The Effects of Mechanical Stimulation on Proteoglycan synthesis by Articular Chondrocytes

Charles D. Bavington

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DECLARATION

i. This thesis is my own work.

ii. The experimental work described in this thesis was carried out by myself, unless otherwise stated.

Charles D. Bavington

ABSTRACT

Osteoarthritis (OA) is a disease of articular cartilage that is characterised by degeneration of the cartilage matrix leading to cartilage destruction and loss, such that the underlying bone is no longer protected from the forces associated with joint articulation. The role of mechanical loading in maintaining normal cartilage structure and in the development and progression of joint disease has been demonstrated in animal studies. Future development of effective treatments for OA requires an understanding of the mechanisms whereby mechanical loading regulates cartilage structure and function.

Loading of cartilage and chondrocyte cultures *in vitro* has demonstrated the importance of mechanical forces in regulating chondrocyte metabolism. The mechanisms whereby mechanical stresses regulate cellular function have been well characterised in other cell types, such as vascular endothelial cells and cardiac myocytes, but are poorly understood in chondrocytes. The aim of this thesis was to investigate the effects of cyclical mechanical stimulation on chondrocyte proteoglycan (PG) metabolism and to explore the mechanisms involved in the transduction of the mechanical stimulus into a biochemical response.

Techniques for the preparation and maintenance of primary cultures of human and bovine articular chondrocytes were established. Chondrocytes were exposed to cyclical pressurisation in an apparatus that functions to produce strain of the base of culture dishes with deformation of attached cells. PG synthesis during pressurisation, assayed by measuring the incorporation of $[^{35}SO_4]$-sulphate into PGs, was increased significantly compared to controls following 6h cyclical pressure-induced strain (PIS). Increases in $[^{35}SO_4]$-sulphate incorporation following cyclical PIS were abolished by blocking stretch-activated ion channels with 10 μM Gadolinium. These experiments provided the first evidence that stretch-activated ion channels are involved in the signal transduction process that leads to accelerated PG synthesis following cyclical deformation of chondrocytes.

Two antibodies (3B3 and 7D4) raised against epitopes within the chondroitin sulphate chains of cartilage PG have been shown to be of particular value in detecting structural changes early in the development of OA. Methods were established for the measurement of these epitopes and normal chondrocyte glycosaminoglycan (GAG) epitopes by flow cytometry. 3B3 and 7D4 epitopes were expressed at low levels in both human and bovine chondrocytes from normal tissue. There was no increase in their expression up to 24 h after stimulation of chondrocytes for 3 h with cyclical PIS.

A new apparatus was developed for the production of cyclical PIS, which could accommodate two strained and two unstrained dishes under conditions of pressurisation. This apparatus proved suitable for further studies of the signal transduction mechanisms involved in responses to PIS. Chondrocytes treated with adhesion-blocking anti-α5 and anti-β1 integrin antibodies had a reduced response to strain, whereas those treated with an adhesion enhancing anti-β1 integrin antibody resulted in an increased response. These results implicate α5β1 integrin, which is the cell surface receptor for fibronectin, as a mechanotransducer in chondrocytes.

Cyclic AMP is a ubiquitous signalling molecule involved in the response of many cell types to mechanical stress. The intracellular concentration of cAMP in chondrocytes was measured by radioimmunoassay. Treatment of chondrocytes with the adenylate cyclase activator Forskolin resulted in a 10 fold increase in cAMP but this was not influenced by PIS. Treatment of chondrocytes with Forskolin over a 24 h period produced a significant increase in PG synthesis. These results suggest that cAMP can be involved in activating PG synthesis but is not increased in response to cyclical PIS.

The studies demonstrate that chondrocytes respond to cyclical deformation by increasing PG synthesis but that the expression of GAG-epitopes associated with OA was not increased. The results also indicate that chondrocytes detect cyclical deformation by mechanisms involving cell surface integrin receptors and the opening of mechanosensitive ion channels.

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Finally, thanks to Dr. Brent Williams and Mark Dockrell for teaching me the radioimmunoassay for cyclic AMP and providing invaluable advice and ideas.

CONTENTS

1. ABBREVIATIONS

aa - amino acid

AC - Adenylyl cyclase

Ang II - angiotensin II

bFGF - basic fibroblastic growth factor

BSA - bovine serum albumin

cAMP - cyclic adenosine monophosphate

CHAPS - 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate

cGMP - Cyclic guanosine monophosphate

CREB - cAMP response element-binding protein

CREM - cAMP response element modulator protein

CS - chondroitin sulphate

CS-4 - chondroitin-4-sulphate

CS-6 - chondroitin-6-sulphate

CSK - cytoskeleton

DAG - diacylglycerol

DMEM - Dulbecco's modified Eagle's medium

DMMB - dimethylmethylene blue

DMSO - dimethyl sulphoxide

DS - dermatan sulphate

EC - endothelial cells

ECM - extracellular matrix

EDCF - endothelium derived constricting factor

EDHF - endothelium derived hyperpolarising factor

EDRF - endothelium derived relaxing factor

EDTA - Ethylenediamine-tetraacetic acid

ET-1 - endothelin-1

FAK - focal adhesion kinase

FCS - Fetal calf serum

bFGF - basic fibroblastic growth factor

GAG - glycosaminoglycan

GCET - 4 M Guanidine-HCl (GnHCl), 0.5 % CHAPS, 10 μM EDTA, 3 μM Tris HCl

Gd^{3+} - Gadolinium

GEF - guanine nucleotide exchange factor

GPI - glycophosphatidylinositol

G-protein - guanine nucleotide-binding protein

Grb2 - growth-factor-receptor-bound protein 2

h - hours

HA - hyaluronic acid

HABR - hyaluronic acid binding region

HMA - hexamethylene amiloride

IEG - immediate early gene

IGF-I - insulin like growth factor I

IL- 1 - interleukin 1

IL-6 - interleukin 6

IP_3 - inositol 1,4,5-trisphosphate

KS - keratan sulphate

MAPK - mitogen activated protein kinase

NFκ-B - nuclear factor κ-B

NO - nitric oxide

eNOS - endothelial nitric oxide synthase

OA - osteoarthritis

PAPS - 3'-phospho adenosine 5'-phosphosulphate

PBS - phosphate buffered saline

PBS-BSA - phosphate buffered saline, 0.1 % azide, 0.2% BSA

PDGF - platlet derived growth factor

PG - proteoglycan

PGI_2 - prostacyclin

PGE_2 - prostaglandin E_2

PIS - pressure-induced strain

PKC - protein kinase C

PKA protein kinase A

PLA - phospholipase A

PLC - phospholipase C

PLD - phospholipase D

PTH - parathyroid hormone

Ras - rat sarcoma virus

RIA - radioimmunoassay

RT°C - room temperature (23-27 °C)

SA - stretch activated

sem - standard error of mean

SMC - smooth muscle cell

SOS - son of sevenless (*Drosphila* gene product)

Src - Rous sarcoma virus oncogene

TGF-β1 - transforming growth factor-β1

TIMP - tissue inhibitor of mettaloproteinases

TXA_2 - thromboxane A_2

2. OVERVIEW

Articular cartilage covers the heads of long bones in synovial joints, providing an essentially frictionless articulating surface that is resistant to the considerable sheer and compressive forces produced by normal joint movement. Osteoarthritis (OA) is a disease of joints that is characterised by degeneration and regeneration of the articular cartilage matrix eventually leading to cartilage destruction and loss, such that the underlying bone is no longer protected from the forces associated with joint loading and articulation. Causative factors leading to the development of OA can be classed either as those that render cartilage abnormal or those that lead to abnormal loading of cartilage (Fig 2.1) (1). In both types, a resultant imbalance between the mechanical forces on the tissue and the tissue's response leads to progression of disease and eventual cartilage loss (1). OA is the single largest cause of physical disability in the elderly (2). Our basic understanding of the organisation and regulation of the cartilage matrix is limited and needs to be extended before the processes that lead to the development and progression of OA can be further investigated.

The practical and ethical difficulties involved in studying human cartilage biochemistry *in vivo* and *ex-vivo* have lead to the extensive use of experimental animals to study both normal (3,4) and OA (5,6) matrix regulation. No animal model exactly replicates every aspect of human cartilage composition and metabolism, but scale effects mean that the mechanical forces involved in animal joints are similar to those in human joints (7) and taken together they are a valuable tool. Animal studies have demonstrated the importance of mechanical loading for the maintenance of normal cartilage integrity. Immobilisation or disuse of joints leads to reduction in matrix synthesis, whereas increased joint loading or exercise cause increased matrix synthesis (reviewed in (8)). These normal responses to loading are altered in animal models of OA where experimental intervention can be used to induce

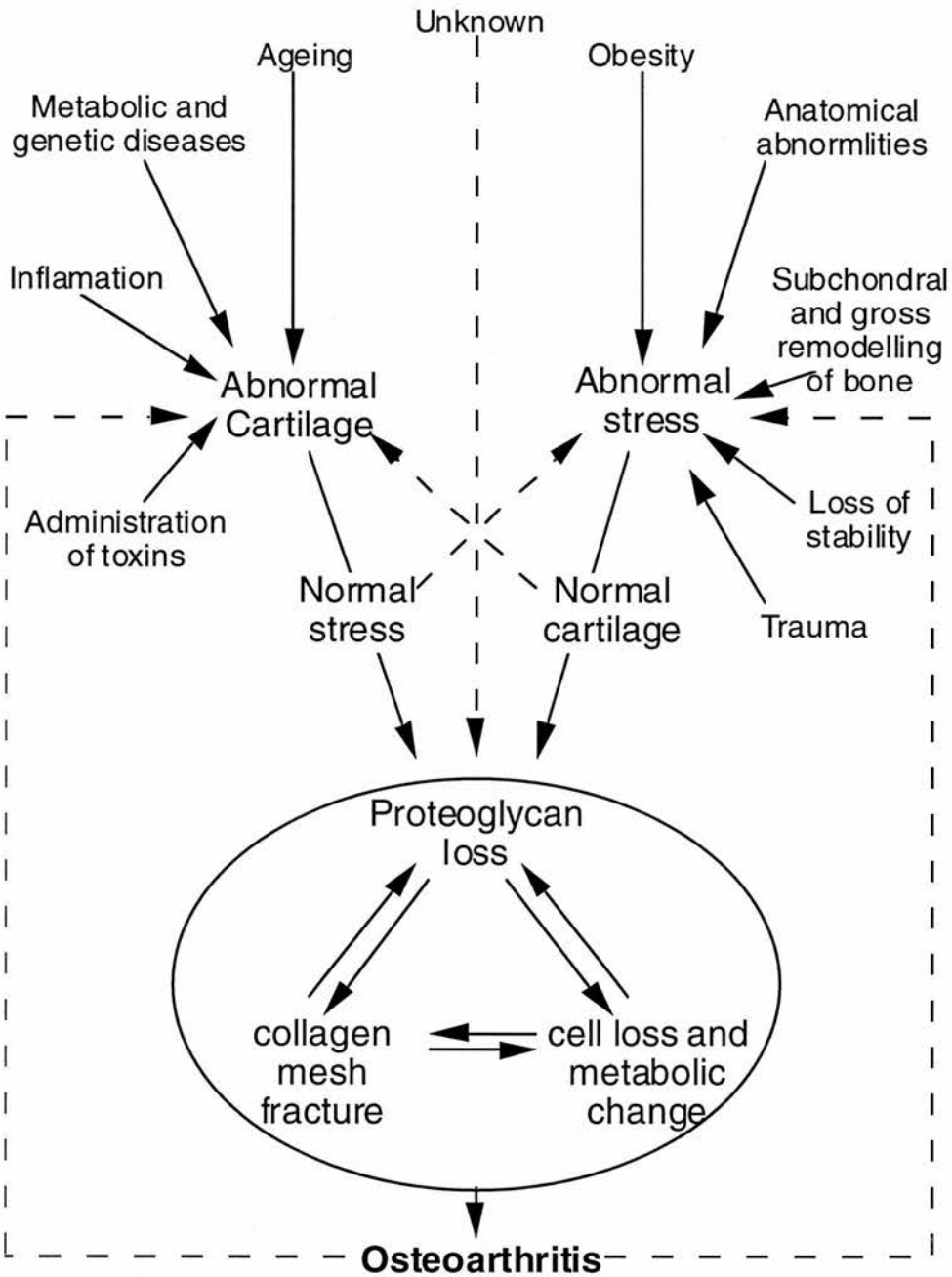


Figure 2.1 Development of OA. Causes may be categorized as those producing either abnormal cartilage or abnormal loading. In either case there is an imbalance between tissue composition and the stress to which it is subjected which produces changes in the matrix structure and production by cells. Adapted from Stockwell 1991 (1).

abnormal joint loading or abnormal cartilage matrix which is rapidly followed by degeneration of the matrix in a manner similar to OA (reviewed in (9)). In order to understand the involvement of mechanical loading of cartilage in the development and progression of joint disease we must first understand its role in normal matrix regulation.

