-length peptide with a molecular weight of 350 kDa, with or without mechanical stimulation (Figure 5.16).

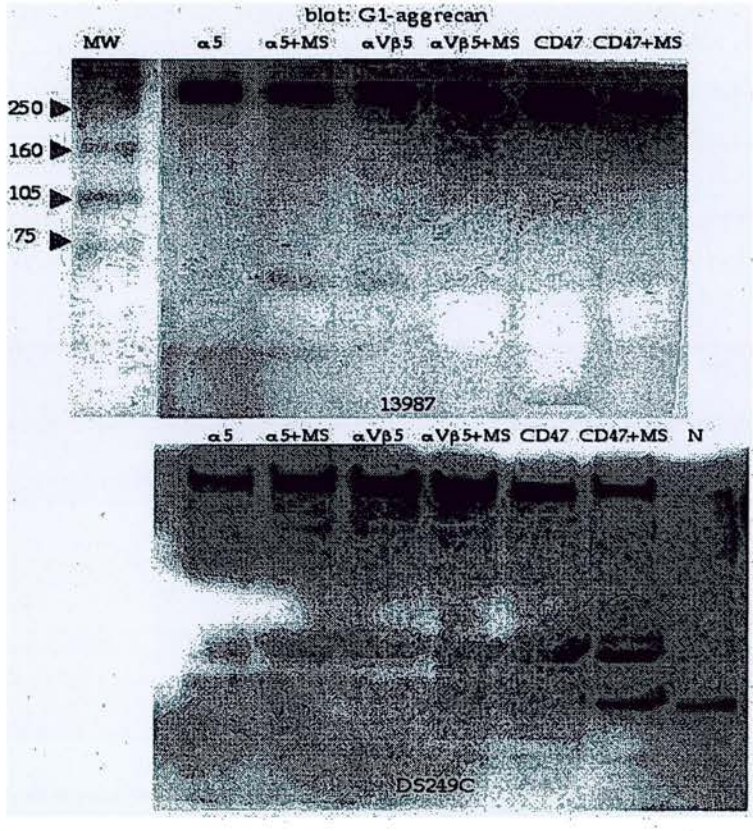


Figure 5.16 Western blots of aggrecan core proteins following 0.33 Hz cyclical mechanical stimulation in the presence of anti-integrin antibodies in normal chondrocytes

Normal chondrocytes (day book number 13987, DS249C) were subjected to 0.33Hz mechanical stimulation. Anti-integrin (α5, αVβ5, and CD47) antibodies were added to 0.5 per cent serum containing medium 30 minutes prior to the experiments. Medium samples from resting chondrocytes and stimulated chondrocytes (MS) were collected and precipitated. Extracts were deglycosylated and 1 μg GAG per lane was blotted with anti-G1 aggrecan antibody 1:400. The only protein species immuno-reactive with G1-aggrecan antibody released from resting and stimulated (MS) chondrocytes was greater than 250 kDa in size (~350 kDa) and was homogeneous. Anti-α5, αVβ5, and CD47 antibodies did not modulate this trend. The blot shown is representative of a series of experiments including three different donors of normal cartilage.

N: negative control (0.5 per cent serum containing medium)

When monoclonal antibodies for C6S containing peptides (A) and C0S containing peptides (C) were used, there was no difference between mechanically stimulated (MS) and unstimulated (CT) chondrocytes. However when the monoclonal antibody for C4S containing peptides was used (B), a clear band with a molecular weight of greater than 250KDa could be detected in medium samples after mechanical stimulation at 4000μ strain (Figure 5.17). The density of this band increased when the mechanical strain was increased eight fold at 32000μ strain (see arrow).

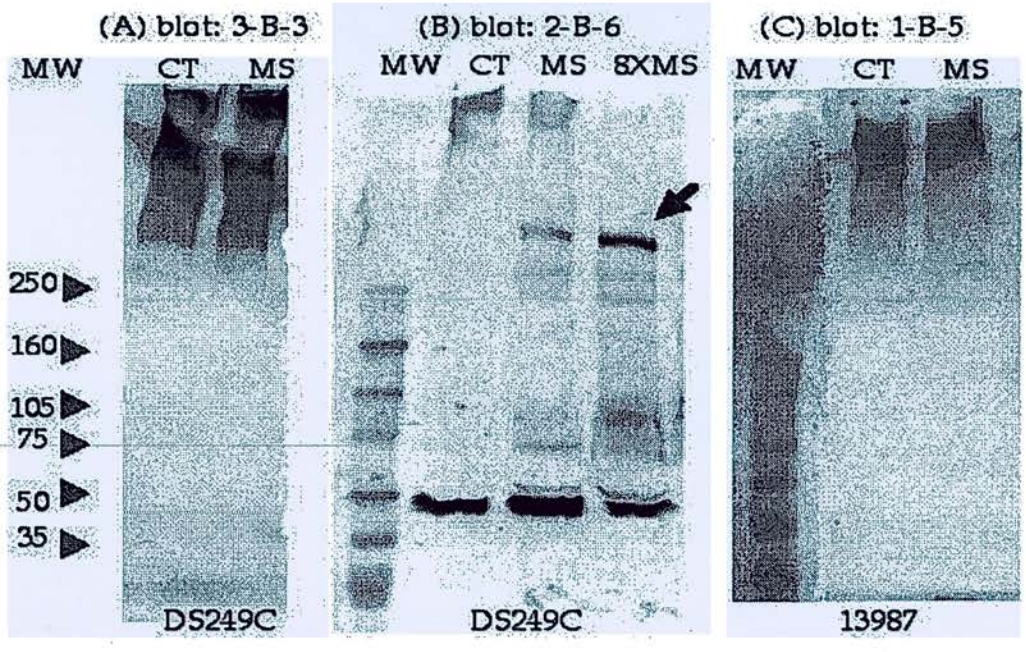


Figure 5.17 Western blots of aggrecan following 0.33 Hz cyclical mechanical stimulation using monoclonal antibodies in normal chondrocytes

Normal chondrocytes (day book number DS249C and 13987) were subjected to 0.33Hz mechanical stimulation. Medium samples from resting chondrocytes (CT) and stimulated chondrocytes (MS) were collected and precipitated. Extracts were deglycosylated and 1 μg GAG per lane was blotted with 3-B-3 monoclonal antibodies 1:200, 2 μg GAG per lane was blotted with 2-B-6 1:100, and 1 μg GAG per lane was blotted with 1-B-5 monoclonal antibodies 1:5000. Resting (CT) and stimulated (MS) chondrocytes released only large molecular weight core protein containing C6S and C0S stubs (A, C). However, However when the monoclonal antibody for C4S containing peptides was used (B), a clear band with a molecular weight of greater than 250 KDa could be detected in medium samples after mechanical stimulation at 4000μ strain. The density of this band increased when the mechanical strain was increased eight fold at 32000μ strain (see black arrow). The blot shown is representative a series of experiments including three different donors of normal cartilage.

5.3.3.2 Results obtained from OA chondrocytes

The results obtained here were different depending on the grade of OA.

In OA grade I, when using anti-aggrecan G1 domain antibody: (1) the only peptide present after 24 hours was a full-length peptide with a molecular weight of 350 kDa, both in control (CT; without mechanical stimulation) and following MS (Figure 5.18), (2) in the presence of the anti-α5 integrin antibody, mechanical stimulation increased the density of the 350 kDa band detected with anti-aggrecan G1 domain antibody (Figure 5.19, see arrows), (3) in the presence of anti-αVβ5 integrin antibody, the density of the 350 kDa molecular weight band detected with anti-aggrecan G1 domain antibody, varied following mechanical stimulation, (4) in the presence of anti-CD47 antibody, there were no changes in the 350 kDa band following mechanical stimulation (Figure 5.19).

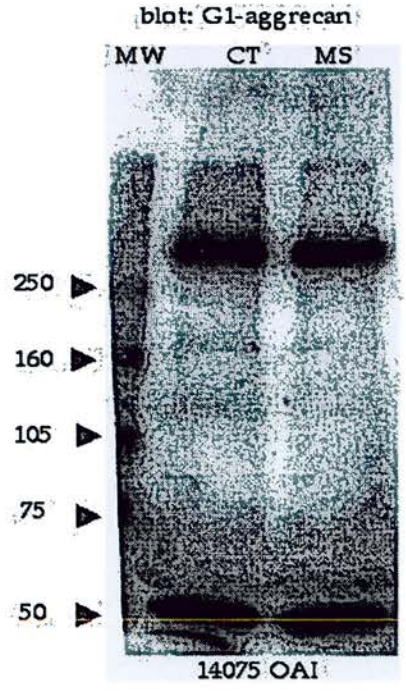


Figure 5.18 Western blots of aggrecan core proteins following 0.33 Hz cyclical mechanical stimulation in OA grade I chondrocytes

OA chondrocytes (day book number 14075 OAI) were subjected to 0.33Hz mechanical stimulation. Medium samples from resting chondrocytes (CT) and stimulated chondrocytes (MS) in medium supplemented with 0.5 per cent FCS were collected and precipitated. Extracts were deglycosylated and 1 μg GAG per lane was blotted with anti-G1 aggrecan antibody 1:400. Resting (CT) and stimulated (MS) chondrocytes released only large molecular weight core proteins with a molecular weight of 350 kDa but did not show any noticeable differences. The blot shown is representative of a series of experiments including three different donors of OA cartilage.

CT: resting chondrocytes (without mechanical stimulation)
MS: mechanically stimulated

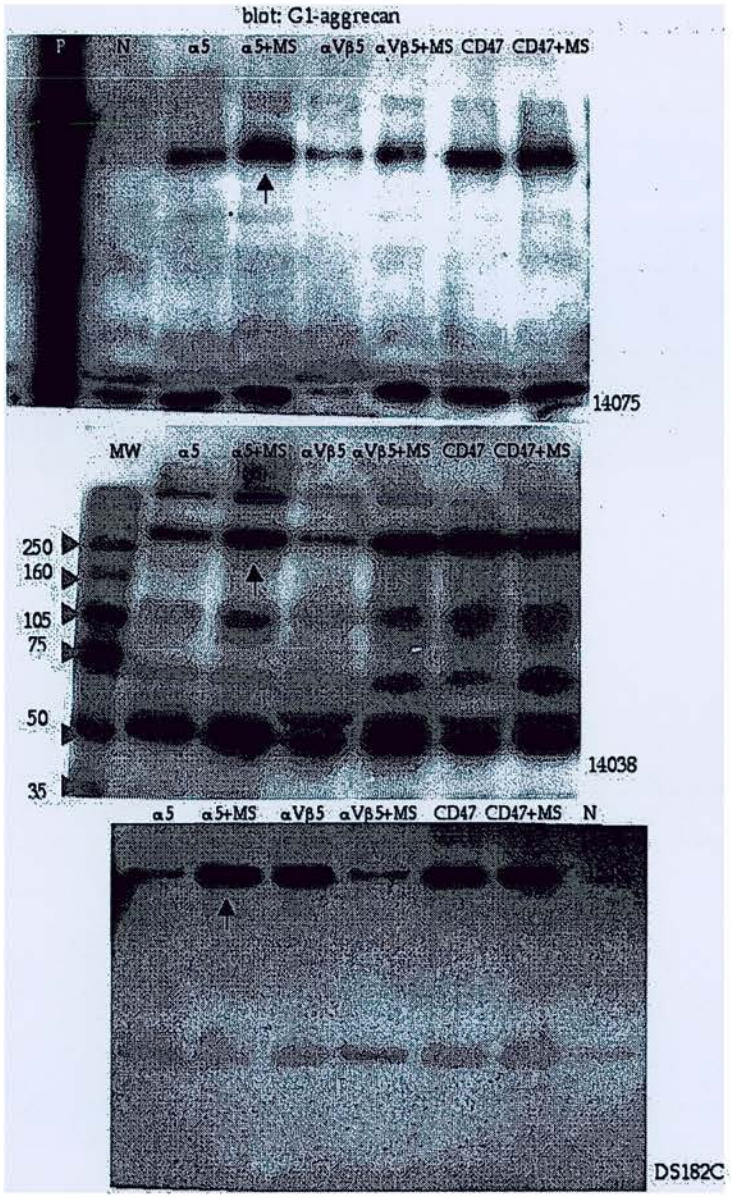


Figure 5.19 Western blots of aggrecan core proteins following 0.33 Hz cyclical mechanical stimulation in the presence of anti-integrin antibodies in OA grade I chondrocytes

OA grade I chondrocytes (day book number 14075,14038, and DS182C) were subjected to 0.33Hz mechanical stimulation. Anti-integrin (α5, αVβ5, and CD47) antibodies were added to 0.5 per cent serum containing medium 30 minutes prior to the experiments. Extracts were deglycosylated and 1 μg GAG per lane was blotted with anti-G1 aggrecan antibody 1:400. Resting and stimulated (MS) chondrocytes released only large molecular weight core protein (350 kDa). In the presence of anti-α5 integrin antibodies, mechanical stimulation increased the density of the 350 kDa molecular mass band detected with anti-aggrecan G1 domain antibody (see arrow).

P: (positive control) 1 μg GAG extracted directly from OA cartilage (14038 OAI)
N: (negative control) 1 ml 0.5 per cent serum containing medium

In OA grade II, when using the anti-aggrecan G1 domain antibody: (1) large molecular weight core protein with a molecular weight of 350 kDa were absent in some control samples, as well as in some mechanically stimulated samples (Figure 5.20), (2) smaller bands with molecular weight of ~160 kDa were more prominent in both control and mechanically stimulated samples (Figure 5.20, 5.21), (3) in the presence of anti-α5 integrin antibodies, mechanical stimulation increased the density of the 350 kDa molecular mass band detectable with anti-aggrecan G1 domain antibody (Figure 5.21, see arrows), (4) in the presence of anti-α5β1 integrin and anti-CD47 antibodies, the density of the 350 kDa molecular mass band detected with anti-aggrecan G1 domain antibody varied (Figure 5.21).

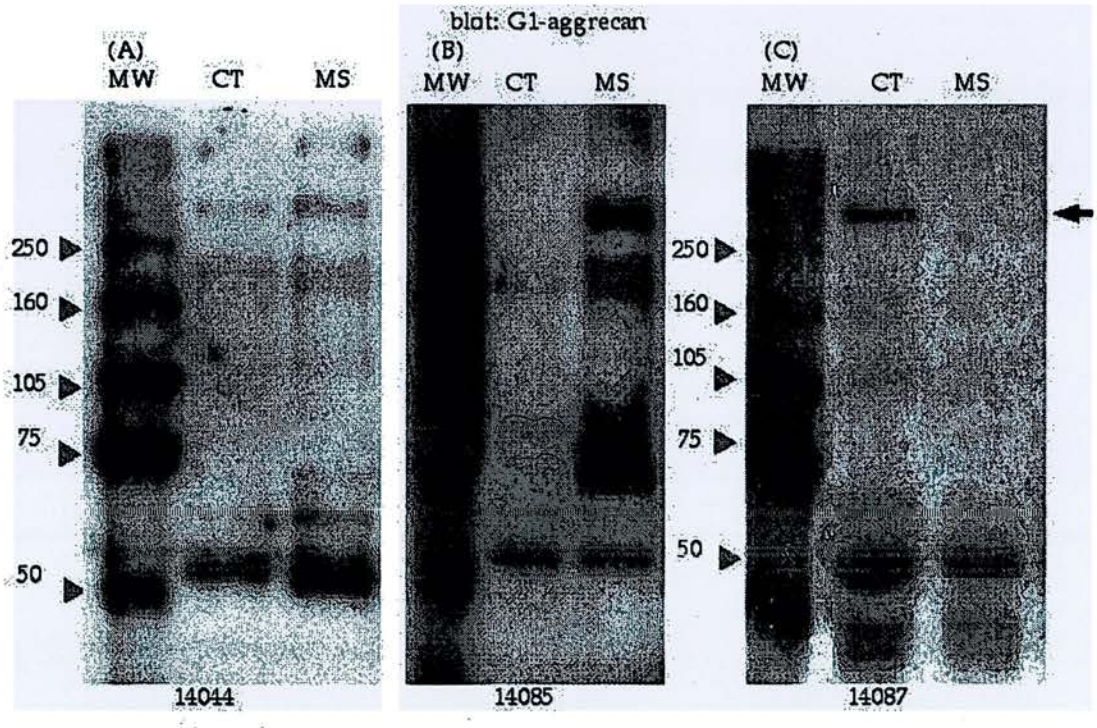


Figure 5.20 Western blots of aggrecan core proteins following 0.33 Hz cyclical mechanical stimulation in OA grade II chondrocytes

OA II chondrocytes (day book number 14035, 14087, and DS130C) were subjected to 0.33 Hz mechanical stimulation. Medium samples from resting chondrocytes (CT) and stimulated chondrocytes (MS) cultured in IMDM supplemented with 0.5 per cent FCS were collected and precipitated. Extracts were deglycosylated and 1 μg GAG per lane was blotted with anti-G1 aggrecan antibody 1:400. Large molecular weight core protein with a molecular weight 350 kDa (see black arrow) was observed less frequently than in normal or OA grade I chondrocytes, and this was not due to mechanical stimulation. Also smaller bands with a molecular weight of ~160 kDa were more prominent than in normal or OA grade I.

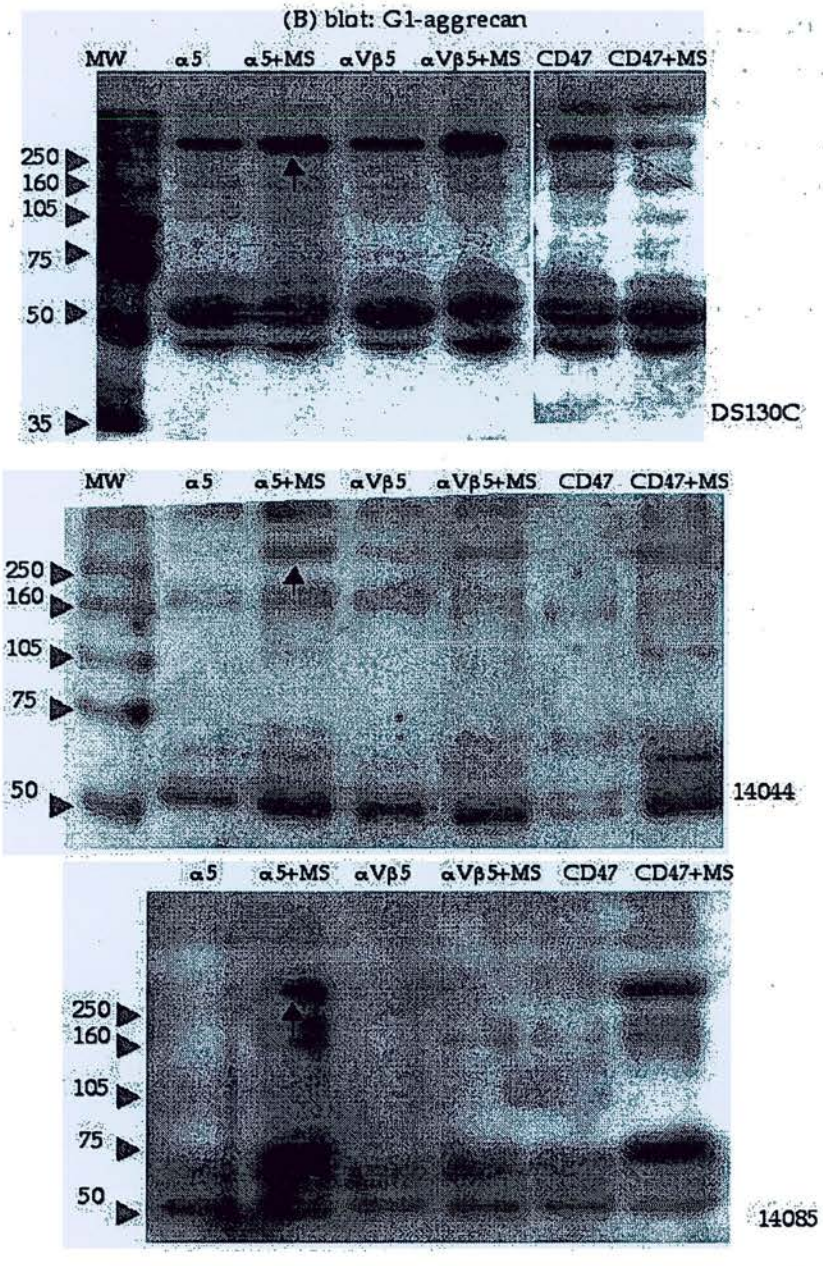


Figure 5.21 Western blots of aggrecan core proteins following 0.33 Hz cyclical mechanical stimulation in the presence of anti-integrin antibodies in OA grade II chondrocytes

Resting and stimulated (MS) chondrocytes did not release large molecular weight core protein (350 kDa) as frequently as normal or OA grade I chondrocytes. In the presence of anti-α5 integrin antibodies, mechanical stimulation increased the 350 kDa molecular mass reactivity with the anti-aggrecan G1 domain antibody but not in the control samples (see white arrows). Smaller bands with molecular weight of ~160 kDa were more prominent than in normal or OA grade I.

In OA grade III, when using the anti-aggrecan G1 domain antibody: (1) large molecular weight core protein was not detected in control samples, nor in mechanically stimulated samples (Figure 5.22), (2) in the presence of anti-integrin antibodies there were no differences in expression of aggrecan species between control samples and mechanically stimulated samples (Figure 5.23).

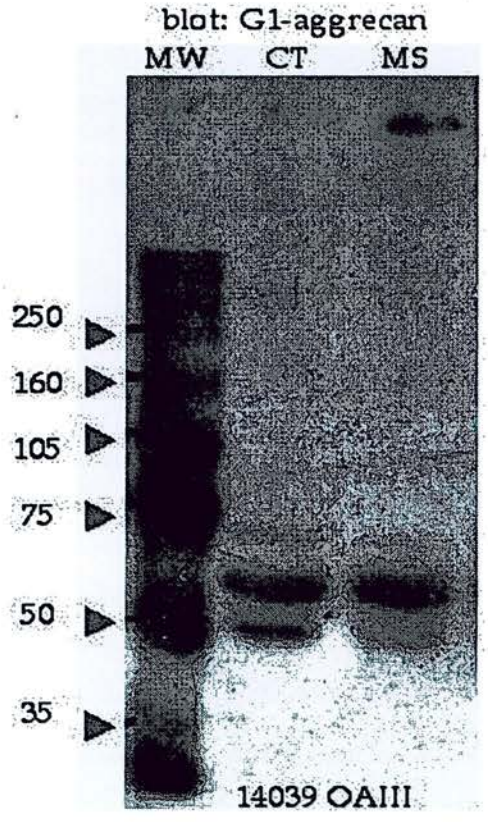


Figure 5.22 Western blots of aggrecan core proteins following 0.33 Hz cyclical mechanical stimulation in OA grade III chondrocytes

OA chondrocytes (day book number 14039 OA grade III) were subjected to 0.33 Hz mechanical stimulation. Medium samples from resting chondrocytes (CT) and stimulated chondrocytes (MS) cultured in IMDM supplemented with 0.5 per cent FCS were collected and precipitated. Extracts were deglycosylated and 1 μg GAG per lane was blotted with anti-G1 aggrecan antibody 1:400. Large molecular weight core proteins with a molecular weight of 350 kDa were not detected in OA grade III chondrocytes, and this absence was not due to mechanical stimulation.

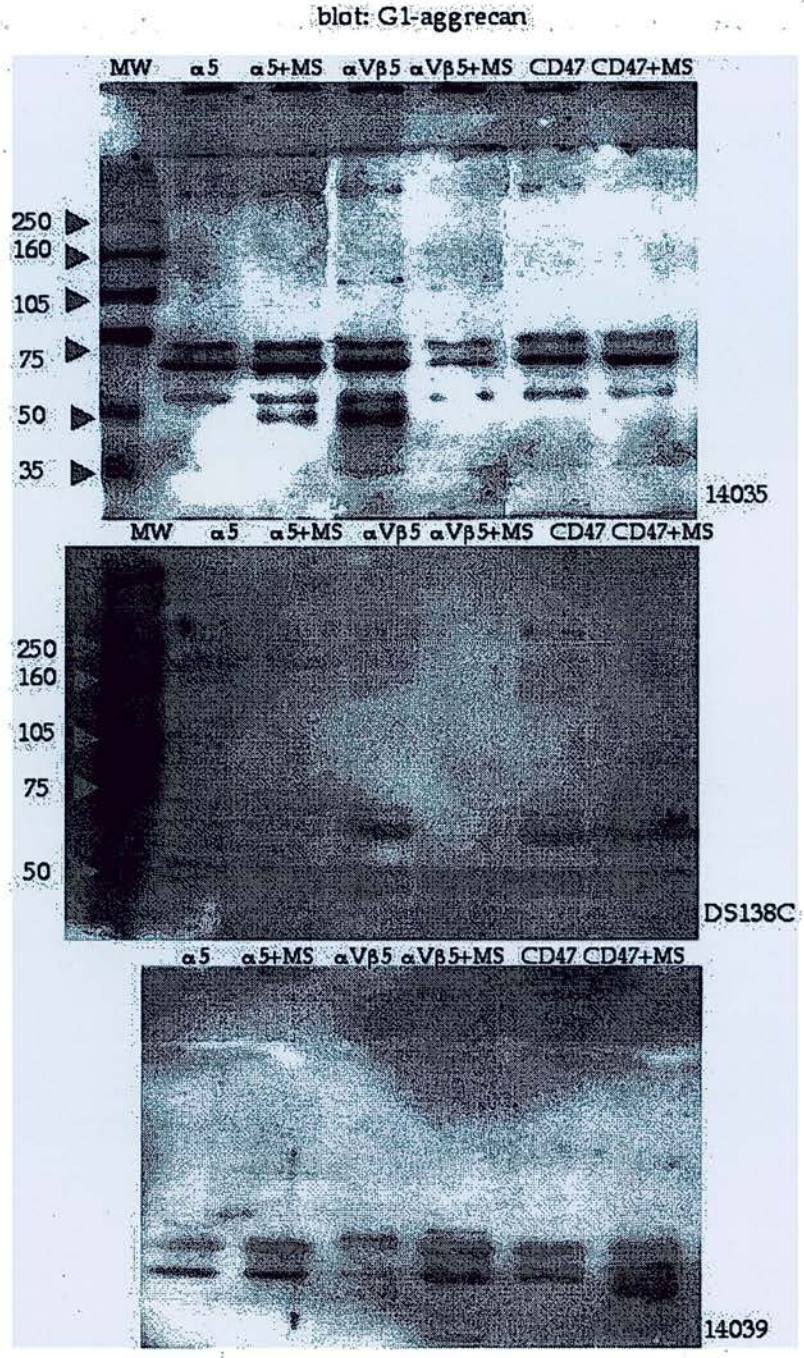


Figure 5.23 Western blots of aggrecan core proteins following 0.33 Hz cyclical mechanical stimulation in the presence of anti-integrin antibodies in OA grade III chondrocytes

Resting and stimulated (MS) chondrocytes released little large molecular weight core protein (350 kDa) and anti-α5, αVβ5, and CD47 antibodies did not modulate this trend.

When using monoclonal antibodies, In OA grade I, there were no differences between mechanically stimulated and unstimulated (CT) samples of C6S, C4S, C0S containing peptides (Figure 5.24, 5.25, 5.26). In OA grade II, there were no differences between mechanically stimulated and unstimulated chondrocytes in C6S and C0S containing peptides using monoclonal antibodies. However, eight times greater strain (8XMS) increased the density of a small molecular weight band ~50 kDa detectable with a 2-B-6 antibody (Figure 5.25). In OA grade III, only weak bands could be detected in both mechanically stimulated samples and unstimulated samples with monoclonal antibodies to C6S, C4S, and C0S (Figure 5.25). However, higher strain (8XMS) resulted in a heavier band of a small molecular weight of ~50 kDa detectable with a 2-B-6 monoclonal antibody (Figure 5.25).

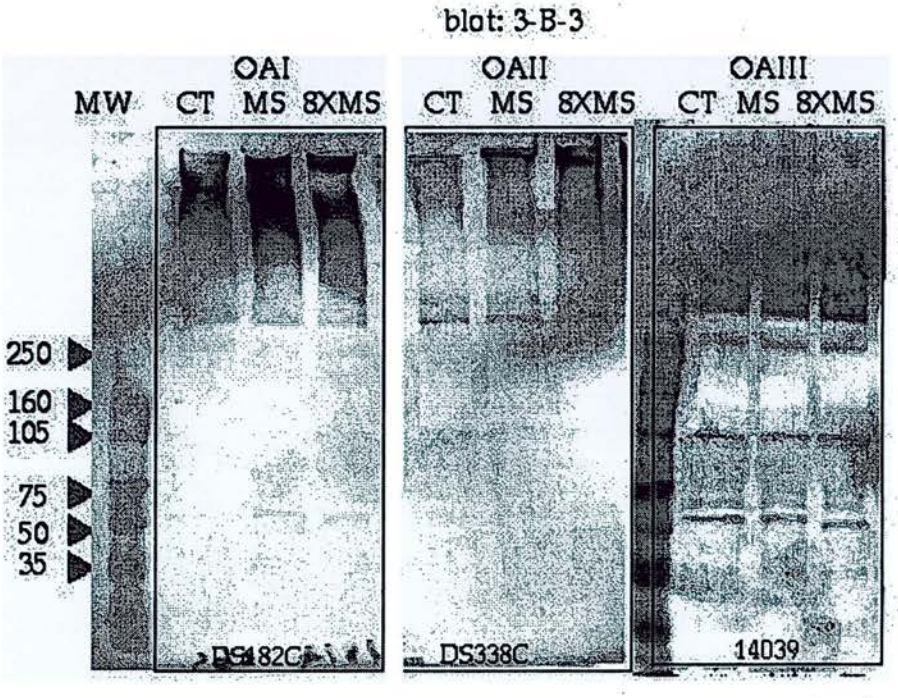


Figure 5.24 Western blots of aggrecan following 0.33 Hz cyclical mechanical stimulation detected by 3-B-3 monoclonal antibody in OA chondrocytes

OA chondrocytes were subjected to 0.33 Hz mechanical stimulation. Medium samples from resting chondrocytes (CT) and stimulated chondrocytes (MS) were collected and precipitated. Extracts were deglycosylated, and 1 μg per lane of GAG was blotted with the 3-B-3 monoclonal antibody 1:200. Resting (CT) and stimulated (MS) chondrocytes released only large molecular weight core proteins containing C6S aggrecan in early OA (OA grade I and OA grade II) but fragments appeared in OA grade III.

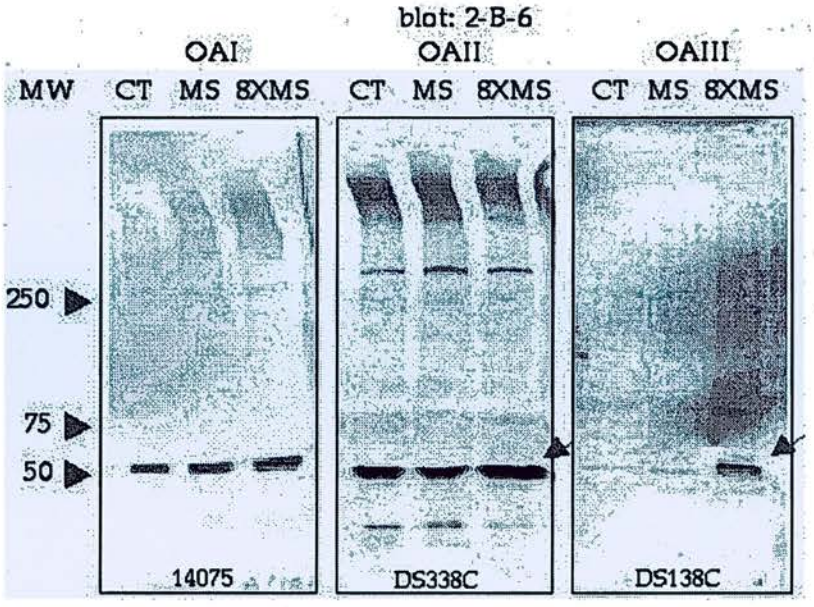


Figure 5.25 Western blots of aggrecan following 0.33 Hz cyclical mechanical stimulation detected by 2-B-6 monoclonal antibody in OA chondrocytes

OA chondrocytes were subjected to 0.33 Hz mechanical stimulation. Medium samples from resting chondrocytes (CT) and stimulated chondrocytes (MS) were collected and precipitated. Extracts were deglycosylated, and 2 μg per lane of GAG was blotted with the 2-B-6 monoclonal antibody 1:100. Resting (CT) and stimulated (MS) chondrocytes released smaller C4S containing aggrecan with molecular weight's density of ~50 kDa. Eight times greater strain (32000μ strain) (8XMS) slightly increased the density of the ~50 kDa bands in OA grade II and III (see grey arrows).

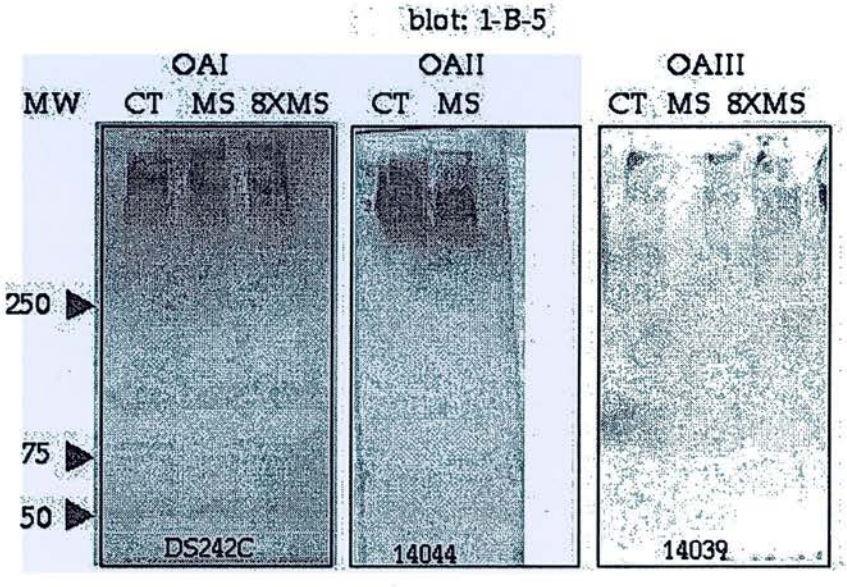


Figure 5.26 Western blots of aggrecan following 0.33 Hz cyclical mechanical stimulation detected by 1-B-5 monoclonal antibody in OA chondrocytes

OA chondrocytes were subjected to 0.33 Hz mechanical stimulation. Medium samples from resting chondrocytes (CT) and stimulated chondrocytes (MS) were collected and precipitated. Extracts were deglycosylated, and 1 μg per lane of GAG was blotted with monoclonal antibody 1-B-5 1:5000. Resting (CT) and stimulated (MS) chondrocytes released only large molecular weight core proteins. The density of the large molecular weight peptides were decreased in the OA grade III chondrocytes.

5.3.3.3 Results obtained from RA chondrocytes

When using the anti-aggrecan G1 domain antibody, the only G1-containing peptide detected, after 24 hours synthesis by RA chondrocytes, was a full-length one with a molecular weight of 350 kDa, apparently not degraded (Figure 5.27). There was no significant alteration in the peptides metabolism after the cyclic mechanical stimulation (Figure 5.28).