This thesis sets out to establish how chondrocytes detect mechanical forces and how the resulting mechanical signals lead to altered metabolic responses. In order to achieve these objectives I have:

i) investigated the effect of cyclical pressurisation on chondrocyte metabolism using a custom built apparatus.

ii) identified possible chondrocyte mechanoreceptors

ii) identified intracellular signal transduction pathways in chondrocytes which mediate the alterations in metabolic function that occur in response to mechanical forces.

The main focus of the study was on chondrocyte proteoglycan synthesis, which is altered in the early stages of OA (1) and hence may be involved in disease development.

The layout of this thesis can be seen in the list of contents on page ii. Briefly, the overview is followed by a comprehensive review of literature describing the structure and function of cartilage, changes which occur in osteoarthritis, and the effect of experimental loading of cartilage and other cell types *in vivo* and *in vitro* . The general methods chapter then describes techniques which are common to all the subsequent chapters; these provide methods, results, and conclusions for distinct subjects of investigation. The results are discussed as a whole and conclusions drawn in the final discussion chapter.

3. LITERATURE REVIEW

3.1. Structure of Articular Cartilage

Macroscopically articular cartilage has a homogenous appearance since it is avascular and non-inervated (Fig 3.1) (1). Tissue sections reveal that it consists of a matrix with sparsely distributed chondrocytes existing in isolation or in small clusters, the cells occupying 1 - 10 % of the total cartilage volume (10). Articular cartilage matrix consists principally of water (70-80%), collagen (10-30%), and proteoglycan (PG, 3-10%) (11). The PGs exist predominantly as large aggregates (Mr. up to 5 x 10^8 Da) bound to glycosaminoglycan (GAG) chains of hyaluronic acid (HA) (12). The collagen forms a tough meshwork which entraps the PG aggregates preventing them from fully hydrating, and creating a fibre reinforced water-gel (1).

Cartilage varies in thickness from a few micrometers to a few millimetres (7), depending on the joint and the animal concerned. In large human synovial joints such as the knee, normal adult cartilage is 2-4 mm thick (10). Cartilage thickness varies not only from joint to joint but also from area to area within joints (7,13), loading patterns contributing to these variations. Weight bearing and moderate exercise has been shown to increase articular cartilage thickness (14,15), whereas immobilisation or excessive exercise can decrease it (14,16,17), demonstrating the importance of load to cartilage integrity.

Articular cartilage is normally divided into four zones (Figs 3.1 & 3.2) parallel to the surface (18):

(I) Superficial zone; adjacent to joint cavity, where collagen fibrils are arranged tangentially to the surface. The cells are discoidal with their long axis parallel to the surface.

(II) Intermediate (transitional) zone; the coiled collagen fibrils are randomly oriented in an interlacing meshwork, and the cells are spheroidal and evenly spaced.

(III) Deep zone; the cells are large, rounded and aligned in vertical columns. The collagen fibres are arranged radially to the surface.

(IV) Calcified zone; adjacent to the subchondral bone. There are few cells and the matrix is mineralised with crystals of calcium salts. The border between the deep and calcified zone is visible in histological sections and is called the tide mark (18).

In human articular cartilage the superficial zone and calcified zones each occupy about 5-10% of the total cartilage thickness and the other layers each occupy about 40-45% (10).

The zonal differences in cartilage matrix structure are determined by the embedded chondrocytes which produce that matrix. Studies of chondrocytes from these zones have demonstrated differences in cell density, cell proliferation, cell size (Figs 3.1 & 3.2), PG synthesis, collagen synthesis, and responses to the pro-inflammatory cytokine interleukin 1 (IL-1) (19,20). In human cartilage, chondrocyte density is 2-4 fold higher in the superficial zone than in the deep zone (1). Chondrocytes show negligible proliferation in mature cartilage but bovine chondrocytes obtained from the deep zone, show increased capacity for proliferation in culture in response to growth factors compared to those obtained from the superficial and intermediate zones (19). Rates of PG synthesis (20,21) and collagen (22) synthesis both increase progressively from the superficial to the deep zone. The accelerated rates of synthesis of matrix components may be responsible for higher GAG content (13) and thicker collagen fibrils seen in the deeper layers (19). The superficial layer has a higher water content and reduced GAG concentration (10). There are also structural differences between the PG produced by the different

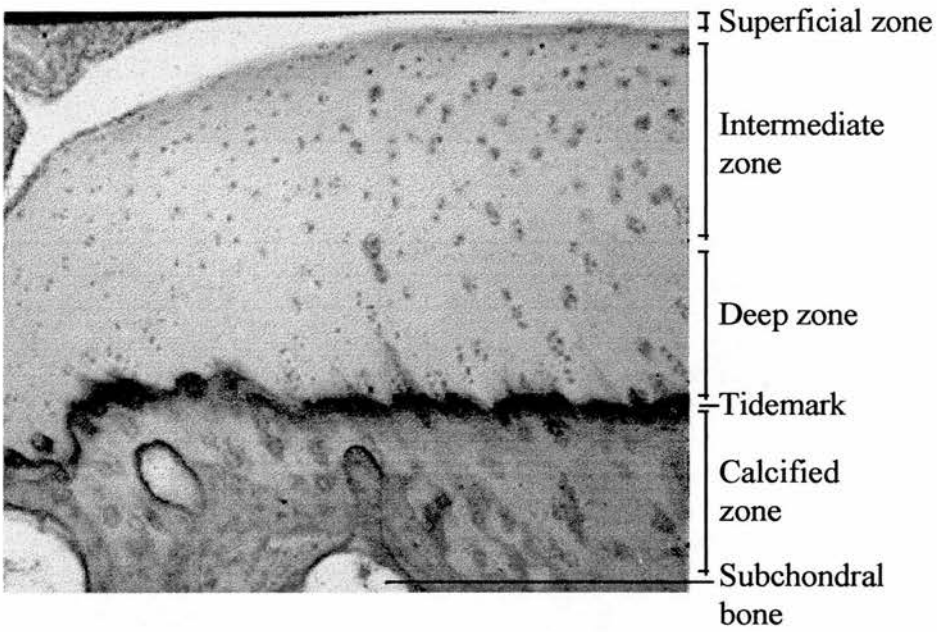


Figure 3.1 Section through normal articular cartilage. Cartilage taken from a normal knee joint sectioned and stained to show the different cartilage layers.

chondrocyte populations (19,23). These zonal variations may be related to the differences in cell shape 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. Inter-zonal variations are not the only evidence of heterogeneity in cartilage. The matrix of the intermediate and deep zones can be further subdivided into three regions: the pericellular, the territorial, and the interterritorial (10) (Fig. 3.2). The unit of specialised pericellular matrix and the chondrocyte from which it is derived form a structure termed the chondron which contains one or more cells and which can be isolated from cartilage (24). There is a fine collagen mesh (10) containing the cell and a dynamic PG structure assembled on HA which binds to its receptor on the cell membrane (25). The role of the pericellular matrix is difficult to study since it is destroyed by commonly used isolation procedures but it may function to mediate chondrocyte matrix interactions and the assembly of new matrix (24).

The territorial matrix lies between the chondron and the bulk of the cartilage or interterritorial matrix. Its collagen structure is intermediate between the fine meshwork of the pericellular matrix and the coarse fibres of the interterritorial matrix (26).

The interterritorial matrix forms the bulk of the articular cartilage. It has the classically described structure of a collagen meshwork with embedded PG-HA aggregates displaying the zonal variations described above.

3.2. The Chondrocytes

Chondrocytes are the sole cell type found in normal cartilage. Chondrocytes from different sources of cartilage vary considerably in size, number and activity (27), and those from articular cartilage form a heterogeneous population as described above. Adult human humeral head cartilage contains about 400 cells mm^{-3} in the superficial layer and about 160 cells mm^{-3} in the deeper layers (28). Bovine articular cartilage

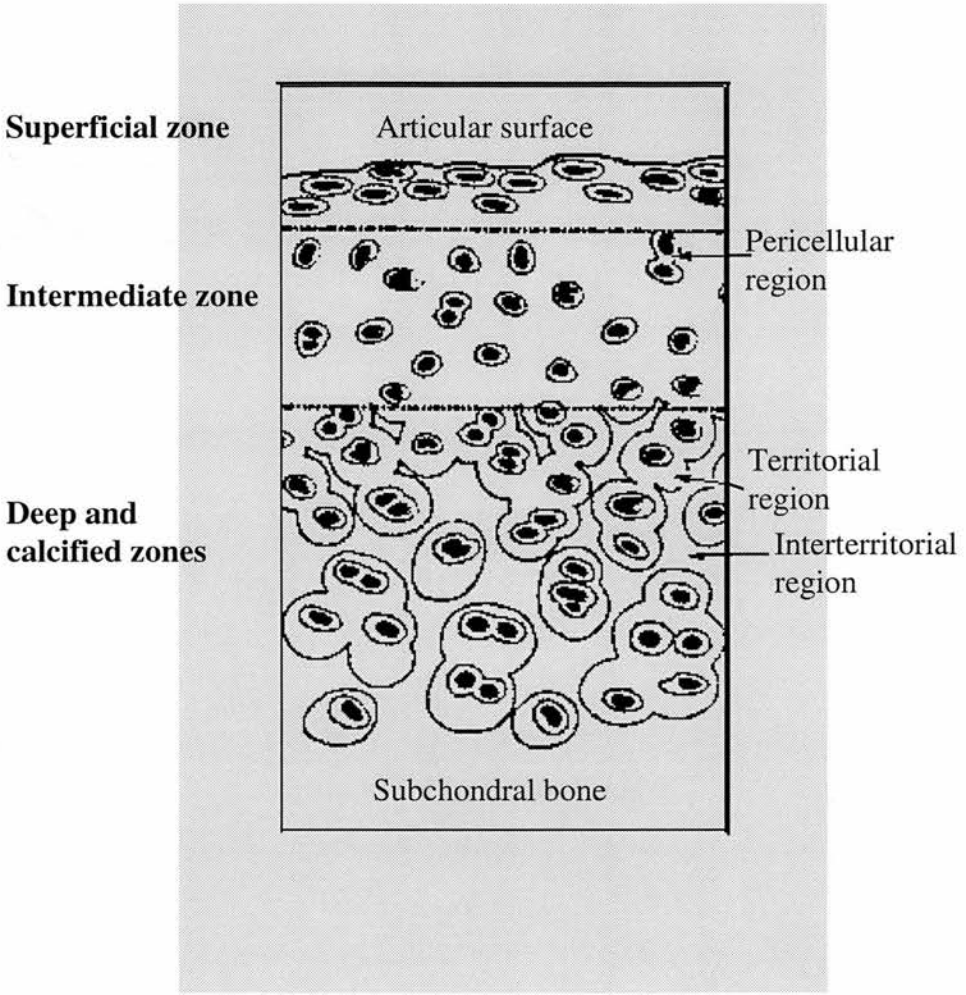


Figure 3.2 Diagrammatic reprsentation of the zones and regions of articular cartilage.

from the metatarsophalangeal joints of immature animals (8 - 11 months) contains about 40 cells mm^{-3} (28). Chondrocytes are discoidal or ovoid in shape with scalloped edges, and they contain extensive endoplasmic reticulum and Golgi apparatus (27). Measurements of chondrocyte size vary considerably depending on the microscopic technique used; superficial cells from human articular cartilage are about 14 μm long and 3 μm high, deeper cells are about 19 μm by 12 μm (28).

The chondrocytes' primary function is to maintain the surrounding matrix which is turning over continuously. In normal adult tissue collagen turnover is negligible (29) but PGs have a half-life of 10 - 20 days (30). The cells detect changes in the composition of the surrounding matrix, by unknown mechanisms, in order to maintain the relative concentration of its components (28). Chondrocytes respond to damage of the cartilage matrix by increasing synthesis of matrix collagen and PGs, but this does not result in repair of the lost cartilage as assembly of a normal matrix does not take place (31).

Chondrocyte structure and function can be studied in cartilage explant or isolated chondrocyte cell culture systems. Articular cartilage can easily be maintained in explant form in culture medium (32) so that the chondrocytes remain in contact with native cartilage matrix without the need for exposure to enzymatic treatments. 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 (32). 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. Chondrocytes are isolated from tissue by digestion with collagenase following which they can be cultured in monolayer, in artificial culture matrices such as agarose and alginate, or floating in spinner culture (33). Monolayer cultures are associated with some loss of chondrocyte phenotype (27,34) with time in culture and following passage. Removal from the cartilage matrix and attachment to tissue culture dishes result in change of cell shape and cell

volume, loss of cell-matrix interactions, and alteration in the chemical and osmotic environments, all of which may define the cells' phenotype *in vivo* (27). Culture systems such as agarose gel (21) and alginate gel (35,36) reduce the effect of chondrocyte isolation on phenotype by suspending the cell in an artificial matrix in which its shape is maintained and in which it does not form abnormal cell-substrate attachments. These methods are preferable to monolayer culture but are not practical for techniques, such as those in cell-stretch studies, that require cells to be immobilised on a substrate. Where it is essential to use monolayer cultures of chondrocytes, phenotypic changes can be kept to a minimum by using primary cultures which have been cultured for only short periods of time (27). Recent studies show that expression of cartilage-specific molecules, such as type II collagen, by chondrocytes is maintained in long-term monolayer culture despite other changes that are traditionally taken to indicate loss of phenotype (37).

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 (33). *In vivo* , chondrocytes experience very low oxygen tension (about 1 kPa compared to 20 kPa *in vitro* (38)) and metabolism is primarily by anaerobic glycolysis (38,39). Nutrient and growth factor diffusion are limited *in vivo* because of the small pore size in tissue (38). In cartilage explant culture, diffusion distances are reduced and the 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 (40). In isolated chondrocyte cell cultures the cells are bathed in nutrient medium and have negligible surrounding matrix. As a consequence both the rate and regulation of metabolism are considerably altered compared to chondrocytes *in vivo* (33).

3.3. Composition of articular cartilage

The cartilage matrix is composed of water, PGs, collagens, and minor glycoproteins (Fig. 3.3). The PGs can be structurally subdivided into different species, either on the basis of their core protein structure or the composition of the GAGs bound to the core protein. A number of different GAGs are produced in cartilage and are of particular interest since their relative concentrations and structures are significantly altered in ageing and disease (2).

3.3.1. Glycosaminoglycans

A glycosaminoglycan is a heteropolymer consisting of repeating disaccharide subunits containing hexosamine and hexuronic acid or hexose (12). These are linear molecules which are highly charged due to the presence of carboxyl groups and sulphate esters (12). GAG's 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 (2). The major GAG's found in cartilage PG's are Chondroitin sulphate (CS) and Keratan Sulphate (KS) , although Hyaluronic acid and dermatan sulphate (DS) are also found in articular cartilage (12) (Fig. 3.4).

Chondroitin Sulphate

The repeating unit in CS is glucuronic acid linked β-(1,3) to N-acetylgalactosamine. Linear CS chains are polymers of this disaccharide unit joined by a β-(1,4) linkage (Fig.3.4). The amine residues within the n-acetylgalactosamine can be sulphated in the -4 or -6 positions and these are referred to as chondroitin-4-sulphate (CS-4) and chondroitin-6-sulphate (CS-6). A GAG chain commonly exhibits regions of 4-sulphation followed by regions of 6-sulphation, but unsulphated and disulphated residues are also seen (12). The average CS chain consists of 25-30 disaccharides (about 20 kDa). CS-4 predominates in young cartilage and CS-6 in adult cartilage

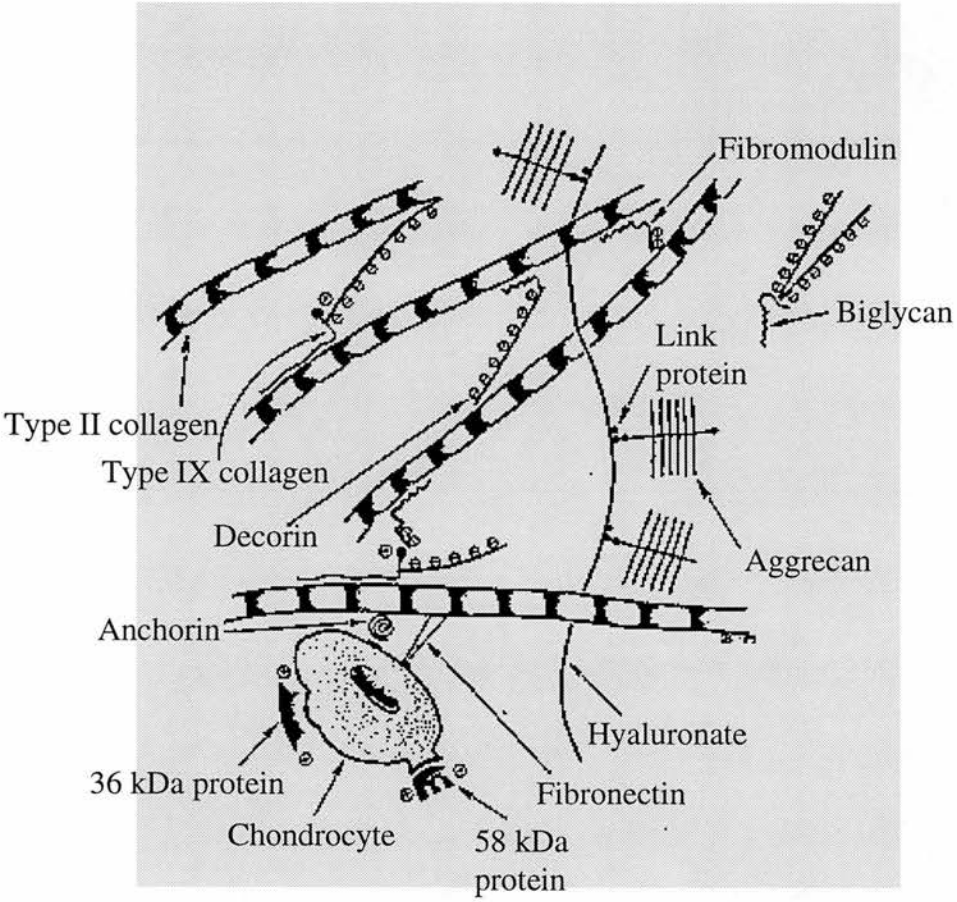


Figure 3.3 Schematic illustration of the major constituents of the cartilage matrix. Adapted from Oldberg *et al.* (56).

(41,42). In osteoarthritis the proportion of CS-4 increases (43), and following moderate exercise the proportion of CS-6 increases (42).

Keratan sulphate

There are two forms of KS in mammalian tissues, KS I is found in cornea and KS II is the type found in skeletal tissues (12). The disaccharide repeating unit is galactose linked β-(1,4) to N-acetylglucosamine (Fig. 3.4); KS is unique in containing no uronic acid (12). Most KS chains contain about 13 repeating units (about 5-10 kDa) linked β-(1,3) to each other. KS II is sulphated to a variable degree at C-6 of the N-acetylglucosamine residue (11). The proportion of KS in articular cartilage increases with depth from the articular surface (23) and during maturation and ageing (2,23,44).

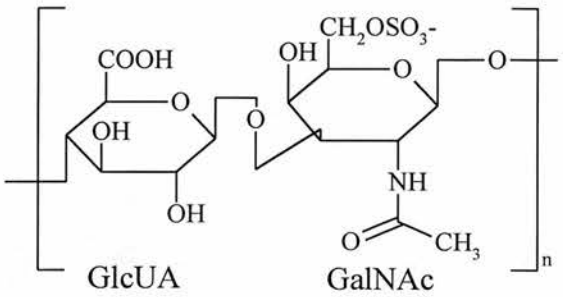
Dermatan sulphate

This GAG has a structure similar to that of CS except that some of the glucuronic acid residues are epimerised and converted to iduronic acid. The iduronic acid residues can be sulphated at C-2 and the N-acetylgalactosamine at C-4 (45). The glycosidic bond is changed to α-(1,3). In articular cartilage about 40% of the hexuronate can be iduronate instead of glucuronate (46).

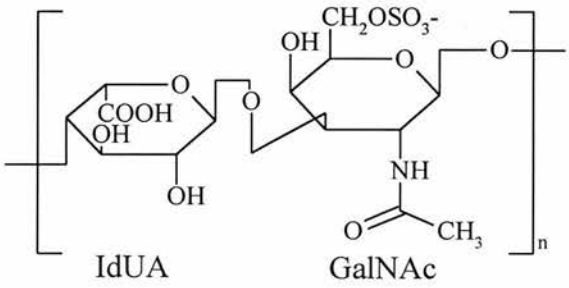
Hyaluronic acid

HA is the only unsulphated GAG found in cartilage and consists of repeating units of glucuronic acid linked β-(1,3) to N-acetylglucosamine. The disaccharides are linked β-(1,4) to each other to form long, unbranched chains of varying molecular weight (500-1600 kDa) (12). In articular cartilage it forms very large aggregates with PG's which bind a to minimum of five disaccharide units (47).

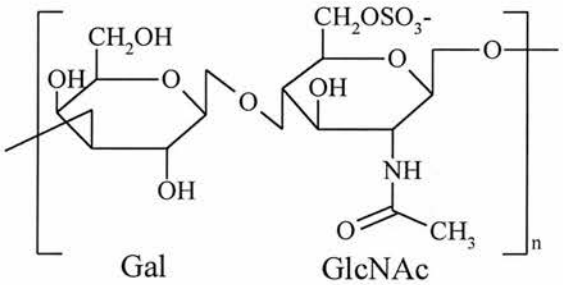
Chondroitin Sulphate



Dermatan sulphate



Keratan sulphate



Hyaluronic acid

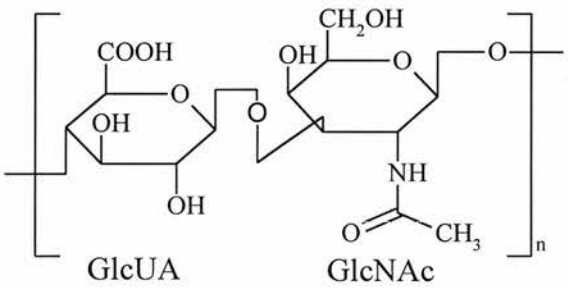


Figure 3.4 Structure of the disaccharide repeating units of cartilage GAGs. Sulphated GAGs are shown sulphated in the six position. Gal - galactose, GalNAc - n-acetyl galactosamine, GlcNAc - n-acetyl glucosamine, GlcUa - glucuronic acid, IdUa - iduronic acid.

3.3.2 N- and O-linked oligosaccharide chains

In addition to the GAGs described, N- and O-linked oligosaccharides are covalently bound to proteoglycan core protein. N-linked oligosaccharides have a complex triantennary mannose structure and form the majority of N-linked moieties (12). These N-linked oligosaccharides are relatively abundant in cartilage PG, in particular around the hyaluronic acid binding region, but their function is unknown.

There are three distinct O-linked oligosaccharides distributed along the whole PG core protein. They have no known function but they may represent incomplete KS II molecules since they have a similar structure to the linkage region of KS II (12).