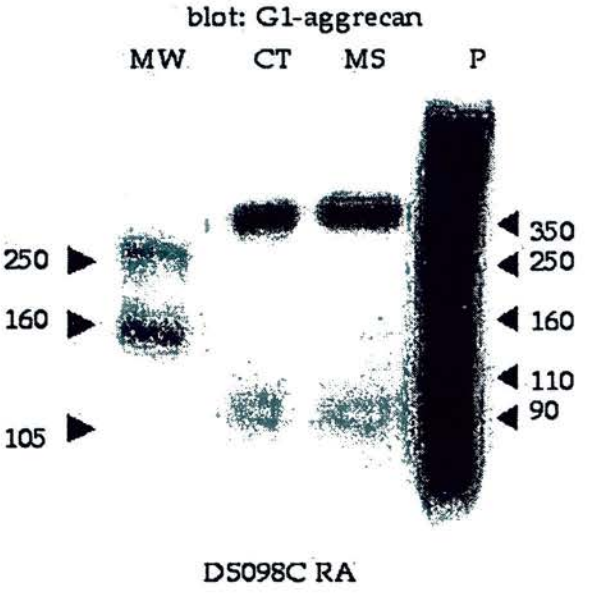


Figure 5.27 Western blots of aggrecan core proteins following 0.33 Hz cyclical mechanical stimulation of RA chondrocytes
RA chondrocytes (day book number DS098C) were subjected to 0.33 Hz mechanical stimulation. Medium samples from resting chondrocytes (CT) and stimulated chondrocytes (MS) cultured in medium supplemented with 0.5 per cent FCS were collected and precipitated. Extracts were deglycosylated and 1 μg per lane of GAG was blotted with anti-G1 aggrecan antibody 1:400. Resting (CT) and stimulated (MS) chondrocytes both released only large molecular weight core proteins with a molecular weight of ~350 kDa. The blot shown is representative of a series of experiments including three different donors of RA cartilage.

CT: resting chondrocytes (without mechanical stimulation)
MS: mechanically stimulated
P: 1 μg GAG extracted directly from RA cartilage (DS098C) (positive control)

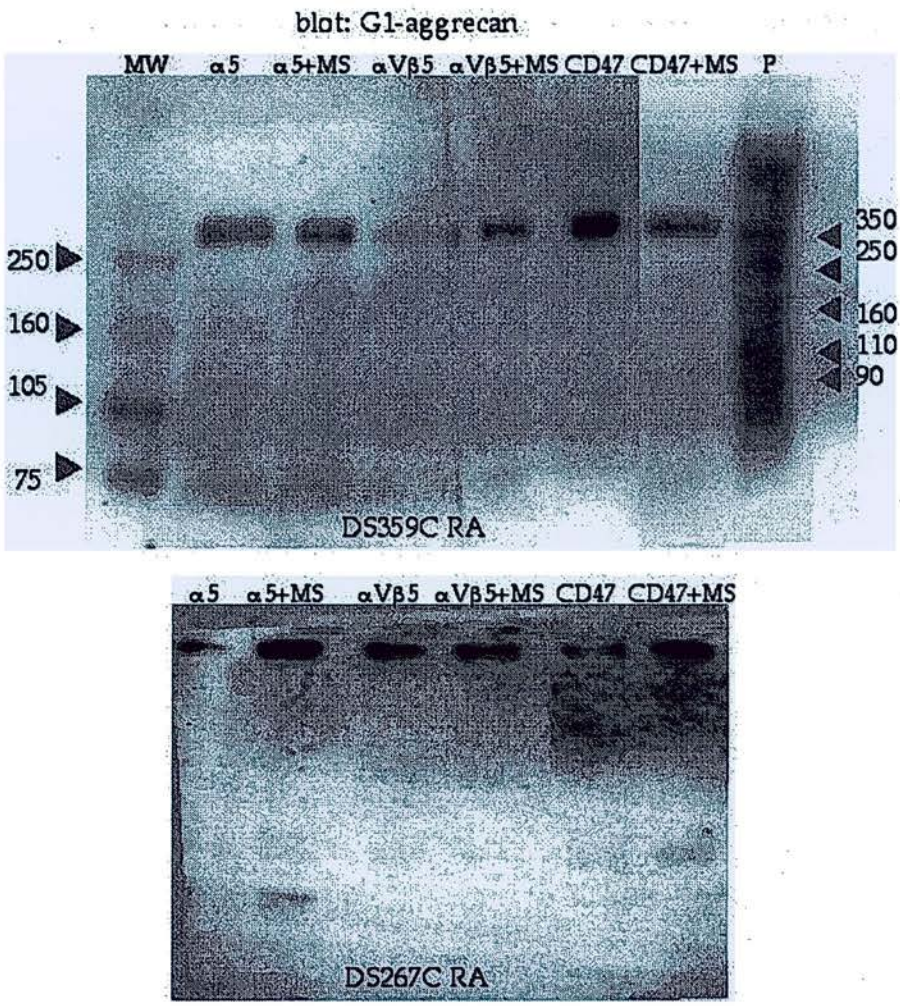


Figure 5.28 Western blots of aggrecan core proteins following 0.33 Hz cyclical mechanical stimulation in the presence of anti-integrin antibodies in RA chondrocytes

RA chondrocytes (day book number DS098C) were subjected to 0.33 Hz mechanical stimulation. Anti-integrin (α5, αVβ5, and CD47) antibodies were added to 0.5 per cent serum containing medium, 30 minutes prior to the experiments. Medium samples from resting chondrocytes and stimulated chondrocytes (MS) were collected and precipitated. Extracts were deglycosylated and 1 μg per lane of GAG was blotted with anti-G1 aggrecan antibody 1:400. Resting and stimulated (MS) chondrocytes released only large molecular weight core proteins (~350 kDa), and anti-α5, αVβ5, and CD47 antibodies did not modulate this trend. The blot shown is representative of a series of experiments including three different donors of normal cartilage.

P: 1 μg per lane of GAG extracted directly from RA cartilage (DS098C)

Using monoclonal antibodies, there was no difference between mechanically stimulated and unstimulated chondrocytes in C6S, C4S, C0S containing peptides. The intensity of the band expressing the C4S epitope was significantly greater in smaller bands with molecular weight of ~50 kDa, than in larger bands in RA (Figure 5.29).

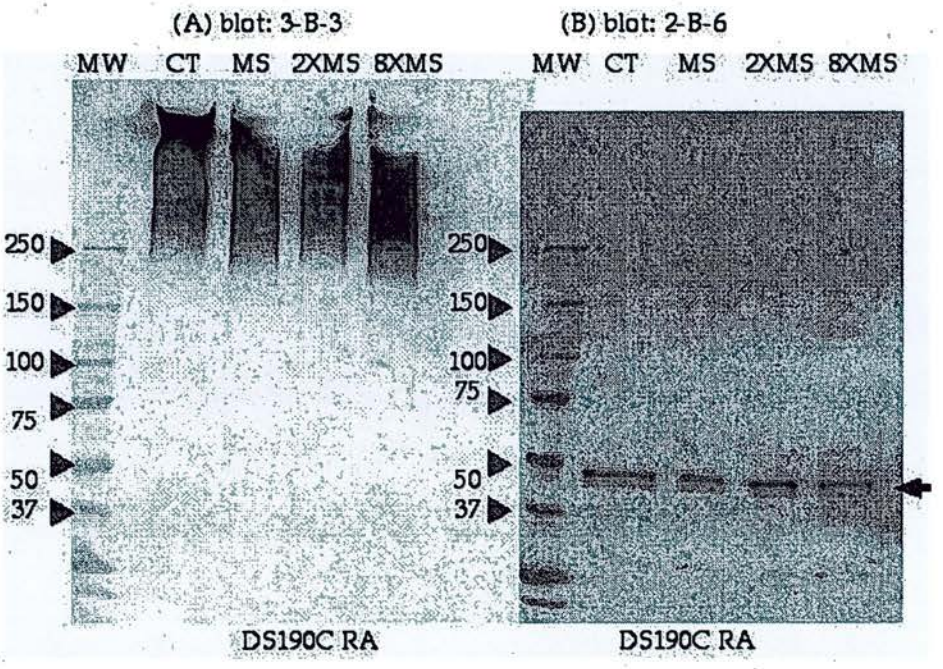


Figure 5.29 Western blots of aggrecan following 0.33 Hz cyclical mechanical stimulation detected with monoclonal antibodies in RA chondrocytes

RA chondrocytes (day book number DS190C) were subjected to 0.33 Hz mechanical stimulation. Medium samples from resting chondrocytes (CT) and stimulated chondrocytes (MS) were collected and precipitated. Extracts were deglycosylated and 1 μg per lane of GAG was blotted with 3-B-3 and 2-B-6 monoclonal antibodies 1:200 and 1:100 respectively. Resting (CT) and stimulated (MS) chondrocytes released only large molecular weight core protein containing C6S stubs (A). There were aggrecan species containing C4S of ~50 kDa (see arrow) (B), but fewer aggrecan with high molecular masses above 250 kDa. The blot shown is representative of a series of experiments including three different donors of RA cartilage.

CT: resting chondrocytes (without mechanical stimulation)
MS: mechanically stimulated at 4000μ strain
2XMS: mechanically stimulated at 8000μ strain
8XMS: mechanically stimulated at 32000μ strain

5.3.4 Discussion

The anti-aggrecan G1 domain antibody was used to examine the abundance of different peptides 24 hours after mechanical stimulation. The main peptide detected was a full-length peptide with a molecular weight of 350 kDa, in both controls without mechanical stimulation and mechanically stimulated medium samples from normal chondrocytes (Figure 5.14, 5.15), in the presence or absence of anti-integrin antibodies to α5, αVβ5, and CD47 (Figure 5.16). When monoclonal antibody for C4S containing peptides was used, a clear band with a molecular weight of greater than 250KDa could be detected in medium samples from normal chondrocytes after mechanical stimulation at 4000μ strain, and the density of this band increased when the mechanical strain was increased eight fold at 32000μ strain (Figure 5.17).

The results obtained from OA chondrocytes were different depending on the grade of OA.

In OA grade I, when using anti-aggrecan G1 domain antibody, the only peptide present after 24 hours was a peptide with a molecular weight of 350 kDa, both in control and following mechanical stimulation (Figure 5.18). The density of the band was increased by mechanical stimulation in the presence of the anti-α5 integrin antibody (Figure 5.19).

In OA grade II, when using the anti-aggrecan G1 domain antibody, large molecular weight core proteins of 350 kDa were absent in some control samples, as well as in some mechanically stimulated samples, and smaller bands with molecular weight of ~160 kDa were more prominent in both control and mechanically stimulated samples (Figure 5.20). The density of the band was increased by mechanical stimulation in the presence of the anti-α5 integrin antibody (Figure 5.21). The density of a small molecular weight band of ~50 kDa detected by 2-B-6 monoclonal antibody increased when the mechanical strain was eight fold higher at 32000μ strain (Figure 5.24).

In OA grade III, when using the anti-aggrecan G1 domain antibody, large molecular weight core protein was not detected in control samples, nor in mechanically stimulated samples in the presence or absence of anti-integrin antibodies (Figure 5.22,23). The density of a small molecular weight band of ~50 kDa detected by 2-B-6 monoclonal antibody increased when the mechanical strain was eight fold higher at 32000μ strain (Figure 5.24).

The results obtained from RA chondrocytes were different from normal or OA chondrocytes. When using the anti-aggrecan G1 domain antibody, the only G1-containing peptide detected was a full-length one with a molecular weight of 350 kDa, apparently not degraded. There was no significant alteration in the peptides metabolism after the cyclic mechanical stimulation.

Using monoclonal antibodies, there was no difference between mechanically stimulated and unstimulated chondrocytes.

Mechanical stimuli are known to have a major influence on chondrocyte function. As can be seen in Chapter Three and Four, it has been shown that the 0.33 Hz 4000μ strain of mechanical stimulation increased the synthesis of GAG in normal chondrocytes. α5 integrins, αVβ5 integrins and the integrin associated protein (IAP) CD47 were involved in these processes. On the other hand, GAG synthesis, following mechanical stimulation, did not increase in OA and RA chondrocytes. Judging by the studies in this section, this decrease may be due to decreased anabolic activity in the early stage of OA and RA chondrocytes, and there may be increased proteolytic activity through releasing aggrecan fragments from the late stage of OA chondrocytes.

In vitro studies have been undertaken here in an effort to characterise the processes that occur in chondrocytes, especially those concerning newly synthesized aggrecan in monolayer culture. The only aggrecan core species, immuno-reactive with G1-aggrecan antibody released from resting and mechanically stimulated normal chondrocytes, was ~350 kDa in size and homogeneous. As can be seen in the results, newly synthesized aggrecan is likely to be intact (un-degraded). It also affects that increased PG synthesis following mechanical stimulation is associated with increases in intact aggrecan production.

We found that neither anti-integrin antibodies nor anti-CD47 antibody modulated the aggrecan species released from cultured chondrocytes after mechanical stimulation in normal chondrocytes. Therefore, these antibodies do not stimulate any catabolic response.

Extra-cellular matrix molecules are constantly degraded, broken down, synthesized anew, and reassembled in a cyclical process. Mechanical forces and biochemical factors are known to affect cell-mediated processes (Quinn et al 1999). PG release into medium is thought to be a rapid cell-mediated process, since the release is greatly reduced when the cartilage is subject to freeze-thawing to kill the chondrocytes (Carney et al 1988). In a study of the effects of five kilo gram loading on the synthesis of PGs of normal bovine articular cartilage *in vitro*, Korver et al (1992) showed that loading did not affect the composition of newly synthesized large PGs, when measured by size exclusion chromatography. Also the aggregating capacity of aggrecan did not change after loading (Korver et al 1992). On the other hand, over-compression induces the release of a large aggregating species, with a core protein size of about 200 kDa, which is C-terminally truncated (Quinn et al 1999). This might be the result of an enhanced degradation of the hyaluronan network, rather than the result of accelerated proteolysis, judging by the size of the released aggrecan species.

Whether mechanical stimulation can affect the cell-mediated processes of PG release into medium, directed by regulated protease action (aggrecanase and MMPs), needs to be elucidated. Flannery et al (1999) showed that human OA chondrocytes in four day monolayer cultures express mRNAs of the metalloproteinase enzymes (MMPs and ADAMs). Hughes et al (1997) monitored aggrecanase activity using a recombinant substrate in agarose culture for a period of 120 hours. Cleavage at the Glu^{373}-Ala^{374} inter-globular domain site was confirmed using Western blots with the BC-3 monoclonal antibody. In the system used by Hughes et al, aggrecanase activity was only detected in retinoic acid stimulated cultures, demonstrating that aggrecanase activity is minimal in the normal chondrocyte environment. Aggrecanase-mediated degradation of aggrecan can typically be seen when chondrocyte cultures are stimulated by cytokines such as IL-1 (Sandy et al 2000). In the present study, it was difficult to test whether aggrecanase or MMPs cleavages of aggrecan existed in our system, as only around 2 to 4 μg of GAG per culture dish could be collected during a period of 24 hours. In order to confirm aggrecanase and MMP activity, at least 10 μg of GAG-containing aggrecan catabolites (representing approximately 1 μg of protein) are needed to for western blotting with BC-3 and BC-4 monoclonal antibodies (Hughes et al 1995).

There appear to be a decrease in 2-B-6 positive the high molecular mass bands in stimulated samples from normal chondrocytes, with a specific band observed around 375 kDa. These molecules had not been degraded in the inter-globular domain, as aggrecanase-generated, or MMP-generated, bands will have lower molecular mass (40 to 80 kDa) (Little et al 1999). Judging by the work described by Tortorella et al (2001), this fragment must be C-terminally truncated. It can be identified by specific antibodies corresponding with this protein. The results suggest that cyclical mechanical stimulation affects C4S disaccharide sulphation isomers on the GAGs of the newly synthesized PGs of normal chondrocytes *in vitro*. Vilim et al (1993) showed that the signal given by 2-B-6 was very weak, or not present at all, indicating a very low presence of C4S in human aggrecan isolated from associative extracts from normal human femoral head articular cartilage. It appears that mechanical stimulation increases the number of 2-B-6 positive fragments, but not of 3-B-3 or 1-B-5 positive fragments. However, the 2-B-6 positive fragments may relate to increased turnover as the result of mechanical stimulation. As the signal given by 2-B-6 was weak, 2 μg per lane of GAG was loaded for 2-B-6 blotting, as oppose to 1 μg of GAG for 3-B-3, and the amount of GAG in medium from mechanically stimulated samples was twice that of the unstimulated samples. Saamanen et al (1989) showed that 15 weeks of running exercise increased the level of PG in the cartilage in the weight bearing site, particularly increasing the amount of C6S as compared with C4S levels in the articular cartilage of the knees of young beagle dogs. In human cartilage, the superficial zone is rich in C6S and this increases with age (Mourao et al 1988, Roughley et al 1981), while C4S dominates in the deep zone (Mourao et al 1988). This may indicate that increased C6S is result of cartilage maturation and changes in the level of C4S may be due to increased matrix turnover.

Aggrecan populations in OA samples lacked the large molecular weight bands on electrophoresis. Although was variable in OA grade II, when microscopic grading of OA was higher (advanced OA) the 350 kDa band was constantly absent. Notably, in OA grade III, no 350 kDa bands could be seen and this was not modulated by mechanical stimulation or by the presence of anti-integrin antibodies. Interestingly, blocking α5 integrins increased the intensity of the G1 domain containing a large molecular weight band with molecular weight of 350 kDa, following mechanical stimulation in OA grade I and II (Figure 5.20, 5.21, see arrows). Mechanical stimulation did not increase GAG synthesis in OA chondrocytes in the presence of the anti-α5 integrin antibody (Chapter Four) but it may influence aggrecan species produced by OA chondrocytes. Aggrecan is not itself a ligand for integrins, but fibronectin is known to interact with aggrecan. The aggrecan-binding domains of fibronectin are localized in the third region of the molecule and in the adjoining type III repeats. α5β1 integrin binds to a different region of the fibronectin molecule, the first cell-binding RGD containing domain (Condic et al 1999). This implies that the precise combinations or concentrations of aggrecan and fibronectin molecules, present in the extra-cellular matrix, may modulate cellular activity via integrins. It might be speculated that the mechano-transduction pathways in OA chondrocytes are changed from those operating in normal chondrocytes, and by blocking α5 integrin, downstream signalling events may be significantly altered. There may be a role for α5 integrin in aggrecan synthesis, following mechanical stimulation in early stage of OA chondrocytes.

The concentration of high molecular mass chondroitin-6, -4, -0 sulphate positive epitopes decreased in OA grade III chondrocytes, compared to normal ones, in this study. No significant change in the expression of chondroitin sulphate epitopes were seen in OA chondrocytes exposed to cyclical mechanical stimulation after a 24 hour culture period. This means that cyclical mechanical stimulation did not alter the response of OA chondrocytes. This absence of response to mechanical stimulation was one of the most important and characteristic phenotypic changes found in this study.

Increased level of PGs bearing 3-B-3 (-) and 7-D-4 epitopes in the articular cartilage matrix has been observed with the onset of OA (Caterson et al 1990, Carney et al 1992, Slater et al 1995, Carlson et al 1995). The increase in PGs reacting with 3-B-3 (-) and 7-D-4 antibodies is associated with a hyper-metabolic response of chondrocytes, and has been used as a marker of early OA change (Caterson et al 1990, Carney et al 1992, Slater et al 1995, Carlson et al 1995). However, no such elevated levels of PGs bearing 3-B-3 (+) epitopes were seen in this study in OA grade I chondrocytes (early stage of OA). Increased 3-B-3 (-) level have also been induced in normal bovine cartilage that was cyclically loaded in vitro, suggesting mechanical regulation of synthesis of this epitope (Ostendorf et al 1994). There were no significant increase in synthesis of 3-B-3 (+) epitope in normal, OA or RA chondrocytes following mechanical stimulation in this study. While 3-B-3(-) and 7-D-4 epitopes were evident in the

superficial cells of articular cartilage, there was no difference between joint regions and exercise groups in the level of these markers (Little et al 1997).

This is the first study in which chondrocyte expression of G1-containing aggrecan and CS epitopes have been assessed by Western blotting, using antibodies to aggrecan G1 domain and CS epitopes in RA chondrocytes, following mechanical stimulation. Although this cannot determine intrinsic cellular differences due to local variations in matrix composition and mechanical properties, the results here suggest that RA chondrocytes synthesize aggrecan species differently from normal or OA chondrocytes, and do not show any response to mechanical stimulation.

5.4 Summary

A summary of the Western blot experiments is shown in Table 5.2.

		G1-aggrecan		C6S		C4S		C0S	
		large	small	large	small	large	small	large	small
Normal	NS	+	+	+		+	+	+	
	MS	++*	++*	+		++	+	+	
OA I	NS	+	+	+		+	+	+	
	MS	+	+	+		+	+	+	
OA II	NS	+/±	+/±	+		+	+	+	
	MS	+/±	+/±	+		+	++	+	
OA III	NS	±	++	±	+	±	±	±	
	MS	±	++	±	+	±	±	±	
RA	NS	+	+	+		±	+		
	MS	+	+	+		±	+		

+ expressed

++ strong expression

± weak expression

NS not stimulated

MS mechanically stimulated

* when the same amount of medium were blotted per lane

Table 5.2 A summary of the Western blot experiments

The results clearly showed that increased GAG synthesis in normal chondrocytes is related to increases in aggrecan species. Mechanically-stimulated monolayer-cultured chondrocytes derived from normal human articular cartilage. showed stronger expressions of large and small G1-containing aggrecan species when blotted with the same amount of medium per lane. Electrophoretic patterns of PGs released from normal chondrocytes showed mainly un-degraded intact aggrecan species when the same amount of GAGs were blotted, whereas samples from chondrocytes derived from OA cartilage exhibited an inability to synthesize intact aggrecan species, especially in advanced OA (grade III). Cultured chondrocytes derived from RA cartilage synthesized un-degraded intact aggrecan species.

Expression of aggrecan species and C4S containing epitopes was increased in normal chondrocytes following mechanical stimulation. In the presence of anti-α5 integrin antibody, mechanical stimulation in early stage OA chondrocytes increased intact aggrecan species. There were no differences in the synthesis of aggrecan peptides between mechanically stimulated and unstimulated controls in advanced OA or RA chondrocytes. There were, however, differences in anabolic activity between normal, OA, and RA chondrocytes. Mechanical stimulation alters some anabolic events in normal and early OA chondrocytes, but not in advanced OA or in RA chondrocytes.

Chapter Six

The Effects of Mechanical Stimulation on Proteoglycan Synthesis in Human Articular Chondrocytes in Three-Dimensional Culture

Chondrocytes maintained in monolayer culture differ from chondrocytes in cartilage *in vivo* in cell morphology, cell-to-cell contact, and their matrix environment. The monolayer culture system provides a predominantly two-dimensional culture milieu which is useful for studies of cell stretching. Three-dimensional culture systems in agarose and alginate gel reduce the effects of chondrocyte isolation on phenotype by suspending the cells in an artificial matrix in which their shape is maintained, and in which they do not form abnormal cell-substrate attachments. Moreover, three-dimensional culture facilitates compression studies. This is theoretically preferable to monolayer culture as three-dimensional culture simulates the *in vivo* situation more closely. This study was undertaken to assess whether chondrocyte responses to mechanical stimulation were similar in two-dimensional and three-dimensional cultures.

6.1 Development of protocols for alginate three-dimensional culture

It is important to develop model systems of chondrocyte culture in which mechanical loads can be applied. Alginate 'beads' are generally used for three-dimensional culture as they are technically easy to make and relatively uniform in size. However, recently methods have been developed for making alginate-chondrocyte cylindrical constructs (alginate disks) which are reported to be more suitable for loading study (Wong et al 2001). Preliminary experiments were, therefore, performed to assess the mechanical properties of alginate beads and disks.

6.1.1 Assessment of alginate beads

6.1.1.1 Aim

The aim of this section in this study was to assess the source of the properties of alginate beads.

6.1.1.2 Materials and methods

OA cartilage (sample day book number 14101, 74 year old female) with mixed grading was used. Isolated cells were mixed with the two per cent alginate solution at a cell concentration of $23x10^6$cells/ml alginate. The alginate cell mixture was dropped into a 102 mM $CaCl_2$ solution through a 21 gauge needle to give a final cell density of $25x10^4$cells per bead. After 10 minutes incubation in $CaCl_2$ solution, beads were washed once with PBS, and then maintained in culture medium (IMDM with 10 per cent FCS containing the antibiotic-antimycotic solution). The cells were maintained in a humidified atmosphere of five per cent CO_2 at 37°C with daily changes of medium. Alginate-chondrocyte constructs in beads was tested in a time course of one, two, three and seven days for production of DNA and GAG. The chondrocyte-alginate gels were disrupted and digested by addition of an alginate digestion buffer containing papain (Appendix). The samples were incubated at 60°C for 24 hours. 40 µl of papain-digested sample was added to 250 µl of DMMB solution and the absorbance was determined immediately at 595 nm (Enobakhare et al 1996). Corrections for the alginate background at various dilutions were made as alginate gel alone was used for constructing a standard curve (following personal communication with Dr Ameye, Lausanne). The total content of DNA was quantified using the bisbenzimide fluorescent dye (Hoechst 33258).

6.1.1.3 Results

The mass of an alginate bead from a drop of a 21 gauge needle was on average 0.0107 ± 0.0011 g. Total DNA content increased from 1.85 ± 0.58 µg per bead to 2.28 ± 0.57 µg per bead after seven days (Figure 6.1). Total GAG content within the alginate beads increased from 3.74 ± 1.24 µg per bead to 5.18 ± 0.82 µg per bead (Figure 6.2). GAG content (µg) per DNA (µg) increased from 2.17 ± 0.81 µg per bead to 2.38 ± 0.96 µg per bead. None of the differences here were statistically significant.

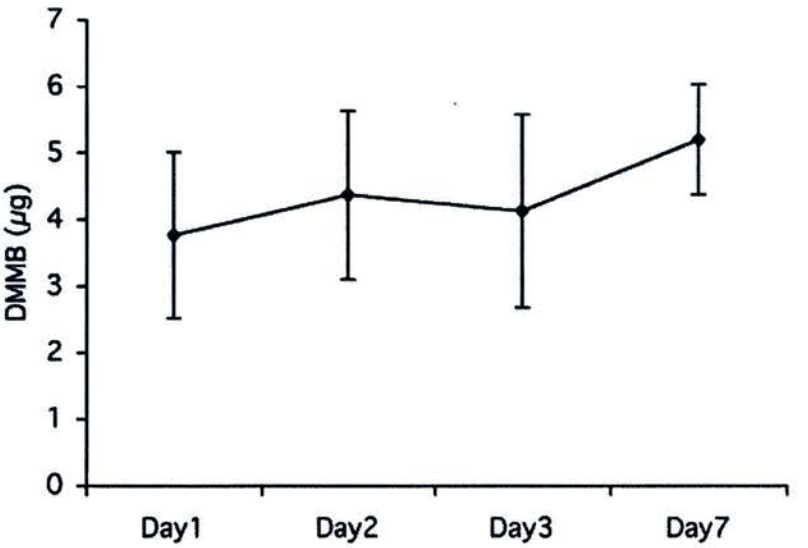


Figure 6.1 Changes of GAG content in alginate beads

One 11 mm^3 of alginate bead contained $25x10^4$ cells. Results expressed as mean ± SD (n=6/time point).

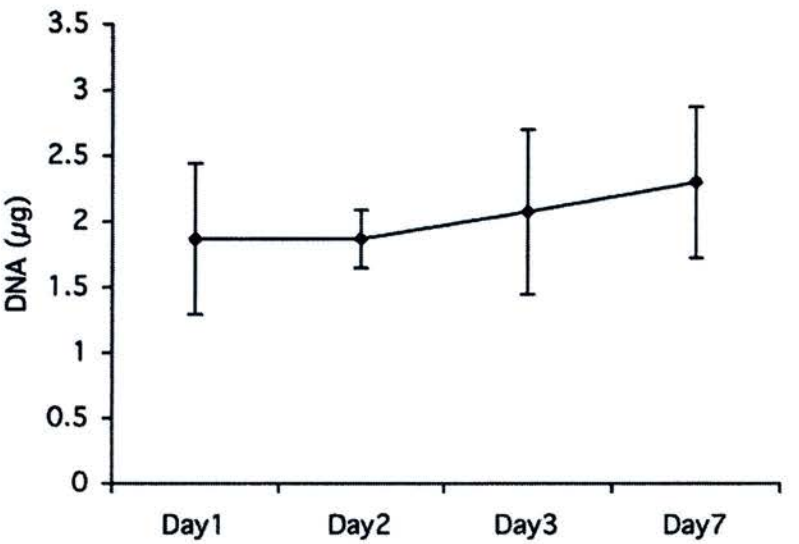


Figure 6.2 Changes in DNA content in alginate beads

Total GAG content was measured as chondroitin sulphate equivalent by the DMMB reactivity method. Results expressed as mean ± SD (n=6/time point).

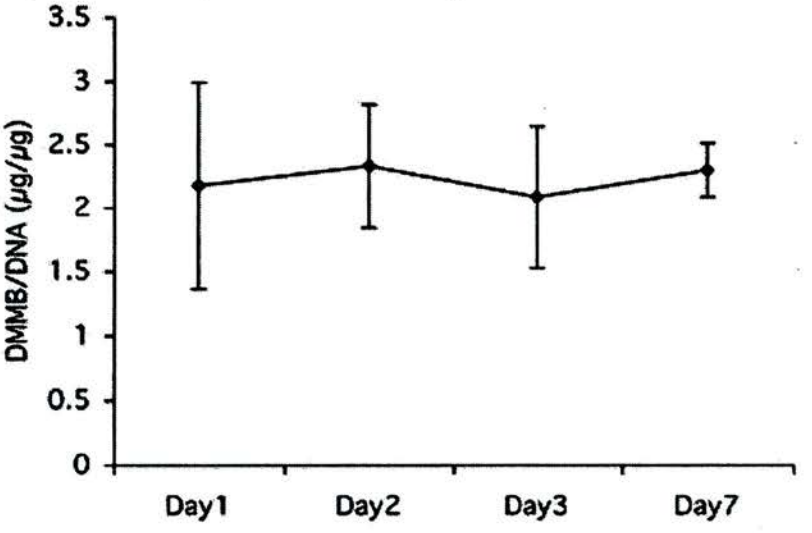


Figure 6.3 Changes in GAG content (μg) per DNA (μg) in alginate beads

Results expressed as mean ± SD (n=6/time point).

6.1.1.4 Discussion

Total DNA and GAG content per bead did not increase significantly from one to seven days. Liu et al (1998) showed that DNA content measured using the bisbenzimidazole fluorescent dye Hoechst 33258 showed no significant increase in the first 10 days of culture. However, Liu et al (1998) showed that GAG content measured with DMMB increased steadily during the first 10 days of culture. It has also been reported that GAG content (μg) measured by the DMMB assay per DNA (μg) increased during seven days of alginate culture in bovine chondrocytes (Ragan et al 2000). The difference may be explained by the fact that chondrocytes used in this study were from aged, OA cartilage.

Three-dimensional culture techniques are used in different fields of biomedical science. Alginate culture can maintain chondrocytes in a spherical conformation,and more differentiated phenotype with expression of cartilage specific proteins (Aydelotte et al 1998). Alginate beads were used firstly for the study of cancer cells (Kupchik et al 1983) and later for studying embryonal sternal chicken chondrocytes (Guo et al 1989). Alginate is a negatively charged un-sulphated co-polymer of L guluronan and D-mannuronan, which polymerises to form a gel in the presence of calcium ions. Chondrocytes can be released easily from the alginate gels by the addition of calcium chelator (Gacesa et al 1998). The spherical morphology and cell viability of chondrocytes were maintained for a long time in alginate

culture (Ragan et al 2000). When cells were encapsulated inside alginate beads, they were evenly dispersed within the semi-solid beads and rapidly exhibited the typical spherical morphology of chondrocytes (Hauselmann 1992). As chondrocytes maintain spherical morphology and differentiated phenotypes in an alginate culture system, this is therefore more likely to produce biologically relevant results than a monolayer culture.

6.1.2 Studies of mechanical compression of alginate beads

6.1.2.1 Aim

These studies here were undertaken to assess the effects of cyclic mechanical compression of the structural integrity of alginate beads.

6.1.2.2 Materials and methods

OA cartilage (day book number DS338C, 73 years old female) with mixed grading was used. Isolated cells were mixed with the two per cent alginate solution at the cell concentration of $23x10^6$cells/ml alginate. The alginate cell mixture was dropped into a 102 mM $CaCl_2$ solution through a 21 gauge needle to give a final cell density $25x10^4$cells/bead. After 10 minutes incubation, beads were washed once with PBS, and then maintained in culture medium (IMDM with 10 per cent FCS containing the antibiotic-antimycotic solution). The cells were maintained in a humidified atmosphere of five per cent CO_2 at 37°C with daily changes of medium. On the third culture day, the culture medium was replaced by serum–free medium one hour prior to testing the mechanical properties of the alginate-chondrocyte beads. A set of three beads was placed in the bottom of a 12-well-plate and cyclically (0.33 Hz) compressed by 10 per cent, 30 per cent, and 50 per cent of the original thickness for 20 minutes in a specially designed apparatus (Figure 2.10). After compression, the beads were left for 24 hours at 37°C. The height of the beads, the GAG content of the beads, and the amount of GAG released into the culture medium were measured. The height of the beads was measured using a 10 times scale magnifier (PEAK®, Japan) (Figure 6.4).

Figure 6.4 PEAK® 10 times scale magnifier

6.1.2.3 Results

The compression testing apparatus did not register the original height (3.0 mm) of the beads, and the material thickness was set at a zero point with the beads marginally compressed. The beads were experimentally compressed by 10 per cent, 30 per cent and 50 per cent in height from the set zero point. The heights of the beads after 5 cycles were 2.5 ± 0.2 mm (10 per cent), 1.5 ± 0.4 mm (30 per cent) and 1 ± 0.2 mm (50 per cent). There was some evidence of split lines inside beads after 50 per cent compression (Figure 6.5) and these beads did not return to their original height after 24 hours incubation.

GAG, synthesized by chondrocytes in the beads after 10, 30, and 50 per cent cyclical mechanical compression, was retained within the beads and the amount of GAG released into the medium did not change significantly after the compression (Figure 6.6).