3.3.3. Proteoglycans

Proteoglycan can be defined as "any macromolecule that has a core protein containing at least one covalently bound glycosaminoglycan chain" (48). Cartilage contains a variety of large and small PGs which have traditionally been thought to form an amorphous ground substance within the more ordered cartilage meshwork. It is now apparent, however, that there may be a degree of order as a result of interaction between GAG's (49). Large PGs predominate in articular cartilage and the majority of these exist in aggregates which are formed by non-covalent bonding to HA (Fig. 3.5) to give the characteristic 'bottle brush' structure that is visible under the electron microscope (50). The GAG chains of the PGs are highly negatively charged due to carboxyl and sulphate groups (12) and this creates a high fixed charge density in PG aggregates. This high concentration of negative charge attracts an equally high concentration of counter ions and the resulting osmotic potential creates a powerful swelling force within the matrix (12). The PG's are, however, prevented from swelling to their maximum hydrated volume by the collagen fibre network, which is kept under tension as a consequence. When the cartilage is compressed, following joint loading, water is squeezed out of the tissue until the raised osmotic

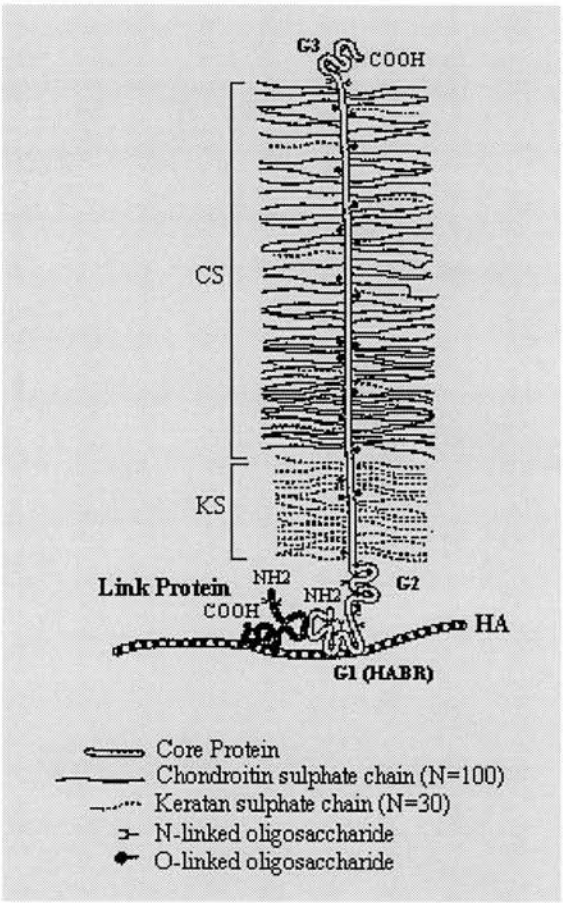


Figure 3.5 Diagrammatic representation of the interaction between aggrecan and HA stabilised by link protein. G1-3 are the three globular domains of the aggrecan core protein. HA - Hyaluronic acid, HABR - HA binding region. Adapted from Rosenberg & Buckwalter (50).

pressure is equal to the applied compression. Thus resistance to compression is largely a function of PG's (12).

Aggrecan

The predominant PG in articular cartilage is aggrecan or large chondroitin sulphate proteoglycan. It accounts for about 10% of the dry weight (45) and about 80% of total cartilage PG (12). 20-100 aggrecan molecules are linked to HA to give aggregates with a molecular mass up to 5 x 10^8 Da and up to 5μm long.

The aggrecan molecule is made up of 87% chondroitin sulphate (CS), 6% keratan sulphate (KS) GAGs, and 7% core protein by weight, and has a molecular weight of about 2600 kDa (45). The molecule (Fig. 3.6) has been shown to have three globular domains (G1-G3) and two extended regions (E1 &E2) (51). The amino-terminal G1 domain or HA-binding region (HABR) is responsible for HA binding. PG-HA interaction is stabilised by link protein, a 40kDa protein which is structurally related to the G1 domain (51). E2 is the major site of GAG attachment, although G1, E1 and G2 are also binding sites for KS and N- & O-linked oligosaccharides. The G3 domain may function to stabilise the molecule within the matrix since it is highly homologous to a lectin specific to galactose and fucose (52).

Small Chondroitin sulphate / 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 / Dermatan sulphate (DS) GAG side chains and its leucine rich core protein consist predominantly of 12 repeats of 24 residues. It has a molecular weight of 100 kDa (53). Decorin and biglycan constitute about 2% of the total cartilage PGs (54).

Decorin has about 55% sequence homology with biglycan but carries only one CS/DS side chain. It has a molecular weight of 67 kDa (53). Both biglycan and decorin interact with other matrix components and may be involved in tissue repair

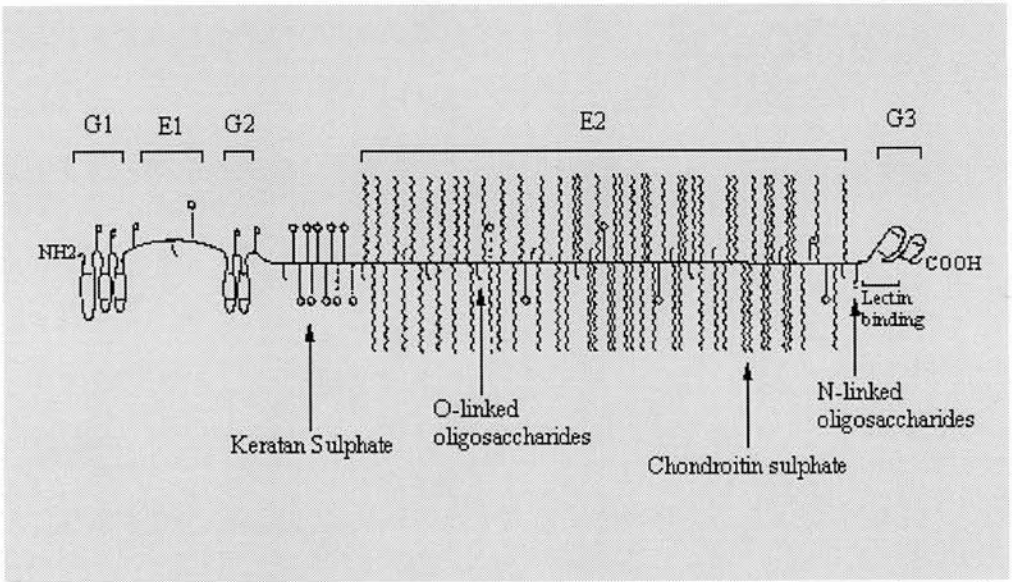


Figure 3.6 Diagrammatic representation of the cartilage proteoglycan Aggrecan and its domain structure. G1 - 3 are the three globular domains of the core protein. E1 & 2 are the two elongated domains. Adapted from Hardingham & Bayliss (2).

mechanisms (55). Decorin is so-named because of its ability to 'decorate' collagen fibres, binding to the triple helix via the core protein and thus having a possible role in the regulation of collagen fibrillogenesis (54). Decorin is also able to bind to fibronectin and the fibronectin cell binding site via its core protein (54).

Fibromodulin is a 59 kDa PG related to both biglycan and decorin. It has a similar core protein repeat sequences which are leucine rich and it is substituted with at least one KS chain. It has the ability to bind to molecules of type I and II collagen, which inhibits subsequent formation of fibrils from these molecules (56).

3.3.4. Collagen

The main structural element within articular cartilage is the meshwork of fibrillar collagen (Fig. 3.7). Fibres in the superficial layer are arranged tangentially in the form of a 'two-dimensional mat' which resists the shear stresses produced by joint articulation (57). In the deep layer the fibres are arranged radially and are anchored in the calcified layer (58). Their main function is to resist the internal swelling pressure of the matrix which can be as high as 200 kPa (59). The collagen fibres in the intermediate zone have an overall radial orientation but they interweave to form a mesh (1).

The collagens are a large family of at least 16 genetically distinct, specialised glycoproteins called types I - XVI. The generally accepted definition of a collagen molecule is that it is a structural protein of the extracellular matrix which contains one or more domains which have the conformation of the collagen triple helix (60). The collagen family can be subdivided into three classes on the basis of their structure and function (Fig. 3.8) (61). Articular cartilage collagen types include members of all three classes (45,62). The principle collagen in articular cartilage (90 %) is type II, but cartilage also contains types VI, IX, X, XI, XII and XIV collagens which together make up about 10% of the total collagen in cartilage (45,62).

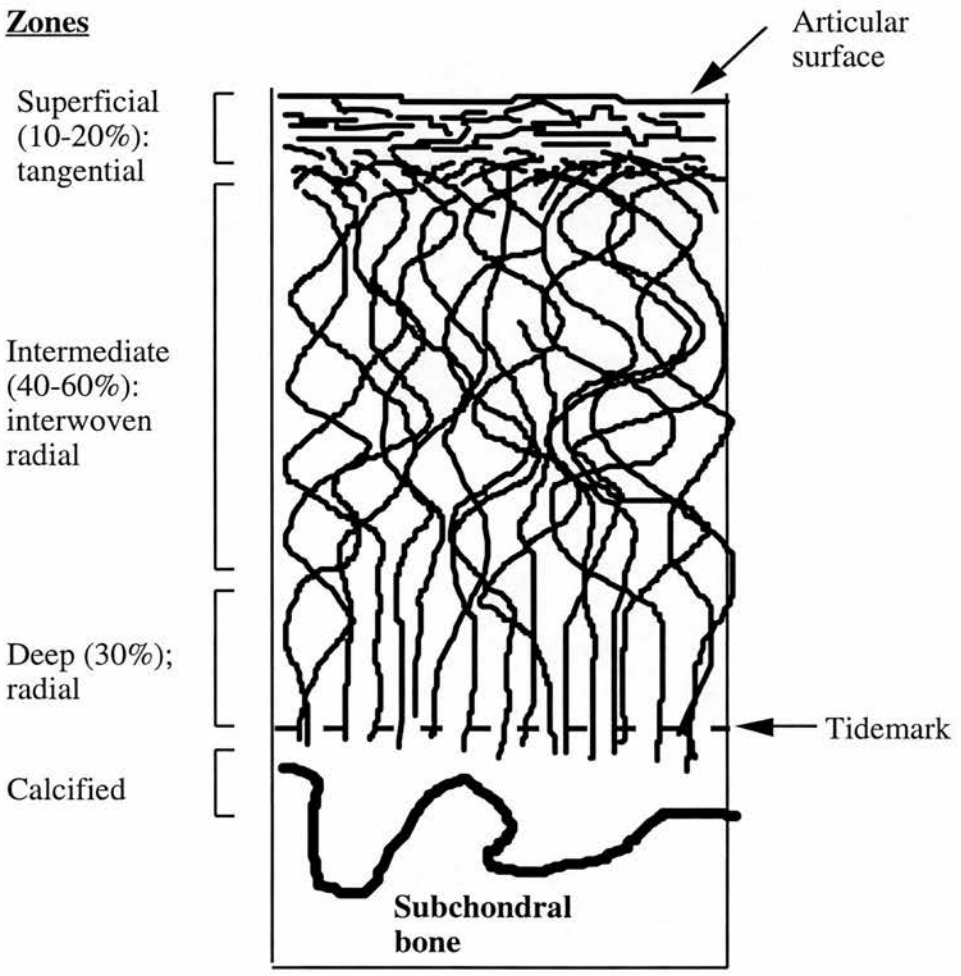


Figure 3.7 Schematic representation of cartilage meshwork organisation in adult articular cartilage. The percentage of the total cartilage thickness occupied by each zone is shown along with the overall collagen fibre orientation within each zone.

The collagen triple helix

The collagen triple helix is formed of three coiled polypeptides wound around each other to form a coiled-coil structure (60,63) (Fig. 3.9). Each polypeptide forms a left-handed helix such that every third residue is at the centre of the triple helix, but shifted by 30° compared to the previous central residue. This results in a right-handed superhelix (60). The amino acid sequence of helical domains of collagens has a characteristic Gly-Xaa-Yaa triplet repeat (where Xaa and Yaa are any other amino acid), such that only glycine residues are on the "inside" of the superhelix since it has no side chain to sterically hinder helix formation. Irregularities in the helix are produced by deviation from the triplet repeat sequence (60). Approximately 20% of the Xaa and Yaa residues are proline or hydroxyproline and are responsible for stabilising the formation of the triple helix. Lysine and hydroxylysine residues are also distributed through specific regions of the molecule forming inter- and intra-collagen covalent bonds (60). The triple helical domains are flanked by globular domains of polypeptide which lie at the carboxy - and amino termini of the polypeptides and between multiple helical domains in some collagen molecules (61). The terminal non-helical domains are the site of inter and intra-polypeptide disulphide bridges involved in formation of the triple helix, and are involved in regulation of the assembly of collagen molecules into fibrils and other structural motifs (64).