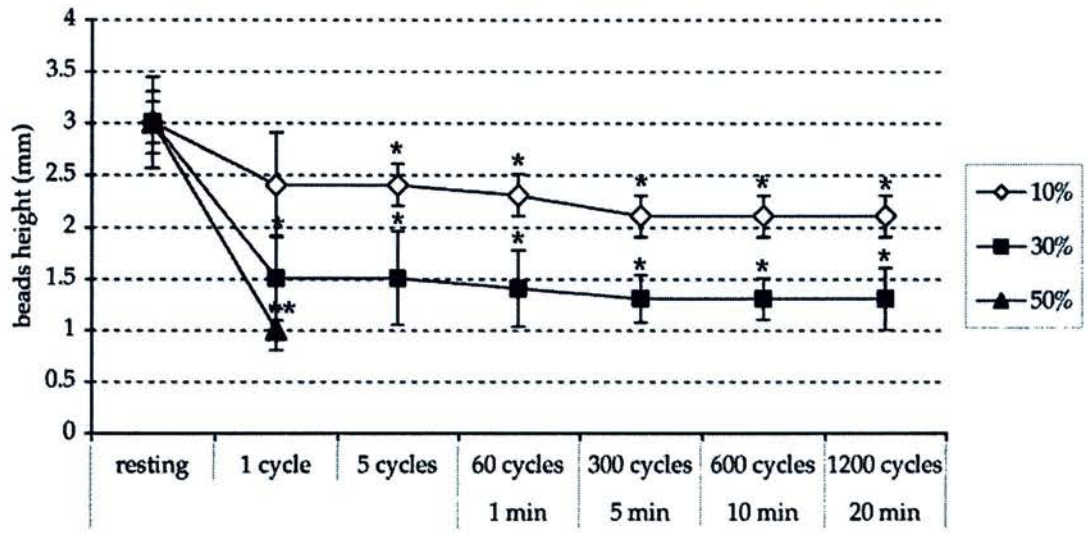


Figure 6.5 Measurement of height in alginate beads following 0.33 Hz mechanical cyclical compression

Cyclical compression of 10 per cent, 30 per cent, and 50 per cent height of the beads (originally 3 mm in height) showed a rapid decrease in the height without returning to original levels within 5 cycle compression. The height was measured using 10 time scale magnifier (PEAK®, Japan) (n=3/time point, mean ± SD) (*$p<0.05$, **$p<0.01$).

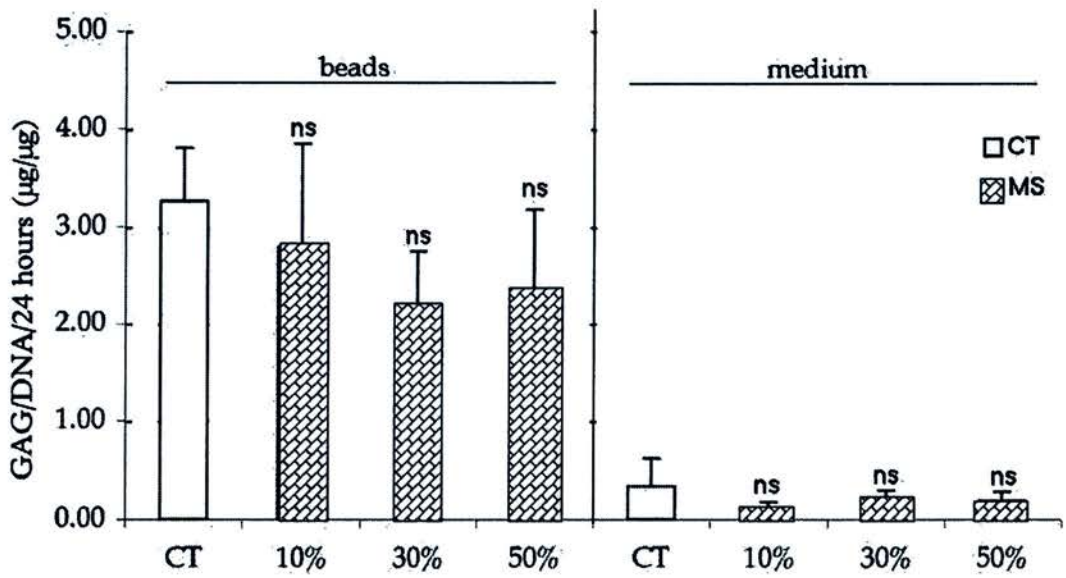


Figure 6.6 Measurement of GAGs in alginate beads and culture medium following mechanical cyclical compression

GAG, synthesized by chondrocytes after 10, 30, and 50 per cent cyclical (0.33 Hz) mechanical compression, was retained within the beads, and the amount of GAG released into the medium did not change significantly after the compression (n=3/stimulation, mean ± SD).

ns: not significant

6.1.2.4 Discussion

The results of these studies showed that the alginate beads decreased in height immediately after one compression stroke. Cyclical compression of 50 per cent broke the three-dimensional structure. The zero point at the top of the beads (around 3 mm in height) in the custom designed compression apparatus was problematic, as the apparatus registered a certain resistance force. Judging by the results obtained, the apparatus registered the zero point only after approximately 20 per cent compression of the beads. This made accurate measurement of the mechanical properties of bead formation technically difficult. There was little further change in the height of the beads after five cycles of compression, indicating the beads become irreversibly flattened by the compression. Measurement of GAGs in the alginate beads and culture medium following cyclical compression showed that newly synthesized GAGs were retained within the alginate and that the amount of GAG released into the medium did not change significantly following compression. Liu et al (1998) previously showed that over 95 per cent of Sulphate labelled PGs were retained within alginate beads during long periods of culture. The majority of matrix molecules become incorporated and assembled into the extra-cellular matrix in the alginate culture system whereas most matrix molecules are released into the culture medium in monolayer cultures (Kamada et al 2002).

These studies looked at the usefulness of chondrocyte culture in three-dimensional alginate beads for studies of mechanical stimulation. The results showed that with the mechanical compression system available, alginate bead cultures were not very suitable for studying the effects of cyclical compression on chondrocytes.

6.1.3 Studies of mechanical compression of alginate disks

6.1.3.1 Aim

In the last five years, there have been a number of publications reporting the use of three-dimensional cultures to examining molecular aspects of matrix formation following mechanical loading (Wong et al 2001). Studies were therefore undertaken to assess how well alginate disks will survive cyclic mechanical compression.

6.1.3.2 Materials and Methods

Two per cent alginate was injected into an ethanol-sterilised, diffusion-permeable, custom-designed casting frame. Disks (10 mm diameter, 2 mm height) were punched out from gelled alginate (Chapter Two). Each disk was transferred to a single well of a 12-well plate containing

1 ml medium. On the third culture day they were cyclically (0.33 Hz) compressed to 30 per cent of the original thickness in time courses up to 20 minutes in the specially designed compression testing apparatus (Figure 2.10). The height of the beads was measured using a 10 times scale magnifier (PEAK®, Japan) (Figure 6.4).

6.1.3.3 Results

The height of the alginate disks started to decrease significantly from an original height of 2.5 ± 0.2 mm to 2.0 ± 0.1 mm following five minutes, 30 per cent, at 0.33 Hz cyclical compression. The disk height decreased by 30 per cent after 10 minutes and 200 cycles of compression, and did not show any further change up to 20 minutes compression (Figure 6.7) (data from Dr K. E. Elliot and B. Halliday, Edinburgh University).

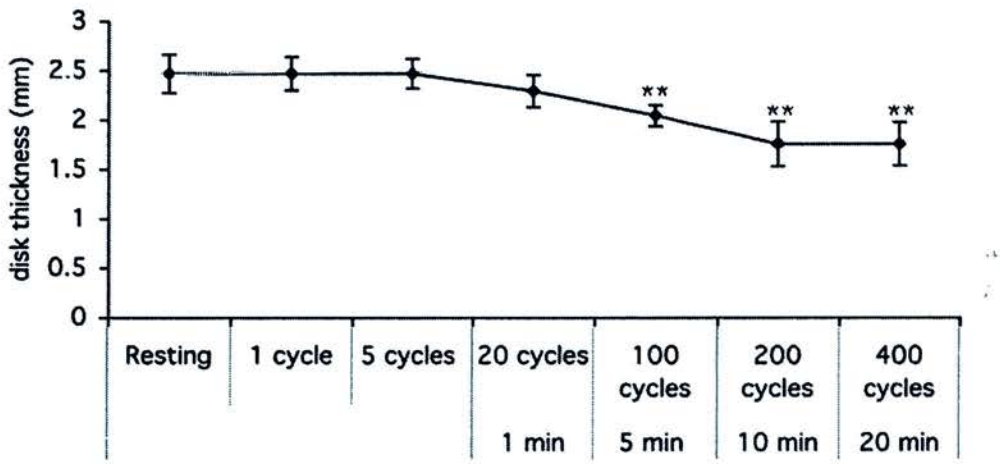


Figure 6.7 Measurement of height of alginate disks following 0.33 Hz mechanical cyclical compression

Cyclical compression of 30 per cent at 0.33Hz of the disks (originally 2.5 mm in height) started to show a significant decrease in height after 5 minutes compression. The height was measured using a 10 times scale magnifier (PEAK®, Japan) (data from Dr K. E. Elliot and B. Halliday) (**$p<0.01$). Results expressed as mean ± SD (n=8/time point).

6.1.3.4 Discussion

The mechanical properties of alginate disks (a cylinder 1 cm in diameter, 0.25 cm in height) were studied under compression in the three-dimensional compression apparatus (Figure 2.10). A set of two disks was placed in the bottom of a 12-well-plate with 1 ml of medium, and cyclically (0.33 Hz) compressed to 30 per cent of the original thickness for 20 minutes. The alginate disks started decreasing in height significantly following five minute of 30 per

cent cyclical compression at 0.33Hz. Then the disk height decreased by 30 per cent at 10 minutes, and did not show any further change up to 20 minutes compression. No split lines inside the disks were observed in the 20 minute time course (unpublished observation by Dr K J Elliot and B Halliday, Edinburgh University) (Figure 6.7).

In this study, a modified custom-designed casting frame was made based on the system, described by Wong et al (2001) as explained in the materials and methods (Chapter Two). Wong et al (2001) developed alginate-chondrocyte cylindrical constructs which were able to support large strains for a long period of time prior to failure. Their system is stable for a long term as alginate disks were subjected to non-cyclical compression tests. No study has been performed for testing alginate disks with cyclical mechanical properties.

Mechanical properties of chondrocyte culture in three per cent agarose gels have been well documented by Lee et al (1998). Static compression of their model system resulted in measurable deformation of cells within the gel, which had a tangent modulus of 10 per cent strain at approximately 110 kPa. In addition, cyclical compression of agarose-chondrocyte constructs have been reported by Lee et al (1997) and Toyoda et al (2003) (Table 1.2). However, the use of the alginate gel system is clearly preferable because alginate is much easier to remove than agarose and this makes it possible to distinguish the molecules that becomes resident in different matrix regions (e.g cell-associated matrix and further-removed matrix) (Kamada et al 2002).

6.2 The effects of cyclic compression on GAG synthesis in human articular chondrocytes derived from cartilage of patients with OA

6.2.1 Aim

The environment of chondrocytes in monolayer is different from that in a three-dimensional culture system. The aim of this section of this study was to assess whether responses of chondrocytes were similar in two-dimensional and three-dimensional culture. This study also explores whether mechanical stimulation influences chondrocyte function in three-dimensional culture.

6.2.2 Materials and methods

Because of normal and RA samples were not available, only OA samples were studied in alginate culture. Chondrocytes were isolated from OA articular cartilage of two males (aged 58

and 69) and eight females with a mean age of 68.8 years (range 59-77) (Appendix). Isolated cells were mixed with a 2 per cent alginate solution at a density of 2 x 10^6cells per ml alginate to give a final cell density of 30 x 10^4 cells per disk (0.157 ml alginate per disk). Alginate-cell mixture was injected into an ethanol-sterilised, diffusion-permeable, custom-designed casting frame. Disks (10 mm diameter, 2 mm height) were punched out from gelled alginate (Chapter Two). Each disk was transferred to a single well of a 12-well plate containing 2 ml medium. On the third culture day, medium with 10 per cent FCS was replaced with serum–free medium, one hour prior to testing the mechanical properties of the alginate-chondrocyte disks. They were placed in the bottom of a 12-well-plate and cyclically (0.33 Hz, 0.1 Hz and 1 Hz) compressed to 30 per cent of the original thickness for 20 minutes in a specially designed compression testing apparatus (Figure 2.10). After compression, the disks were incubated for 24 hours at 37°C. The amount of GAG and DNA in disks was analysed.

6.2.3 Results

Alginate-OA chondrocyte disks, exposed to cyclic compression of 0.1 Hz and 0.3 Hz to 30 per cent of the original thickness for 20 minutes showed a significant decrease in GAG synthesis (*p = 0.024 and **p = 0.019) compared to paired unstimulated controls after 24-hour incubation at 37°C,(Figure 6.8). Disks subjected to 1 Hz cyclic compression showed only a non-significant decrease of 25.6 per cent in GAG synthesis compared to paired controls (Figure 6.8).

6.2.4 Discussion

30 per cent cyclic compression at 0.1 Hz and 0.33 Hz decreased GAG synthesis significantly compared to unstimulated controls, while the decrease was not statistically significant at 1 Hz. The results presented here suggest that cyclic mechanical compression has profound effects on the synthesis of GAG by articular chondrocytes derived from OA cartilage in three dimensional alginate culture.

Using confocal microscopy, 20 per cent compression of 2 per cent alginate-chondrocyte constructs is known to deform cells and nuclei (Knight et al 2002). It is likely that in this study, 30 per cent compression resulted in deformation of chondrocytes and nuclei in 2 per cent alginate disks.

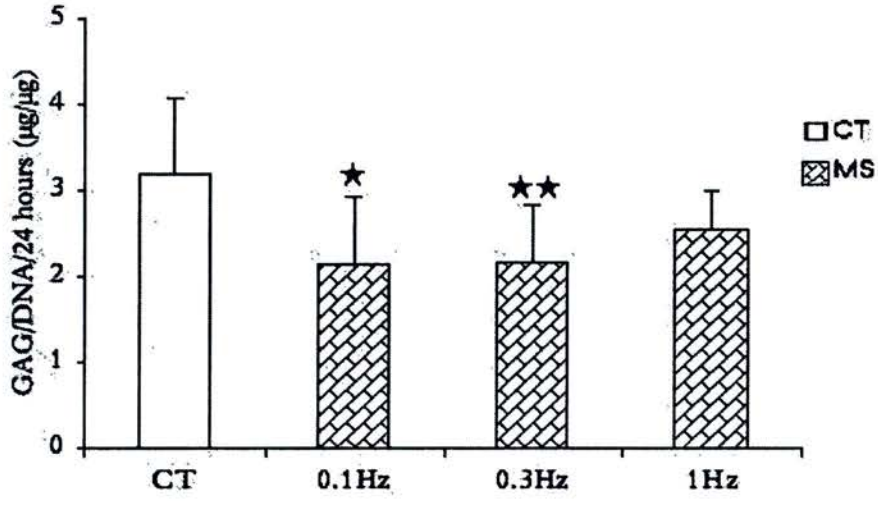


Figure 6.8 The effects of cyclic compression on GAG synthesis in alginate OA chondrocyte disks.

Three-dimensional alginate-cell disks exposed to cyclic compression of 0.1 Hz, 0.33 Hz and 1 Hz to 30 per cent of the original thickness for 20 minutes followed by 24 hour incubation at 37°C showed a significant decrease (*p = 0.024 vs. CT, **p = 0.019 vs. CT). There was a slight decrease of 25.6 per cent at 1 Hz in GAG synthesis compared to paired controls (p=0.086). Values are given as means ± SD (n=8).

There is a possibility that decrease in GAG synthesis induced by cyclic mechanical compression contributes to the OA chondrocyte phenotype. It has long been known that mechanical stimulation can stimulate chondrocytes to produce more PGs. Increases in the synthesis of GAG by chondrocytes may be part of a common response of all, or most, connective tissue cells to mechanical stimulation. In some three-dimensional culture model systems, chondrocytes have been reported to respond bio-synthetically to mechanical load in a manner similar to that observed in organ culture. For example, Demarteau et al (2003) have shown that mechanical loading (5 per cent deformation at 0.1 Hz frequency) increased GAG synthesis in normal human articular chondrocytes cultured in a three-dimensional bio-degradable polymer scaffold, when the GAG content prior to compression was sufficiently high. However, this is the first time that the alginate gel system has been used to examine the effects of cyclic mechanical compression on human articular chondrocytes derived from OA cartilage, and the results were not compared with results from chondrocytes derived from normal cartilage. It would certainly be of interest to undertake experiments with chondrocytes derived from normal human articular cartilage.

Cells cultured in monolayer, which have an altered organisation of their cytoskeleton compared with articular chondrocytes *in vivo*, are likely to respond differently to the application of mechanical stimulation. In this study, mechanical stimulation of monolayer-cultured OA chondrocytes did not show any change in GAG synthesis (Chapter Three), but when OA chondrocytes were embedded in alginate three-dimensional culture, chondrocyte metabolism was influenced by compressive strains. Although the nature of the mechanical stimulation applied to the monolayer-cultured chondrocytes differed from the compressive strain applied to the alginate cultures, it is reasonable that the response to compressive strain observed of chondrocytes in three dimensional alginate are similar to responses of articular chondrocytes *in vivo*.

Review of investigations by other researchers suggest that the duration of pressure and frequency of application are very important. Lee et al (1997) showed that static and 0.3 Hz compressive strain inhibited GAG synthesis, while 1 Hz stimulated synthesis in bovine chondrocytes embedded in agarose. Cyclic strain at 0.33 Hz and 3 Hz but not at 1Hz resulted in reduction in GAG synthesis in superficial cells of bovine articular cartilage while dynamic strain at 1 Hz induced highly significant increases in GAG synthesis in deep cells in agarose culture (Lee et al 1998). However, using three-dimensional agarose culture, 5 MPa static pressure for 4 hours resulted in a significant increase in GAG synthesis in bovine articular chondrocytes (Toyoda et al 2003). 2.8 MPa constant and cyclic (0.015 Hz) hydrostatic fluid pressure enhanced matrix synthesis (C4S containing PG and Keratan sulphate) in bovine chondrocytes in porous three-dimensional sponges without inducing cellular deformation (Mizuno et al 2002). Overall, increasing levels of static compression decreased biosynthesis in a dose-dependent manner (Ragan et al 2000, Lee et al 1997, Buschmann et al 1995), while dynamic compression stimulated biosynthetic activity (Elder et al 2000, 2001, Mauck et al 2000, Buschmann et al 1995). This was similar to the results obtained from cartilage explants (Sah et al 1989).

It is important to note that the application of compressive strain to alginate constructs for 10 to 20 minutes at 30 per cent compression resulted in a 20 per cent decrease in the height of the alginate disks (Figure 6.7) which did not recover even after 24 hours incubation in free-swelling conditions. This means that 10 per cent static compression and 10 per cent dynamic compression were applied to the three-dimensional disks. Cells are spherical after isolation from cartilage and remain so in culture. Compressive strain applied to alginate-cell constructs must induce cellular deformation. Following 10 minute compression, cellular deformation resist for as long as 24 hours. However, mean deformation indices of cells in un-strained and strained constructs following 20 minute compression and during 24-hour culture periods were not measured (Lee et al 1998). They reported that when deformation was measured after 24 and 72 hours in culture, deformation at the later time point was significantly reduced when compared with earlier one, due to the accumulation of extra-cellular matrix surrounding the cells. In this study, cyclic compression was applied after 72 hours in culture, so accumulated

extra-cellular matrix surrounding the cells should also be considered. Compressive strains applied to alginate-cell constructs and articular cartilage are however totally different, as demonstrated by Wang et al (2003). The strain distribution inside chondrocytes seeded in 2 per cent alginate disks is linear throughout, whereas articular cartilage explants under the same loading conditions shows non-linear displacement. The solid matrix of articular cartilage is incompressible when subjected to hydrostatic pressure up to 12 MPa (Bachrach et al 1998).

PGs synthesized by chondrocytes in alginate culture are found in two compartments: a cell-associated matrix and a further-removed matrix (Hauselmann et al 1992). The use of alginate cultures makes it possible to examine molecules that have become resident in different matrix regions. Mok et al (1994) suggested that aggrecan molecules which reside in the cell-associated matrix compartment have a much faster rate of turnover than those in the further-removed matrix areas. Moreover, catabolism of the newly synthesized aggrecan molecules occurs in the cell-associated matrix. Further study of the regulation of PG synthesis by mechanical forces will be greatly facilitated by separating matrix molecules from these compartments and analysing them separately.

6.3 Treatment of human articular chondrocytes in alginate culture with anti-integrin antibodies

6.3.1 Aim

The physiological response of chondrocytes to changes in the cartilage matrix involves signalling pathways in response to multiple stimuli including mechanical stimulation. Activation following mechanical stimulation is likely to be recognized by cell surface integrins which serve to modulate cellular responses. A series of experiments have been undertaken here to establish whether the β1 and and αVβ5 integrins were involved in the responses that lead to decreased GAG synthesis following cyclical mechanical stimulation and 30 per cent compression of the alginate-chondrocyte constructs.

6.3.2 Materials and methods

The amount of newly synthesized GAG was assayed in the presence or absence of monoclonal integrin function blocking antibodies raised against human integrin subunits. Aliquots of stock solutions containing 1 mg per ml mouse IgG, αVβ5 integrin antibody (P1F6 MAB1961 Chemicon International, England), or β1 integrin antibody (JB1A MAB1965 Chemicon

international, England) were added to the medium to give a final concentrations of 1.0 μg per ml, one hour prior to mechanical compression. Human articular chondrocytes derived from patients with OA were then exposed to cyclical pressurization (0.1 Hz, 0.33 Hz and 1 Hz to 30 per cent of the original thickness for 20 minutes at 37 °C). Following pressurization, the alginate-cell construct disks were incubated for 24 hours at 37 °C, and the amount of newly synthesized GAG was determined by DMMB assay and DNA was quantified using the bisbenzimide fluorescent dye (Hoechst 33258).

6.3.3 Results

There were significant decreases in GAG synthesis following 30 per cent compression of 0.1 Hz and 0.33 Hz in control samples in the presence of mouse IgG (mean ± SD: 1.94 ± 0.63 μg GAG/μg DNA at 0.1 Hz, #p = 0.041, 2.15 ± 0.56 μg GAG/μg DNA at 0.33 Hz, ##p = 0.034 one tail/0.068 two tail) compared with non-stimulated controls: 2.92 ± 0.54. In the presence of antibodies to αVβ5 integrin and β1 integrin, this decreased GAG synthesis response was blocked completely (Figure 6.9). Incubation with mouse IgG and antibodies to αVβ5 integrin and β1 without mechanical stimulation had no effect (mean ± SD: 2.60 ± 0.23, 2.27 ± 0.33 μg GAG/μg DNA) indicating that these antibodies alone do not affect GAG production in chondrocytes.

6.3.4 Discussion

The results presented in this study showed that antibodies to αVβ5 and β1 integrins are able to block the events that lead to decreased GAG synthesis following cyclic compression of alginate-chondrocyte culture. Scully et al (2001) have shown that blocking the β1 integrin subunit with anti-β1 antibodies (4B4) reduces PG synthesis in bovine chondrocytes in alginate bead culture. They suggested that type II collagen and β1 integrin interaction modulates the effect of TGF-β on chondrocytes. Lee et al showed that the increased PG synthesis, which is stimulated by TGF-β, can be blocked by anti-β1 antibody (4B4) on day four in alginate bead culture (Lee et al 2002). Attachment of chondrocytes to extra-cellular matrix is a necessary prerequisite for cells to respond to external changes and so to maintain the composition and mechanical properties of articular cartilage. They receive information from the external environment through receptors. The integrins, particularly members of β1 subfamily, appear to be the primary receptors for the attachment of chondrocytes to the extra-cellular matrix (Durr et al 1993, Enomoto-Iwamoto et al 1997, Woods et al 1994). In this study, antibodies to β1 and αVβ5 integrins had little effects on GAG synthesis. However, the decrease in GAG synthesis following mechanical stimulation (cyclic compression strain of 0.1 Hz and 0.33 Hz), a decrease in GAG synthesis was blocked. Chondrocytes from OA articular cartilage were

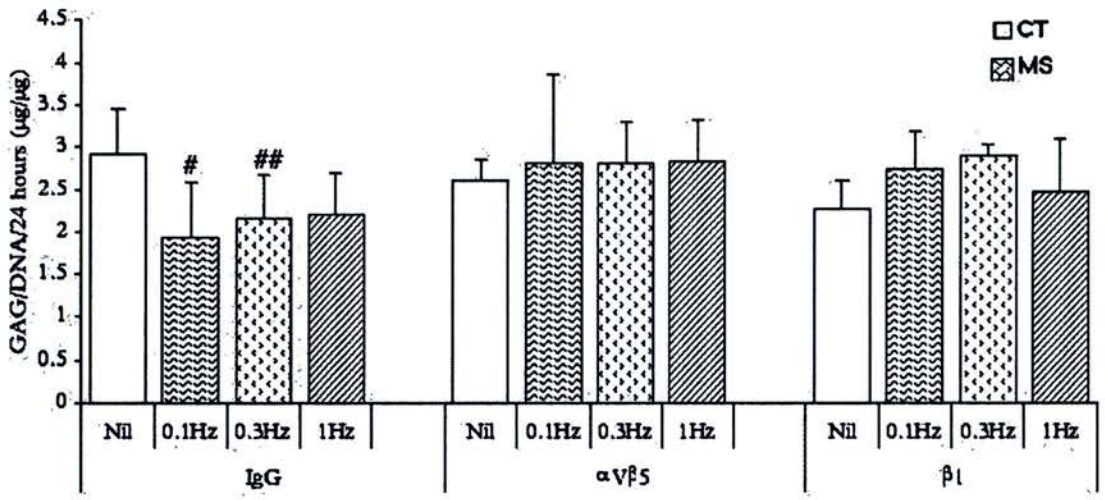


Figure 6.9 The effects of anti-integrin antibodies on GAG synthesis in response to cyclic compression

Cyclic mechanical compression of 0.1 Hz and 0.33 Hz significantly decreased GAG synthesis in alginate-chondrocyte disks in the presence of control mouse IgG (#p = 0.041 vs. IgG, ##p = 0.034 vs. IgG one tail 0.068 two tail). This decrease was blocked by adding anti-αVβ5 integrin antibody P1F6 and anti β1 integrin antibody JB1A. Values are given as means ± SD (n=4).

αVβ5: anti-αVβ5 integrin antibody P1F6
β1: anti-β1 integrin antibody JB1A

isolated and embedded in alginate. In this way, chondrocytes were maintained in milieu which resembles the *in vivo* environment more closely than a monolayer cultures in in extra-cellular matrix composition, mechanical properties, and overall tissue integrity. This may continue to understanding the mechano-transduction pathways leading to changes in PG synthesis following cyclic mechanical stimulation.

The anti-β1 integrin antibody JB1A (MAB1965) is a monoclonal Ig isotype IgG_1 subclass antibody that recognises residues 82-87 of the mature β1 integrin chain. This restricted region of the β 1 integrin serves as the target for regulatory antibodies which inhibit integrin function. JB1A binding to purified peptide was also inhibited by Mn^{2+}. This is due to interference with antibody function rather than to a cation-dependent change in the epitope (Ni and Wilkins 1998). Thus JB1A is a function blocking antibody, which inhibits cell adhesion to collagen and fibronectin or laminin.

Interestingly, bovine chondrocytes in alginate synthesized significantly less collagen when cultured in the presence of cyclic RGD peptide, but there were no effects on PG synthesis (Beekman et al 1997). Covalent bonding of RGD peptide enables the attachment of chondrocytes to alginate. Integrins are likely to be involved in extra-cellular matrix synthesis in alginate culture.

Jobanputra et al (1996) showed that the culture of chondrocytes in monolayer with inflammatory synovial fluid led to an increase in expression of $\alpha 1$, $\alpha 5$ and αV integrins. These changes were diminished in alginate cultures. This implies that cell shape may be important for the expression of integrins. In particular, αV integrin expression may be more susceptible to cell shape changes as αV integrin is found to be intensively expressed in the superficial layer of cartilage where the cells are flattened. Roberts et al (2001) have reported that mechanical compression influences intra-cellular Ca^{2+} signalling in bovine articular chondrocytes seeded agarose culture. The integrin αV subunit has been shown to mediate increases in intra-cellular Ca^{2+} (Yamada and Miyamoto 1995) and there is considerable evidence linking the cytoskeleton with activation of ion channels and intracellular Ca^{2+} signalling (Janmey 1998). Mechano-transduction in chondrocytes is a calcium-dependent pathway (Wright et al. 1996). This study suggests that mechanical loading initiates a mechano-transduction pathway, which is both integrin and calcium dependent, and which eventually leads to changes in chondrocyte metabolism.

The current studies represent the first attempt to examine integrin-mediated GAG synthesis in chondrocytes derived from human articular cartilage from patients with OA following mechanical compression of chondrocytes in alginate disks. Unfortunately it was not possible to obtain normal cartilage for comparison. Clearly this would be important as the results in the previous work with monolayer cultured chondrocytes indicate that there may be significant differences in responses between normal and OA chondrocytes. Further elucidation of these regulatory mechanisms could contribute to a better understanding of the pathogenesis of diseases of articular cartilage. Better understanding of the regulation of chondrocytes metabolism following mechanical stimulation are prerequisite for studies on cartilage repair.

6.4 Summary

A three-dimensional alginate culture system has been developed to investigate the effects of mechanical stimulation of chondrocytes *in vitro*. Chondrocytes were isolated from human articular OA cartilage by enzyme digestion and seeded in two per cent alginate at a final concentration 25~30 x 10^4cells per disk or bead. Accurate measurement of mechanical

properties of bead formation was technically difficult and bead formation of alginate was found to be unsuitable for mechanical loading research. Alginate-chondrocyte disks were subjected to compressive strains of 30 per cent at 0.1 Hz, 0.33 Hz and 1 Hz for 20 minutes following 24 hours of incubation. Mechanical compressive strains of 0.1 Hz and 0.33 Hz resulted in a significant decrease of GAG synthesis from OA chondrocytes. This decrease was blocked in the presence of anti β1 integrin and α5β1 integrin antibodies. This study indicates that distinct mechano-transduction pathways may be induced by mechanical compressive strains via β1 and α5β1 integrins in OA chondrocytes.

Chapter Seven

General Discussions, Conclusions, and Future Prospects

7.1 General Discussions and Conclusions

PG metabolism in articular cartilage is, at least in part, dependent on mechanical stimulation of chondrocytes. This thesis has set out to investigate how mechanical forces regulate matrix synthesis in chondrocytes derived from patients with OA and RA, compared with chondrocytes from normal cartilage. In order to achieve this objective, methods were developed to enable the examination of the effects of cyclical mechanical stimulation on rates of GAG synthesis by chondrocytes in monolayer culture and alginate three-dimensional culture.

This study has demonstrated accelerated GAG synthesis in response to cyclical mechanical stimulation, in human articular chondrocytes derived from normal articular cartilage. The increase in GAG synthesis, measured by the DMMB assay, was around two-fold following 4000μ strain at 0.33 Hz cyclical mechanical stimulation. This is consistent with a previous study (Millward-Sadler et al 2000b) showing that human articular chondrocytes from normal cartilage responded to 4000μ strain at 0.33 Hz cyclical mechanical stimulation by increasing aggrecan mRNA. Western blotting was successfully used to analyse the nature of PGs, released into the culture medium from monolayer cultured chondrocytes following mechanical stimulation. Aggrecan core protein species were detected by using anti-aggrecan G1 domain antibody, and CS epitopes were detected by specific monoclonal antibodies. G1-containing aggrecan species, released from monolayer-cultured chondrocytes, derived from normal human articular cartilage, are increased following mechanical stimulation. Electrophoretic patterns of PGs showed mainly un-degraded large molecular weight aggrecan core species, and also revealed an increased number of C4S-containing species. Thus, following mechanical stimulation, newly-synthesized aggrecan was increased and the concentration of CS was changed. Cyclical mechanical stimulation affected C4S disaccharide sulphation isomers on the GAGs of the newly synthesized PGs of normal chondrocytes. The increased 2-B-6 positive fragments of C4S epitopes may relate to the increased turnover of matrices as the result of mechanical stimulation.

The study has demonstrated the increase in GAG synthesis in chondrocytes from normal cartilage induced by cyclical mechanical stimulation. Chondrocytes from normal cartilage increased GAG synthesis which was blocked by antibodies to anti-α5 integrin antibody (P1D6), or anti-αVβ5 integrin antibody (P1F6) and or anti CD47 antibody (Bric 126).

Antibodies alone had no effect on GAG production by unstimulated cells, suggesting that integrins and integrin associated regulatory molecules are involved in the mechano-transduction pathway, possibly through interplay with the perception of the mechanical signals by the chondrocytes. Anti-α5 integrin antibody (P1D6) is likely to act through modulation of chondrocyte blocking cell binding to a specific extra-cellular matrix ligand such as fibronectin. Anti-αVβ5 integrin antibody (P1F6) was probably blocking cells adhesion to vitronectin in extra-cellular matrix. The affects of the anti CD47 antibody (Bric 126), however, is likely to be through a different route, perhaps by interacting with CD47 binding to α5β1 integrins and disruption of a macro-molecular complex necessary for co-ordinated integrin activation. Increased aggrecan mRNA, following chondrocyte membrane deformation, can be blocked by RGD containing peptides, or anti-α5 integrin antibody (P1D6), or by anti-β1 integrin antibody (P4C10) (Millward-Sadler et al 2000a) consistent with the idea that of the accelerated GAG synthesis is, at least in part, a downstream event of increased aggrecan mRNA. This study is the first to show that αVβ5 integrin and CD47 may also be involved in the mechano-transduction pathway that regulates chondrocyte matrix production.

The attachment of integrins to extra-cellular matrix may be one of the primary events that occurs in response to mechanical stimulation and may be important in the regulation of chondrocyte activity following mechanical stimulation. It is well documented that signal transduction is regulated by several proteins forming a complex in order to function (Brown and Frazier 2001). There are other integrins and membrane receptors in chondrocytes. The application of other anti-integrin antibodies, such as anti-αVβ3 integrin antibody, to chondrocyte cultures might provide more information regarding the role of integrins in the events that lead to increased PG synthesis. This study was the first one to show that αVβ5 integrin and CD47 may be involved in mechano-transduction pathways that involve chondrocyte matrix production.

Cyclical mechanical stimulation applied to articular chondrocytes from patients with OA in monolayer culture did not show any significant change in GAG synthesis, consistent with previous studies in which no change in aggrecan mRNA levels were seen under similar experimental conditions (Millward-Sadler et al 2000a, Salter et al 2002). The results of the DMMB assay showed no difference in the quantity of GAGs, synthesized by stimulated and unstimulated OA chondrocytes cultured in monolayer, in the presence or absence of integrin antibodies. However, the apparent intensity of the large molecular weight 350 KDa band increased following mechanical stimulation of OA grade I and II chondrocytes, in the presence of anti-α5 integrin antibody. It may be speculated that the mechano-transduction pathways in OA chondrocytes are changed from those operating in normal chondrocytes, and by modulating blocking the function of α5 integrin, matrix interactions and/or downstream signalling events may be significantly altered in OA chondrocytes. There may be a role for α5 integrin in aggrecan synthesis, following mechanical stimulation of early stage OA

chondrocytes. Moreover, it may be possible to mitigate against the progression of OA using anti-integrin antibodies.

Cyclical mechanical stimulation by compression of OA chondrocytes in three-dimensional alginate culture (disk formation) resulted in a decrease in GAG synthesis, measured by the DMMB assay. This decrease in GAG synthesis in alginate-chondrocyte construct culture was seen after cyclical compression of 30 per cent at 0.33 Hz and 0.1 Hz. Cyclical mechanical loading is known to act in different ways, affecting different stages in the syntheses of PGs. Short term compression may decrease PG synthesis by inhibiting translational or post-translational steps. Interestingly, in this study, this decrease was blocked by the presence of anti-β1 integrin antibody (JB1A) and anti-αVβ5 integrin antibody (P1F6). It seems likely that cell-matrix linkage, through integrins as mechano-receptors, is also critical for the response of chondrocytes derived from OA cartilage. Moreover, the kinds of integrins and their distribution in OA chondrocytes in monolayer culture, may be different from those expressed in a three-dimensional culture system. Thus the presence of the extra-cellular matrix and the cell-surface receptors has may have been shown to have a major effect on cell response and the extra-cellular matrix can also have a significant influence on the chondrocyte phenotype.

When chondrocytes were mechanically stimulated at 32000μ strains, rather than 4000μ strains, there was an increase in the production of molecular weight 50 KDa PGs of small C4S-containing epitopes, probably small PG decorin, in chondrocytes from OA cartilage. This change was not seen in chondrocytes from normal cartilage. This increased synthesis of C4S containing PGs may be an adaptive change in the a result of an altered mechano-transduction pathway related to disease onset. For instance, Decorin is reported to be increased in high contact stress areas of articular cartilage (Little et al 1996) and in OA cartilage (CS-Szabo et al 1995, 1997), particularly in later stages of OA (Bock et al 2001) when compared to age-matched normal controls.

Previous studies have demonstrated differences in responses to mechanical stimulation, between chondrocytes from normal and OA cartilage. The hyperpolarization response of normal chondrocytes to cyclical mechanical stimulation at 0.33 Hz is associated with the opening of small conductance calcium-dependent K^+ channels and it is mediated via the α5β1 integrin. In contrast, OA chondrocytes following 0.33 Hz cyclical mechanical stimulation show a membrane depolarization as a result of tetrodotoxin-sensitive Na^+ channels. This involves α5β1 integrin but the subsequent cellular response has been shown to be different (Salter et al 2002). The present study suggests the idea that OA cartilage shows significant differences in mechano-responses. This may be part of a general phenotypic switch in disease (Sandell and Aigner 2001), or chondrocytes derived from OA cartilage may have adopted to a new mechanical- environment and their threshold for response to cyclical mechanical stimulation may has been altered.

One of the important findings in this study was that PG synthesis in OA chondrocytes is variable depending on the grades of OA. End stage OA chondrocytes (OA grade III) showed significantly decreased PG synthesis, compared to normal or other grades of OA chondrocytes. This, in fact, relates to an inability to synthesize intact aggrecan species, showing a decrease in the synthesis of large molecular weight aggrecan core protein with a molecular weight of around 350 kDa. In addition, end-stage OA chondrocytes (OA grade II and III) produced smaller bands with molecular weight 50 to 160 kDa. There may be decreased anabolic activity, as well as increased proteolytic activity in this stage of OA chondrocytes.

In normal articular cartilage, there is a balance between matrix synthesis and degradation. In advanced OA, there is a significant depletion of the PG content of the matrix. (Bollet 1963, Anderson 1964, Mankin 1970). The mechanical environment and biological processes responsible for these alterations are difficult to identify. Degenerative and repair process occur concurrently in articular cartilage, and pathological features including changes in matrix composition, chondrocyte clustering, cartilage fissuring and flaking, osteophytes are partly secondary to the effects of mechanical forces. My studies here showed that chondrocytes derived from OA cartilage have altered matrix production in response to mechanical stimulation when compared to normal ones. The response of chondrocytes derived from OA cartilage to mechanical stimulation appears to be defective. An early event in OA cartilage may be a failure of chondrocytes to respond of mechanical stimulation with an increase in PG synthesis, and in later stages of OA, the anabolic capacity of PG synthesis is significantly depleted, leading to cartilage loss. The results of the experiments presented in this thesis are consistent with a hypothesis that the progression of OA may be mediated by a lack of anabolic response to mechanical stimulation in cartilage chondrocytes. The basis for these changes in chondrocytes metabolism are probably the results of both environmentally induced (Little et al 1997) and genotypic changes (Aigner 2004).

To date, no studies have been made to test the effects of mechanical loading and unloading on human articular chondrocytes from RA cartilage. The results in this study showed that chondrocytes, isolated from the articular cartilage of patients with RA exposed to mechanical stimulation, did not demonstrate any significant change in GAG synthesis. In this context, the impaired anabolic response to mechanical stimulation in RA chondrocytes is similar to that of chondrocytes from OA cartilage. However, the amounts of GAG synthesised by unstimulated chondrocytes derived from RA knee joints, were not significantly different from those from normal chondrocytes. G1 domain-containing aggrecan species were also present, unlike in OA grade II or III chondrocytes. Chondrocytes in residual cartilage in RA joints may produce more matrix than chondrocytes in OA grade III cartilage.

There are few studies which have demonstrated the differences between RA chondrocytes and normal or OA chondrocytes in cartilage. Jobanputra et al (1996) have shown the change in expression of integrins in diseased cartilage, indicating a phenotypic change in RA

chondrocytes. However few studies have been undertaken on the role of integrins in RA chondrocytes compared with that of integrins in synovial tissue in this disease. This was the first study to look at the regulation of matrix production via integrin-mediated mechano-transduction pathways in chondrocytes from patients with RA. Anti-integrin antibodies used in this study did not modulate matrix syntheses in RA chondrocytes following mechanical stimulation.

7.2 Future prospects

(i) Further studies of the regulation of the chondrocyte PG synthesis by mechanical forces will be greatly facilitated by the use of a stable matrix for chondrocyte culture systems. A three-dimensional, bio-degradable, and cyclically compressible culture system is required.

(ii) Integrin expression of chondrocytes derived from Normal, OA and RA cartilage cultured in monolayer and 3D needs to be examined.

(iii) $\alpha V\beta 5$ integrin involvement in intracellular signalling following mechanical stimulation needs to be explored.

(iv) Chondrocytes have the capacity to synthesize a number of metalloproteinases (MMPs and ADAM-TS) *in vitro* (Flannery et al 1999). No obvious metalloproteinases activities could be seen in this study. In the future, chondrocytes *in vitro* could be exposed to IL-1 or retinoic acid prior to anabolic cyclical mechanical stimulation in order to assess the counter-effect of mechanical stimulation to metalloproteinase activities.

(v) An increase of fibronectin fragments was detectable in OA cartilage (Burton-Wurster et al 1986, Jones et al 1987, Rees et al 1987, Chevalier et al 1992). Enhanced fibronectin synthesis is partially influenced by biomechanical forces (Steinmeyer et al 1997, Farquhar et al 1996, Burton-Wurster et al 1993). It would be interesting to see if OA chondrocytes produced more fibronectin in response to mechanical stimulation than normal chondrocytes. Similarly, increased levels of mRNA and the protein of small PG decorin in OA have been reported (Cs-Szabo et al 1995, 1997). Decorin synthesis is influenced by mechanical loading (Korver et al 1992, Little et al 1996, Lammi 1994). Investigations of these extra-cellular matrix molecules, such as fibronectin and decorin, following the mechanical stimulation of chondrocytes from OA cartilage, will be important for gaining fuller understanding of cell-matrix interactions. Altered mechano-transduction pathways in OA chondrocytes may explain the increases in these extra-cellular matrix molecules found in abnormal cartilage in OA joints.

(vi) Heterotypic interactions between chondrocytes and synovial cells are known to occur in RA (D'Andrea et al 1998). Synovial pannus formation is initiated with the recognition and adhesion of synovial cells to chondrocytes and to the cartilage matrix mediated by cadherins and integrins. The evidence is that these two cell types develop pathways allowing direct and indirect intracellular communication resulting in increased tissue sensitivity to changes in the extra-cellular matrix environment. To assess chondrocyte mechano-transduction in RA chondrocytes, it will be necessary to use a co-culture with synovial cells.

Appendix I

Antibodies

monoclonal antibody		final concentration
anti-α5 integrin	P1D6 (MAB1956 Chemicon International)	1 μg/ml
anti-αVβ5 integrin	P1F6 (MAB1961 Chemicon International)	1 μg/ml
anti-CD47	Bric 126 (IBGRL Research, UK)	1 μg/ml
anti-β1 integrin	JB1A (MAB1965 Chemicon International)	1 μg/ml
mouse IgG	control	1 μg/ml
anti-aggrecan G1 domain antibody	rabbit antisera anti-G1 domain of human aggrecan	1:400
2-B-6	4 sulfate CS stubs	1:100
3-B-3	6 sulfate CS stubs	1:200
1-B-5	0 sulfate CS stubs	1:5000
5-D-4	keratan sulfate (after only chondroitinase ABC digestion)	1:1000
70.6	anti decorin	1:200
BC-3	aggrecanase cleavaged neoepitope (ARGSVI)	1:500
BC-13	aggrecanase cleavaged neoepitope (NITEGE)	1:100
BC-4	MMP cleavaged neoepitope (VDIPEN)	1:100
BC-14	MMP cleavaged neoepitope (FFGVG)	1:200
anti-rabbit	an alkinephosphatase-conjugated goat anti-rabbit IgG	1:2000
anti-sheep	rabbit antiserum conjugated with horseradish peroxidase (Dako Cytomation, Denmark)	1:1000

Buffer compositions

GuHCl PG extraction buffer

	content	quantity
4 M GuHCl extraction buffer (100 ml)	4 M GuHCl	8 M 50 ml
	4% (w/v) CHAPS (Calbiochem®)	20% 20 ml
	100 mM sodium acetate buffer pH 5.8	1 M 10 ml
	10 mM 6-amino-hexanoic acid (nova biochem)	1 M 1 ml
	10 mM EDTA	0.1M 10 ml
	1 mM PMSF in methanol	100 mM 1 ml
	5 mM benzamidine hydrochloride (AMS,UK)	0.1746 g
	10 mM N-ethyl-maleimide in methanol	1 M 1 ml
	distilled water	8.5 ml

DMMB stock solution and GAG standard solution

	content	quantity
DMMB stock solution (500 ml)	DMMB	0.008 g
	glycine	1.52 g
	NaCl	2.85 g
	HCl	adjust to pH 3.0
	distilled water	adjust to 500 ml
GAG standard solution	bovine chondroitin sulfate A	100 µg/ml

Papain buffer and DMMB solution for alginate

	contents	quantity
Papain buffer (250 ml) stored at 4°C	150 mM NaCl	2.192 g
	55 mM sodium citrate	4.04 g
	5 mM cystein HC	0.197 g
	5 mM EDTA	0.4605 g
DMMB solution for alginate (pH 1.5)(1 litre)	1,9-dimethylene blue	21 mg
	absolute ethanol	5 ml
	sodium formate	2.0 g
	concentrated formic acid to adjust to pH 1.5	

Hoechst dye stock, Hoechst working solution, and DNA standard solution

material	content	quantity
Hoechst dye stock sterilized by filtration through a 0.22 µm filter and stored at 4°C in a light tight container	Hoechst dye 33258	0.1 mg/ml in distilled water
working assay solution sterilized by autoclaving and stored at 4°C, total 50ml	Hoechst dye stock	250 µl
	10 mM Tris-HCl pH 7.4	1 M 500 µl
	1 mM Na2EDTA	100 mM 500 µl
	0.1 mM NaCl	500 mM 20 ml
	distilled water	28.75 ml
DNA standard solution	calf thymus DNA	100 ng/µl

Materials used for SDS-PAGE

	content	quantity
4x sample buffer (16 ml)	distilled water	7.6 ml
	0.5 M Tris-HCl, pH 6.8	2.0 ml
	glycerol (Fisons, UK)	1.6 ml
	10% [w/v] SDS	3.2 ml
	1 M DTT	1.6 ml
	bromophenol blue	8.0 mg
stacking gel 5% (8 ml)	distilled water	5.5 ml
	1.0 M Tris-HCl, pH 6.8	1.0 ml
	10% [w/v] SDS	0.08 ml
	acrylamide/bis [30% stock] (ultra pure grade amresco®, USA)	1.3ml
	10% APS	0.08 ml
	TEMED	0.008 ml
separating gel 7.5% (20 ml)	distilled water	9.6 ml
	1.5 M Tris-HCl, pH 8.8	5 ml
	10% [w/v] SDS	0.2 ml
	acrylamide/bis [30% stock]	5 ml
	10% APS	0.2 ml
	TEMED	0.016 ml
transfer buffer pH 8.3 (1 litre) (pH not adjust)	0.0025 M Tris	3.03 g
	0.19 M glycine	14.4 g
	15% (v/v) methanol	150 ml
10X Tris-glycine electrode buffer pH 8.3 (2 litre) (not adjust pH)	0.025 M Tris	60 g
	0.19 M glycine	288 g
	0.1% [w/v] SDS	20 g

Materials used for SDS-PAGE for epitope monoclonal antibodies

	content	quantity
2x sample buffer (16 ml)	distilled water	
	0.125 M Tris-HCl, pH 6.8	
	20% glycerol (Fisons, UK)	
	4% [w/v] SDS	
	10% mercaptoethanol	
	0.01% bromophenol blue	
transfer buffer pH 8.3 (1 litre) 10X Tris-glycine electrode buffer pH 8.3 (2 litre) (pH not adjust) (re-used up to 5 times)	0.0025 M Tris	3.03 g
	0.19 M glycine	14.4 g
	20% (v/v) methanol	200 ml
	0.025 M Tris	60 g
	0.19 M glycine	288 g
	0.1% [w/v] SDS	20 g
running buffer (1 litre)	25 mM Tris (TRIZMA)	3.02 g
	192 mM glycine	14.4 g
	0.1% SDS	1.0 g
TSA buffer (2 litre) (pH 7.4 with HCl)	50 mM Tris	12.12 g
	200 mM NaCl	23.38 g
	0.02% sodium azide	0.6 g
AP buffer (200 ml) (pH 9.55)	100 mM Tris	2.42 g
	100 mM NaCl	1.168 g
	5 mM MgCl2	0.2033 g

Deglycosylation buffer

material	contents	quantity
deglycosylation buffer (50 µl)	0.1 unit/ml keratanase II	5 µl
	proteolytic inhibitors	5 µl
	1 unit/ml chondroitinase ABC	5 µl
	1 M sodium acetate pH 7.3	5 µl
	1 M Tris-HCl pH 7.3	5 µl
	distilled water	25 µl
proteolytic inhibitors (1 ml)	50 mM PMSF in 100% ethanol	100 mM 1 µl
	100 mM N-ethyl-maleimide in 0.1 M Tris-HCl pH 7.4	1 M 2 µl
	36 mM pepstein A in 0.1 M Tris-HCl pH 7.4	20 µl
	100 mM EDTA in 0.1 M Tris-HCl pH 7.4	1 M 2 µl
	0.1 M Tris-HCl pH 7.4	1 M 75 µl

OA grading system (Macroscopic)

grade	OA cartilage changes judged by the naked eye
0	normal cartilage with smooth surface
I	superficial flaking
II	softening, such as sponge like cartilage (i.e. not denuding bone)
III	extensive fibrillation and fissuring of cartilage, bone exposure
IV	total cartilage loss and bone exposure, eburnation and bone grooving

Modified from OA grading system of McElligott and Collins (1960)

Frequencies used in experiments

frequency (Hz)	helium gas flow on (seconds)	helium gas flow off (seconds)
0.1	2	7.8
0.33	2	1
1	0.5	0.5

Appendix II

Original data: Chapters 3-5

sample					OARSI 2005			data					
grade	donor	procedure	culture period (days)	gender/age	grade	stage	score	reagent	pre-medium µg/ml/16-hour	post-medium µg/ml/40-hour	GAG µg/24 hour/dish (3ml)	DNA µg/dish (250000 cells)	GAG/DNA (µg/µg)
normal	14013	AKA	13	M/76	1	1	1	Nil	6.334	21.255	2.351	2.043	1.151
								Nil+MS	9.112	55.334	8.333	2.055	4.055
								IgG	7.669	27.780	3.255	1.471	2.213
								IgG+MS	9.504	53.330	7.815	2.466	3.169
								a5	11.888	25.259	1.485	1.907	0.779
								a5+MS	10.944	29.333	2.583	2.447	1.056
								aVb5	10.222	20.518	1.037	1.962	0.529
								aVb5+MS	15.778	27.852	0.837	2.192	0.382
								CD47	14.112	30.444	1.855	2.454	0.756
								CD47+MS	8.556	31.628	3.759	1.917	1.961
normal	14039	TKR	10	M/39	0	0	0	Nil	12.667	25.354	1.271	2.005	0.634
								Nil+MS	6.943	26.650	3.247	2.340	1.388
								IgG	13.911	25.998	1.026	2.710	0.379
								IgG+MS	8.544	28.880	3.213	2.480	1.295
								a5	4.545	15.150	1.667	2.550	0.654
								a5+MS	4.911	17.900	2.107	1.940	1.086
								aVb5	8.400	23.900	2.260	2.980	0.758
								aVb5+MS	4.100	23.850	3.540	2.153	1.644
								CD47	6.900	28.900	3.710	3.720	0.997
								CD47+MS	5.650	20.400	2.385	3.810	0.626

sample					OARSI 2005			data					
grade	donor	procedure	culture period (days)	gender/age	grade	stage	score	reagent	pre-medium µg/ml/16-hour	post-medium µg/ml/40-hour	GAG µg/24 hour/dish (3ml)	DNA µg/dish (250000 cells)	GAG/DNA (µg/µg)
normal	14039	TKR	10	M/39	0	0	0	Nil	12.667	25.354	1.271	2.005	0.634
								Nil+MS	6.943	26.650	3.247	2.340	1.388
								IgG	5.333	23.722	3.145	1.611	1.952
								IgG+MS	7.278	46.500	7.117	2.385	2.984
								a5	10.944	44.000	5.517	3.072	1.796
								a5+MS	5.944	37.889	5.795	2.812	2.061
								aVb5	5.111	34.278	5.322	1.782	2.987
								aVb5+MS	7.889	41.778	5.989	2.691	2.226
								CD47	7.056	45.667	7.017	3.061	2.292
								CD47+MS	4.278	23.722	3.461	1.315	2.632
normal	14080	TKR	10	F/66	0	0	0	Nil	9.848	29.000	2.846	2.431	1.171
								Nil+MS	10.182	45.428	6.031	2.201	2.740
								IgG	12.576	30.428	2.313	2.165	1.068
								IgG+MS	10.485	37.572	4.369	1.422	3.072
								a5	13.182	37.572	3.560	2.612	1.363
								a5+MS	10.455	30.428	2.949	3.119	0.946
								aVb5	13.182	29.714	1.988	3.347	0.594
								aVb5+MS	13.788	38.286	3.521	3.210	1.097
								CD47	11.121	21.572	0.978	2.130	0.459
								CD47+MS	9.848	25.074	2.060	2.070	0.995
OA I	14023	TKR	9	F/76	2	2	4	Nil	3.727	17.364	2.355	1.829	1.287
1								Nil+MS	7.818	20.091	1.673	1.453	1.151
								IgG	6.636	15.091	1.027	1.274	0.806
								IgG+MS	9.273	28.273	2.873	2.003	1.434

								a5	10.545	23.273	1.491	1.207	1.235
								a5+MS	6.818	15.091	0.973	1.773	0.549
								aVb5	6.818	17.364	1.427	1.773	0.805
								aVb5+MS	8.273	13.273	0.173	2.014	0.086
								CD47	10.091	18.273	0.627	2.027	0.309
								CD47+MS	15.454	28.727	1.109	2.417	0.459
OA I	14051	TKR	10	F/57	no slides	no slides	no slides	Nil	6.429	20.286	2.129	2.279	0.934
2								Nil+MS	8.000	16.357	0.871	1.035	0.842
								IgG	8.286	19.214	1.357	1.898	0.715
								IgG+MS	7.786	27.786	3.221	2.022	1.593
								a5	6.500	17.786	1.607	2.279	0.705
								a5+MS	7.571	26.000	2.929	2.035	1.439
								aVb5	6.857	21.000	2.143	2.346	0.913
								aVb5+MS	11.286	31.000	2.814	1.764	1.595
								CD47	10.857	38.143	4.372	2.426	1.802
								CD47+MS	8.429	24.214	2.314	1.678	1.379
OA I	14035	TKR	7	M/71	slides fault	fault	fault	Nil	8.313	26.625	2.831	1.574	1.799
3								Nil+MS	6.875	26.335	3.205	1.645	1.948
								IgG	7.063	20.063	1.894	2.564	0.739
								IgG+MS	10.063	26.313	2.244	2.100	1.068
								a5	8.313	21.625	1.831	2.328	0.787
								a5+MS	8.938	27.875	2.894	2.207	1.311
								aVb5	7.125	26.000	3.063	2.308	1.327
								aVb5+MS	8.625	19.750	1.363	2.808	0.485
								CD47	8.438	16.313	0.731	2.305	0.317
								CD47+MS	6.188	11.313	0.406	1.669	0.243

sample					OARSI 2005			data					
grade	donor	procedure	culture period (days)	gender/age	grade	stage	score	reagent	pre-medium µg/ml/16-hour	post-medium µg/ml/40-hour	GAG µg/24 hour/dish (3ml)	DNA µg/dish (250000 cells)	GAG/DNA (µg/µg)
OA I	14038	TKR	7	M/64	2	1	2	Nil	3.591	21.545	3.232	2.896	1.116
4								Nil+MS	7.909	25.409	2.709	1.432	1.892
								IgG	7.000	16.773	1.255	2.356	0.533
								IgG+MS	6.318	28.136	3.732	1.705	2.189
								a5	6.545	24.045	2.846	3.468	0.821
								a5+MS	7.000	20.636	2.027	2.087	0.971
								aVb5	9.045	25.182	2.323	2.511	0.925
								aVb5+MS	9.273	22.909	1.800	2.239	0.804
								CD47	6.318	12.909	0.686	2.040	0.336
								CD47+MS	9.727	24.955	2.073	2.539	0.816
OA I	DS170C	TKR	8	M/70	1	1	1	Nil	4.054	26.591	4.102	2.409	1.703
5								Nil+MS	4.719	25.227	3.630	1.500	2.420
								IgG	6.002	26.136	3.427	2.136	1.604
								IgG+MS	6.002	24.091	3.018	2.318	1.302
								a5	8.900	36.591	4.648	1.818	2.557
								a5+MS	6.144	35.909	5.339	2.25	2.373
								aVb5	5.574	36.364	5.601	1.954	2.866
								aVb5+MS	3.769	30.000	4.869	1.704	2.858
								CD47	3.198	28.409	4.722	2.045	2.309
								CD47+MS	6.049	27.955	3.776	2.045	1.847
OA I	14081	TKR	6	F/72	no	no	no	Nil	10.083	33.000	3.575	2.272	1.574
6								Nil+MS	10.083	22.167	1.409	1.198	1.176
								IgG	12.167	29.667	2.283	1.438	1.588

								IgG+MS	3.417	15.083	1.992	2.414	0.825
								a5	6.056	17.786	1.740	2.279	0.764
								a5+MS	7.857	26.000	2.843	2.035	1.397
								aVb5	5.083	25.917	3.659	3.33	1.099
								aVb5+MS	9.25	31.330	3.491	2.387	1.463
								CD47	8.166	23.000	2.150	2.591	0.830
								CD47+MS	17.25	36.330	2.091	2.272	0.920
OA II	DS037C	TKR	9	M/71	2	1	2	Nil	7.687	28.625	3.419	2.298	1.488
1								Nil+MS	0.813	10.500	1.856	2.833	0.655
								IgG	2.687	20.813	3.357	2.064	1.626
								IgG+MS	6.347	14.875	1.071	3.098	0.346
								a5	0.817	6.437	1.042	2.77	0.376
								a5+MS	5.5	16.750	1.700	1.79	0.950
								aVb5	13.313	20.813	0.169	1.458	0.116
								aVb5+MS	12.063	21.438	0.669	2.694	0.248
								CD47	5.125	14.563	1.375	2.134	0.644
								CD47+MS	11.125	28.000	2.263	2.224	1.017
OA II	DS130C	TKR	9	M/57	3	4	12	Nil	14.333	38.778	3.456	2.099	1.646
2								Nil+MS	12.944	37.389	3.595	3.141	1.144
								IgG	13.778	27.111	1.289	2.062	0.625
								IgG+MS	15.167	39.333	3.317	2.796	1.186
								a5	17.944	44.056	3.428	1.997	1.717
								a5+MS	15.167	29.333	1.317	1.516	0.868
								aVb5	14.333	48.222	5.345	2.91	1.837
								aVb5+MS	11.833	50.722	6.595	1.879	3.510
								CD47	14.056	42.944	4.372	2.098	2.084
								CD47+MS	10.444	26.000	2.067	1.278	1.617

sample					OARSI 2005			data					
grade	donor	procedure	culture period (days)	gender/age	grade	stage	score	reagent	pre-medium µg/ml/16-hour	post-medium µg/ml/40-hour	GAG µg/24 hour/dish (3ml)	DNA µg/dish (250000 cells)	GAG/DNA (µg/µg)
OA II	14071	TKR	8	F/51	2	4	8	Nil	3.611	19.642	2.845	1.853	1.535
3								Nil+MS	6.389	20.418	2.167	1.909	1.135
								IgG	6.944	16.675	1.252	1.754	0.714
								IgG+MS	1.111	13.818	2.430	1.656	1.468
								a5	3.333	17.853	2.571	1.779	1.445
								a5+MS	1.944	23.431	4.103	2.024	2.027
								aVb5	7.222	19.036	1.641	1.841	0.891
								aVb5+MS	5.889	21.216	2.477	1.966	1.260
								CD47	1.389	19.718	3.527	1.886	1.870
								CD47+MS	5.556	19.152	2.164	1.959	1.104
OA II	DS069C	TKR	9	F/65	2	4	8	Nil	7.609	15.435	0.804	1.949	0.413
4								Nil+MS	3.478	15.435	2.044	1.741	1.174
								IgG	4.783	14.438	1.453	1.601	0.907
								IgG+MS	8.478	16.078	0.672	2.107	0.319
								a5	4.565	20.217	2.674	2.416	1.107
								a5+MS	10.432	20.435	0.957	2.095	0.457
								aVb5	7.826	17.714	1.195	2.477	0.482
								aVb5+MS	7.391	15.435	0.870	2.281	0.381
								CD47	11.739	19.783	0.435	1.273	0.342
								CD47+MS	9.565	18.696	0.870	2.002	0.434
OA II	14078	TKR	8	F/75	2	1	2	Nil	12.915	24.975	1.121	2.616	0.428
5								Nil+MS	9.397	25.402	2.261	1.277	1.771
								IgG	7.638	21.206	1.950	2.284	0.854

								IgG+MS	6.884	28.492	3.633	2.244	1.619
								a5	9.407	16.935	0.565	1.885	0.300
								a5+MS	10.965	27.990	2.309	1.58	1.461
								aVb5	6.915	18.191	1.564	1.591	0.983
								aVb5+MS	6.382	12.161	0.518	1.636	0.316
								CD47	5.879	25.477	3.332	3.113	1.070
								CD47+MS	3.116	17.688	2.603	2.43	1.071
OA II	14044	TKR	7	F/71	3	3	9	Nil	6.712	28.332	3.653	2.042	1.789
6								Nil+MS	6.087	23.098	2.794	1.135	2.461
								IgG	4.516	10.32	0.709	1.898	0.374
								IgG+MS	5.803	27.705	3.800	3.021	1.258
								a5	8.379	17.901	1.067	2.279	0.468
								a5+MS	3.056	14.853	2.054	2.135	0.962
								aVb5	5.868	26.943	3.628	2.455	1.478
								aVb5+MS	11.286	41.346	4.883	2.543	1.920
								CD47	5.251	22.241	2.873	2.426	1.184
								CD47+MS	14.429	39.940	3.659	2.311	1.583
OA III	DS079C	TKR	10	M/64	4	4	16	Nil	20.250	31.063	0.138	2.371	0.058
1								Nil+MS	20.750	34.188	0.613	2.073	0.296
								IgG	19.240	31.480	0.524	2.419	0.217
								IgG+MS	18.673	29.228	0.244	1.874	0.130
								a5	15.438	28.250	1.019	1.899	0.536
								a5+MS	18.250	27.938	0.113	1.845	0.061
								aVb5	16.375	31.375	1.363	2.749	0.496
								aVb5+MS	15.813	26.688	0.594	1.973	0.301
								CD47	20.188	32.000	0.344	2.959	0.116
								CD47+MS	12.938	25.750	1.269	2.496	0.508

sample					OARSI 2005			data					
grade	donor	procedure	culture period (days)	gender/age	grade	stage	score	reagent	pre-medium µg/ml/16-hour	post-medium µg/ml/40-hour	GAG µg/24 hour/dish (3ml)	DNA µg/dish (250000 cells)	GAG/DNA (µg/µg)
OA III	14035	TKR	7	M/71	4	4	16	Nil	0.095	9.650	1.902	2.810	0.677
2								Nil+MS	1.524	4.509	0.445	1.035	0.430
								IgG	5.095	17.577	1.987	2.860	0.695
								IgG+MS	0.000	2.342	0.468	2.332	0.201
								a5	5.095	18.653	2.202	2.224	0.990
								a5+MS	7.000	12.800	0.460	2.041	0.225
								aVb5	1.286	11.859	1.986	2.218	0.895
								aVb5+MS	6.048	15.189	1.223	2.761	0.443
								CD47	10.810	24.139	1.585	2.121	0.747
								CD47+MS	5.048	16.619	1.809	2.174	0.832
OA III	DS138C	TKR	7	M/79	3	3	9	Nil	5.750	13.234	0.922	1.045	0.882
3								Nil+MS	0.000	2.000	0.400	1.859	0.215
								IgG	3.667	5.756	0.051	1.237	0.041
								IgG+MS	0.750	6.853	1.146	1.911	0.599
								a5	3.667	7.417	0.383	1.718	0.223
								a5+MS	4.284	9.852	0.685	1.718	0.399
								aVb5	6.076	9.692	0.116	2.077	0.056
								aVb5+MS	0.330	2.833	0.468	1.329	0.352
								CD47	3.250	9.083	0.842	2.322	0.362
								CD47+MS	2.400	7.417	0.763	2.445	0.312
OA III	14039	TKR	8	M/39	1	1	1	Nil	5.737	12.579	0.795	2.297	0.346
4								Nil+MS	7.053	17.263	1.337	1.888	0.708
								IgG	6.895	12.842	0.500	1.776	0.281
								IgG+MS	12.579	27.053	1.637	2.811	0.582

								a5	13.526	22.842	0.511	1.623	0.315
								a5+MS	6.526	14.158	0.874	2.661	0.328
								aVb5	3.368	12.579	1.505	2.523	0.597
								aVb5+MS	11.526	18.105	0.163	1.688	0.097
								CD47	10.316	18.105	0.526	1.948	0.270
								CD47+MS	5.474	12.579	0.874	1.278	0.684
grade	donor	procedure	culture period (days)	gender/age	synovial overgrowth			reagent	pre-medium µg/ml/16-hour	post-medium µg/ml/40-hour	GAG µg/24 hour/dish (3ml)	DNA µg/dish (250000 cells)	GAG/DNA (µg/µg)
RA	DS105C	TKR	8	M/37	±			Nil	14.271	31.859	2.091	2.445	0.855
1								Nil+MS	10.503	17.286	0.306	1.834	0.167
								IgG	23.568	36.131	0.156	2.941	0.053
								IgG+MS	6.231	14.271	0.985	1.969	0.500
								a5	16.030	35.926	2.376	2.052	1.158
								a5+MS	28.593	51.960	1.814	1.535	1.182
								aVb5	8.241	13.518	0.231	1.784	0.130
								aVb5+MS	10.000	24.322	1.864	1.509	1.236
								CD47	12.010	22.313	0.860	2.159	0.398
								CD47+MS	11.508	17.538	0.055	1.283	0.043
RA	DS098C	TKR	9	F/55	±			Nil	20.550	38.500	1.535	1.941	0.791
2								Nil+MS	13.050	26.500	1.385	2.147	0.645
								IgG	20.800	36.500	1.060	1.353	0.783
								IgG+MS	30.800	52.500	1.260	1.811	0.696
								a5	17.300	50.500	4.910	1.784	2.752
								a5+MS	27.800	53.050	2.270	1.353	1.678
								aVb5	24.800	60.550	4.670	2.312	2.020
								aVb5+MS	30.550	46.800	0.195	1.307	0.149
								CD47	30.550	58.800	2.595	2.278	1.139
								CD47+MS	30.550	57.050	2.245	1.229	1.827

grade	donor	procedure	culture period (days)	gender/age	synovial overgrowth	reagent	pre-medium µg/ml/16-hour	post-medium µg/ml/40-hour	GAG µg/24 hour/dish (3ml)	DNA µg/dish (250000 cells)	GAG/DNA (µg/µg)
RA	DS052C	TKR	7	F/63	+	Nil	17.611	56.778	6.072	2.734	2.221
3						Nil+MS	16.778	55.944	6.155	3.733	1.649
						IgG	20.667	56.222	5.044	3.042	1.658
						IgG+MS	15.667	56.500	6.600	3.172	2.081
						a5	7.056	29.833	3.850	2.344	1.642
						a5+MS	20.944	54.556	4.628	1.888	2.451
						aVb5	21.778	55.111	4.489	2.893	1.552
						aVb5+MS	13.722	54.556	6.795	2.590	2.623
						CD47	18.167	57.056	5.961	2.999	1.988
						CD47+MS	10.389	21.611	1.206	1.889	0.638
RA	S140C	TKR	9	F/67	-	Nil	3.474	11.368	1.231	1.199	1.027
4						Nil+MS	1.895	6.895	0.811	1.594	0.508
						IgG	5.482	10.316	0.419	2.317	0.181
						IgG+MS	9.000	19.263	1.153	1.912	0.603
						a5	10.053	19.526	0.889	1.648	0.540
						a5+MS	5.482	14.789	1.313	1.967	0.668
						aVb5	5.316	10.579	0.521	1.641	0.317
						aVb5+MS	5.579	9.526	0.232	1.327	0.174
						CD47	2.684	12.158	1.626	2.311	0.704
						CD47+MS	4.789	16.632	1.890	2.391	0.790
RA	DS157C	TKR	7	F/68	+	Nil	10.498	38.000	4.451	2.319	1.919
5						Nil+MS	7.474	20.985	1.955	1.556	1.256
						IgG					
						IgG+MS	1.684	16.421	2.779	3.399	0.818
						a5	1.608	28.263	5.170	4.139	1.249

						a5+MS	1.684	35.105	6.516	2.339	2.786
						aVb5	1.507	35.105	6.569	3.135	2.095
						aVb5+MS	3.526	29.842	4.911	2.353	2.087
						CD47	6.947	26.684	3.253	3.185	1.021
						CD47+MS	13.000	39.482	3.996	2.220	1.800
RA	DS190C	TKR	9	F/59	-	Nil	4.667	12.667	1.133	2.734	0.415
6						Nil+MS	0.333	12.667	2.434	1.836	1.325
						IgG	2.000	19.333	3.267	2.110	1.548
						IgG+MS	1.166	27.667	5.184	2.105	2.463
						a5	3.000	13.167	1.733	2.052	0.845
						a5+MS	1.667	9.333	1.367	2.275	0.601
						aVb5	5.832	29.167	4.084	2.413	1.692
						aVb5+MS	2.167	32.667	5.883	3.080	1.910
						CD47	5.833	25.637	3.378	3.333	1.013
						CD47+MS	2.833	20.500	3.250	3.180	1.022

Original data: Chapter 6

grade	donor	procedure	gender/age	reagent	GAG µg/disc/3days	DNA µg/disc (300000 cells)	GAG/DNA (µg/µg/disc)
OA	14101	TKR	F72	beads			
				beads			
				beads			
				beads			
OA	DS338C	TKR	F/73	CT	13.6	3.495	3.89
				0.1Hz	7.267	3.057	2.38
				0.3Hz	11.333	3.445	3.29
				1Hz	11.735	5.85	2.01

grade	donor	procedure	gender/age	reagent	GAG µg/disc/3days	DNA µg/disc (300000 cells)	GAG/DNA (µg/µg/disc)
OA	DS360C	TKR	M/69	CT	10.38	3.46	3.00
				0.1Hz	11.56	4.48	2.58
				0.3Hz	6.68	3.93	1.70
				1Hz	10.56	4.11	2.57
OA	14105	TKR	F/67	CT	12.37	1.53	8.09
				0.1Hz	7.00	2.25	3.11
				0.3Hz	13.50	14.80	0.91
				1Hz	14.37	1.94	7.40
OA	DS398C	TKR	M/58	CT	9.91	3.41	2.91
				0.1Hz	8.78	4.78	1.84
				0.3Hz	13.41	6.71	2.00
				1Hz	11.83	4.71	2.51
				1Hz+IgG	14.52	6.16	2.36
				1Hz+aVb5	13.24	4.09	3.23
				1Hz+b1	12.84	3.90	3.29
				IgG	13.35	4.09	3.26
				aVb5	10.82	4.68	2.31
				b1	10.34	4.37	2.36
OA	DS406C	TKR	F/69	CT	11.18	4.53	2.47
				0.1Hz+IgG	5.95	5.61	1.06
				0.3Hz+IgG	9.44	5.53	1.71

				1Hz+IgG	10.42	5.40	1.93
OA	14108	TKR	F/64	CT	12.25	5.49	2.23
				0.1Hz	10.22	8.37	1.22
				0.3Hz	15.28	13.31	1.15
				1Hz	6.63	2.42	2.74
OA	14109	TKR	F/77	CT	16.35	3.44	4.75
				0.1Hz	9.57	4.57	2.09
				0.3Hz	10.46	4.42	2.37
				1Hz	12.17	5.10	2.39
				0.1Hz+IgG	9.00	4.57	1.97
				0.1Hz+aVb5	11.54	5.32	2.17
				0.1Hz+b1	11.85	4.80	2.47
				0.3Hz+IgG	12.42	5.17	2.40
				0.3Hz+aVb5	12.80	4.27	3.00
				0.3Hz+b1	10.02	3.29	3.04
				1Hz+IgG	9.19	5.02	1.83
				1Hz+aVb5	13.94	4.80	2.91
				1Hz+b1	11.28	6.15	1.83
				IgG	9.75	3.89	2.51
				aVb5	8.01	3.14	2.55
				b1	10.27	5.77	1.78
OA	14110	TKR	F/77	CT	10.13	4.22	2.40
				0.1Hz	10.67	4.56	2.34
				0.3Hz	8.87	3.84	2.31
				1Hz	9.55	3.40	2.81
				0.1Hz+IgG	9.27	4.27	2.17

grade	donor	procedure	gender/age	reagent	GAG µg/disc/3days	DNA µg/disc (300000 cells)	GAG/DNA (µg/µg/disc)
				0.1Hz+aVb5	9.20	4.17	2.21
				0.1Hz+b1	8.87	3.57	2.48
				0.3Hz+IgG	8.80	3.20	2.75
				0.3Hz+aVb5	9.67	4.37	2.21
				0.3Hz+b1	10.87	3.88	2.80
				1Hz+IgG	7.27	3.83	1.90
				1Hz+aVb5	7.73	3.69	2.10
				1Hz+b1	8.60	3.98	2.16
				IgG	10.07	2.72	3.71
				aVb5	10.87	3.79	2.87
				b1	10.93	4.42	2.48
OA	14112	TKR	F/71	CT	17.86	4.70	3.80
				0.1Hz	16.66	4.73	3.52
				0.3Hz	10.15	3.76	2.70
				1Hz	11.16	3.36	3.32
				0.1Hz+IgG	12.89	5.05	2.56
				0.1Hz+aVb5	17.46	4.33	4.03
				0.1Hz+b1	13.29	4.08	3.26
				0.3Hz+IgG	10.39	5.89	1.76
				0.3Hz+aVb5	14.46	4.57	3.17
				0.3Hz+b1	12.35	4.36	2.83
				1Hz+IgG	14.03	4.69	2.99
				1Hz+aVb5	13.59	4.47	3.04
				1Hz+b1	12.48	4.88	2.56

			IgG	10.42	3.95	2.64
			aVb5	11.01	4.08	2.70
			b1	12.10	4.89	2.48

References

Abbaszade I, Liu RQ, *et al.* (1999) Cloning and characterization of ADAMTS11, an aggrecanase from the ADAMTS family. *J Biol Chem* **274**, 23443-50.