The side chains of the Xaa and Yaa residues are on the outer surface of the superhelix enabling collagen molecules to interact laterally with other collagen molecules (60). These intramolecular interactions create collagen structures such as fibres and sheets which are the framework for extracellular matrices. It is now clear that many different types of collagen interact so that collagen structures can be thought of as molecular alloys (64).

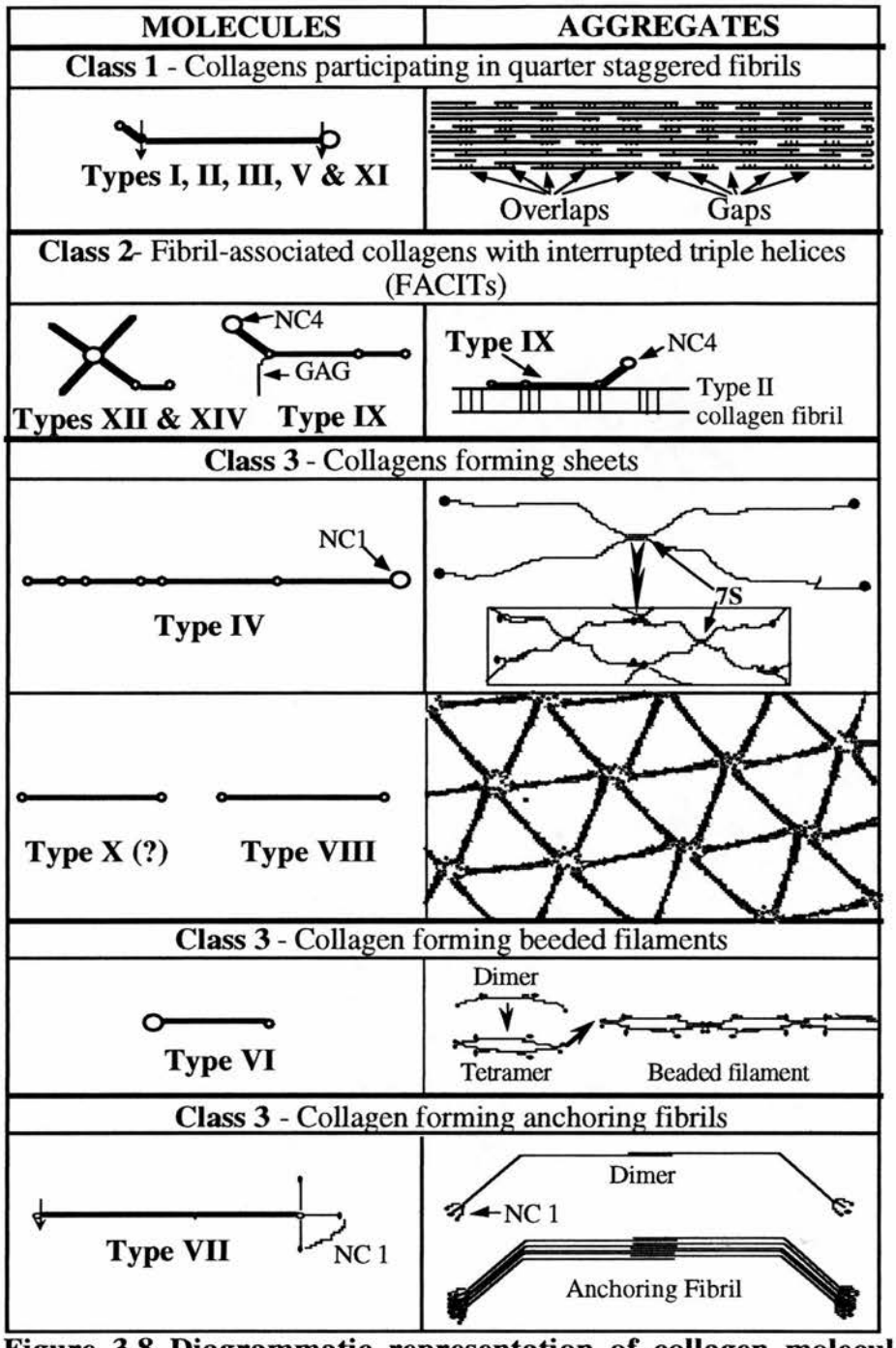


Figure 3.8 Diagrammatic representation of collagen molecules and aggregates. Molecules show collagenous (triple helical) domains as heavy lines and non-collagenous (NC) globular domains as open circles. Type VII has linear portions of its NC1 domain shown as a thinner line. Sites of propeptide cleavage prior to aggregation are shown by small arrows. Adapted from van der Rest and Garbone (64).

Collagen synthesis

The biosynthetic pathway leading to the formation of fibrillar collagen (class 1) is well understood while the analogous events involved in the synthesis of other collagens are less well documented. Reference will be made here to type II collagen since it is the major collagen of cartilage. Information regarding other types will be provided where available.

Type II is a homotrimeric molecule consisting of three α1(II) chains (49,63). The molecule is first synthesised as a precursor or procollagen which is enzymatically processed after secretion by the cell in order to allow fibril assembly (49,63). The polypeptides are synthesised in the rough endoplasmic reticulum where co-translational hydroxylation of proline and lysine residues occurs (61) through the action of prolyl and lysyl hydroxylases. These enzymes require for their activity ferrous iron, ascorbate, and α-ketoglutarate. Glycosylation of hydroxylysyl residues also occurs cotranslationally. Following production of the completed proα chains they are aligned and cross-linked by disulphide bonding in the C-terminal propart which triggers triple helix formation (49,61). The completed procollagen molecules are then exported to the cell surface where the pro-parts of the molecule are removed enzymatically prior to microfibrillar assembly (64). This assembly is based upon lateral interaction between homologous regions within the triple helical domains and is stabilised by lysine-derived cross-linking (64). The molecules are staggered by one quarter of their length, giving rise to a characteristic banding pattern visible under the electron microscope. Microfibrils are normally assembled from five collagen molecules which then aggregate laterally and end-to-end to form collagen fibres (61).

Class 1 collagens

The class 1 collagens (Fig. 3.8) have a characteristic domain structure and form quarter-staggered fibrils (49,64). In articular cartilage the class 1 collagen is type II collagen which is associated with type XI collagen. Most other connective tissues

contain type I collagen which may be associated with types III and V(63). The main triple helical domain of type I collagen is flanked at one end by the C-terminal propeptide, which prevents mature fibril assembly, and at the other end by a short (approximately 30 amino acid (aa) residues) linear domain followed by a second short (33-54 aa residues) triple helical domain (49,64). The final N-terminal propeptide is highly variable in size, but is large in type XI and is only removed slowly by proteolytic cleavage (61).

The type II collagen fibres of articular cartilage are of varying thickness and organisation according to the zone from which they are taken (see 3.1 above). These structural variations are the result of regulated fibrillogenesis either through regulation of the proteolytic enzymes involved in propeptide cleavage or through the synthesis of other types of collagen and PGs which directly influence fibril thickness, or are involved in fibre cross-linking (62).

Class 2 collagens

Also known as FACITs (*F*ibril *A*ssociated *C*ollagens with *I*nterrupted *T*riple helix), these collagens (Fig 3.8) are able to interact with the surface of fibrils of other collagen types and modify their properties (64). Types IX, XII, and XIV form this class and are all found in articular cartilage (45,62). These are non-fibrillar collagens which have three functional regions: one or two helical domains which interact with fibrils, a second triple helical region of only one domain which projects out from the fibril, and a large N-terminal non-helical region which flanks the second helical region and may interact with other matrix elements or cells (64).

Type IX is the best described of these collagens. It is a heterotrimer with three helical domains. Two of these are responsible for interaction with type II fibrils in cartilage which are stabilised by the formation of covalent crosslinks (61,64). The third domain projects out from the fibril and is flanked by a large N-terminal globular domain. This probably serves as a means of attaching new functional

groups, such as carbohydrate groups, to the fibrils (64). Type IX collagen has also been shown to be associated with a covalently-linked CS chain (61).

Types XII and XIV are homotrimeric collagens with only two triple helical domains and a very large N-terminal linear domain (61,64). The function of these collagens in cartilage is unknown.

Class 3 collagens

These collagens serve unique functions (Fig. 3.8). Type IV collagen forms sheets and is the major component of basement membranes (63). Type VIII collagen forms a regular hexagonal lattice found in Descemet's membrane of the cornea (64). Type X is synthesised by hypertrophic chondrocytes during endochondral ossification and persists in adult cartilage in the calcified zone (64) and superficial layer (65). It has a single triple helical domain flanked by two globular domains. Its function is unknown but it must be assumed that it is related to its highly specific distribution (61,64).

Type VI collagen is a heterotrimeric molecule with a very short triple helical domain bounded by two large globular domains (63,66). Type VI molecules aggregate head-to-tail to form dimers which then assemble into tetramers. These in turn form linear aggregates called beaded filaments (64,66). The filaments are stabilised by disulphide bridges and often bundle together and form a fine meshwork (64,66). The function of type VI collagen is uncertain, but it is found in all connective tissues and in cartilage it forms part of the distinctive pericellular matrix (24). Type VI collagen has 11 arginine - glycine - glutamine (RGD) sequences (64) which mediate binding of fibronectin to chondrocyte integrin receptors (67) suggesting that its pericellular distribution may be due to integrin binding.

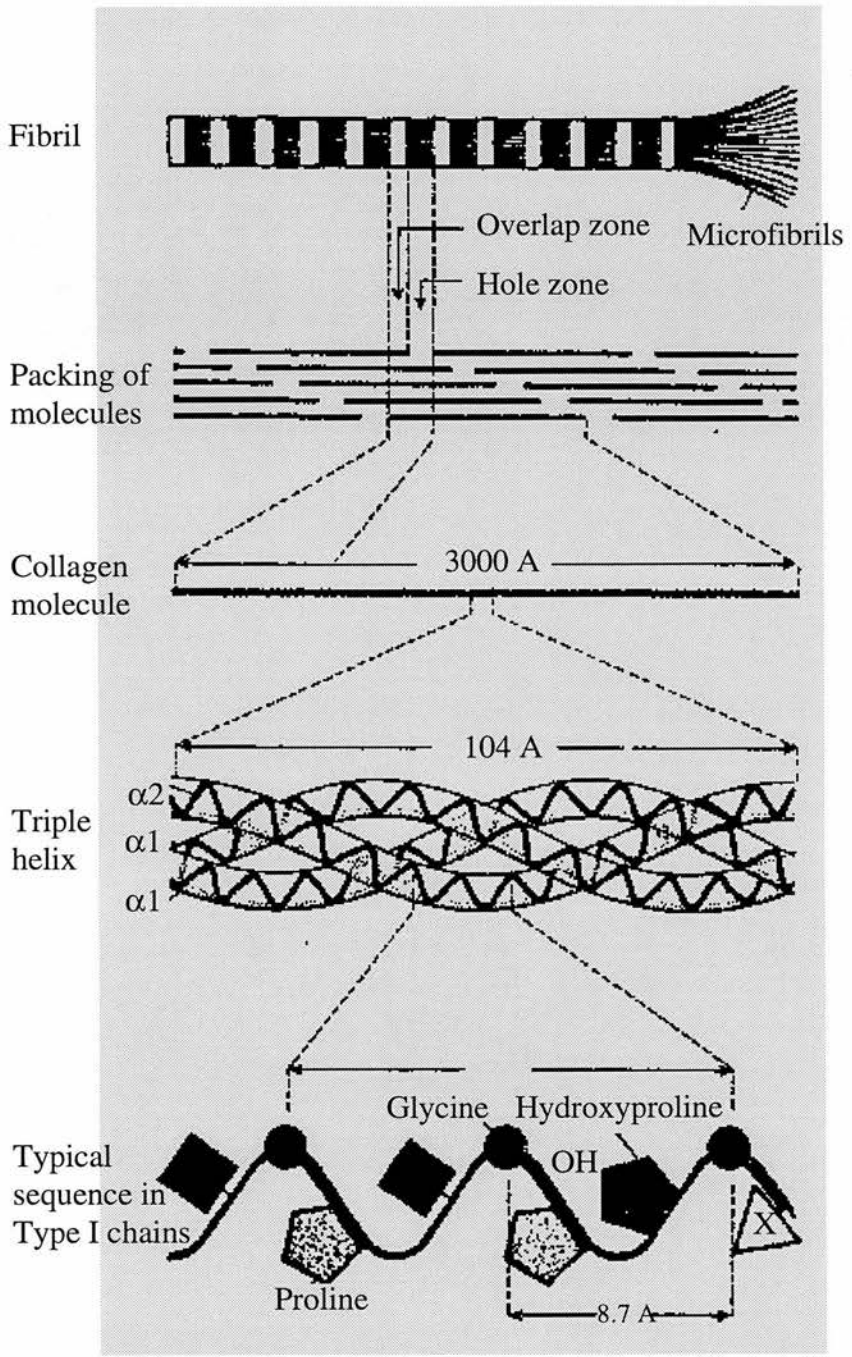


Figure 3.9 Schematic representation of the structure of a fibril of Type I collagen. Diagram represents the folding of the triple helix and the packing of molecules into fibrils. X represents any amino acid. Adapted from The textbook of Rheumatology (4th edition), p24.