Afoke NY, Byers PD, Hutton WC (1987) Contact pressures in the human hip joint. *J Bone Joint Surg Br* **69**, 536-41.

Agarwal S, Long P, Gassner R, Piesco NP, Buckley MJ (2001) Cyclic tensile strain suppresses catabolic effects of interleukin-1beta in fibrochondrocytes from the temporomandibular joint. *Arthritis Rheum* **44**, 608-17.

Aigner T (2004) Functional genomics of osteoarthritis and rheumatoid disease. *Rheuma21st.*

Aigner T, Bertling W, Stoss H, Weseloh G, von der Mark K (1993) Independent expression of fibril-forming collagens I, II, and III in chondrocytes of human osteoarthritic cartilage. *J Clin Invest* **91**, 829-37.

Aigner T, Dudhia J (1997) Phenotypic modulation of chondrocytes as a potential therapeutic target in osteoarthritis: a hypothesis. *Ann Rheum Dis* **56**, 287-91.

Aigner T, McKenna L (2002) Molecular pathology and pathobiology of osteoarthritic cartilage. *Cell Mol Life Sci* **59**, 5-18.

Aigner T, Zhu Y, Chansky HH, Matsen FA, 3rd, Maloney WJ, Sandell LJ (1999) Reexpression of type IIA procollagen by adult articular chondrocytes in osteoarthritic cartilage. *Arthritis Rheum* **42**, 1443-50.

Allen DM, Mao JJ (2004) Heterogeneous nanostructural and nanoelastic properties of pericellular and interterritorial matrices of chondrocytes by atomic force microscopy. *J Struct Biol* **145**, 196-204.

Alonso M, Hidalgo J, Hendricks L, Velasco A (1996) Degradation of aggrecan precursors within a specialized subcompartment of the chicken chondrocyte endoplasmic reticulum. *Biochem J* **316 (Pt 2)**, 487-95.

Amin AR, Abramson SB (1998) The role of nitric oxide in articular cartilage breakdown in osteoarthritis. *Curr Opin Rheumatol* **10**, 263-8.

Amin AR, Attur M, Patel RN, Thakker GD, Marshall PJ, Rediske J, Stuchin SA, Patel IR, Abramson SB (1997) Superinduction of cyclooxygenase-2 activity in human osteoarthritis-affected cartilage. Influence of nitric oxide. *J Clin Invest* **99**, 1231-7.

Amin AR, Di Cesare PE, Vyas P, Attur M, Tzeng E, Billiar TR, Stuchin SA, Abramson SB (1995) The expression and regulation of nitric oxide synthase in human osteoarthritis-affected chondrocytes: evidence for up-regulated neuronal nitric oxide synthase. *J Exp Med* **182**, 2097-102.

Anderson CE, Ludowieg J, Harper HA, Engleman EP (1964) The Composition of the Organic Component of Human Articular Cartilage. Relationship to Age and Degenerative Joint Disease. *J Bone Joint Surg Am* **46**, 1176-83.

Archer CW, McDowell J, Bayliss MT, Stephens MD, Bentley G (1990) Phenotypic modulation in sub-populations of human articular chondrocytes in vitro. *J Cell Sci* **97 (Pt 2)**, 361-71.

Argraves WS, McKeown-Longo PJ, Goetinck PF (1981) Absence of proteoglycan core protein in the cartilage mutant nanomelia. *FEBS Lett* **131**, 265-8.

Arner EC, Hughes CE, Decicco CP, Caterson B, Tortorella MD (1998) Cytokine-induced cartilage proteoglycan degradation is mediated by aggrecanase. *Osteoarthritis Cartilage* **6**, 214-28.

Arner EC, Tortorella MD (1995) Signal transduction through chondrocyte integrin receptors induces matrix metalloproteinase synthesis and synergizes with interleukin-1. *Arthritis Rheum* **38**, 1304-14.

Arokoski J, Kiviranta I, Jurvelin J, Tammi M, Helminen HJ (1993) Long-distance running causes site-dependent decrease of cartilage glycosaminoglycan content in the knee joints of beagle dogs. *Arthritis Rheum* **36**, 1451-9.

Arroyo AG, Garcia-Pardo A, Sanchez-Madrid F (1993) A high affinity conformational state on VLA integrin heterodimers induced by an anti-beta 1 chain monoclonal antibody. *J Biol Chem* **268**, 9863-8.

Arroyo AG, Sanchez-Mateos P, Campanero MR, Martin-Padura I, Dejana E, Sanchez-Madrid F (1992) Regulation of the VLA integrin-ligand interactions through the beta 1 subunit. *J Cell Biol* **117**, 659-70.

Aspberg A, Miura R, Bourdoulous S, Shimonaka M, Heinegard D, Schachner M, Ruoslahti E, Yamaguchi Y (1997) The C-type lectin domains of lecticans, a family of aggregating chondroitin sulfate proteoglycans, bind tenascin-R by protein-protein interactions independent of carbohydrate moiety. *Proc Natl Acad Sci U S A* **94**, 10116-21.

Attur MG, Dave MN, Clancy RM, Patel IR, Abramson SB, Amin AR (2000) Functional genomic analysis in arthritis-affected cartilage: yin-yang regulation of inflammatory mediators by alpha 5 beta 1 and alpha V beta 3 integrins. *J Immunol* **164**, 2684-91.

Attur MG, Patel IR, Patel RN, Abramson SB, Amin AR (1998) Autocrine production of IL-1 beta by human osteoarthritis-affected cartilage and differential regulation of endogenous nitric oxide, IL-6, prostaglandin E2, and IL-8. *Proc Assoc Am Physicians* **110**, 65-72.

Avent N, Judson PA, *et al.* (1988) Monoclonal antibodies that recognize different membrane proteins that are deficient in Rhnull human erythrocytes. One group of antibodies reacts with a variety of cells and tissues whereas the other group is erythroid-specific. *Biochem J* **251**, 499-505.

Ayad S, Boot-handford, R. P., Humphries, M.J., (1994). In 'The Extracellular Matrix Facts Book'. (Academic Press: London)

Aydelotte MB, Schumacher, B.L., Kuettner, K.E. (1992) Heterogeneity of articular chondrocytes. In 'Articular cartilage and OA'. (Ed. KE Kuettner) pp. 237–249. (Raven Press: New York)

Aydelotte MB, Greenhill RR, Kuettner KE (1988) Differences between sub-populations of cultured bovine articular chondrocytes. II. Proteoglycan metabolism. *Connect Tissue Res* **18**, 223-34.

Aydelotte MB, Raiss RX, Caterson B, Kuettner KE (1992) Influence of interleukin-1 on the morphology and proteoglycan metabolism of cultured bovine articular chondrocytes. *Connect Tissue Res* **28**, 143-59.

Bachrach NM, Mow VC, Guilak F (1998) Incompressibility of the solid matrix of articular cartilage under high hydrostatic pressures. *J Biomech* **31**, 445-51.

Baeten D, Demetter P, *et al.* (2000) Comparative study of the synovial histology in rheumatoid arthritis, spondyloarthropathy, and osteoarthritis: influence of disease duration and activity. *Ann Rheum Dis* **59**, 945-53.

Baldwin CT, Reginato AM, Prockop DJ (1989) A new epidermal growth factor-like domain in the human core protein for the large cartilage-specific proteoglycan. Evidence for alternative splicing of the domain. *J Biol Chem* **264**, 15747-50.

Barnett CH, Davies, D.V., McConaill, M.A. (1961) 'Synovial Joints: Their Structure and Mechanics. London: Longmans.' (Longmans: London)

Bauer JS, Varner J, Schreiner C, Kornberg L, Nicholas R, Juliano RL (1993) Functional role of the cytoplasmic domain of the integrin alpha 5 subunit. *J Cell Biol* **122**, 209-21.

Bavington C, Wright M, Jobanputra P, Lin H, Morrison P, Brennan FR, Salter DM, Nuki G (1996) Accelerated proteoglycan synthesis in human chondrocytes following cyclical pressurization is mediated by beta1 integrin and stretch activated channels. *Br J Rheumatol* **35 suppl A1**, 110.

Bayliss MT, Ali SY (1978) Age-related changes in the composition and structure of human articular cartilage proteoglycans. *Biochem J* **176**, 683-93.

Bayliss MT, Urban JP, Johnstone B, Holm S (1986) In vitro method for measuring synthesis rates in the inter-vertebral disc. *J Orthop Res* **4**, 10-7.

Beekman B, Verzijl N, Bank RA, von der Mark K, TeKoppele JM (1997) Synthesis of collagen by bovine chondrocytes cultured in alginate; post-translational modifications and cell-matrix interaction. *Exp Cell Res* **237**, 135-41.

Benya PD (1990) Chondrocyte Culture: Introduction and survey of techniques. In 'Methods in cartilage research'. (Ed. A Maroudas, Kuettner, K.) pp. 85-89. (Academic Press: London)

Benya PD, Padilla SR, Nimni ME (1978) Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell* **15**, 1313-21.

Benya PD, Shaffer JD (1982) Dedifferentiated chondrocytes re-express the differentiated collagen phenotype when cultured in agarose gels. *Cell* **30**, 215-24.

Bewsey KE, Wen C, Purple C, Homandberg GA (1996) Fibronectin fragments induce the expression of stromelysin-1 mRNA and protein in bovine chondrocytes in monolayer culture. *Biochim Biophys Acta* **1317**, 55-64.

Bidanset DJ, LeBaron R, Rosenberg L, Murphy-Ullrich JE, Hook M (1992) Regulation of cell substrate adhesion: effects of small galactosaminoglycan-containing proteoglycans. *J Cell Biol* **118**, 1523-31.

Billinghurst RC, Dahlberg L, *et al.* (1997) Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J Clin Invest* **99**, 1534-45.

Blobe GC, Schiemann WP, Lodish HF (2000) Role of transforming growth factor beta in human disease. *N Engl J Med* **342**, 1350-8.

Blystone SD, Lindberg FP, LaFlamme SE, Brown EJ (1995) Integrin beta 3 cytoplasmic tail is necessary and sufficient for regulation of alpha 5 beta 1 phagocytosis by alpha v beta 3 and integrin-associated protein. *J Cell Biol* **130**, 745-54.

Bock HC, Michaeli P, Bode C, Schultz W, Kresse H, Herken R, Miosge N (2001) The small proteoglycans decorin and biglycan in human articular cartilage of late-stage osteoarthritis. *Osteoarthritis Cartilage* **9**, 654-63.

Bodary SC, McLean JW (1990) The integrin beta 1 subunit associates with the vitronectin receptor alpha v subunit to form a novel vitronectin receptor in a human embryonic kidney cell line. *J Biol Chem* **265**, 5938-41.

Bollet AJ (1967) Connective tissue polysaccharide metabolism and the pathogenesis of osteoarthritis. *Adv Intern Med* **13**, 33-60.

Bollet AJ, Handy JR, Sturgill BC (1963) Chondroitin sulfate concentration and protein-polysaccharide composition of articular cartilage in osteoarthritis. *J Clin Invest* **42**, 853-9.

Bollet AJ, Nance J (1966) Biochemical findings in normal and osteoarthritic articular cartilage.II. Chondroitin sulfate concentration and chain length. water, and ash content. *J Clin Invest* **45**, 1170-1177.

Bonaventure J, Kadhom N, Cohen-Solal L, Ng KH, Bourguignon J, Lasselin C, Freisinger P (1994) Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. *Exp Cell Res* **212**, 97-104.

Brand HS, de Koning MH, van Kampen GP, van der Korst JK (1991) Age related changes in the turnover of proteoglycans from explants of bovine articular cartilage. *J Rheumatol* **18**, 599-605.

Brandt KD (1986) Workshop on etiopathogenesis of osteoarthritis. *J Rheumatol* **13**, 1127-1159.

Brandt KD, Myers SL, Burr D, Albrecht M (1991) Osteoarthritic changes in canine articular cartilage, subchondral bone, and synovium fifty-four months after transection of the anterior cruciate ligament. *Arthritis Rheum* **34**, 1560-70.

Brennan MJ, Oldberg A, Pierschbacher MD, Ruoslahti E (1984) Chondroitin/dermatan sulfate proteoglycan in human fetal membranes. Demonstration of an antigenically similar proteoglycan in fibroblasts. *J Biol Chem* **259**, 13742-50.

Brighton CT, Kitajima T, Hunt RM (1984) Zonal analysis of cytoplasmic components of articular cartilage chondrocytes. *Arthritis Rheum* **27**, 1290-9.

Brighton CT, Lane JM, Koh JK (1974) In vitro rabbit articular cartilage organ model. II. 35S incorporation in various oxygen tensions. *Arthritis Rheum* **17**, 245-52.

Brinckerhoff CE (1991) Joint destruction in arthritis: metalloproteinases in the spotlight. *Arthritis Rheum* **34**, 1073-5.

Brocklehurst R, Bayliss MT, Maroudas A, Coysh HL, Freeman MA, Revell PA, Ali SY (1984) The composition of normal and osteoarthritic articular cartilage from human knee joints. With special reference to unicompartmental replacement and osteotomy of the knee. *J Bone Joint Surg Am* **66**, 95-106.

Brown E, Hooper L, Ho T, Gresham H (1990) Integrin-associated protein: a 50-kD plasma membrane antigen physically and functionally associated with integrins. *J Cell Biol* **111**, 2785-94.

Brown EJ, Frazier WA (2001) Integrin-associated protein (CD47) and its ligands. *Trends Cell Biol* **11**, 130-5.

Brown RA, Jones KL (1990) The synthesis and accumulation of fibronectin by human articular cartilage. *J Rheumatol* **17**, 65-72.

Bulstra SK, Buurman WA, Walenkamp GH, Van der Linden AJ (1989) Metabolic characteristics of in vitro cultured human chondrocytes in relation to the histopathologic grade of osteoarthritis. *Clin Orthop*, 294-302.

Burg MA, Tillet E, Timpl R, Stallcup WB (1996) Binding of the NG2 proteoglycan to type VI collagen and other extracellular matrix molecules. *J Biol Chem* **271**, 26110-6.

Burridge K, Chrzanowska-Wodnicka M (1996) Focal adhesions, contractility, and signaling. *Annu Rev Cell Dev Biol* **12**, 463-518.

Burridge K, Turner CE, Romer LH (1992) Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J Cell Biol* **119**, 893-903.

Burrows L, Clark K, Mould AP, Humphries MJ (1999) Fine mapping of inhibitory anti-alpha5 monoclonal antibody epitopes that differentially affect integrin-ligand binding. *Biochem J* **344 Pt 2**, 527-33.

Burrows RB, Sanantonio, J. D., Cintron, C. (1994) Corneal type-IV collagen tetramers bind to native corneal decorin. *Invest Opthalmol Visual Sci*, 1353-1355.

Burton-Wurster N, Butler M, Harter S, Colombo C, Quintavalla J, Swartzendurber D, Arsenis C, Lust G (1986) Presence of fibronectin in articular cartilage in two animal models of osteoarthritis. *J Rheumatol* **13**, 175-82.

Burton-Wurster N, Lust G (1990) Fibronectin and proteoglycan synthesis in long term cultures of cartilage explants in Ham's F12 supplemented with insulin and calcium: effects of the addition of TGF-beta. *Arch Biochem Biophys* **283**, 27-33.

Burton-Wurster N, Vernier-Singer M, Farquhar T, Lust G (1993) Effect of compressive loading and unloading on the synthesis of total protein, proteoglycan, and fibronectin by canine cartilage explants. *J Orthop Res* **11**, 717-29.

Buschmann MD, Gluzband YA, Grodzinsky AJ, Hunziker EB (1995) Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. *J Cell Sci* **108 (Pt 4)** 1497-508.

Bush PG, Hall AC (2003) The volume and morphology of chondrocytes within non-degenerate and degenerate human articular cartilage. *Osteoarthritis Cartilage.* **11**,242-51.

Byers PD, Maroudase A, Oztop F, Stockwell RA, Venn MF (1977) Histological and biochemical studies on cartilage from osteoarthrotic femoral heads with special reference to surface characteristics. *Connect Tissue Res* **5**, 41-9.

Calabro A, Hascall VC (1994) Effects of brefeldin A on aggrecan core protein synthesis and maturation in rat chondrosarcoma cells. *J Biol Chem* **269**, 22771-8.

Campbell MA, Handley CJ (1987) The effect of retinoic acid on proteoglycan turnover in bovine articular cartilage cultures. *Arch Biochem Biophys* **258**, 143-55.

Campbell MA, Handley CJ, Hascall VC, Campbell RA, Lowther DA (1984) Turnover of proteoglycans in cultures of bovine articular cartilage. *Arch Biochem Biophys* **234**, 275-89.

Cancedda R, Descalzi Cancedda F, Castagnola P (1995) Chondrocyte differentiation. *Int Rev Cytol* **159**, 265-358.

Cao L, Lee V, Adams ME, Kiani C, Zhang Y, Hu W, Yang BB (1999) beta-Integrin-collagen interaction reduces chondrocyte apoptosis. *Matrix Biol* **18**, 343-55.

Cao M, Westerhausen-Larson A, Niyibizi C, Kavalkovich K, Georgescu HI, Rizzo CF, Hebda PA, Stefanovic-Racic M, Evans CH (1997) Nitric oxide inhibits the synthesis of type-II collagen without altering Col2A1 mRNA abundance: prolyl hydroxylase as a possible target. *Biochem J* **324 (Pt 1)**, 305-10.

Cao Z, Huang K, Horwitz AF (1998) Identification of a domain on the integrin alpha5 subunit implicated in cell spreading and signaling. *J Biol Chem* **273**, 31670-9.

Carlson CS, Loeser RF, Johnstone B, Tulli HM, Dobson DB, Caterson B (1995) Osteoarthritis in cynomolgus macaques. II. Detection of modulated proteoglycan epitopes in cartilage and synovial fluid. *J Orthop Res* **13**, 399-409.

Carney SL, Billingham ME, Caterson B, Ratcliffe A, Bayliss MT, Hardingham TE, Muir H (1992) Changes in proteoglycan turnover in experimental canine osteoarthritic cartilage. *Matrix* **12**, 137-47.

Carney SL, Billingham ME, Muir H, Sandy JD (1985) Structure of newly synthesised (35S)-proteoglycans and (35S)-proteoglycan turnover products of cartilage explant cultures from dogs with experimental osteoarthritis. *J Orthop Res* **3**, 140-7.

Carney SL, Muir H (1988) The structure and function of cartilage proteoglycans. *Physiol Rev* **68**, 858-910.

Carroll G (1989) Measurement of sulphated glycosaminoglycans and proteoglycan fragments in arthritic synovial fluid. *Ann Rheum Dis* **48**, 17-24.

Caterson B, Christner JE, Baker JR, Couchman JR (1985) Production and characterization of monoclonal antibodies directed against connective tissue proteoglycans. *Fed Proc* **44**, 386-93.

Caterson B, Flannery CR, Hughes CE, Little CB (2000) Mechanisms involved in cartilage proteoglycan catabolism. *Matrix Biol* **19**, 333-44.

Caterson B, Griffin J, Mahmoodian F, Sorrell JM (1990) Monoclonal antibodies against chondroitin sulphate isomers: their use as probes for investigating proteoglycan metabolism. *Biochem Soc Trans* **18**, 820-3.

Cheresh DA, Berliner SA, Vicente V, Ruggeri ZM (1989) Recognition of distinct adhesive sites on fibrinogen by related integrins on platelets and endothelial cells. *Cell* **58**, 945-53.

Cheresh DA, Smith JW, Cooper HM, Quaranta V (1989) A novel vitronectin receptor integrin (alpha v beta x) is responsible for distinct adhesive properties of carcinoma cells. *Cell* **57**, 59-69.

Chevalier X (1993) Fibronectin, cartilage, and osteoarthritis. *Semin Arthritis Rheum* **22**, 307-18.

Chevalier X, Groult N, Labat-Robert J (1992) Biosynthesis and distribution of fibronectin in normal and osteoarthritic human cartilage. *Clin Physiol Biochem* **9**, 1-6.

Chiquet M (1999) Regulation of extracellular matrix gene expression by mechanical stress. *Matrix Biol* **18**, 417-26.

Chung J, Wang XQ, Lindberg FP, Frazier WA (1999) Thrombospondin-1 acts via IAP/CD47 to synergize with collagen in alpha2beta1-mediated platelet activation. *Blood* **94**, 642-8.

Clancy RM, Amin AR, Abramson SB (1998) The role of nitric oxide in inflammation and immunity. *Arthritis Rheum* **41**, 1141-51.

Clancy RM, Rediske J, Tang X, Nijher N, Frenkel S, Philips M, Abramson SB (1997) Outside-in signaling in the chondrocyte. Nitric oxide disrupts fibronectin-induced assembly of a subplasmalemmal actin/rho A/focal adhesion kinase signaling complex. *J Clin Invest* **100**, 1789-96.

Clark EA, Brugge JS (1995) Integrins and signal transduction pathways: the road taken. *Science* **268**, 233-9.

Clemmensen I, Holund B, Andersen RB (1983) Fibrin and fibronectin in rheumatoid synovial membrane and rheumatoid synovial fluid. *Arthritis Rheum* **26**, 479-85.

Cole AA, Kuettner KE (2002) Molecular basis for differences between human joints. *Cell Mol Life Sci* 59, 19-26.

Condic ML, Snow DM, Letourneau PC (1999) Embryonic neurons adapt to the inhibitory proteoglycan aggrecan by increasing integrin expression. *J Neurosci* 19, 10036-43.

Cooper D, Lindberg FP, Gamble JR, Brown EJ, Vadas MA (1995) Transendothelial migration of neutrophils involves integrin-associated protein (CD47). *Proc Natl Acad Sci U S A* 92, 3978-82.

Coster L, Fransson LA (1981) Isolation and characterization of dermatan sulphate proteoglycans from bovine sclera. *Biochem J* 193, 143-53.

Cs-Szabo G, Melching LI, Roughley PJ, Glant TT (1997) Changes in messenger RNA and protein levels of proteoglycans and link protein in human osteoarthritic cartilage samples. *Arthritis Rheum* 40, 1037-45.

Cs-Szabo G, Roughley PJ, Plaas AH, Glant TT (1995) Large and small proteoglycans of osteoarthritic and rheumatoid articular cartilage. *Arthritis Rheum* 38, 660-8.

Cutolo M, Picasso M, Ponassi M, Sun MZ, Balza E (1992) Tenascin and fibronectin distribution in human normal and pathological synovium. *J Rheumatol* 19, 1439-47.

D'Andrea P, Calabrese A, Grandolfo M (1998) Intercellular calcium signalling between chondrocytes and synovial cells in co-culture. *Biochem J* 329 (Pt 3), 681-7.

Damsky C, Tremble P, Werb Z (1992) Signal transduction via the fibronectin receptor: do integrins regulate matrix remodeling? *Matrix Suppl* 1, 184-91.

Damsky CH, Werb Z (1992) Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr Opin Cell Biol* 4, 772-81.

Davies PF, Tripathi SC (1993) Mechanical stress mechanisms and the cell. An endothelial paradigm. *Circ Res* 72, 239-45.

Davisson T, Kunig S, Chen A, Sah R, Ratcliffe A (2002) Static and dynamic compression modulate matrix metabolism in tissue engineered cartilage. *J Orthop Res* 20, 842-8.

De Luca S, Heinegard D, Hascall VC (1977) Chemical and physical changes in proteoglycans during development of chick limb bud chondrocytes grown in vitro. *J Biol Chem* 252, 6600-8.

de Wit PJ, Brandwagt BF, *et al.* (2002) The molecular basis of co-evolution between Cladosporium fulvum and tomato. *Antonie Van Leeuwenhoek* 81, 409-12.

De Witt MT, Handley CJ, Oakes BW, Lowther DA (1984) In vitro response of chondrocytes to mechanical loading. The effect of short term mechanical tension. *Connect Tissue Res* 12, 97-109.

Dedhar S, Hannigan GE (1996) Integrin cytoplasmic interactions and bidirectional transmembrane signalling. *Curr Opin Cell Biol* 8, 657-69.

Defilippi P, Silengo L, Tarone G (1992) Alpha 6 beta 1 integrin (laminin receptor) is down-regulated by tumor necrosis factor alpha and interleukin-1 beta in human endothelial cells. *J Biol Chem* 267, 18303-7.

Defilippi P, Truffa G, Stefanuto G, Altruda F, Silengo L, Tarone G (1991) Tumor necrosis factor alpha and interferon gamma modulate the expression of the vitronectin receptor (integrin beta 3) in human endothelial cells. *J Biol Chem* 266, 7638-45.

DeGroot J, Verzijl N, Bank RA, Lafeber FP, Bijlsma JW, TeKoppele JM (1999) Age-related decrease in proteoglycan synthesis of human articular chondrocytes: the role of nonenzymatic glycation. *Arthritis Rheum* **42**, 1003-9.

Delehedde M, Lyon M, Sergeant N, Rahmoune H, Fernig DG (2001) Proteoglycans: pericellular and cell surface multireceptors that integrate external stimuli in the mammary gland. *J Mammary Gland Biol Neoplasia* **6**, 253-73.

Demarteau O, Wendt D, Braccini A, Jakob M, Schafer D, Heberer M, Martin I (2003) Dynamic compression of cartilage constructs engineered from expanded human articular chondrocytes. *Biochem Biophys Res Commun* **310**, 580-8.

Denhardt DT, Noda M (1998) Osteopontin expression and function: role in bone remodeling. *J Cell Biochem Suppl* **30-31**, 92-102.

Dinarello CA (1996) Biologic basis for interleukin-1 in disease. *Blood* **87**, 2095-147.

Dinarello CA (2000) The role of the interleukin-1-receptor antagonist in blocking inflammation mediated by interleukin-1. *N Engl J Med* **343**, 732-4.

Disatnik MH, Rando TA (1999) Integrin-mediated muscle cell spreading. The role of protein kinase c in outside-in and inside-out signaling and evidence of integrin cross-talk. *J Biol Chem* **274**, 32486-92.

Doege K, Sasaki M, Horigan E, Hassell JR, Yamada Y (1987) Complete primary structure of the rat cartilage proteoglycan core protein deduced from cDNA clones. *J Biol Chem* **262**, 17757-67.

Domowicz MS, Pirok EW, 3rd, Novak TE, Schwartz NB (2000) Role of the C-terminal G3 domain in sorting and secretion of aggrecan core protein and ubiquitin-mediated degradation of accumulated mutant precursors. *J Biol Chem* **275**, 35098-105.

Dourado GS, Adams ME, Matyas JR, Huang D (1996) Expression of biglycan, decorin and fibromodulin in the hypertrophic phase of experimental osteoarthritis. *Osteoarthritis Cartilage* **4**, 187-96.

Dozin B, Malpeli M, Camardella L, Cancedda R, Pietrangelo A (2002) Response of young, aged and osteoarthritic human articular chondrocytes to inflammatory cytokines: molecular and cellular aspects. *Matrix Biol* **21**, 449-59.

Dreier R, Wallace S, Fuchs S, Bruckner P, Grassel S (2001) Paracrine interactions of chondrocytes and macrophages in cartilage degradation: articular chondrocytes provide factors that activate macrophage-derived pro-gelatinase B (pro-MMP-9). *J Cell Sci* **114**, 3813-22.

Dudhia J, Davidson CM, Wells TM, Vynios DH, Hardingham TE, Bayliss MT (1996) Age-related changes in the content of the C-terminal region of aggrecan in human articular cartilage. *Biochem J* **313 (Pt 3)**, 933-40.

Durr J, Goodman S, Potocnik A, von der Mark H, von der Mark K (1993) Localization of beta 1-integrins in human cartilage and their role in chondrocyte adhesion to collagen and fibronectin. *Exp Cell Res* **207**, 235-44.

Dutu A, Vlaicu-Rus V, Bolosiu HD, Parasca I, Cristea A (1986) Fibronectin in plasma and synovial fluid of patients with rheumatic diseases. *Med Interne* **24**, 61-8.

Eckstein F, Faber S, Muhlbauer R, Hohe J, Englmeier KH, Reiser M, Putz R (2002) Functional adaptation of human joints to mechanical stimuli. *Osteoarthritis Cartilage* **10**, 44-50.

Eger W, Schumacher BL, Mollenhauer J, Kuettner KE, Cole AA (2002) Human knee and ankle cartilage explants: catabolic differences. *J Orthop Res* **20**, 526-34.

Ehrlich M, G., Mankin H, J. (1980) Biochemical changes in osteoarthritis. In 'The aetiopathogenesis of osteoarthritis'. (Ed. G Nuki) pp. 29-36)

Elder SH, Goldstein SA, Kimura JH, Soslowsky LJ, Spengler DM (2001) Chondrocyte differentiation is modulated by frequency and duration of cyclic compressive loading. *Ann Biomed Eng* **29**, 476-82.

Elder SH, Kimura JH, Soslowsky LJ, Lavagnino M, Goldstein SA (2000) Effect of compressive loading on chondrocyte differentiation in agarose cultures of chick limb-bud cells. *J Orthop Res* **18**, 78-86.

Elices MJ, Tsai V, *et al.* (1994) Expression and functional significance of alternatively spliced CS1 fibronectin in rheumatoid arthritis microvasculature. *J Clin Invest* **93**, 405-16.

Enobakhare BO, Bader DL, Lee DA (1996) Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue. *Anal Biochem* **243**, 189-91.

Enomoto-Iwamoto M, Iwamoto M, Nakashima K, Mukudai Y, Boettiger D, Pacifici M, Kurisu K, Suzuki F (1997) Involvement of alpha5beta1 integrin in matrix interactions and proliferation of chondrocytes. *J Bone Miner Res* **12**, 1124-32.

Farndale RW, Buttle DJ, Barrett AJ (1986) Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* **883**, 173-7.

Farndale RW, Sayers CA, Barrett AJ (1982) A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. *Connect Tissue Res* **9**, 247-8.

Farquhar T, Xia Y, Mann K, Bertram J, Burton-Wurster N, Jelinski L, Lust G (1996) Swelling and fibronectin accumulation in articular cartilage explants after cyclical impact. *J Orthop Res* **14**, 417-23.

Faull RJ, Kovach NL, Harlan JM, Ginsberg MH (1993) Affinity modulation of integrin alpha 5 beta 1: regulation of the functional response by soluble fibronectin. *J Cell Biol* **121**, 155-62.