3.3.5. Non-collagenous matrix proteins

In addition to the major structural elements of the cartilage matrix already described, a number of minor proteins and glycoproteins can also be isolated from cartilage, many of which may function in mediating interactions between chondrocytes and matrix components (68). Link protein is an exception in that it stabilises PG aggregates as already described (51).

Fibronectin is a minor glycoprotein constituent of cartilage which is distributed pericellularly and also throughout the matrix (69). It mediates chondrocyte adhesion to matrix components (68). It has been shown to be upregulated in OA cartilage (70). Anchorin binds type II collagen and may mediate the anchoring of chondrocytes within the collagen meshwork (68,71). Cartilage oligomeric protein which has been isolated from articular cartilage does not appear to be strongly linked to other matrix molecules and has no known function (68). Two un-named proteins of molecular weight 58 kDa and 36 kDa, have also been isolated from cartilage. Their function is uncertain as yet but they may bind to the chondrocyte surface (68).

3.4. Proteoglycan metabolism in articular cartilage

In mature, normal articular cartilage there is very little collagen turnover but considerable enzymatic breakdown and synthesis of PG (72). For functional PG aggregates to form, HA and link protein must be synthesised simultaneously in order to allow extracellular assembly to take place (73,74). PG synthesis occurs intracellularly in the endoplasmic reticulum and Golgi apparatus (73,75) but synthesis of hyaluronate occurs on the cell membrane from where it is exported directly (76).

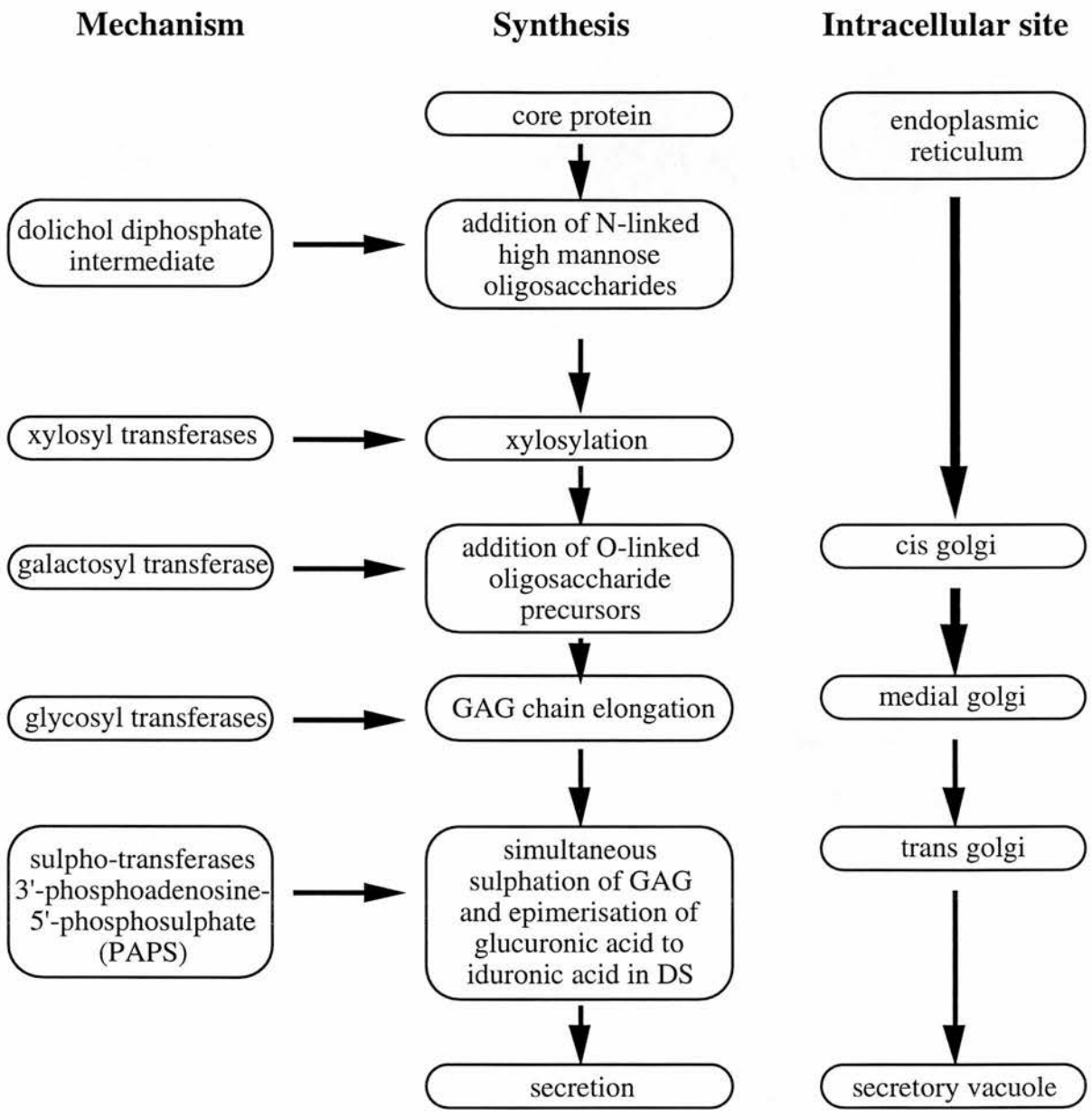


Figure 3.10 Post-transcriptional events in the biosynthesis of PGs. Adapted from Poole (73).

3.4.1. Proteoglycan synthesis

Synthesis of the protein and carbohydrate moieties of PG occur sequentially (77-79), although the initial steps of GAG chain synthesis may occur while the core protein is still being synthesised (80) (Fig. 3.10). The core protein is translated from mRNA by ribosomes on the rough endoplasmic reticulum (81), a process which has a half time of approximately 60 min and represents 70-90% of the time taken to secrete a completed PG molecule (78,82). Following translocation to the trans-golgi cisternae more than 7000 post-translational modifications are performed, in the 10-15 min remaining before secretion by exocytosis (78,79,82). 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 (83).

N-linked oligosaccharides and O-linked oligosaccharides are added to the PG precursor at an early stage, possibly cotranslationally, in the endoplasmic reticulum (75). The N-linked oligosaccharides are added in the form of dolichol-bound high mannose primers which are processed after translocation of core protein to the golgi apparatus (75). The O-linked oligosaccharide precursors are linked to the protein core by galactosyl-transferases and then extended by other transferases to be completed at about the same time as the CS chains (84).

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 ER and cis-golgi (85-87). Elongation of GAG chains by addition of alternate glucuronic acid and N-acetylgalactosamine then takes place in the medial and trans-golgi apparatus through the action of specific transferases (85). Sulphation probably takes place in the trans golgi network during chain polymerisation and is mediated by sulpho-transferases (75,88) which add sulphate from 3'-phosphoadenosine 5'-phosphosulphate (PAPS)

(88,89). Terminal sulphation may terminate chain polymerisation but little is known about regulation of chain length, which can vary significantly (88,90).

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 oligosaccharide (75). 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(83). The proportion of KS in completed PG's is variable but the sum of O-linked oligosaccharides and KS is relatively constant, indicating their common origin (75). The mechanism that determines whether KS or oligosaccharide polymerisation occurs is unknown (75).

Hyaluronate synthesis is performed by hyaluronate synthetase which is found on the inner side of the cell membrane (76). It functions to link N-acetylglucosamine and glucuronic acid sequentially, the product being extruded directly into the extracellular matrix. The incomplete HA 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. It is not known what determines chain length but it is likely to depend on increasing interactions with matrix components as polymerisation proceeds (76). Extracellular assembly of proteoglycan aggregates on HA 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 (91). The bulk of new HA, when released from its synthetase, diffuses away from the cell into the territorial matrix, but some will remain in the pericellular matrix bound to the cell-surface HA receptor CD44 (25).

3.4.2. Proteoglycan catabolism

In normal adult cartilage there is a dynamic balance between PG synthesis and degradation whereby the chondrocytes are able to maintain a uniform concentration of PG within the cartilage matrix (92). In disease this balance is upset such that the

rate of degradation exceeds the rate of synthesis and normal tissue function is compromised. The variation in PG concentration within cartilage and across joints (19) indicates that sensitive mechanisms exist to regulate proteoglycan turnover which are dependent on the chondrocytes' physicochemical environment. It is not known precisely how PGs are lost from cartilage or how chondrocytes detect these changes, but it is clear that in disease, these mechanisms malfunction.

Experimentally pro-inflammatory cytokines, such as Il-1, have been shown to stimulate matrix degradation and decrease PG synthesis (93) whereas growth factors, such as TGF - β, have the opposite effect (92). As has already been indicated, PG and HA synthesis are closely co-ordinated, and it is possible that cell surface molecules that interact with HA may regulate synthetic and catabolic pathways (92). Mechanical loading of chondrocytes has also been shown to be critical in the regulation of PG turnover, but the mechanism by which this occurs has not been defined (93). It is possible that all regulatory factors alter matrix turnover by a common mechanism such as the regulation of the many degradative enzymes and their inhibitors that are found in cartilage (92). In order to understand the processes involved in disease of cartilage, the regulation of normal matrix turnover which predicts tissue composition and hence function must first be established.

3.4.3. Zonal and topographical variation of cartilage composition and metabolism

The relative concentration of matrix components varies with depth from the surface (19,94 - 106); this has been shown to be regulated by the embedded chondrocytes (19). CS content increases with increasing depth from the surface reaching a maximum in the intermediate zone (94,95,97,102). The CS chain length may also increase with depth (94,95,102). The KS content similarly increases with distance from the joint surface (95,100,101,107). The superficial layer is rich in HA (98,108), collagen (19,22) and water but contains little link protein (98). The surface layers

also demonstrate the most prominent expression of the DS-PG decorin (109). The degree of aggregation of PG's is reduced in the deeper layers, probably due to the reduced concentration of HA (94,95).

The zonal variation in matrix composition is reflected in the metabolism of chondrocytes from the each zone (21). Cells from the superficial layer have lower rates of synthesis of PG's and collagen (18,22,103,106,110,111) when compared to cells in deeper layers. Turnover of PG is significantly higher in chondrocytes of the superficial layer which may be due to the lower aggregation of PG's synthesised by these cells (21).These metabolic differences are maintained by isolated chondrocytes from the various zones of articular cartilage in a variety of culture systems (21,106,110) demonstrating that they are an intrinsic property of the chondrocytes and not secondary to zonal variations in matrix composition (19). The factors that determine the metabolic status of chondrocytes are unknown but may be related to cell shape (19).