Fava R, Olsen N, Keski-Oja J, Moses H, Pincus T (1989) Active and latent forms of transforming growth factor beta activity in synovial effusions. *J Exp Med* **169**, 291-6.

Fellini SA, Hascall VC, Kimura JH (1984) Localization of proteoglycan core protein in subcellular fractions isolated from rat chondrosarcoma chondrocytes. *J Biol Chem* **259**, 4634-41.

Firestein GS, Zvaifler NJ (1990) How important are T cells in chronic rheumatoid synovitis? *Arthritis Rheum* **33**, 768-73.

Fisher LW, Hawkins GR, Tuross N, Termine JD (1987) Purification and partial characterization of small proteoglycans I and II, bone sialoproteins I and II, and osteonectin from the mineral compartment of developing human bone. *J Biol Chem* **262**, 9702-8.

Fisher LW, Termine JD, *et al.* (1983) Proteoglycans of developing bone. *J Biol Chem* **258**, 6588-94.

Flannery C, Stanescu V, Morgelin M, Boynton R, Gordy J, Sandy J (1992) Variability in the G3 domain content of bovine aggrecan from cartilage extracts and chondrocyte cultures. *Arch Biochem Biophys* **297**, 52-60.

Flannery CR, Lark MW, Sandy JD (1992) Identification of a stromelysin cleavage site within the interglobular domain of human aggrecan. Evidence for proteolysis at this site in vivo in human articular cartilage. *J Biol Chem* **267**, 1008-14.

Flannery CR, Little CB, Caterson B, Hughes CE (1999) Effects of culture conditions and exposure to catabolic stimulators (IL-1 and retinoic acid) on the expression of matrix metalloproteinases (MMPs) and disintegrin metalloproteinases (ADAMs) by articular cartilage chondrocytes. *Matrix Biol* **18**, 225-37.

Flannery CR, Little CB, Hughes CE, Caterson B (1999) Expression of ADAMTS homologues in articular cartilage. *Biochem Biophys Res Commun* **260**, 318-22.

Flannery CR, Little CB, Hughes CE, Curtis CL, Caterson B, Jones SA (2000) IL-6 and its soluble receptor augment aggrecanase-mediated proteoglycan catabolism in articular cartilage. *Matrix Biol* **19**, 549-53.

Fornaro M, Languino LR (1997) Alternatively spliced variants: a new view of the integrin cytoplasmic domain. *Matrix Biol* **16**, 185-93.

Franzen A, Heinegard D (1984) Characterization of proteoglycans from the calcified matrix of bovine bone. *Biochem J* **224**, 59-66.

Frazier WA, Gao AG, Dimitry J, Chung J, Brown EJ, Lindberg FP, Linder ME (1999) The thrombospondin receptor integrin-associated protein (CD47) functionally couples to heterotrimeric Gi. *J Biol Chem* **274**, 8554-60.

Fujii K, Tsuji M, Tajima M (1999) Rheumatoid arthritis: a synovial disease? *Ann Rheum Dis* **58**, 727-30.

Fujimoto TT, Katsutani S, Shimomura T, Fujimura K (2003) Thrombospondin-bound integrin-associated protein (CD47) physically and functionally modifies integrin alphaIIbbeta3 by its extracellular domain. *J Biol Chem* **278**, 26655-65.

Funderburgh JL, Funderburgh ML, Mann MM, Corpuz L, Roth MR (2001) Proteoglycan expression during transforming growth factor beta -induced keratocyte-myofibroblast transdifferentiation. *J Biol Chem* **276**, 44173-8.

Gacesa P (1998) Bacterial alginate biosynthesis--recent progress and future prospects. *Microbiology* **144 (Pt 5)**, 1133-43.

Gallagher JT (1989) The extended family of proteoglycans: social residents of the pericellular zone. *Curr Opin Cell Biol* **1**, 1201-18.

Gao AG, Lindberg FP, Dimitry JM, Brown EJ, Frazier WA (1996a) Thrombospondin modulates alpha v beta 3 function through integrin-associated protein. *J Cell Biol* **135**, 533-44.

Gao AG, Lindberg FP, Finn MB, Blystone SD, Brown EJ, Frazier WA (1996b) Integrin-associated protein is a receptor for the C-terminal domain of thrombospondin. *J Biol Chem* **271**, 21-4.

Gay S, Gay RE, Koopman WJ (1993) Molecular and cellular mechanisms of joint destruction in rheumatoid arthritis: two cellular mechanisms explain joint destruction? *Ann Rheum Dis* **52 Suppl 1**, S39-47.

Giancotti FG (1997) Integrin signaling: specificity and control of cell survival and cell cycle progression. *Curr Opin Cell Biol* **9**, 691-700.

Giuffrida A, Scarpa S, Birarelli P, Modesti A (2004) The interaction of tenascin-C with fibronectin modulates the migration and specific metalloprotease activity in human mesothelioma cell lines of different histotype. *Int J Oncol* **25**, 745-50.

Glossl J, Beck M, Kresse H (1984) Biosynthesis of proteodermatan sulfate in cultured human fibroblasts. *J Biol Chem* **259**, 14144-50.

Godman GC, Lane N (1964) On the Site of Sulfation in the Chondrocyte. *J Cell Biol* **21**, 353-66.

Goldring MB (2000) The role of the chondrocyte in osteoarthritis. *Arthritis Rheum* **43**, 1916-26.

Goldring MB, Sandell LJ, Stephenson ML, Krane SM (1986) Immune interferon suppresses levels of procollagen mRNA and type II collagen synthesis in cultured human articular and costal chondrocytes. *J Biol Chem* **261**, 9049-55.

Gough AK, Lilley J, Eyre S, Holder RL, Emery P (1994) Generalised bone loss in patients with early rheumatoid arthritis. *Lancet* **344**, 23-7.

Graf R, Apenberg S, Freyberg M, Friedl P (2003) A common mechanism for the mechanosensitive regulation of apoptosis in different cell types and for different mechanical stimuli. *Apoptosis* **8**, 531-8.

Gray ML, Pizzanelli AM, Grodzinsky AJ, Lee RC (1988) Mechanical and physiochemical determinants of the chondrocyte biosynthetic response. *J Orthop Res* **6**, 777-92.

Green JM, Zhelesnyak A, Chung J, Lindberg FP, Sarfati M, Frazier WA, Brown EJ (1999) Role of cholesterol in formation and function of a signaling complex involving alphavbeta3, integrin-associated protein (CD47), and heterotrimeric G proteins. *J Cell Biol* **146**, 673-82.

Grierson JP, Meldolesi J (1995) Shear stress-induced [Ca2+]i transients and oscillations in mouse fibroblasts are mediated by endogenously released ATP. *J Biol Chem* **270**, 4451-6.

Grodzinsky AJ (2004) Mechanical regulation of chondrocyte gene expression and biosynthesis in cartilage explants: links to molecular mechanics (abstract). In 'Molecular approaches to osteoarthritis'. London pp. 48-50

Groux H, Huet S, Valentin H, Pham D, Bernard A (1989) Suppressor effects and cyclic AMP accumulation by the CD29 molecule of CD4+ lymphocytes. *Nature* **339**, 152-4.

Gudi SRP, Frangos, J.A., (1994) Mechanical signal transduction and G proteins. In 'Cell mechanics and cellular engineering'. (Ed.VC Mow, Guilak, F., Tran-Son-Tay, R., Hochmuth, R.M.) pp. 295-307. (Springer-Verlag: New York)

Guerassimov A, Zhang Y, Cartman A, Rosenberg LC, Esdaile J, Fitzcharles MA, Poole AR (1999) Immune responses to cartilage link protein and the G1 domain of proteoglycan aggrecan in patients with osteoarthritis. *Arthritis Rheum* **42**, 527-33.

Guilak F, Donahue, H.J, Zell, R.A., Grande, D., McLeod, K.J., Rubin, C.T. (1994) Deformation-induced calcium signalling in articular chondrocytes. In 'Cell Mechanics and Cellular Engineering'. (Ed.VC Mow, Guilak, F., Tran-Son-Tay, R., Hochmuth, R.M.) pp. 380-397. (Springer-Verlag: New York)

Guo JF, Jourdian GW, MacCallum DK (1989) Culture and growth characteristics of chondrocytes encapsulated in alginate beads. *Connect Tissue Res* **19**, 277-97.

Hagg R, Bruckner P, Hedbom E (1998) Cartilage fibrils of mammals are biochemically heterogeneous: differential distribution of decorin and collagen IX. *J Cell Biol* **142**, 285-94.

Hall AC, Urban JP, Gehl KA (1991) The effects of hydrostatic pressure on matrix synthesis in articular cartilage. *J Orthop Res* **9**, 1-10.

Handley CJ, Ng, C.K., Curtis, A.J. (1990) Short- and long-term explant culture of cartilage: Introduction. In 'Methods in cartilage research'. (Ed. A Maroudas, Kuettner, K.) pp. 105-108. (Academic Press: London)

Handley CJ, Buttle DJ (1995) Assay of proteoglycan degradation. *Methods Enzymol* **248**, 47-58.

Haralson MA, Hassell. J.R. (1995) The extracellular matrix-an overview. In 'Extracellular Matrix'. (Ed. MA Haralson, Hassell. J.R.) pp. pp 1-30. (Oxford University Press: New York)

Hardingham T (1981) Proteoglycans: their structure, interactions and molecular organization in cartilage. *Biochem Soc Trans* **9**, 489-97.

Hardingham T, Bayliss M (1990) Proteoglycans of articular cartilage: changes in aging and in joint disease. *Semin Arthritis Rheum* **20**, 12-33.

Hardingham TE, Fosang AJ (1992) Proteoglycans: many forms and many functions. *Faseb J* **6**, 861-70.

Hardingham TE, Fosang AJ, Dudhia J (1994) The structure, function and turnover of aggrecan, the large aggregating proteoglycan from cartilage. *Eur J Clin Chem Clin Biochem* **32**, 249-57.

Hascall GK, Yanagishita, M., Calabro, A., Midura, R., Rada, J.A., Hassell, J.R. (1995) Isolation and charactrization of proteoglycan core protein. In 'Extracellular Matrix'. (Ed. MA Haralson, Hassell. J.R.) pp. 221-240. (Oxford University Press: New York)

Hascall GK, Kimura JH (1981) The ultrastructure of cultures from the Swarm rat chondrosarcoma. *Anat Rec* **200**, 287-92.

Hascall VC (1981) Proteoglycans: Structure and function. In 'Biology of carbohydrates'. (Ed. MH Ginsberg, Robbins, P.) pp. 1-50. (John Wiley & Sons: New York)

Hascall VC, Heinegard D (1974) Aggregation of cartilage proteoglycans. I. The role of hyaluronic acid. *J Biol Chem* **249**, 4232-41.

Hascall VC, Jr., Kimura JH (1981) Biosynthesis, secretion, and aggregation of proteoglycans by rat chondrosarcoma chondrocytes. *Ala J Med Sci* **18**, 29-35.

Hascall VC, Kimura JH (1982) Proteoglycans: isolation and characterization. *Methods Enzymol* **82 Pt A**, 769-800.

Hassell JR, Kimura JH, Hascall VC (1986) Proteoglycan core protein families. *Annu Rev Biochem* **55**, 539-67.

Hasty KA, Pourmotabbed TF, Goldberg GI, Thompson JP, Spinella DG, Stevens RM, Mainardi CL (1990) Human neutrophil collagenase. A distinct gene product with homology to other matrix metalloproteinases. *J Biol Chem* **265**, 11421-4.

Hauselmann HJ, Aydelotte MB, Schumacher BL, Kuettner KE, Gitelis SH, Thonar EJ (1992) Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads. *Matrix* **12**, 116-29.

Hauselmann HJ, Fernandes RJ, Mok SS, Schmid TM, Block JA, Aydelotte MB, Kuettner KE, Thonar EJ (1994) Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. *J Cell Sci* **107 (Pt 1)**, 17-27.

Haynes BF, Hale LP, Patton KL, Martin ME, McCallum RM (1991) Measurement of an adhesion molecule as an indicator of inflammatory disease activity. Up-regulation of the receptor for hyaluronate (CD44) in rheumatoid arthritis. *Arthritis Rheum* 34, 1434-43.

Hedbom E, Hauselmann HJ (2002) Molecular aspects of pathogenesis in osteoarthritis: the role of inflammation. *Cell Mol Life Sci* 59, 45-53.

Hedbom E, Heinegard D (1993) Binding of fibromodulin and decorin to separate sites on fibrillar collagens. *J Biol Chem* 268, 27307-12.

Heinegard D, Oldberg A (1989) Structure and biology of cartilage and bone matrix noncollagenous macromolecules. *Faseb J* 3, 2042-51.

Heino J, Ignotz RA, Hemler ME, Crouse C, Massague J (1989) Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. *J Biol Chem* 264, 380-8.

Helminen HJ (1987) 'Joint loading : biology and health of articular structures.' (John Wright: Bristol)

Hermann P, Armant M, *et al.* (1999) The vitronectin receptor and its associated CD47 molecule mediates proinflammatory cytokine synthesis in human monocytes by interaction with soluble CD23. *J Cell Biol* 144, 767-75.

Hickery MS, Bayliss MT (1998) Interleukin-1 induced nitric oxide inhibits sulphation of glycosaminoglycan chains in human articular chondrocytes. *Biochim Biophys Acta* 1425, 282-90.

Hodge WA, Fijan RS, Carlson KL, Burgess RG, Harris WH, Mann RW (1986) Contact pressures in the human hip joint measured in vivo. *Proc Natl Acad Sci U S A* 83, 2879-83.

Hollander AP, Atkins RM, Eastwood DM, Dieppe PA, Elson CJ (1991) Human cartilage is degraded by rheumatoid arthritis synovial fluid but not by recombinant cytokines in vitro. *Clin Exp Immunol* 83, 52-7.

Hollander AP, Heathfield TF, Webber C, Iwata Y, Bourne R, Rorabeck C, Poole AR (1994) Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. *J Clin Invest* 93, 1722-32.

Holm S, Maroudas A, Urban JP, Selstam G, Nachemson A (1981) Nutrition of the intervertebral disc: solute transport and metabolism. *Connect Tissue Res* 8, 101-19.

Holmes MW, Bayliss MT, Muir H (1988) Hyaluronic acid in human articular cartilage. Age-related changes in content and size. *Biochem J* 250, 435-41.

Holmvall K, Camper L, Johansson S, Kimura JH, Lundgren-Akerlund E (1995) Chondrocyte and chondrosarcoma cell integrins with affinity for collagen type II and their response to mechanical stress. *Exp Cell Res* 221, 496-503.

Homandberg GA (1999) Potential regulation of cartilage metabolism in osteoarthritis by fibronectin fragments. *Front Biosci* 4, D713-30.

Homandberg GA, Costa V, Ummadi V, Pichika R (2002) Antisense oligonucleotides to the integrin receptor subunit alpha(5) decrease fibronectin fragment mediated cartilage chondrolysis. *Osteoarthritis Cartilage* 10, 381-93.

Homandberg GA, Hui F (1994) Arg-Gly-Asp-Ser peptide analogs suppress cartilage chondrolytic activities of integrin-binding and nonbinding fibronectin fragments. *Arch Biochem Biophys* 310, 40-8.

Homandberg GA, Hui F (1994) High concentrations of fibronectin fragments cause short-term catabolic effects in cartilage tissue while lower concentrations cause continuous anabolic effects. *Arch Biochem Biophys* **311**, 213-8.

Homandberg GA, Meyers R, Xie DL (1992) Fibronectin fragments cause chondrolysis of bovine articular cartilage slices in culture. *J Biol Chem* **267**, 3597-604.

Horowitz SH (1986) Ganglioside therapy in diabetic neuropathy. *Muscle Nerve* **9**, 531-6.

Hughes CE, Buttner FH, Eidenmuller B, Caterson B, Bartnik E (1997) Utilization of a recombinant substrate rAgg1 to study the biochemical properties of aggrecanase in cell culture systems. *J Biol Chem* **272**, 20269-74.

Hughes CE, Caterson B, Fosang AJ, Roughley PJ, Mort JS (1995) Monoclonal antibodies that specifically recognize neoepitope sequences generated by 'aggrecanase' and matrix metalloproteinase cleavage of aggrecan: application to catabolism in situ and in vitro. *Biochem J* **305 (Pt 3)**, 799-804.

Hughes CE, Little CB, Buttner FH, Bartnik E, Caterson B (1998) Differential expression of aggrecanase and matrix metalloproteinase activity in chondrocytes isolated from bovine and porcine articular cartilage. *J Biol Chem* **273**, 30576-82.

Hughes DE, Salter DM, Dedhar S, Simpson R (1993) Integrin expression in human bone. *J Bone Miner Res* **8**, 527-33.

Hughes PE, Pfaff M (1998) Integrin affinity modulation. *Trends Cell Biol* **8**, 359-64.

Huhtala P, Humphries MJ, McCarthy JB, Tremble PM, Werb Z, Damsky CH (1995) Cooperative signaling by alpha 5 beta 1 and alpha 4 beta 1 integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. *J Cell Biol* **129**, 867-79.

Hulth A, Lindberg L, Telhag H (1972) Mitosis in human osteoarthritic cartilage. *Clin Orthop* **84**, 197-9.

Humbel R (1974) Identification and quantitation of keratan sulfate in urine. *Clin Chim Acta* **52**, 173-7.

Hung CT, Pollack SR, Reilly TM, Brighton CT (1995) Real-time calcium response of cultured bone cells to fluid flow. *Clin Orthop*, 256-69.

Hunziker EB, Quinn TM, Hauselmann HJ (2002) Quantitative structural organization of normal adult human articular cartilage. *Osteoarthritis Cartilage* **10**, 564-72.

Hynes RO (1987) Integrins: a family of cell surface receptors. *Cell* **48**, 549-54.

Hynes RO (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.

Hynes RO (1996) Targeted mutations in cell adhesion genes: what have we learned from them? *Dev Biol* **180**, 402-12.

Ignotz RA, Massague J (1986) Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol Chem* **261**, 4337-45.

Ikenoue T, Trindade MC, Lee MS, Lin EY, Schurman DJ, Goodman SB, Smith RL (2003) Mechanoregulation of human articular chondrocyte aggrecan and type II collagen expression by intermittent hydrostatic pressure in vitro. *J Orthop Res* **21**, 110-6.

Ilic MZ, Handley CJ, Robinson HC, Mok MT (1992) Mechanism of catabolism of aggrecan by articular cartilage. *Arch Biochem Biophys* **294**, 115-22.

Imai K (1997) [Matrix metalloproteinases and cancer cell invasion and metastasis]. *Tanpakushitsu Kakusan Koso* **42**, 1694-700.

Inerot S, Heinegard D, Audell L, Olsson SE (1978) Articular cartilage proteoglycans in aging and osteoarthritis. *Biochem J* **169**, 143-56.

Ingber D (1991) Integrins as mechanochemical transducers. *Curr Opin Cell Biol* **3**, 841-8.

Iozzo RV (1999) The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins. *J Biol Chem* **274**, 18843-6.

Ishibashi Y, Claus S, Relman DA (1994) Bordetella pertussis filamentous hemagglutinin interacts with a leukocyte signal transduction complex and stimulates bacterial adherence to monocyte CR3 (CD11b/CD18). *J Exp Med* **180**, 1225-33.

Ishikawa H, Hirata S, Nishibayashi Y, Imura S, Kubo H, Ohno O (1994) The role of adhesion molecules in synovial pannus formation in rheumatoid arthritis. *Clin Orthop Relat Res*, 297-303.

Ishikawa H, Ohno O, Saura R, Matsubara T, Kuroda T, Hirohata K (1991) Cytokine enhancement of monocyte/synovial cell attachment to the surface of cartilage: a possible trigger of pannus formation in arthritis. *Rheumatol Int* **11**, 31-6.

Jalali S, del Pozo MA, Chen K, Miao H, Li Y, Schwartz MA, Shyy JY, Chien S (2001) Integrin-mediated mechanotransduction requires its dynamic interaction with specific extracellular matrix (ECM) ligands. *Proc Natl Acad Sci U S A* **98**, 1042-6.

Janmey PA (1998) The cytoskeleton and cell signaling: component localization and mechanical coupling. *Physiol Rev* **78**, 763-81.

Jarvelainen HT, Kinsella MG, Wight TN, Sandell LJ (1991) Differential expression of small chondroitin/dermatan sulfate proteoglycans, PG-I/biglycan and PG-II/decorin, by vascular smooth muscle and endothelial cells in culture. *J Biol Chem* **266**, 23274-81.

Jin G, Sah RL, Li YS, Lotz M, Shyy JY, Chien S (2000) Biomechanical regulation of matrix metalloproteinase-9 in cultured chondrocytes. *J Orthop Res* **18**, 899-908.

Jobanputra P, Corrigall V, Kingsley G, Panayi G (1992) Cellular responses to human chondrocytes: absence of allogeneic responses in the presence of HLA-DR and ICAM-1. *Clin Exp Immunol* **90**, 336-44.

Jobanputra P, Lin H, Jenkins K, Bavington C, Brennan FR, Nuki G, Salter DM, Godolphin JL (1996) Modulation of human chondrocyte integrins by inflammatory synovial fluid. *Arthritis Rheum* **39**, 1430-2.

Johnson BA, Haines GK, Harlow LA, Koch AE (1993) Adhesion molecule expression in human synovial tissue. *Arthritis Rheum* **36**, 137-46.

Jones KL, Brown M, Ali SY, Brown RA (1987) An immunohistochemical study of fibronectin in human osteoarthritic and disease free articular cartilage. *Ann Rheum Dis* **46**, 809-15.

Joosten LA, Lubberts E, Helsen MM, Saxne T, Coenen-de Roo CJ, Heinegard D, van den Berg WB (1999) Protection against cartilage and bone destruction by systemic interleukin-4 treatment in established murine type II collagen-induced arthritis. *Arthritis Res* **1**, 81-91.

Jubb RW (1984) Differential responses of human articular cartilage to retinol. *Ann Rheum Dis* 43, 833-40.

Juliano RL, Haskill S (1993) Signal transduction from the extracellular matrix. *J Cell Biol* 120, 577-85.

Kamada H, Masuda K, D'Souza AL, Lenz ME, Pietryla D, Otten L, Thonar EJ (2002) Age-related differences in the accumulation and size of hyaluronan in alginate culture. *Arch Biochem Biophys* 408, 192-9.

Kaplan G, Gaudernack G (1982) In vitro differentiation of human monocytes. Differences in monocyte phenotypes induced by cultivation on glass or on collagen. *J Exp Med* 156, 1101-14.

Kempson GE, Muir H, Pollard C, Tuke M (1973) The tensile properties of the cartilage of human femoral condyles related to the content of collagen and glycosaminoglycans. *Biochim Biophys Acta* 297, 456-72.

Kiani C, Lee V, Cao L, Chen L, Wu Y, Zhang Y, Adams ME, Yang BB (2001) Roles of aggrecan domains in biosynthesis, modification by glycosaminoglycans and product secretion. *Biochem J* 354, 199-207.

Kim JE, Jeong HW, Nam JO, Lee BH, Choi JY, Park RW, Park JY, Kim IS (2002) Identification of motifs in the fasciclin domains of the transforming growth factor-beta-induced matrix protein betaig-h3 that interact with the alphavbeta5 integrin. *J Biol Chem* 277, 46159-65.

Kim YJ, Grodzinsky AJ, Plaas AH (1996) Compression of cartilage results in differential effects on biosynthetic pathways for aggrecan, link protein, and hyaluronan. *Arch Biochem Biophys* 328, 331-40.

Kim YJ, Sah RL, Doong JY, Grodzinsky AJ (1988) Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Anal Biochem* 174, 168-76.

Kim YJ, Sah RL, Grodzinsky AJ, Plaas AH, Sandy JD (1994) Mechanical regulation of cartilage biosynthetic behavior: physical stimuli. *Arch Biochem Biophys* 311, 1-12.

Kimura JH, Kuettner, K.E (1986) Studies on the synthesis and assembly of cartilage proteoglycan. In 'Articular cartilage biochemistry'. (Ed. K Kuettner, Schleyerbach, R., Hascall, V.C.) pp. 113-124. (Raven Press: New York)

Kimura JH, Thonar EJ, Hascall VC, Reiner A, Poole AR (1981) Identification of core protein, an intermediate in proteoglycan biosynthesis in cultured chondrocytes from the Swarm rat chondrosarcoma. *J Biol Chem* 256, 7890-7.

Kinsella MG, Wight TN (1988) Isolation and characterization of dermatan sulfate proteoglycans synthesized by cultured bovine aortic endothelial cells. *J Biol Chem* 263, 19222-31.

Kinsella MG, Wight TN (1990) Formation of high molecular weight dermatan sulfate proteoglycan in bovine aortic endothelial cell cultures. Evidence for transglutaminase-catalyzed cross-linking to fibronectin. *J Biol Chem* 265, 17891-8.

Kiviranta I, Jurvelin J, Tammi M, Saamanen AM, Helminen HJ (1987b) Weight bearing controls glycosaminoglycan concentration and articular cartilage thickness in the knee joints of young beagle dogs. *Arthritis Rheum* 30, 801-9.

Kiviranta I, Tammi M, Jurvelin J, Helminen HJ (1987a) Topographical variation of glycosaminoglycan content and cartilage thickness in canine knee (stifle) joint cartilage. Application of the microspectrophotometric method. *J Anat* 150, 265-76.

Kiviranta I, Tammi M, Jurvelin J, Saamanen AM, Helminen HJ (1988) Moderate running exercise augments glycosaminoglycans and thickness of articular cartilage in the knee joint of young beagle dogs. *J Orthop Res* 6, 188-95.

Kjellen L, Lindahl U (1991) Proteoglycans: structures and interactions. *Annu Rev Biochem* 60, 443-75.

Klein-Nulend J, Veldhuijzen JP, van de Stadt RJ, van Kampen GPJ, Kuijer R, Burger EH (1996) Influence of intermittent compressive force on proteoglycan content in calcifying growth plate cartilage *in vitro*. *J Biol Chem* 262:15490-5.

Knight MM, Lee DA, Bader DL (1998) The influence of elaborated pericellular matrix on the deformation of isolated articular chondrocytes cultured in agarose. *Biochim Biophys Acta* 1405, 67-77.

Knight MM, van de Breevaart Bravenboer J, Lee DA, van Osch GJ, Weinans H, Bader DL (2002) Cell and nucleus deformation in compressed chondrocyte-alginate constructs: temporal changes and calculation of cell modulus. *Biochim Biophys Acta* 1570, 1-8.

Knoeppel DW, Liu J, Meyers EA, Shore SG (1998) Heterometallic One-Dimensional Arrays Containing Cyanide-Bridged Lanthanide(III) and Transition Metals. *Inorg Chem* 37, 4828-4837.

Knudson CB (1993) Hyaluronan receptor-directed assembly of chondrocyte pericellular matrix. *J Cell Biol* 120, 825-34.

Ko KS, McCulloch CA (2001) Intercellular mechanotransduction: cellular circuits that coordinate tissue responses to mechanical loading. *Biochem Biophys Res Commun* 285, 1077-83.

Kolettas E, Buluwela L, Bayliss MT, Muir HI (1995) Expression of cartilage-specific molecules is retained on long-term culture of human articular chondrocytes. *J Cell Sci* 108 (Pt 5), 1991-9.

Koopman WJ, Gay S (1988) The MRL-lpr/lpr mouse. A model for the study of rheumatoid arthritis. *Scand J Rheumatol Suppl* 75, 284-9.

Kornberg LJ, Earp HS, Turner CE, Prockop C, Juliano RL (1991) Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of beta 1 integrins. *Proc Natl Acad Sci U S A* 88, 8392-6.

Korver GH, van de Stadt RJ, van Kampen GP, van der Korst JK (1990) Composition of proteoglycans synthesized in different layers of cultured anatomically intact articular cartilage. *Matrix* 10, 394-401.

Korver TH, van de Stadt RJ, Kiljan E, van Kampen GP, van der Korst JK (1992) Effects of loading on the synthesis of proteoglycans in different layers of anatomically intact articular cartilage in vitro. *J Rheumatol* 19, 905-12.

Koshino T, Wada S, Ara Y, Saito T (2003) Regeneration of degenerated articular cartilage after high tibial valgus osteotomy for medial compartmental osteoarthritis of the knee. *Knee* 10, 229-36.

Kozaci LD, Buttle DJ, Hollander AP (1997) Degradation of type II collagen, but not proteoglycan, correlates with matrix metalloproteinase activity in cartilage explant cultures. *Arthritis Rheum* 40, 164-74.

Kuettner KE (1992) Biochemistry of articular cartilage in health and disease. *Clin Biochem* 25, 155-63.

Kuettner KE, Aydelotte MB, Thonar EJ (1991) Articular cartilage matrix and structure: a minireview. *J Rheumatol Suppl* **27**, 46-8.

Kuno K, Kanada N, Nakashima E, Fujiki F, Ichimura F, Matsushima K (1997) Molecular cloning of a gene encoding a new type of metalloproteinase-disintegrin family protein with thrombospondin motifs as an inflammation associated gene. *J Biol Chem* **272**, 556-62.

Kupchik HZ, Langer RS, Haberern C, El Deriny S, O'Brien M (1983) A new method for the three-dimensional in vitro growth of human cancer cells. *Exp Cell Res* **147**, 454-60.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-5.

Lafeber F, Veldhuijzen JP, Vanroy JL, Huber-Bruning O, Bijlsma JW (1992a) Intermittent hydrostatic compressive force stimulates exclusively the proteoglycan synthesis of osteoarthritic human cartilage. *Br J Rheumatol* **31**, 437-42.

Lafeber FP, van der Kraan PM, van Roy HL, Vitters EL, Huber-Bruning O, van den Berg WB, Bijlsma JW (1992b) Local changes in proteoglycan synthesis during culture are different for normal and osteoarthritic cartilage. *Am J Pathol* **140**, 1421-9.

Lafyatis R, Thompson NL, *et al.* (1989) Transforming growth factor-beta production by synovial tissues from rheumatoid patients and streptococcal cell wall arthritic rats. Studies on secretion by synovial fibroblast-like cells and immunohistologic localization. *J Immunol* **143**, 1142-8.

Lam K, Zhang L, Yamada KM, Lafrenie RM (2001) Adhesion of epithelial cells to fibronectin or collagen I induces alterations in gene expression via a protein kinase C-dependent mechanism. *J Cell Physiol* **189**, 79-90.

Lammi MJ, Inkinen R, Parkkinen JJ, Hakkinen T, Jortikka M, Nelimarkka LO, Jarvelainen HT, Tammi MI (1994) Expression of reduced amounts of structurally altered aggrecan in articular cartilage chondrocytes exposed to high hydrostatic pressure. *Biochem J* **304** (**Pt 3**), 723-30.

Langelier E, Suetterlin R, Hoemann CD, Aebi U, Buschmann MD (2000) The chondrocyte cytoskeleton in mature articular cartilage: structure and distribution of actin, tubulin, and vimentin filaments. *J Histochem Cytochem* **48**, 1307-20.

Lapadula G, Iannone F, Zuccaro C, Grattagliano V, Covelli M, Patella V, Lo Bianco G, Pipitone V (1997) Integrin expression on chondrocytes: correlations with the degree of cartilage damage in human osteoarthritis. *Clin Exp Rheumatol* **15**, 247-54.

Lapadula G, Iannone F, Zuccaro C, Grattagliano V, Covelli M, Patella V, Lo Bianco G, Pipitone V (1998) Chondrocyte phenotyping in human osteoarthritis. *Clin Rheumatol* **17**, 99-104.

Lark MW, Bayne EK, *et al.* (1997) Aggrecan degradation in human cartilage. Evidence for both matrix metalloproteinase and aggrecanase activity in normal, osteoarthritic, and rheumatoid joints. *J Clin Invest* **100**, 93-106.

Larsson T, Aspden RM, Heinegard D (1991) Effects of mechanical load on cartilage matrix biosynthesis in vitro. *Matrix* **11** 388-94.

Lawrence RC, Helmick CG, *et al.* (1998) Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. *Arthritis Rheum* **41**, 778-99.

Leavesley DI, Ferguson GD, Wayner EA, Cheresh DA (1992) Requirement of the integrin beta 3 subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen. *J Cell Biol* **117**, 1101-7.

Lee DA, Bader DL (1995) The development and characterization of an in vitro system to study strain-induced cell deformation in isolated chondrocytes. *In Vitro Cell Dev Biol Anim* **31**, 828-35.

Lee DA, Bader DL (1997) Compressive strains at physiological frequencies influence the metabolism of chondrocytes seeded in agarose. *J Orthop Res* **15**, 181-8.

Lee DA, Frean SP, Lees P, Bader DL (1998) Dynamic mechanical compression influences nitric oxide production by articular chondrocytes seeded in agarose. *Biochem Biophys Res Commun* **251**, 580-5.

Lee DA, Noguchi T, Knight MM, O'Donnell L, Bentley G, Bader DL (1998) Response of chondrocyte subpopulations cultured within unloaded and loaded agarose. *J Orthop Res* **16**, 726-33.

Lee ER, Lamplugh L, Leblond CP, Mordier S, Magny MC, Mort JS (1998) Immunolocalization of the cleavage of the aggrecan core protein at the Asn341-Phe342 bond, as an indicator of the location of the metalloproteinases active in the lysis of the rat growth plate. *Anat Rec* **252**, 117-32.

Lee RC, Rich JB, Kelley KM, Weiman DS, Mathews MB (1982) A comparison of in vitro cellular responses to mechanical and electrical stimulation. *Am Surg* **48**, 567-74.

Lewandowska K, Choi HU, Rosenberg LC, Zardi L, Culp LA (1987) Fibronectin-mediated adhesion of fibroblasts: inhibition by dermatan sulfate proteoglycan and evidence for a cryptic glycosaminoglycan-binding domain. *J Cell Biol* **105**, 1443-54.

Lewis JM, Schwartz MA (1995) Mapping in vivo associations of cytoplasmic proteins with integrin beta 1 cytoplasmic domain mutants. *Mol Biol Cell* **6**, 151-60.

Li WQ, Dehnade F, Zafarullah M (2001) Oncostatin M-induced matrix metalloproteinase and tissue inhibitor of metalloproteinase-3 genes expression in chondrocytes requires Janus kinase/STAT signaling pathway. *J Immunol* **166**, 3491-8.

Lindberg FP, Bullard DC, Caver TE, Gresham HD, Beaudet AL, Brown EJ (1996) Decreased resistance to bacterial infection and granulocyte defects in IAP-deficient mice. *Science* **274**, 795-8.

Lindberg FP, Gresham HD, Schwarz E, Brown EJ (1993) Molecular cloning of integrin-associated protein: an immunoglobulin family member with multiple membrane-spanning domains implicated in alpha v beta 3-dependent ligand binding. *J Cell Biol* **123**, 485-96.

Lindberg FP, Lublin DM, Telen MJ, Veile RA, Miller YE, Donis-Keller H, Brown EJ (1994) Rh-related antigen CD47 is the signal-transducer integrin-associated protein. *J Biol Chem* **269**, 1567-70.

Lipman JM (1989) Fluorophotometric quantitation of DNA in articular cartilage utilizing Hoechst 33258. *Anal Biochem* **176**, 128-31.