As well as zonal variations in cartilage composition and metabolism there are topographical differences within the joint. In weight-bearing regions the cartilage is thicker and has elevated collagen and PG content, and contains more KS, DS and HA than adjacent unloaded regions (14,98,108,112-115). These observations point to the critical role of mechanical stimulation in the regulation of chondrocyte function.

3.5. Loading of articular cartilage

Loading of articular cartilage *in vivo* results from weight bearing and joint movement. Loading can be mimicked *in vitro* either by mechanical compression of cartilage explants or by exposing chondrocyte cultures to hydrostatic pressure or stretch. Numerous studies have examined the effect of loading on articular cartilage composition and metabolism both *in vivo* (reviewed in (3)) and *in vitro* (reviewed in (116)). Joint loading leads to a number of physicochemical changes within the cartilage including mechanical deformation, altered hydrostatic pressure, loss of

water, streaming potentials, and altered pH and osmolality (117). Hydrostatic pressure has been measured in a human hip joint using implanted pressure transducers. This study has shown average pressures of 1.5 to 3.7 MPa and peak pressures on standing of 10-20 MPa (118-120). Scale effects indicate that similar increases would be expected in other species (7). The contact pressures generated during joint loading can result in an average compression of the tissue by up to 13 % of its uncompressed thickness (121) leading to matrix deformation (122,123) and deformation of chondrocytes (124,125). Modulation of cartilage composition and metabolism has been shown to be dependent on the amplitude and frequency of the load applied, the most striking differences being found between static (continuous) and dynamic (intermittent) load (116).

3.5.1. The effect of articular cartilage loading *in vivo*

In vivo loading studies in animals can be divided into those that have looked at the effect of (a) reduced loading, (b) increased loading, and (c) running exercise (3). Although the majority of these studies have been carried out in quadrupeds the similarity of the stress levels encountered in various species suggests that the experimental findings have relevance to human articular cartilage (7).

Reduced loading

Reduced loading has been achieved most commonly by immobilisation of one limb by splinting (120) or by the application of rigid casts (14,17,120,126). Partial amputation, such that free movement of a joint still occurs without weight bearing is an alternative approach (127). In most of the studies, the contra-lateral limb is exposed to elevated load and sham operated animals act as controls (3). The precise nature of the immobilisation (e.g. in extension or flexion) can significantly alter the results obtained, but in general reduced load is deleterious to cartilage integrity leading to tissue atrophy (3). Reduced loading leads to reduced GAG content

(14,17,120,126), elevated water content (17,120,127), reduced cartilage stiffness (128), reduced GAG synthesis (17,120,126), and reduced aggregation of PG (120), without effect on collagen content or structure (3). These effects are also seen in reduced loading where motion is still possible (120,127) indicating that reduction in compressive force is more important than joint movement (3). The softened matrix produced following reduced loading can be regenerated after low levels of exercise (17,129) or continuous passive motion (130), but is susceptible to damage by severe exercise or loading (129,130).

Increased loading

The contra-lateral limb has been used in immobilisation or amputation studies to investigate the effect of increased load on articular cartilage. This model has the disadvantage that abnormal loading patterns are produced because of the abnormality of walking on three rather than four limbs. Elevated weight bearing leads to unchanged (127) or small increases in cartilage GAG content (14) and increased synthesis (126) with local variation in the degree of response when load bearing and non-load bearing areas are compared (14). Hence, elevated loading enhances the cartilage matrix structure whilst unloading produces a softer more 'watery' matrix that is susceptible to damage (3).

Running exercise

Running exercise in experimental animals has effects on articular cartilage similar to both reduced and elevated loading (3). Strenuous running exercise regimes reduce GAG content (16,131) and produce local tissue damage at the contact sites of the cartilage (129). In contrast moderate running exercise enhances GAG content and cartilage thickness locally (15,42). In both situations PG synthesis is elevated (129,131). Many of the changes seen following running exercise are localised to the

immediate contact areas demonstrating the importance of mechanical factors in the maintenance of cartilage matrix integrity (3).

3.5.2. The effects of loading articular cartilage and chondrocytes *in vitro*

The loading studies described so far have a number of disadvantages which have lead to the development of systems for cartilage loading *in vitro*. The very nature of *in vivo* loading means that measurements of matrix structure, composition, or synthesis must be performed after the loading regime is completed when cartilage can be removed for analysis. *In vitro* systems allow real time measurements of physical parameters such as cell shape and intracellular ion fluxes (124,132-134), and metabolic labelling during loading. The precise magnitude, distribution, and frequency of load in *in vivo* studies is ill defined and shows wide variation between studies and species (reviewed in (3)). In contrast, loads of known magnitude and frequency can be applied to a defined area of cartilage in *vitro*, in a precisely maintained physicochemical environment. However, *in vitro* loading studies place articular cartilage in a non-physiological environment, exposing the tissue to conditions never experienced *in vivo*. Nevretheless, limitations of *in vivo* studies, make controlled *in vitro* loading studies the method of choice for investigating the mechanisms of cartilage matrix regulation.

Loading studies can be divided into three groups:

(a) those looking at the mechanical compression of cartilage tissue explants or chondrocytes cultured in a three-dimensional agarose matrix,

(b) those looking at the effect of hydrostatic pressure on cartilage explants or chondrocytes in a number of culture systems,

(c) those looking at the effect of stretch on chondrocyte cultures.

The detailed effects of all types of chondrocyte and cartilage loading studies which are described in Chapter 5 (p.74) of this thesis, are summarised in this section.

The effect of mechanical compression of cartilage explants and chondrocyte agarose cultures

These studies have examined the effect of loading by mechanically compressing full-thickness plugs or explants of cartilage which are cut from the joint *post mortem*. In general static compression leads to reduced matrix PG synthesis over hours or days but the degree of response is dependent on the amplitude of the applied load (135-144). Cultures in three-dimensional agarose gels responded to continuous compression by reducing PG (135,141) and protein synthesis (135). Static compression of explants also reduces protein synthesis (138,143,145), in particular of fibronectin (138), core protein and link protein (142). In contrast fibronectin accumulation is stimulated by cyclical impaction of explants (146). Very few studies have investigated the effect of mechanical compression on cartilage collagen synthesis, probably due to its low turnover rate (72), and no studies have demonstrated the modulation of collagen synthesis or turnover by static compression.

Compression of cartilage plugs or chondrocyte/agarose cultures produces a number of events in the matrix (Table 3.1) (117). The rapid events may be responsible for stimulating matrix synthesis (117,143) in the initial period of static compression (142) or during dynamic compression (135,143,147), whereas the later events may act to produce the longer term reduction in matrix synthesis (143,147).

Table 3.1 The sequence of events following the application of load to articular cartilage. Adapted from Urban and Hall (117).

Very rapid events (ms)	1. Rise in hydrostatic pressure 2. Tissue and chondrocyte deformation 3. Fluid flow resulting in streaming potentials
Slower events (min - h)	4. Fluid loss 5. Increased GAG concentration 6. Changes in ionic composition of interstitial fluid; (a) increased cation (Na^+, K^+, Ca^{2+}, H^+) concentrations (b) increased osmolality (c) decreased anion (Cl^-, HCO_3^-, SO_4^{2-}) concentrations

Dynamic mechanical compression of cartilage explants leads to increased matrix PG synthesis, although this varies with the frequency of the loading cycle (135-139,141,148-153). In general frequencies approaching 0.1 Hz are stimulatory to PG synthesis (135-137,139,143,144,148-152) whereas lower frequencies are inhibitory (135-137,139,143,152). The effect of dynamic loading on cartilage explant PG synthesis is summarised in Table 5.2 (see section 5.1.1., p75). Protein synthesis is also stimulated by cyclical compression of explants (135-137,139), but collagen synthesis has been shown to decrease (154) and tritiated hydroxyproline residues are lost at an increased rate from cyclically loaded explants (155) indicating increased collagen breakdown.

The effect of hydrostatic pressure on cartilage explants and chondrocyte cultures

Relatively few studies have examined the effect of hydrostatic pressure on articular cartilage or chondrocyte function (Tables 5.3 & 5.4 in section 5.1.2.). However in the studies that have been performed, the results for the response to cyclical and static pressurisation are broadly in agreement with those seen following mechanical compression. Cyclical pressure stimulated PG synthesis in cartilage explants (156-159) and chondrocyte cultures (158,160-162) but the response was very sensitive to

the magnitude and frequency of the stimulus. Static hydrostatic pressurisation of cartilage explants reduced PG synthesis at 10 - 50 MPa (156,163) and stimulated PG synthesis at 1-10 MPa (156,163,164). Collagen mRNA levels have been shown to be elevated by cyclical pressurisation of explants (165) and continuous pressurisation of a chondrocyte-like cell line (163). Cyclical pressurisation was also shown to elevate intracellular cAMP levels (166) and induce changes in the golgi apparatus (167) and cytoskeleton (168) of cultured chondrocytes. Changes in hydrostatic pressure are clearly an important component of the forces generated following mechanical loading that regulate chondrocyte function. The sensitivity in response to different pressurisation regimes indicates that the cells may have in-built threshold levels that are defined by the tissue source.

The effect of stretch and shear-stress on matrix synthesis by chondrocyte cultures

Chondrocytes can be grown on flexible membranes or coverslips and then exposed to stretch by deformation of the culture substrate (169-172) which mimics the deformation of the cartilage substrate under load *in vivo*. Both intermittent (169,171), and continuous (170) stretch cause significant increases in sulphate incorporation, as well as an elevation of intracellular cAMP (169,171) and DNA(171). The results are summarised in Table 5.5 (see section 5.1.3., p. 82). Intermittent stretch also increases type II collagen mRNA and PG core protein mRNA levels in cultured chondrocytes (172). Fluid induced shear stress of chondrocytes equivalent to a force of 1.6 Pa on the cell membrane induces a two fold increase in $[^{35}SO_4]$-sulphate incorporation, increases the release of prostaglandin E2, as well as causing cells to elongate and align with the direction of stress (173) and inducing a 10-fold increase in interleukin-6 (IL-6) mRNA and protein (174) and TIMP-1 mRNA (175). Chondrocytes may be exposed to shear stress in vivo due to fluid flow or the effects described may be due to cell deformation.

It is clear that chondrocytes *in situ* in cartilage respond to interacting mechanical forces and are sensitive to both the frequency and magnitude of these forces. It may be that different mechanical stimuli activate interacting signal transduction mechanisms, the net effect of which is seen in the final metabolic response. In order to gain insight into the complex mechanotransduction pathways activated in chondrocytes it is of value to examine the extensive investigations which have been performed to elucidate mechanotransduction in other cell types .

3.6. Loading of non-cartilaginous cell types

Many cell types are exposed to mechanical forces which play a central role in the regulation of cell function. Cellular mechanisms activated on stimulation by these forces allow organisms to interact successfully with their environment. The role of mechanical forces in the regulation of cell function in many cell types, including endothelial cells lining blood vessels, smooth muscle cells, cardiac myocytes, lung epithelial cells, and bone cells, have been investigated. The endothelial and smooth muscle cells of blood vessels are exposed to regular fluctuations in pressure, stretch, and fluid flow; the effect of these forces has been the subject of extensive study. Bone cells have the same embryonic origins as chondrocytes and their primary function is to regulate the structure of the surrounding bone so that it is able to withstand the forces to which it is exposed. Bone cells exist in a similar high-stress environment to chondrocytes and so a knowledge of bone cell responses to experimental loading will be of value in studies of chondrocyte loading.