Little CB, Flannery CR, Hughes CE, Mort JS, Roughley PJ, Dent C, Caterson B (1999) Aggrecanase versus matrix metalloproteinases in the catabolism of the interglobular domain of aggrecan in vitro. *Biochem J* **344 Pt 1**, 61-8.

Little CB, Ghosh P (1997) Variation in proteoglycan metabolism by articular chondrocytes in different joint regions is determined by post-natal mechanical loading. *Osteoarthritis Cartilage* **5**, 49-62.

Little CB, Ghosh P, Bellenger CR (1996) Topographic variation in biglycan and decorin synthesis by articular cartilage in the early stages of osteoarthritis: an experimental study in sheep. *J Orthop Res* **14**, 433-44.

Little CB, Hughes CE, *et al.* (2002) Matrix metalloproteinases are involved in C-terminal and interglobular domain processing of cartilage aggrecan in late stage cartilage degradation. *Matrix Biol* **21**, 271-88.

Liu H, Lee YW, Dean MF (1998) Re-expression of differentiated proteoglycan phenotype by dedifferentiated human chondrocytes during culture in alginate beads. *Biochim Biophys Acta* **1425**, 505-15.

Liu H, McKenna LA, Dean MF (2000) An N-terminal peptide from link protein can stimulate biosynthesis of collagen by human articular cartilage. *Arch Biochem Biophys* **378**, 116-22.

Liu J, Sekiya I, Asai K, Tada T, Kato T, Matsui N (2001) Effects of mechanical vibration on DNA and proteoglycan synthesis in cultured articular chondrocytes. *Modern Rheumatology* **11**, 40-46.

Liu W, Burton-Wurster N, Glant TT, Tashman S, Sumner DR, Kamath RV, Lust G, Kimura JH, Cs-Szabo G (2003) Spontaneous and experimental osteoarthritis in dog: similarities and differences in proteoglycan levels. *J Orthop Res* **21**, 730-7.

Liu Y, Merlin D, Burst SL, Pochet M, Madara JL, Parkos CA (2001) The role of CD47 in neutrophil transmigration. Increased rate of migration correlates with increased cell surface expression of CD47. *J Biol Chem* **276**, 40156-66.

Lodish H, Berk A, Zipursky LS, Matsudaira P, Baltimore D, Darnell J (1995) 'Molecular Cell Biology.' (W. H. Freeman and Company: New York)

Loeser RF (1993) Integrin-mediated attachment of articular chondrocytes to extracellular matrix proteins. *Arthritis Rheum* **36**, 1103-10.

Loeser RF (1994) Modulation of integrin-mediated attachment of chondrocytes to extracellular matrix proteins by cations, retinoic acid, and transforming growth factor beta. *Exp Cell Res* **211**, 17-23.

Loeser RF (2000) Chondrocyte integrin expression and function. *Biorheology* **37**, 109-16.

Loeser RF, Carlson CS, McGee MP (1995) Expression of beta 1 integrins by cultured articular chondrocytes and in osteoarthritic cartilage. *Exp Cell Res* **217**, 248-57.

Loeser RF, Shanker G, Carlson CS, Gardin JF, Shelton BJ, Sonntag WE (2000) Reduction in the chondrocyte response to insulin-like growth factor 1 in aging and osteoarthritis: studies in a non-human primate model of naturally occurring disease. *Arthritis Rheum* **43**, 2110-20.

Loftus JC, Liddington RC (1997) New insights into integrin-ligand interaction. *J Clin Invest* **100**, S77-81.

Lohmander LS, Kimura, J.H. (1986) Biosynthesis of cartilage proteoglycan. In 'Articular cartilage biochemistry'. (Ed. K Kuettner, Schleyerbach, R., Hascall, V.C.) pp. 93-111. (Raven Press: New York)

Lohmander LS (2004) Osteoarthritis - the broad view. In 'Molecular Approaches to Osteoarthritis
Kennedy Institute of Rheumatology Symposium'. London. (Ed. Hea Nagase) pp. 12-13

Lohmander LS, Hascall VC, Yanagishita M, Kuettner KE, Kimura JH (1986) Post-translational events in proteoglycan synthesis: kinetics of synthesis of chondroitin sulfate and oligosaccharides on the core protein. *Arch Biochem Biophys* **250**, 211-27.

Lohmander LS, Neame PJ, Sandy JD (1993) The structure of aggrecan fragments in human synovial fluid. Evidence that aggrecanase mediates cartilage degradation in inflammatory joint disease, joint injury, and osteoarthritis. *Arthritis Rheum* **36**, 1214-22.

Lotz M, Guerne PA (1991) Interleukin-6 induces the synthesis of tissue inhibitor of metalloproteinases-1/erythroid potentiating activity (TIMP-1/EPA). *J Biol Chem* **266**, 2017-20.

Lust G, Burton-Wurster N, Leipold H (1987) Fibronectin as a marker for osteoarthritis. *J Rheumatol* **14 Spec No**, 28-9.

MacLeod JN, Burton-Wurster N, Gu DN, Lust G (1996) Fibronectin mRNA splice variant in articular cartilage lacks bases encoding the V, III-15, and I-10 protein segments. *J Biol Chem* **271**, 18954-60.

Maechim G, Stockwell, R.A. (1979) The Matrix. In 'Adult Articular Cartilage'. (Ed. MAR Freeman) pp. 1-50. (Pitman Medical: Kent)

Malemud CJ, Papay RS, Hering TM, Holderbaum D, Goldberg VM, Haqqi TM (1995) Phenotypic modulation of newly synthesized proteoglycans in human cartilage and chondrocytes. *Osteoarthritis Cartilage* **3**, 227-38.

Malfait AM, Liu RQ, Ijiri K, Komiya S, Tortorella MD (2002) Inhibition of ADAM-TS4 and ADAM-TS5 prevents aggrecan degradation in osteoarthritic cartilage. *J Biol Chem* **277**, 22201-8.

Mankin HJ, Dorfman H, Lippiello L, Zarins A (1971a) Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. *J Bone Joint Surg Am* **53**, 523-37.

Mankin HJ, Lippiello L (1971) The glycosaminoglycans of normal and arthritic cartilage. *J Clin Invest* **50**, 1712-9.

Mankin HJ, Lippiello L (1971b) The glycosaminoglycans of normal and arthritic cartilage. *J Clin Invest* **50**, 1712-9.

Maroudas A (1974) Sulphate diffusion and incorporation into human articular cartilage. *Biochim Biophys Acta* **338**, 265-279.

Maroudas A (1980a) Metabolism of cartilaginous tissues: a quantitative approach. In 'Studies in Joint Disease'. (Ed. A Maroudas, Holborow, E.J.) pp. 59-86. (Pitman Medical: Tunbridge Wells)

Maroudas A, Bayliss MT, Venn MF (1980) Further studies on the composition of human femoral head cartilage. *Ann Rheum Dis* **39**, 514-23.

Maroudas A, Venn MF (1980) Biochemical and physicochemical studies on osteoarthritic cartilage from the human femoral head. In 'The aetiopathogenesis of osteoarthritis'. (Ed. G Nuki))

Martel-Pelletier J, McCollum R, Fujimoto N, Obata K, Cloutier JM, Pelletier JP (1994) Excess of metalloproteases over tissue inhibitor of metalloprotease may contribute to cartilage degradation in osteoarthritis and rheumatoid arthritis. *Lab Invest* **70**, 807-15.

Martel-Pelletier J, Pelletier JP (1994) [Importance of interleukin-I receptors in osteoarthritis]. *Rev Rheum Ed Fr* **61**, 109S-113S.

Martel-Pelletier J, Pelletier JP, Cloutier JM, Malemud CJ (1987) Newly synthesized and endogenous proteoglycans in human osteoarthritic knee cartilage. *J Rheumatol* **14**, 321-8.

Matsukawa M, Fukuda K, Yamasaki K, Yoshida K, Munakata H, Hamanishi C (2004) Enhancement of nitric oxide and proteoglycan synthesis due to cyclic tensile strain loaded on chondrocytes attached to fibronectin. *Inflamm Res* **53**, 239-44.

Mauck RL, Soltz MA, Wang CC, Wong DD, Chao PH, Valhmu WB, Hung CT, Ateshian GA (2000) Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. *J Biomech Eng* **122**, 252-60.

Mawby WJ, Holmes CH, Anstee DJ, Spring FA, Tanner MJ (1994) Isolation and characterization of CD47 glycoprotein: a multispanning membrane protein which is the same as integrin-associated protein (IAP) and the ovarian tumour marker OA3. *Biochem J* **304 (Pt 2)**, 525-30.

Mazzetti I, Grigolo B, Pulsatelli L, Dolzani P, Silvestri T, Roseti L, Meliconi R, Facchini A (2001) Differential roles of nitric oxide and oxygen radicals in chondrocytes affected by osteoarthritis and rheumatoid arthritis. *Clin Sci (London)* **101**, 593-9.

McCartney-Francis N, Allen JB, Mizel DE, Albina JE, Xie QW, Nathan CF, Wahl SM (1993) Suppression of arthritis by an inhibitor of nitric oxide synthase. *J Exp Med* **178**, 749-54.

McDevitt CA, Pahl JA, Ayad S, Miller RR, Uratsuji M, Andrish JT (1988) Experimental osteoarthritic articular cartilage is enriched in guanidine soluble type VI collagen. *Biochem Biophys Res Commun* **157**, 250-5.

McElligott TF, Collins DH (1960) Chondrocyte function of human articular and costal cartilage compared by measuring the in vitro uptake of labelled (35S) sulphate. *Ann Rheum Dis* **19**, 31-41.

McLean JW, Vestal DJ, Cheresh DA, Bodary SC (1990) cDNA sequence of the human integrin beta 5 subunit. *J Biol Chem* **265**, 17126-31.

McQuillan DJ, Handley CJ, Robinson HC, Ng K, Tzaicos C (1986) The relation of RNA synthesis to chondroitin sulphate biosynthesis in cultured bovine cartilage. *Biochem J* **235**, 499-505.

Meachim G, Collins DH (1962) Cell counts of normal and osteoarthritic articular cartilage in relation to the uptake of sulphate (35SO4) in vitro. *Ann Rheum Dis* **21**, 45-50.

Mehraban F, Kuo SY, Riera H, Chang C, Moskowitz RW (1994) Prostromelysin and procollagenase genes are differentially up-regulated in chondrocytes from the knees of rabbits with experimental osteoarthritis. *Arthritis Rheum* **37**, 1189-97.

Melching LI, Roughley PJ (1989) The synthesis of dermatan sulphate proteoglycans by fetal and adult human articular cartilage. *Biochem J* **261**, 501-8.

Melchiorri C, Meliconi R, Frizziero L, Silvestri T, Pulsatelli L, Mazzetti I, Borzi RM, Uguccioni M, Facchini A (1998) Enhanced and coordinated in vivo expression of inflammatory cytokines and nitric oxide synthase by chondrocytes from patients with osteoarthritis. *Arthritis Rheum* **41**, 2165-74.

Milam SB, Magnuson VL, Steffensen B, Chen D, Klebe RJ (1991) IL-1 beta and prostaglandins regulate integrin mRNA expression. *J Cell Physiol* **149**, 173-83.

Millward-Sadler SJ, Salter DM (2004) Integrin-dependent signal cascades in chondrocyte mechanotransduction. *Ann Biomed Eng* **32**, 435-46.

Millward-Sadler SJ, Wright MO, Davies LW, Nuki G, Salter DM (2000) Mechanotransduction via integrins and interleukin-4 results in altered aggrecan and matrix metalloproteinase 3 gene expression in normal, but not osteoarthritic, human articular chondrocytes. *Arthritis Rheum* **43**, 2091-9.

Millward-Sadler SJ, Wright MO, Lee H, Caldwell H, Nuki G, Salter DM (2000) Altered electrophysiological responses to mechanical stimulation and abnormal signalling through alpha5beta1 integrin in chondrocytes from osteoarthritic cartilage. *Osteoarthritis Cartilage* **8**, 272-8.

Millward-Sadler SJ, Wright MO, Lee H, Nishida K, Caldwell H, Nuki G, Salter DM (1999) Integrin-regulated secretion of interleukin 4: A novel pathway of mechanotransduction in human articular chondrocytes. *J Cell Biol* **145**, 183-9.

Miosge N, Flachsbart K, Goetz W, Schultz W, Kresse H, Herken R (1994) Light and electron microscopical immunohistochemical localization of the small proteoglycan core proteins decorin and biglycan in human knee joint cartilage. *Histochem J* **26**, 939-45.

Mitrovic D (1985) The mechanism of cartilage destruction in rheumatoid arthritis. *Arthritis Rheum* **28**, 1192-5.

Miyamoto I, Nagase S (1980) Isolation and characterization of proteodermatansulfate from rat skin. *J Biochem (Tokyo)* **88**, 1793-803.

Miyamoto S, Teramoto H, Coso OA, Gutkind JS, Burbelo PD, Akiyama SK, Yamada KM (1995) Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J Cell Biol* **131**, 791-805.

Mizuno S, Tateishi T, Ushida T, Glowacki J (2002) Hydrostatic fluid pressure enhances matrix synthesis and accumulation by bovine chondrocytes in three-dimensional culture. *J Cell Physiol* **193**, 319-27.

Mok SS, Masuda K, Hauselmann HJ, Aydelotte MB, Thonar EJ (1994) Aggrecan synthesized by mature bovine chondrocytes suspended in alginate. Identification of two distinct metabolic matrix pools. *J Biol Chem* **269**, 33021-7.

Moldovan F, Pelletier JP, Hambor J, Cloutier JM, Martel-Pelletier J (1997) Collagenase-3 (matrix metalloprotease 13) is preferentially localized in the deep layer of human arthritic cartilage in situ: in vitro mimicking effect by transforming growth factor beta. *Arthritis Rheum* **40**, 1653-61.

Moos V, Fickert S, Muller B, Weber U, Sieper J (1999) Immunohistological analysis of cytokine expression in human osteoarthritic and healthy cartilage. *J Rheumatol* **26**, 870-9.

Mourao PA (1988) Distribution of chondroitin 4-sulfate and chondroitin 6-sulfate in human articular and growth cartilage. *Arthritis Rheum* **31**, 1028-33.

Moyle M, Napier MA, McLean JW (1991) Cloning and expression of a divergent integrin subunit beta 8. *J Biol Chem* **266**, 19650-8.

Mudgett JS, Hutchinson NI, *et al.* (1998) Susceptibility of stromelysin 1-deficient mice to collagen-induced arthritis and cartilage destruction. *Arthritis Rheum* **41**, 110-21.

Muir H (1973) Biochemistry. In 'Adult articular cartilage'. (Ed. MAR Freeman) pp. 100-130. (Pitman Medical: London)

Muir H (1995) The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. *Bioessays* **17**, 1039-48.

Muller PK, Lemmen C, Gay S, Gauss V, Kuhn K (1977) Immunochemical and biochemical study of collagen synthesis by chondrocytes in culture. *Exp Cell Res* **108**, 47-55.

Murphy G, Knauper V, Atkinson S, Butler G, English W, Hutton M, Stracke J, Clark I (2002) Matrix metalloproteinases in arthritic disease. *Arthritis Res* **4 Suppl 3**, S39-49.
Murrell GA, Jang D, Williams RJ (1995) Nitric oxide activates metalloprotease enzymes in articular cartilage. *Biochem Biophys Res Commun* **206**, 15-21.

Nakache M, Gaub HE (1988) Hydrodynamic hyperpolarization of endothelial cells. *Proc Natl Acad Sci U S A* **85**, 1841-3.

Nakahara H, Nomizu M, Akiyama SK, Yamada Y, Yeh Y, Chen WT (1996) A mechanism for regulation of melanoma invasion. Ligation of alpha6beta1 integrin by laminin G peptides. *J Biol Chem* **271**, 27221-4.

Naruse K, Sokabe M (1993) Involvement of stretch-activated ion channels in Ca2+ mobilization to mechanical stretch in endothelial cells. *Am J Physiol* **264**, C1037-44.

Neame PJ, Barry FP (1993) The link proteins. *Experientia* **49**, 393-402.

Nebe B, Rychly J, Knopp A, Bohn W (1995) Mechanical induction of beta 1-integrin-mediated calcium signaling in a hepatocyte cell line. *Exp Cell Res* **218**, 479-84.

Neri D, Montigiani S, Kirkham PM (1996) Biophysical methods for the determination of antibody-antigen affinities. *Trends Biotechnol* **14**, 465-70.

Ni H, Wilkins JA (1998) Localisation of a novel adhesion blocking epitope on the human beta 1 integrin chain. *Cell Adhes Commun* **5**, 257-71.

Nietfeld JJ, Wilbrink B, Helle M, van Roy JL, den Otter W, Swaak AJ, Huber-Bruning O (1990) Interleukin-1-induced interleukin-6 is required for the inhibition of proteoglycan synthesis by interleukin-1 in human articular cartilage. *Arthritis Rheum* **33**, 1695-701.

Nikkari L, Aho H, Yli-Jama T, Larjava H, Jalkanen M, Heino J (1993) Expression of integrin family of cell adhesion receptors in rheumatoid synovium. Alpha 6 integrin subunit in normal and hyperplastic synovial lining cell layer. *Am J Pathol* **142**, 1019-27.

Nikkari L, Haapasalmi K, Aho H, Torvinen A, Sheppard D, Larjava H, Heino J (1995) Localization of the alpha v subfamily of integrins and their putative ligands in synovial lining cell layer. *J Rheumatol* **22**, 16-23.

Nimni M, Deshmukh K (1973) Differences in collagen metabolism between normal and osteoarthritic human articular cartilage. *Science* **181**, 751-2.

Nishimoto SK, Kajiwara T, Ledger PW, Tanzer ML (1982) Effects of the ionophore monensin on type II collagen and proteoglycan synthesis and secretion by cultured chondrocytes. *J Biol Chem* **257**, 11712-6.

O'Toole TE, Katagiri Y, Faull RJ, Peter K, Tamura R, Quaranta V, Loftus JC, Shattil SJ, Ginsberg MH (1994) Integrin cytoplasmic domains mediate inside-out signal transduction. *J Cell Biol* **124**, 1047-59.

Ohata H, Seito N, Aizawa H, Nobe K, Momose K (1995) Sensitizing effect of lysophosphatidic acid on mechanoreceptor-linked response in cytosolic free Ca2+ concentration in cultured smooth muscle cells. *Biochem Biophys Res Commun* **208**, 19-25.

Ohta S, Imai K, Yamashita K, Matsumoto T, Azumano I, Okada Y (1998) Expression of matrix metalloproteinase 7 (matrilysin) in human osteoarthritic cartilage. *Lab Invest* **78**, 79-87.

Okada Y, Takeuchi N, Tomita K, Nakanishi I, Nagase H (1989) Immunolocalization of matrix metalloproteinase 3 (stromelysin) in rheumatoid synovioblasts (B cells): correlation with rheumatoid arthritis. *Ann Rheum Dis* **48**, 645-53.

Okimura A, Okada Y, *et al.* (1997) Enhancement of cartilage matrix protein synthesis in arthritic cartilage. *Arthritis Rheum* **40**, 1029-36.

Olin AI, Morgelin M, Sasaki T, Timpl R, Heinegard D, Aspberg A (2001) The proteoglycans aggrecan and Versican form networks with fibulin-2 through their lectin domain binding. *J Biol Chem* **276**, 1253-61.

Orazizadeh M (2004) Expression and function of integrin-associated proteins in human articular cartilage. PhD thesis, The University of Edinburgh.

Ostendorf RH, van de Stadt RJ, van Kampen GP (1994) Intermittent loading induces the expression of 3-B-3(-) epitope in cultured bovine articular cartilage. *J Rheumatol* **21**, 287-92.

Ostergaard K, Petersen J, Andersen CB, Bendtzen K, Salter DM (1997) Histologic/ histochemical grading system for osteoarthritic articular cartilage: reproducibility and validity. *Arthritis Rheum* **40**, 1766-71.

Ostergaard K, Salter DM (1998) Immunohistochemistry in the study of normal and osteoarthritic articular cartilage. *Prog Histochem Cytochem* **33**, 93-165.

Ostergaard K, Salter DM, Petersen J, Bendtzen K, Hvolris J, Andersen CB (1998) Expression of alpha and beta subunits of the integrin superfamily in articular cartilage from macroscopically normal and osteoarthritic human femoral heads. *Ann Rheum Dis* **57**, 303-8.

Otey CA, Pavalko FM, Burridge K (1990) An interaction between alpha-actinin and the beta 1 integrin subunit in vitro. *J Cell Biol* **111**, 721-9.

Owsianik WD, Kundi A, Whitehead JN, Kraag GR, Goldsmith C (1980) Radiological articular involvement in the dominant hand in rheumatoid arthritis. *Ann Rheum Dis* **39**, 508-10.

Pacifici R, Carano A, Santoro SA, Rifas L, Jeffrey JJ, Malone JD, McCracken R, Avioli LV (1991) Bone matrix constituents stimulate interleukin-1 release from human blood mononuclear cells. *J Clin Invest* **87**, 221-8.

Page Thomas DP, King B, Stephens T, Dingle JT (1991) In vivo studies of cartilage regeneration after damage induced by catabolin/interleukin-1. *Ann Rheum Dis* **50**, 75-80.

Palmoski M, Perricone E, Brandt KD (1979) Development and reversal of a proteoglycan aggregation defect in normal canine knee cartilage after immobilization. *Arthritis Rheum* **22**, 508-17.

Palmoski MJ, Brandt KD (1984) Effects of static and cyclic compressive loading on articular cartilage plugs in vitro. *Arthritis Rheum* **27**, 675-81.

Parkkinen JJ, Lammi, M.I., Tammi, M.I., Helminen, H.J. (1994) Proteoglycan synthesis and cytoskeleton in hydrostatically loaded chondrocytes. In 'Cell Mechanics and Cellular Engineering'. (Ed. VC Mow, Guilak, F., Tran-Son-Tay, R., Hochmuth, R.M.) pp. 420-436. (Springer-Verlag: New York)

Parkkinen JJ, Lammi MJ, Helminen HJ, Tammi M (1992) Local stimulation of proteoglycan synthesis in articular cartilage explants by dynamic compression in vitro. *J Orthop Res* **10**, 610-20.

Parkos CA, Colgan SP, Liang TW, Nusrat A, Bacarra AE, Carnes DK, Madara JL (1996) CD47 mediates post-adhesive events required for neutrophil migration across polarized intestinal epithelia. *J Cell Biol* **132**, 437-50.

Parsons JT, Parsons SJ (1997) Src family protein tyrosine kinases: cooperating with growth factor and adhesion signaling pathways. *Curr Opin Cell Biol* **9**, 187-92.

Pasqualini R, Bodorova J, Ye S, Hemler ME (1993) A study of the structure, function and distribution of beta 5 integrins using novel anti-beta 5 monoclonal antibodies. *J Cell Sci* **105 (Pt 1)**, 101-11.

Pasqualini R, Hemler ME (1994) Contrasting roles for integrin beta 1 and beta 5 cytoplasmic domains in subcellular localization, cell proliferation, and cell migration. *J Cell Biol* **125**, 447-60.

Paulsson M, Morgelin M, Wiedemann H, Beardmore-Gray M, Dunham D, Hardingham T, Heinegard D, Timpl R, Engel J (1987) Extended and globular protein domains in cartilage proteoglycans. *Biochem J* **245**, 763-72.

Pearson D, Sasse J (1992) Differential regulation of biglycan and decorin by retinoic acid in bovine chondrocytes. *J Biol Chem* **267**, 25364-70.

Pelletier JP, Jovanovic D, Fernandes JC, Manning P, Connor JR, Currie MG, Di Battista JA, Martel-Pelletier J (1998) Reduced progression of experimental osteoarthritis in vivo by selective inhibition of inducible nitric oxide synthase. *Arthritis Rheum* **41**, 1275-86.

Pettipher ER, Henderson B, Hardingham T, Ratcliffe A (1989) Cartilage proteoglycan depletion in acute and chronic antigen-induced arthritis. *Arthritis Rheum* **32**, 601-7.

Pettipher ER, Higgs GA, Henderson B (1986) Arthritogenic activity of interleukin 1. *Agents Actions* **19**, 337-8.

Plaas AH, West LA, Wong-Palms S, Nelson FR (1998) Glycosaminoglycan sulfation in human osteoarthritis. Disease-related alterations at the non-reducing termini of chondroitin and dermatan sulfate. *J Biol Chem* **273**, 12642-9.

Plaas AH, Wong-Palms S, Roughley PJ, Midura RJ, Hascall VC (1997) Chemical and immunological assay of the nonreducing terminal residues of chondroitin sulfate from human aggrecan. *J Biol Chem* **272**, 20603-10.

Plopper GE, McNamee HP, Dike LE, Bojanowski K, Ingber DE (1995) Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. *Mol Biol Cell* **6**, 1349-65.

Pond MJ, Nuki G (1973) Experimentally-induced osteoarthritis in the dog. *Ann Rheum Dis* **32**, 387-8.

Poole AR (1986) Proteoglycans in health and disease: structures and functions. *Biochem J* **236**, 1-14.

Poole AR, Rosenberg LC, Reiner A, Ionescu M, Bogoch E, Roughley PJ (1996) Contents and distributions of the proteoglycans decorin and biglycan in normal and osteoarthritic human articular cartilage. *J Orthop Res* **14**, 681-9.

Poole CA (1997) Articular cartilage chondrons: form, function and failure. *J Anat* **191 (Pt 1)**, 1-13.

Poole CA, Glant TT, Schofield JR (1991) Chondrons from articular cartilage. (IV). Immunolocalization of proteoglycan epitopes in isolated canine tibial chondrons. *J Histochem Cytochem* **39**, 1175-87.

Porter JC, Hogg N (1998) Integrins take partners: cross-talk between integrins and other membrane receptors. *Trends Cell Biol* **8**, 390-6.

Prehm P (1986) Mechanisms, Localisation, and inhibition of hyarulonate synthesis. In 'Articular cartilage biochemistry'. (Ed. K Kuettner, Schleyerbach, R., Hascall, V.C.) pp. 81-92. (Raven Press: New York)

Pritzker KP, Gay S, Jimenez SA, Ostergaard K, Pelletier JP, Revell PA, Salter D, van den Berg WB (2006) Osteoarthritis cartilage histopathology: grading and staging. *Osteoarthritis Cartilage* **14**, 13-29.

Pruzanski W, Bogoch E, Katz A, Wloch M, Stefanski E, Grouix B, Sakotic G, Vadas P (1995) Induction of release of secretory nonpancreatic phospholipase A2 from human articular chondrocytes. *J Rheumatol* **22**, 2114-9.

Prydz K, Dalen KT (2000) Synthesis and sorting of proteoglycans. *J Cell Sci* **113 Pt 2**, 193-205.

Pulai JI, Del Carlo M, Jr., Loeser RF (2002) The alpha5beta1 integrin provides matrix survival signals for normal and osteoarthritic human articular chondrocytes in vitro. *Arthritis Rheum* **46**, 1528-35.

Pulsatelli L, Dolzani P, Piacentini A, Silvestri T, Ruggeri R, Gualtieri G, Meliconi R, Facchini A (1999) Chemokine production by human chondrocytes. *J Rheumatol* **26**, 1992-2001.

Pytela R, Pierschbacher MD, Ginsberg MH, Plow EF, Ruoslahti E (1986) Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp--specific adhesion receptors. *Science* **231**, 1559-62.

Pytela R, Pierschbacher MD, Ruoslahti E (1985) Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell* **40**, 191-8.

Quinn TM, Maung AA, Grodzinsky AJ, Hunziker EB, Sandy JD (1999) Physical and biological regulation of proteoglycan turnover around chondrocytes in cartilage explants. Implications for tissue degradation and repair. *Ann NY Acad Sci* **878**, 420-41.

Ragan PM, Chin VI, Hung HH, Masuda K, Thonar EJ, Arner EC, Grodzinsky AJ, Sandy JD (2000) Chondrocyte extracellular matrix synthesis and turnover are influenced by static compression in a new alginate disk culture system. *Arch Biochem Biophys* **383**, 256-64.

Ralston SH (1997) The Michael Mason Prize Essay 1997. Nitric oxide and bone: what a gas! *Br J Rheumatol* **36**, 831-8.

Ramaswamy H, Hemler ME (1990) Cloning, primary structure and properties of a novel human integrin beta subunit. *EMBO J* **9**, 1561-8.

Ratcliffe A, Tyler JA, Hardingham TE (1986) Articular cartilage cultured with interleukin 1. Increased release of link protein, hyaluronate-binding region and other proteoglycan fragments. *Biochem J* **238**, 571-80.

Rees JA, Ali SY, Brown RA (1987) Ultrastructural localisation of fibronectin in human osteoarthritic articular cartilage. *Ann Rheum Dis* **46**, 816-22.

Rees SG, Flannery CR, Little CB, Hughes CE, Caterson B, Dent CM (2000) Catabolism of aggrecan, decorin and biglycan in tendon. *Biochem J* **350 Pt 1**, 181-8.

Reinhold MI, Lindberg FP, Kersh GJ, Allen PM, Brown EJ (1997) Costimulation of T cell activation by integrin-associated protein (CD47) is an adhesion-dependent, CD28-independent signaling pathway. *J Exp Med* **185**, 1-11.

Reinhold MI, Lindberg FP, Plas D, Reynolds S, Peters MG, Brown EJ (1995) In vivo expression of alternatively spliced forms of integrin-associated protein (CD47). *J Cell Sci* **108** (**Pt 11**), 3419-25.

Rencic A, Gehris AL, Lewis SD, Hume EL, Bennett VD (1995) Splicing patterns of fibronectin mRNA from normal and osteoarthritic human articular cartilage. *Osteoarthritis Cartilage* **3**, 187-96.

Riikonen T, Westermarck J, Koivisto L, Broberg A, Kahari VM, Heino J (1995) Integrin alpha 2 beta 1 is a positive regulator of collagenase (MMP-1) and collagen alpha 1(I) gene expression. *J Biol Chem* **270**, 13548-52.

Rinaldi N, Barth T, Henne C, Mechterscheimer G, Moller P (1994) Synoviocytes in chronic synovitis in situ and cytokine stimulated synovial cells in vitro neo-express alpha 1, alpha 3 and alpha 5 chains of beta 1 integrins. *Virchows Arch* **425**, 171-80.

Rizkalla G, Reiner A, Bogoch E, Poole AR (1992) Studies of the articular cartilage proteoglycan aggrecan in health and osteoarthritis. Evidence for molecular heterogeneity and extensive molecular changes in disease. *J Clin Invest* **90**, 2268-77.

Roberts SR, Knight MM, Lee DA, Bader DL (2001) Mechanical compression influences intracellular Ca2+ signaling in chondrocytes seeded in agarose constructs. *J Appl Physiol* **90**, 1385-91.

Ronday HK, van der Laan WH, *et al.* (2001) Human granzyme B mediates cartilage proteoglycan degradation and is expressed at the invasive front of the synovium in rheumatoid arthritis. *Rheumatology (Oxford)* **40**, 55-61.

Rosales C, Brown EJ (1992) Signal transduction by neutrophil immunoglobulin G Fc receptors. Dissociation of intracytoplasmic calcium concentration rise from inositol 1,4,5-trisphosphate. *J Biol Chem* **267**, 5265-71.

Rosenberg LC, Choi HU, Tang LH, Johnson TL, Pal S, Webber C, Reiner A, Poole AR (1985) Isolation of dermatan sulfate proteoglycans from mature bovine articular cartilages. *J Biol Chem* **260**, 6304-13.

Roughley PJ, White RJ (1989) Dermatan sulphate proteoglycans of human articular cartilage. The properties of dermatan sulphate proteoglycans I and II. *Biochem J* **262**, 823-7.

Roughley PJ, White RJ, Poole AR (1985) Identification of a hyaluronic acid-binding protein that interferes with the preparation of high-buoyant-density proteoglycan aggregates from adult human articular cartilage. *Biochem J* **231**, 129-38.

Roughley PJ, White RJ, Santer V (1981) Comparison of proteoglycans extracted from high and low weight-bearing human articular cartilage, with particular reference to sialic acid content. *J Biol Chem* **256**, 12699-704.

Ruknudin A, Sachs F, Bustamante JO (1993) Stretch-activated ion channels in tissue-cultured chick heart. *Am J Physiol* **264**, H960-72.

Ruoslahti E (1996) RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol* **12**, 697-715.

Ruoslahti E, Pierschbacher MD (1987) New perspectives in cell adhesion: RGD and integrins. *Science* **238**, 491-7.

Ruoslahti E, Yamaguchi Y (1991) Proteoglycans as modulators of growth factor activities. *Cell* **64**, 867-9.

Ruwhof C, van der Laarse A (2000) Mechanical stress-induced cardiac hypertrophy: mechanisms and signal transduction pathways. *Cardiovasc Res* **47**, 23-37.

Saamanen AM, Tammi M, Jurvelin J, Kiviranta I, Helminen HJ (1990) Proteoglycan alterations following immobilization and remobilization in the articular cartilage of young canine knee (stifle) joint. *J Orthop Res* **8**, 863-73.

Saamanen AM, Tammi M, Kiviranta I, Jurvelin J, Helminen HJ (1989) Levels of chondroitin-6-sulfate and nonaggregating proteoglycans at articular cartilage contact sites in the knees of young dogs subjected to moderate running exercise. *Arthritis Rheum* **32**, 1282-92.

Sage EH, Bornstein P (1991) Extracellular proteins that modulate cell-matrix interactions. SPARC, tenascin, and thrombospondin. *J Biol Chem* **266**, 14831-4.

Sah RL, Doong JY, Grodzinsky AJ, Plaas AH, Sandy JD (1991) Effects of compression on the loss of newly synthesized proteoglycans and proteins from cartilage explants. *Arch Biochem Biophys* **286**, 20-9.

Sah RL, Kim YJ, Doong JY, Grodzinsky AJ, Plaas AH, Sandy JD (1989) Biosynthetic response of cartilage explants to dynamic compression. *J Orthop Res* **7**, 619-36.

Salter DM, Godolphin JL, Gourlay MS (1995) Chondrocyte heterogeneity: immunohistologically defined variation of integrin expression at different sites in human fetal knees. *J Histochem Cytochem* **43**, 447-57.

Salter DM, Hughes DE, Simpson R, Gardner DL (1992) Integrin expression by human articular chondrocytes. *Br J Rheumatol* **31**, 231-4.

Salter DM, Millward-Sadler SJ, Nuki G, Wright MO (2002) Differential responses of chondrocytes from normal and osteoarthritic human articular cartilage to mechanical stimulation. *Biorheology* **39**, 97-108.

Salter DM, Robb JE, Wright MO (1997) Electrophysiological responses of human bone cells to mechanical stimulation: evidence for specific integrin function in mechanotransduction. *J Bone Miner Res* **12**, 1133-41.

Sampaio Lde O, Bayliss MT, Hardingham TE, Muir H (1988) Dermatan sulphate proteoglycan from human articular cartilage. Variation in its content with age and its structural comparison with a small chondroitin sulphate proteoglycan from pig laryngeal cartilage. *Biochem J* **254**, 757-64.

Sanchez-Mateos P, Arroyo AG, Balboa MA, Sanchez-Madrid F (1993) Post-receptor occupancy events in leukocytes during beta 1 integrin-ligand interactions. *Eur J Immunol* **23**, 2642-8.

Sandberg M, Vuorio E (1987) Localization of types I, II, and III collagen mRNAs in developing human skeletal tissues by in situ hybridization. *J Cell Biol* **104**, 1077-84.

Sandell LJ, Aigner T (2001) Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res* **3**, 107-13.

Sandy J (1992) Extracellualr Metabolism of Aggrecan. In 'Articular Cartilage and Osteoarthritis'. (Ed. Kea Kuettner) pp. 21-33. (Raven Press, Ltd.,: New York)

Sandy JD, Adams ME, Billingham ME, Plaas A, Muir H (1984) In vivo and in vitro stimulation of chondrocyte biosynthetic activity in early experimental osteoarthritis. *Arthritis Rheum* **27**, 388-97.

Sandy JD, Brown HL, Lowther DA (1978) Degradation of proteoglycan in articular cartilage. *Biochim Biophys Acta* **543**, 536-44.

Sandy JD, Flannery CR, Neame PJ, Lohmander LS (1992) The structure of aggrecan fragments in human synovial fluid. Evidence for the involvement in osteoarthritis of a novel proteinase which cleaves the Glu 373-Ala 374 bond of the interglobular domain. *J Clin Invest* **89**, 1512-6.

Sandy JD, Neame PJ, Boynton RE, Flannery CR (1991) Catabolism of aggrecan in cartilage explants. Identification of a major cleavage site within the interglobular domain. *J Biol Chem* **266**, 8683-5.

Sandy JD, Plaas AH (1986) Age-related changes in the kinetics of release of proteoglycans from normal rabbit cartilage explants. *J Orthop Res* **4**, 263-72.

Sandy JD, Plaas AH (1989) Studies on the hyaluronate binding properties of newly synthesized proteoglycans purified from articular chondrocyte cultures. *Arch Biochem Biophys* **271**, 300-14.

Sandy JD, Thompson V, Doege K, Verscharen C (2000) The intermediates of aggrecanase-dependent cleavage of aggrecan in rat chondrosarcoma cells treated with interleukin-1. *Biochem J* **351**, 161-6.

Sandy JD, Verscharen C (2001) Analysis of aggrecan in human knee cartilage and synovial fluid indicates that aggrecanase (ADAMTS) activity is responsible for the catabolic turnover and loss of whole aggrecan whereas other protease activity is required for C-terminal processing in vivo. *Biochem J* **358**, 615-26.

Santala P, Heino J (1991) Regulation of integrin-type cell adhesion receptors by cytokines. *J Biol Chem* **266**, 23505-9.

Sarkissian M, Lafyatis R (1999) Integrin engagement regulates proliferation and collagenase expression of rheumatoid synovial fibroblasts. *J Immunol* **162**, 1772-9.

Schaller MD, Parsons JT (1994) Focal adhesion kinase and associated proteins. *Curr Opin Cell Biol* **6**, 705-10.

Schmidt G, Hausser H, Kresse H (1991) Interaction of the small proteoglycan decorin with fibronectin. Involvement of the sequence NKISK of the core protein. *Biochem J* **280** (**Pt 2**), 411-4.

Schmidt G, Robenek H, Harrach B, Glossl J, Nolte V, Hormann H, Richter H, Kresse H (1987) Interaction of small dermatan sulfate proteoglycan from fibroblasts with fibronectin. *J Cell Biol* **104**, 1683-91.

Schneiderman R, Keret D, Maroudas A (1986) Effects of mechanical and osmotic pressure on the rate of glycosaminoglycan synthesis in the human adult femoral head cartilage: an in vitro study. *J Orthop Res* **4**, 393-408.

Scholzen T, Solursh M, Suzuki S, Reiter R, Morgan JL, Buchberg AM, Siracusa LD, Iozzo RV (1994) The murine decorin. Complete cDNA cloning, genomic organization, chromosomal assignment, and expression during organogenesis and tissue differentiation. *J Biol Chem* **269**, 28270-81.

Schwartz MA (1993) Spreading of human endothelial cells on fibronectin or vitronectin triggers elevation of intracellular free calcium. *J Cell Biol* **120**, 1003-10.

Schwartz MA, Brown EJ, Fazeli B (1993) A 50-kDa integrin-associated protein is required for integrin-regulated calcium entry in endothelial cells. *J Biol Chem* **268**, 19931-4.

Schwartz MA, Denninghoff K (1994) Alpha v integrins mediate the rise in intracellular calcium in endothelial cells on fibronectin even though they play a minor role in adhesion. *J Biol Chem* **269**, 11133-7.

Schwartz MA, Lechene C, Ingber DE (1991) Insoluble fibronectin activates the Na/H antiporter by clustering and immobilizing integrin alpha 5 beta 1, independent of cell shape. *Proc Natl Acad Sci U S A* **88**, 7849-53.

Schwartz MA, Schaller MD, Ginsberg MH (1995) Integrins: emerging paradigms of signal transduction. *Annu Rev Cell Dev Biol* **11**, 549-99.

Scott DL, Wainwright AC, Walton KW, Williamson N (1981) Significance of fibronectin in rheumatoid arthritis and osteoarthrosis. *Ann Rheum Dis* **40**, 142-53.

Scott JE (1996) Proteodermatan and proteokeratan sulfate (decorin, lumican/fibromodulin) proteins are horseshoe shaped. Implications for their interactions with collagen. *Biochemistry* **35**, 8795-9.

Scott JE, Orford CR (1981) Dermatan sulphate-rich proteoglycan associates with rat tail-tendon collagen at the d band in the gap region. *Biochem J* **197**, 213-6.

Scully SP, Lee JW, Ghert PMA, Qi W (2001) The role of the extracellular matrix in articular chondrocyte regulation. *Clin Orthop*, S72-89.

Seki S, Takeda K, Abo T (1995) [The function and role of extrathymic T cells]. *Nippon Rinsho* **53**, 2846-57.

Shahein YE, de Andres DF, Perez de la Lastra JM (2002) Molecular cloning and functional characterization of the pig homologue of integrin-associated protein (IAP/CD47). *Immunology* **106**, 564-76.

Shakibaei M, Merker HJ (1999) Beta1-integrins in the cartilage matrix. *Cell Tissue Res* **296**, 565-73.

Sheppard D, Rozzo C, Starr L, Quaranta V, Erle DJ, Pytela R (1990) Complete amino acid sequence of a novel integrin beta subunit (beta 6) identified in epithelial cells using the polymerase chain reaction. *J Biol Chem* **265**, 11502-7.

Shiozawa S, Yoshihara R, Kuroki Y, Fujita T, Shiozawa K, Imura S (1992) Pathogenic importance of fibronectin in the superficial region of articular cartilage as a local factor for the induction of pannus extension on rheumatoid articular cartilage. *Ann Rheum Dis* **51**, 869-73.

Shlopov BV, Gumanovskaya ML, Hasty KA (2000) Autocrine regulation of collagenase 3 (matrix metalloproteinase 13) during osteoarthritis. *Arthritis Rheum* **43**, 195-205.

Shlopov BV, Lie WR, Mainardi CL, Cole AA, Chubinskaya S, Hasty KA (1997) Osteoarthritic lesions: involvement of three different collagenases. *Arthritis Rheum* **40**, 2065-74.

Shyy JY, Chien S (1997) Role of integrins in cellular responses to mechanical stress and adhesion. *Curr Opin Cell Biol* **9**, 707-13.

Siczkowski M, Watt FM (1990) Subpopulations of chondrocytes from different zones of pig articular cartilage. Isolation, growth and proteoglycan synthesis in culture. *J Cell Sci* **97 (Pt 2)**, 349-60.

Sigurdson W, Ruknudin A, Sachs F (1992) Calcium imaging of mechanically induced fluxes in tissue-cultured chick heart: role of stretch-activated ion channels. *Am J Physiol* **262**, H1110-5.

Sigurdson WJ, Sachs F, Diamond SL (1993) Mechanical perturbation of cultured human endothelial cells causes rapid increases of intracellular calcium. *Am J Physiol* **264**, H1745-52.

Simon WH (1970) Scale effects in animal joints. I. Articular cartilage thickness and compressive stress. *Arthritis Rheum* **13**, 244-56.

Slater RR, Jr., Bayliss MT, Lachiewicz PF, Visco DM, Caterson B (1995) Monoclonal antibodies that detect biochemical markers of arthritis in humans. *Arthritis Rheum* **38**, 655-9.

Smeets TJ, Dayer JM, Kraan MC, Versendaal J, Chicheportiche R, Breedveld FC, Tak PP (2000) The effects of interferon-beta treatment of synovial inflammation and expression of metalloproteinases in patients with rheumatoid arthritis. *Arthritis Rheum* **43**, 270-4.

Smilenov LB, Mikhailov A, Pelham RJ, Marcantonio EE, Gundersen GG (1999) Focal adhesion motility revealed in stationary fibroblasts. *Science* **286**, 1172-4.

Smith RL, Donlon BS, *et al.* (1995) Effects of fluid-induced shear on articular chondrocyte morphology and metabolism in vitro. *J Orthop Res* **13**, 824-31.

Smith RL, Rusk SF, Ellison BE, Wessells P, Tsuchiya K, Carter DR, Caler WE, Sandell LJ, Schurman DJ (1996) In vitro stimulation of articular chondrocyte mRNA and extracellular matrix synthesis by hydrostatic pressure. *J Orthop Res* **14**, 53-60.

Sonnenberg A, Modderman PW, Hogervorst F (1988) Laminin receptor on platelets is the integrin VLA-6. *Nature* **336**, 487-9.

Springer TA (1997) Folding of the N-terminal, ligand-binding region of integrin alpha-subunits into a beta-propeller domain. *Proc Natl Acad Sci U S A* **94**, 65-72.

Stanton H, Rogerson FM, *et al.* (2005) ADAMTS5 is the major aggrecanase in mouse cartilage in vivo and in vitro. *Nature* **434**, 648-52.

Steinmeyer J, Ackermann B, Raiss RX (1997) Intermittent cyclic loading of cartilage explants modulates fibronectin metabolism. *Osteoarthritis Cartilage* **5**, 331-41.

Stockwell RA (1971) The interrelationship of cell density and cartilage thickness in mammalian articular cartilage. *J Anat* **109**, 411-21.

Streuli C (1999) Extracellular matrix remodelling and cellular differentiation. *Curr Opin Cell Biol* **11**, 634-40.

Studer R, Jaffurs D, Stefanovic-Racic M, Robbins PD, Evans CH (1999) Nitric oxide in osteoarthritis. *Osteoarthritis Cartilage* **7**, 377-9.

Sugiura Y, Niimi T, Sato S, Yoshinouchi T, Banno S, Naniwa T, Maeda H, Shimizu S, Ueda R (2002) Transforming growth factor beta1 gene polymorphism in rheumatoid arthritis. *Ann Rheum Dis* **61**, 826-8.

Sugumaran G, Silbert JE (1991) Subfractionation of chick embryo epiphyseal cartilage Golgi. Localization of enzymes involved in the synthesis of the polysaccharide portion of proteochondroitin sulfate. *J Biol Chem* **266**, 9565-9.

Sun HB, Yokota H (2001) Altered mRNA level of matrix metalloproteinase-13 in MH7A synovial cells under mechanical loading and unloading. *Bone* **28**, 399-403.

Sun HB, Yokota H (2002) Reduction of cytokine-induced expression and activity of MMP-1 and MMP-13 by mechanical strain in MH7A rheumatoid synovial cells. *Matrix Biol* **21**, 263-70.

Suzuki S, Huang ZS, Tanihara H (1990) Cloning of an integrin beta subunit exhibiting high homology with integrin beta 3 subunit. *Proc Natl Acad Sci U S A* **87**, 5354-8.

Suzuki S, Naitoh Y (1990) Amino acid sequence of a novel integrin beta 4 subunit and primary expression of the mRNA in epithelial cells. *EMBO J* **9**, 757-63.

Svensson L, Heinegard D, Oldberg A (1995) Decorin-binding sites for collagen type I are mainly located in leucine-rich repeats 4-5. *J Biol Chem* **270**, 20712-6.

Sweet MB, Thonar EJ, Immelman AR, Solomon L (1977) Biochemical changes in progressive osteoarthrosis. *Ann Rheum Dis* **36**, 387-98.

Sztrolovics R, Alini M, Roughley PJ, Mort JS (1997) Aggrecan degradation in human intervertebral disc and articular cartilage. *Biochem J* **326 (Pt 1)**, 235-41.

Sztrolovics R, White RJ, Roughley PJ, Mort JS (2002) The mechanism of aggrecan release from cartilage differs with tissue origin and the agent used to stimulate catabolism. *Biochem J* **362**, 465-72.

Tagil M, Aspenberg P (1999) Cartilage induction by controlled mechanical stimulation in vivo. *J Orthop Res* **17**, 200-4.

Tak PP, Taylor PC, Breedveld FC, Smeets TJ, Daha MR, Kluin PM, Meinders AE, Maini RN (1996) Decrease in cellularity and expression of adhesion molecules by anti-tumor necrosis factor alpha monoclonal antibody treatment in patients with rheumatoid arthritis. *Arthritis Rheum* **39**, 1077-81.

Tak PP, van der Lubbe PA, *et al.* (1995) Reduction of synovial inflammation after anti-CD4 monoclonal antibody treatment in early rheumatoid arthritis. *Arthritis Rheum* **38**, 1457-65.

Takagi J, DeBottis DP, Erickson HP, Springer TA (2002) The role of the specificity-determining loop of the integrin beta subunit I-like domain in autonomous expression, association with the alpha subunit, and ligand binding. *Biochemistry* **41**, 4339-47.

Takagi T, Okamoto R, Suzuki K, Hayashi T, Sato M, Kurosaka N, Koshino T (2001) Up-regulation of CD44 in rheumatoid chondrocytes. *Scand J Rheumatol* **30**, 110-3.

Taketazu F, Kato M, *et al.* (1994) Enhanced expression of transforming growth factor-beta s and transforming growth factor-beta type II receptor in the synovial tissues of patients with rheumatoid arthritis. *Lab Invest* **70**, 620-30.

Tardif G, Reboul P, Pelletier JP, Geng C, Cloutier JM, Martel-Pelletier J (1996) Normal expression of type 1 insulin-like growth factor receptor by human osteoarthritic chondrocytes with increased expression and synthesis of insulin-like growth factor binding proteins. *Arthritis Rheum* **39**, 968-78.

Taverna D, Disatnik MH, Rayburn H, Bronson RT, Yang J, Rando TA, Hynes RO (1998) Dystrophic muscle in mice chimeric for expression of alpha5 integrin. *J Cell Biol* **143**, 849-59.

Taylor KB, Jeffree GM (1969) A new basic metachromatic dye, I:9-dimethyl methylene blue. *Histochem J* **1**, 199-204.

Taylor PC, Peters AM, Paleolog E, Chapman PT, Elliott MJ, McCloskey R, Feldmann M, Maini RN (2000) Reduction of chemokine levels and leukocyte traffic to joints by tumor necrosis factor alpha blockade in patients with rheumatoid arthritis. *Arthritis Rheum* **43**, 38-47.

Telhag H (1972) Mitosis of chondrocytes in experimental "osteoarthritis" in rabbits. *Clin Orthop* **86**, 224-9.

Telser A, Robinson HC, Dorfman A (1966) The biosynthesis of chondroitin sulfate. *Arch Biochem Biophys* **116**, 458-65.

Teshima R, Treadwell BV, Trahan CA, Mankin HJ (1983) Comparative rates of proteoglycan synthesis and size of proteoglycans in normal and osteoarthritic chondrocytes. *Arthritis Rheum* **26**, 1225-30.

Thonar EJ, Buckwalter JA, Kuettner KE (1986) Maturation-related differences in the structure and composition of proteoglycans synthesized by chondrocytes from bovine articular cartilage. *J Biol Chem* **261**, 2467-74.

Thonar EJ, Lohmander LS, Kimura JH, Fellini SA, Yanagishita M, Hascall VC (1983) Biosynthesis of O-linked oligosaccharides on proteoglycans by chondrocytes from the swarm rat chondrosarcoma. *J Biol Chem* **258**, 11564-70.

Ticchioni M, Deckert M, Mary F, Bernard G, Brown EJ, Bernard A (1997) Integrin-associated protein (CD47) is a comitogenic molecule on CD3-activated human T cells. *J Immunol* **158**, 677-84.

Tonon R, D'Andrea P (2000) Interleukin-1beta increases the functional expression of connexin 43 in articular chondrocytes: evidence for a Ca2+-dependent mechanism. *J Bone Miner Res* **15**, 1669-77.

Tortorella MD, Malfait AM, Deccico C, Arner E (2001) The role of ADAM-TS4 (aggrecanase-1) and ADAM-TS5 (aggrecanase-2) in a model of cartilage degradation. *Osteoarthritis Cartilage* **9**, 539-52.

Toyoda T, Seedhom BB, Yao JQ, Kirkham J, Brookes S, Bonass WA (2003) Hydrostatic pressure modulates proteoglycan metabolism in chondrocytes seeded in agarose. *Arthritis Rheum* **48**, 2865-72.

Trickey WR, Lee GM, Guilak F (2000) Viscoelastic properties of chondrocytes from normal and osteoarthritic human cartilage. *J Orthop Res* **18**, 891-8.

Tsao PW, Mousa SA (1995) Thrombospondin mediates calcium mobilization in fibroblasts via its Arg-Gly-Asp and carboxyl-terminal domains. *J Biol Chem* **270**, 23747-53.

Tyler CW, Apkarian PA (1985) Effects of contrast, orientation and binocularity in the pattern evoked potential. *Vision Res* **25**, 755-66.

Uchida A, Yamashita K, Hashimoto K, Shimomura Y (1988) The effect of mechanical stress on cultured growth cartilage cells. *Connect Tissue Res* **17**, 305-11.

Uldbjerg N, Malmstrom A, Ekman G, Sheehan J, Ulmsten U, Wingerup L (1983) Isolation and characterization of dermatan sulphate proteoglycan from human uterine cervix. *Biochem J* **209**, 497-503.

Upholt WB, Vertel BM, Dorfman A (1979) Translation and characterization of messenger RNAs in differentiating chicken cartilage. *Proc Natl Acad Sci U S A* **76**, 4847-51.

Urban JP (1994) The chondrocyte: a cell under pressure. *Br J Rheumatol* **33**, 901-8.

Urban JPG, Hall AC (1994) The effect of hydrostatic and osmotic pressures on chondrocyte metanolism. In 'Cell mechanics and cellular engineering'. (Ed. VC Mow, Guilak, F., Tran-Son-Tay, R., Hochmuth, R.M.) pp. 398-419. (Springer-Verlag: New York)

Valhmu WB, Stazzone EJ, Bachrach NM, Saed-Nejad F, Fischer SG, Mow VC, Ratcliffe A (1998) Load-controlled compression of articular cartilage induces a transient stimulation of aggrecan gene expression. *Arch Biochem Biophys* **353**, 29-36.

van de Loo FA, Arntz OJ, van Enckevort FH, van Lent PL, van den Berg WB (1998) Reduced cartilage proteoglycan loss during zymosan-induced gonarthritis in NOS2-deficient mice and in anti-interleukin-1-treated wild-type mice with unabated joint inflammation. *Arthritis Rheum* **41**, 634-46.

van den Berg WB (2001) Uncoupling of inflammatory and destructive mechanisms in arthritis. *Semin Arthritis Rheum* **30**, 7-16.

van den Berg WB, van Lent PL (1996) The role of macrophages in chronic arthritis. *Immunobiology* **195**, 614-23.

van der Flier A, Sonnenberg A (2001) Function and interactions of integrins. *Cell Tissue Res* **305**, 285-98.

van Kampen GP, Veldhuijzen JP, Kuijer R, van de Stadt RJ, Schipper CA (1985) Cartilage response to mechanical force in high-density chondrocyte cultures. *Arthritis Rheum* **28**, 419-24.

van Meurs J, van Lent P, *et al.* (1999) Cleavage of aggrecan at the Asn341-Phe342 site coincides with the initiation of collagen damage in murine antigen-induced arthritis: a pivotal role for stromelysin 1 in matrix metalloproteinase activity. *Arthritis Rheum* **42**, 2071-84.

van Meurs JB, van Lent PL, Holthuysen AE, Singer, II, Bayne EK, van den Berg WB (1999) Kinetics of aggrecanase- and metalloproteinase-induced neoepitopes in various stages of cartilage destruction in murine arthritis. *Arthritis Rheum* **42**, 1128-39.

van Meurs JB, van Lent PL, van de Loo AA, Holthuysen AE, Bayne EK, Singer, II, van den Berg WB (1999) Increased vulnerability of postarthritic cartilage to a second arthritic insult: accelerated MMP activity in a flare up of arthritis. *Ann Rheum Dis* **58**, 350-6.

Van Nhieu GT, Krukonis ES, Reszka AA, Horwitz AF, Isberg RR (1996) Mutations in the cytoplasmic domain of the integrin beta1 chain indicate a role for endocytosis factors in bacterial internalization. *J Biol Chem* **271**, 7665-72.

van Roon JA, van Roy JL, Gmelig-Meyling FH, Lafeber FP, Bijlsma JW (1996) Prevention and reversal of cartilage degradation in rheumatoid arthritis by interleukin-10 and interleukin-4. *Arthritis Rheum* **39**, 829-35.

Van Strijp JA, Russell DG, Tuomanen E, Brown EJ, Wright SD (1993) Ligand specificity of purified complement receptor type three (CD11b/CD18, alpha m beta 2, Mac-1). Indirect effects of an Arg-Gly-Asp (RGD) sequence. *J Immunol* **151**, 3324-36.

van't Hof RJ, Hocking L, Wright PK, Ralston SH (2000) Nitric oxide is a mediator of apoptosis in the rheumatoid joint. *Rheumatology (Oxford)* **39**, 1004-8.

Vartio T, Vaheri A, Von Essen R, Isomaki H, Stenman S (1981) Fibronectin in synovial fluid and tissue in rheumatoid arthritis. *Eur J Clin Invest* **11**, 207-12.

Vasan N (1980) Proteoglycans in normal and severely osteoarthritic human cartilage. *Biochem J* **187**, 781-7.

Veale DJ, Reece RJ, *et al.* (1999) Intra-articular primatised anti-CD4: efficacy in resistant rheumatoid knees. A study of combined arthroscopy, magnetic resonance imaging, and histology. *Ann Rheum Dis* **58**, 342-9.

Velasco A, Hidalgo J, Perez-Vilar J, Garcia-Herdugo G, Navas P (1988) Detection of glycosaminoglycans in the Golgi complex of chondrocytes. *Eur J Cell Biol* **47**, 241-50.

Veldhuijzen JP, Huisman AH, Vermeiden JPW, Prahl-Andersen B (1987) The growth of cartilage cells *in vitro* and the effect of intermittent compressive force. A histological evaluation. *Connect Tissue Res* **16**, 187-96.

Venn G, Billingham ME, Hardingham TE (1995) Increased proteoglycan synthesis in cartilage in experimental canine osteoarthritis does not reflect a permanent change in chondrocyte phenotype. *Arthritis Rheum* **38**, 525-32.

Venn M, Maroudas A (1977) Chemical composition and swelling of normal and osteoarthrotic femoral head cartilage. I. Chemical composition. *Ann Rheum Dis* **36**, 121-9.

Verbruggen G, Malfait AM, Veys EM, Gyselbrecht L, Lambert J, Almqvist KF (1993) Influence of interferon-gamma on isolated chondrocytes from human articular cartilage. Dose dependent inhibition of cell proliferation and proteoglycan synthesis. *J Rheumatol* **20**, 1020-6.

Vignon E, Arlot M, Patricot LM, Vignon G (1976) The cell density of human femoral head cartilage. *Clin Orthop Relat Res,*, 303-8.

Vilim V, Fosang AJ (1993) Characterization of proteoglycans isolated from associative extracts of human articular cartilage. *Biochem J* **293 (Pt 1)**, 165-72.

Vogel BE, Tarone G, Giancotti FG, Gailit J, Ruoslahti E (1990) A novel fibronectin receptor with an unexpected subunit composition (alpha V beta 1). *J Biol Chem* **265**, 5934-7.

Vogel KG, Evanko SP (1987) Proteoglycans of fetal bovine tendon. *J Biol Chem* **262**, 13607-13.

Vogel KG, Paulsson M, Heinegard D (1984) Specific inhibition of type I and type II collagen fibrillogenesis by the small proteoglycan of tendon. *Biochem J* **223**, 587-97.

Vogel KG, Trotter JA (1987) The effect of proteoglycans on the morphology of collagen fibrils formed in vitro. *Coll Relat Res* **7**, 105-14.

von der Mark K, Gauss V, von der Mark H, Muller P (1977) Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. *Nature* **267**, 531-2.

Vornehm SI, Dudhia J, Von der Mark K, Aigner T (1996) Expression of collagen types IX and XI and other major cartilage matrix components by human fetal chondrocytes in vivo. *Matrix Biol* **15**, 91-8.

Vuori K, Ruoslahti E (1993) Activation of protein kinase C precedes alpha 5 beta 1 integrin-mediated cell spreading on fibronectin. *J Biol Chem* **268**, 21459-62.

Wang AZ, Wang JC, Fisher GW, Diamond HS (1997) Interleukin-1beta-stimulated invasion of articular cartilage by rheumatoid synovial fibroblasts is inhibited by antibodies to specific integrin receptors and by collagenase inhibitors. *Arthritis Rheum* **40**, 1298-307.

Wang CC, Chahine NO, Hung CT, Ateshian GA (2003) Optical determination of anisotropic material properties of bovine articular cartilage in compression. *J Biomech* **36**, 339-53.

Wang N, Butler JP, Ingber DE (1993) Mechanotransduction across the cell surface and through the cytoskeleton. *Science* **260**, 1124-7.

Wang XQ, Frazier WA (1998) The thrombospondin receptor CD47 (IAP) modulates and associates with alpha2 beta1 integrin in vascular smooth muscle cells. *Mol Biol Cell* **9**, 865-74.

Watanabe H, Cheung SC, Itano N, Kimata K, Yamada Y (1997) Identification of hyaluronan-binding domains of aggrecan. *J Biol Chem* **272**, 28057-65.

Watson PA, Krupinski J, Kempinski AM, Frankenfield CD (1994) Molecular cloning and characterization of the type VII isoform of mammalian adenylyl cyclase expressed widely in mouse tissues and in S49 mouse lymphoma cells. *J Biol Chem* **269**, 28893-8.

Wayner EA, Carter WG, Piotrowicz RS, Kunicki TJ (1988) The function of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins Ic-IIa. *J Cell Biol* **107**, 1881-91.

Wayner EA, Orlando RA, Cheresh DA (1991) Integrins alpha v beta 3 and alpha v beta 5 contribute to cell attachment to vitronectin but differentially distribute on the cell surface. *J Cell Biol* **113**, 919-29.

Weinacker A, Chen A, Agrez M, Cone RI, Nishimura S, Wayner E, Pytela R, Sheppard D (1994) Role of the integrin alpha v beta 6 in cell attachment to fibronectin. Heterologous expression of intact and secreted forms of the receptor. *J Biol Chem* **269**, 6940-8.

Werb Z, Tremble PM, Behrendtsen O, Crowley E, Damsky CH (1989) Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *J Cell Biol* **109**, 877-89.

Wernicke D, Seyfert C, Hinzmann B, Gromnica-Ihle E (1996) Cloning of collagenase 3 from the synovial membrane and its expression in rheumatoid arthritis and osteoarthritis. *J Rheumatol* **23**, 590-5.

Whinna HC, Choi HU, Rosenberg LC, Church FC (1993) Interaction of heparin cofactor II with biglycan and decorin. *J Biol Chem* **268**, 3920-4.

Wight TN, Kinsella MG, Qwarnstrom EE (1992) The role of proteoglycans in cell adhesion, migration and proliferation. *Curr Opin Cell Biol* **4**, 793-801.

Wislman NJ, Fletcher TE (1978) Cilia of neonatal articular chondrocytes: incidence and morphology. *Anat Rec.* **190**, 871-89.

Wiltink A, Nijweide PJ, Scheenen WJ, Ypey DL, Van Duijn B (1995) Cell membrane stretch in osteoclasts triggers a self-reinforcing Ca2+ entry pathway. *Pflugers Arch* **429**, 663-71.

Winnemoller M, Schmidt G, Kresse H (1991) Influence of decorin on fibroblast adhesion to fibronectin. *Eur J Cell Biol* **54**, 10-7.

Wirtz HR, Dobbs LG (1990) Calcium mobilization and exocytosis after one mechanical stretch of lung epithelial cells. *Science* **250**, 1266-9.

Witsch-Prehm P, Miehlke R, Kresse H (1992) Presence of small proteoglycan fragments in normal and arthritic human cartilage. *Arthritis Rheum* **35**, 1042-52.

Wong M, Siegrist M, Goodwin K (2003) Cyclic tensile strain and cyclic hydrostatic pressure differentially regulate expression of hypertrophic markers in primary chondrocytes. *Bone* **33**, 685-93.

Wong M, Siegrist M, Wang X, Hunziker E (2001) Development of mechanically stable alginate/chondrocyte constructs: effects of guluronic acid content and matrix synthesis. *J Orthop Res* **19**, 493-9.

Wong M, Wuethrich P, Buschmann MD, Eggli P, Hunziker E (1997) Chondrocyte biosynthesis correlates with local tissue strain in statically compressed adult articular cartilage. *J Orthop Res* **15**, 189-96.

Woods VL, Jr., Schreck PJ, Gesink DS, Pacheco HO, Amiel D, Akeson WH, Lotz M (1994) Integrin expression by human articular chondrocytes. *Arthritis Rheum* **37**, 537-44.

Woolley DE, Tetlow LC (1997) Observations on the microenvironmental nature of cartilage degradation in rheumatoid arthritis. *Ann Rheum Dis* **56**, 151-61.

Wright M, Jobanputra P, Bavington C, Salter DM, Nuki G (1996) Effects of intermittent pressure-induced strain on the electrophysiology of cultured human chondrocytes: evidence for the presence of stretch-activated membrane ion channels. *Clin Sci (London)* **90**, 61-71.

Wright MO, Nishida K, Bavington C, Godolphin JL, Dunne E, Walmsley S, Jobanputra P, Nuki G, Salter DM (1997) Hyperpolarisation of cultured human chondrocytes following cyclical pressure-induced strain: evidence of a role for alpha 5 beta 1 integrin as a chondrocyte mechanoreceptor. *J Orthop Res* **15**, 742-7.

Wright MO, Stockwell RA, Nuki G (1992) Response of plasma membrane to applied hydrostatic pressure in chondrocytes and fibroblasts. *Connect Tissue Res* **28**, 49-70.

Wu C, Keivens VM, O'Toole TE, McDonald JA, Ginsberg MH (1995) Integrin activation and cytoskeletal interaction are essential for the assembly of a fibronectin matrix. *Cell* **83**, 715-24.

Wu QQ, Chen Q (2000) Mechanoregulation of chondrocyte proliferation, maturation, and hypertrophy: ion-channel dependent transduction of matrix deformation signals. *Exp Cell Res* **256**, 383-91.

Wurster NB, Lust G (1984) Synthesis of fibronectin in normal and osteoarthritic articular cartilage. *Biochim Biophys Acta* **800**, 52-8.

Xia SL, Ferrier J (1995) Calcium signal induced by mechanical perturbation of osteoclasts. *J Cell Physiol* **163**, 493-501.

Xie D, Homandberg GA (1993) Fibronectin fragments bind to and penetrate cartilage tissue resulting in proteinase expression and cartilage damage. *Biochim Biophys Acta* **1182**, 189-96.

Xu J, Liu M, Liu J, Caniggia I, Post M (1996) Mechanical strain induces constitutive and regulated secretion of glycosaminoglycans and proteoglycans in fetal lung cells. *J Cell Sci* **109** (**Pt 6**), 1605-13.

Xu Z, Buckley MJ, Evans CH, Agarwal S (2000) Cyclic tensile strain acts as an antagonist of IL-1 beta actions in chondrocytes. *J Immunol* **165**, 453-60.

Yamada A, Nojima Y, Sugita K, Dang NH, Schlossman SF, Morimoto C (1991) Cross-linking of VLA/CD29 molecule has a co-mitogenic effect with anti-CD3 on CD4 cell activation in serum-free culture system. *Eur J Immunol* **21**, 319-25.

Yamada KM, Miyamoto S (1995) Integrin transmembrane signaling and cytoskeletal control. *Curr Opin Cell Biol* **7**, 681-9.

Yamaguchi Y, Mann DM, Ruoslahti E (1990) Negative regulation of transforming growth factor-beta by the proteoglycan decorin. *Nature* **346**, 281-4.

Yamaguchi Y, Ruoslahti E (1988) Expression of human proteoglycan in Chinese hamster ovary cells inhibits cell proliferation. *Nature* **336**, 244-6.

Yang BL, Cao L, Kiani C, Lee V, Zhang Y, Adams ME, Yang BB (2000) Tandem repeats are involved in G1 domain inhibition of versican expression and secretion and the G3 domain enhances glycosaminoglycan modification and product secretion via the complement-binding protein-like motif. *J Biol Chem* **275**, 21255-61.

Yang JT, Rayburn H, Hynes RO (1993) Embryonic mesodermal defects in alpha 5 integrin-deficient mice. *Development* **119**, 1093-105.

Yang XC, Sachs F (1989) Block of stretch-activated ion channels in Xenopus oocytes by gadolinium and calcium ions. *Science* **243**, 1068-71.

Yano Y, Geibel J, Sumpio BE (1997) Cyclic strain induces reorganization of integrin alpha 5 beta 1 and alpha 2 beta 1 in human umbilical vein endothelial cells. *J Cell Biochem* **64**, 505-13.

Yellowley CE, Jacobs CR, Donahue HJ (1999) Mechanisms contributing to fluid-flow-induced Ca2+ mobilization in articular chondrocytes. *J Cell Physiol* **180**, 402-8.

Yonezawa I, Kato K, Yagita H, Yamauchi Y, Okumura K (1996) VLA-5-mediated interaction with fibronectin induces cytokine production by human chondrocytes. *Biochem Biophys Res Commun* **219**, 261-5.

Youssef PP, Haynes DR, Triantafillou S, Parker A, Gamble JR, Roberts-Thomson PJ, Ahern MJ, Smith MD (1997) Effects of pulse methylprednisolone on inflammatory mediators in peripheral blood, synovial fluid, and synovial membrane in rheumatoid arthritis. *Arthritis Rheum* **40**, 1400-8.

Zaucke F, Dinser R, Maurer P, Paulsson M (2001) Cartilage oligomeric matrix protein (COMP) and collagen IX are sensitive markers for the differentiation state of articular primary chondrocytes. *Biochem J* **358**, 17-24.

Zhang Y, Guerassimov A, Leroux JY, Cartman A, Webber C, Lalic R, de Miguel E, Rosenberg LC, Poole AR (1998) Induction of arthritis in BALB/c mice by cartilage link protein: involvement of distinct regions recognized by T and B lymphocytes. *Am J Pathol* **153**, 1283-91.

Zhou M, Brown EJ (1993) Leukocyte response integrin and integrin-associated protein act as a signal transduction unit in generation of a phagocyte respiratory burst. *J Exp Med* **178**, 1165-74.