3.6.1. The effects of mechanical stress on vascular endothelial and smooth muscle cells

The endothelium is a confluent lining monolayer of thin, flattened, rhomboid cells located between the flowing blood and the deeper layers of the vascular wall (176). Normal endothelial cells (EC) maintain a balance in blood vessels between growth

promotion and inhibition, vasoconstriction and vasodilation of smooth muscle, platelet adherence and nonadherence, and anticoagulation and procoagulation (176). They are exposed to significant fluid forces and their response to mechanical forces forms an integral part of normal physiological control of vascular tone (177). Vascular fluid flow causes frictional shear stress of EC, cyclical increases in luminal pressure, and resultant stretch of the vascular wall. The responses to fluid flow that occur result from the integrated effect of these three stimuli (177). ECs are attached to the subendothelium, a highly organised matrix consisting of collagens, elastin, fibronectin, laminin, GAGs, thrombospondin, vitronectin, and von Willibrand's factor (176). The constituents of this matrix which provide structural integrity, mechanical strength and elasticity to the vascular wall are synthesised by the overlying endothelium (176). The role of mechanical stress in the regulation of EC function has been the subject of considerable research (reviewed in (177)). It is only summarised here, with a particular emphasis on mechanisms that may be of relevance to the study of loading of articular cartilage. The evidence available regarding the signalling mechanisms that mediate EC responses to mechanical stress can provide valuable indicators of the areas which should be investigated in the study of chondrocytes' response to mechanical loading.

Studies of EC morphology have demonstrated a relationship between shape and orientation of these cells and the direction of blood flow (177). *In vitro* studies have demonstrated that EC, which are polygonal and form a 'cobblestone' pattern in culture, align and elongate with the direction of fluid flow (177), perpendicular to the direction of stretch (178), and elongate with random orientation in response to hydrostatic pressure (179). These changes in cell morphology are generated by reorganisation of the cytoskeleton which is responsible for controlling cell shape (177). The filamentous actin redistributes from the periphery to form bundles of central stress fibres (179,180) associated at their ends with new focal contacts, which are also the site of clustering of the cytoskeletal proteins vinculin and talin, and of

the cell surface receptor $\alpha5\beta1$ integrins (177,181). The EC surface adhesion molecule ICAM-1 is selectively upregulated by laminar flow induced shear stress which has no effect on expression of the cell surface receptor E-selectin (182). In addition to these morphological and structural alterations, the application of shear stress, stretch and hydrostatic pressure has been shown to cause rearrangement of, and an increase in the production of the subendothelial matrix components collagen, elastin, fibronectin, and laminin (181,183).

Mechanical stress of the vascular endothelium alters cell proliferation (179) and the production of a number of vasoactive substances (176,177,184). Relaxation of the vascular wall is brought about by the action of nitric oxide (NO), prostaglandin I_2 (PGI_2 or prostacyclin), prostaglandin E_2 (PGE_2) , and endothelium derived hyperpolarising factors (EDHFs) on the smooth muscle cells (SMC) of the blood vessel wall which are responsible for vascular tone (176). NO is the principle endothelium derived relaxing factor (EDRF), it is released rapidly in response to increases in shear stress (177) and its production is also elevated by chronic increases in flow and strain which increase expression of endothelial nitric oxide synthase (eNOS) (184), the enzyme responsible for NO production. PGI_2 is also released in response to increased fluid flow; it is less potent than NO but its effects are of longer duration than those of NO, which is broken down very rapidly (177). In addition to causing vasodilation NO and PGI_2 act to inhibit platelet aggregation, and PGI_2 also acts as a profibrinolytic agent (176). Contraction of the vascular wall is mediated by endothelium derived constricting factors (EDCFs) such as endothelin-1(ET-1), angiotensin II, and thromboxane A_2 (TXA_2), as well as reduced levels of vasodilators (176). The application of increased shear stress on EC causes down regulation of ET-1 which is a potent and long-lasting vasoconstrictor (176). Changes of cell proliferation in the vascular wall are important in vascular remodelling which occurs in response to long term changes in fluid flow and pressure and is mediated by the production of growth factors such as platelet-derived growth factor (PDGF),

transforming growth factor-β1 (TGF-β1), and basic fibroblastic growth factor (bFGF) (179,185). In vitro studies have shown increased PDGF mRNA (186) and increased release of TGF-β1 in EC exposed to shear stress (187), and the increased release of bFGF in response to elevated hydrostatic pressure (179), all of which act as growth promoters for the underlying SMCs (185).

Responses of the vascular wall to mechanical stress also involve the SMCs which are exposed to stretch and hydrostatic pressure changes that result from fluid flow, but not to shear stress since they are protected by the lining endothelium (176). Thus the final response of the vascular wall is a combination of EC mediated effects, many of which act on the SMCs, and SMC mediated responses. SMCs exposed to cyclic strain produce PDGF which has an autocrine effect on DNA levels and cell growth (188). Stretch has also been shown to increase collagen synthesis rates and alter SMC orientation (189), and also to reduce actin expression (190). The effect on DNA synthesis rates is mediated by attachment of the cell to ECM proteins including vitronectin, fibronectin, and collagen and this attachment is in turn mediated by specific cell surface integrin receptors (191). This indicates that integrins provide one possible signal transduction pathway involved in SMC response to strain. SMC have also been shown to undergo hyperpolarisation in response to strain possibly mediated by the action of EDHF, NO, and PGI_2 (192). In addition adenylate cyclase activity is also altered in response to strain (193).

Signal transduction mechanisms in EC and SMC have identified a number of elements of importance to mechanical signalling which may lead to cellular responses of the type described above. These include cell attachment proteins (181,191), cell adhesion molecules (182,191), membrane ion channels (192,194,195), Adenylate cyclase (AC) (196), phospholipase C (PLC) (197,198), intracellular calcium stores (199), mitogen associated protein kinase (MAPK) (177), nuclear factor κ-B (NFκ-B) (200,201), nuclear factor activator protein 1 (AP1) (200), immediate early gene expression (200), specific shear-stress response elements

within some gene promoters (201), and the release of a number of soluble factors (179,184,187,188,194,202) as described above.

3.6.2. The effects of mechanical stress on cardiac myocytes

Cardiac and skeletal muscles rapidly change their mass and phenotype in response to mechanical loading (203). Mechanical stress has been shown to play a key role in cardiac hypertrophy (204) and *in vitro* loading studies have demonstrated that stretch leads to the induction of immediate early genes (IEG) (197,205), increased protein and collagen synthesis (205), and myocyte hypertrophy (205). It has also been shown that stretch rapidly induces angiotensin II (Ang II) gene expression (206) and secretion (207) from cytosolic granules of myocytes (208) and that stretch-conditioned medium causes hypertrophy and immediate early gene expression in unstretched myocytes (207,208). Antagonists for the Ang II receptor subtype AT_1 inhibit these responses to conditioned-medium (208). The Ang II anatagonist [Sar^1, Ile^8] inhibits immediate early gene induction following loading (209). The Ang II receptor antagonists saralasin and CV-11974 both block the increase in MAPK kinase and MAPK activity that follow stretch or application of stretch-conditioned medium (207). The identity of the primary mechanotransduction mechanism in myocytes is unknown but a single SA ion channel has been identified (210). The induction of IEG expression by stretch has been shown to be unaffected by stretch-activated (SA) ion channel blockers or agents which disrupt the cytoskeleton, and there is no evidence that integrins are involved (197). A number of studies have shown the involvement of a cascade of kinase activity leading to the activation of protein kinase C (PKC), MAP kinase, and S6 kinase which act to induce IEG expression (197,207,211,212), and this signal transduction cascade is shared by Ang II (197,207,211,212). Thus the release of an autocrine factor would seam to be a major mechanism responsible for the response of cardiac myocytes to mechanical stress.

3.6.3. The effects of mechanical stress on lung epithelial cells

Mechanical forces have been shown to be involved in regulating lung structure, function and metabolism (213). Intermittent stretch of foetal lung cells at a frequency, amplitude and periodicity similar to that of normal foetal breathing enhances foetal lung cell division and DNA synthesis (214). These responses may be mediated by PGI_2 which is released by continuous and intermittent pulsatile stretch, and which may in turn cause the increase in cyclic adenosine monophosphate (cAMP) that follows stretch (215). It is unlikely however that cAMP is involved in the increase in lung cell proliferation that follows cyclical stretch since inhibitors of cAMP production and cAMP-mediated signal transduction have no effect on this response (216). Studies in which lung cells have been stretched indicate that proliferation results from activation of phospholipase C (PLC) and D (PLD) to produce inositol trisphosphate (IP3) and diacylglycerol (DAG) and activation of PKC (216). DNA synthesis is subsequently activated by PKC, and PKC is rapidly redistributed from the cytosol to the membrane following strain (216). The strain-induced activation of DNA synthesis and PKC, and mechanical stress-induced increase in $[Ca^{2+}]_i$, is blocked by Gadolinium (Gd^{3+}) and an intracellular calcium chelator, indicating the possible role of calcium influx through stretch-activated ion channels (214,217). Stimulation of lung cells with cyclical stretch has also been shown to cause a rapid increase in PDGF-B (PDGF-β subunit) and PDGF-β receptor mRNA levels, which results in a significant rise in both PDGF-BB (PDGF-β dimer) and PDGF-β receptor proteins after 24h. Blockade of this response with antisense oligonucleotides blocks stretch-induced cell proliferation (218). Thus, lung cell proliferation is induced by mechanical stress via a pathway that may involve the activation of PKC and the subsequent release of PDGF (216).

3.6.4. The effects of mechanical stress on fibroblasts

Fibroblasts exposed to mechanical strain show increased proliferation (219,220), and production of collagen and fibronectin (220,221). A SA ion channel has been identified in fibroblasts (222) and shear-stress has been shown to induce a calcium flux that is mediated by the release of ATP and the subsequent upregulation of IP_3 (199). A number of studies have demonstrated that fibroblasts grown in an artificial matrix generate tension, in a manner analogous to wound contraction (223). Fibroblasts exposed to tension produced in this way show greater cell proliferation, greater protein and collagen synthesis, and higher mRNA levels for collagen, fibronectin and actin (223). It has also been demonstrated that dislodging the matrix so that the fibroblast-induced stress is relaxed, leads to the production of arachidonic acid followed by Ca^{2+} influx, production of cAMP and activation of PKA (224). These events precede the disappearance of stress fibres and surface fibronectin (224,225), and the activation of exocytosis of actin, annexins II and VI, and β1 integrin receptors(225).

3.6.5. The effects of mechanical forces on bone cells

Bone functions as the main rigid support element of the body and provides the anchorage for muscles, to facilitate muscle contraction and movement of different skeletal elements. Weight bearing and movement impose considerable physical stresses on bone and are crucial to the regulation of bone structure. Loading of bone through exercise has been shown to increase bone thickness, whereas unloading through immobilisation or space-flight induced weightlessness cause loss of bone and osteoporosis (226). These observations have lead to extensive *in vitro* studies of the effects of defined mechanical stress on bone and bone cell cultures.

The predominant cell type in bone is the osteocyte which, together with bone lining cells, comprise about 95 % of bone cells. Osteocytes are entombed within the calcified bone matrix and are connected to other osteocytes, and to osteoblasts and

lining cells, by cell process extending through canaliculi of unmineralised bone matrix (227). These processes form a three-dimensional cellular network linked by gap junctions. Osteoblasts are responsible for deposition of new bone and differentiate to become osteocytes when they entomb themselves in mineralised bone matrix (226). The osteoblasts form a small percentage (5 %) of the cells lining the bone surface and are formed from osteoprogenitor cells in the blood (226). The remaining bone lining cells have a fibroblastic phenotype. Bone resorption occurs when remodelling is required in response to alterations in the stress patterns in the bone. It results from the action of osteoclasts which originate from hemopoietic stem cells in the bone marrow (226). These three cell types are responsible for maintaining a balance between bone deposition and bone resorption, depending on their mechanical and hormonal environments (226). It is thought that the osteocytes may act as the main mechanosensors in bone and regulate the function of osteoblasts and osteoclasts (227,228), though both of these cell types have been shown to respond to mechanical stress.

Many studies have shown that multidirectional supraphysiological stretch of osteoblasts causes release of PGE_2 followed by osteoblast proliferation. It also results in increased bone resorption by osteoclasts and decreased matrix deposition by osteoblasts (226,229). This has been shown to be a trigger response requiring a minimum number of stretch cycles per day but do not demonstrate any dose-response characteristics (229). Stretching of osteoblasts in cell culture has been shown to lead to reorganisation of the cytoskeleton, a rapid elevation of cAMP and inositol trisphosphate (IP_3) levels, and the activation and redistribution of PKC to the cell membrane (230). Stretch activated ion channels and intracellular calcium fluxes have also been shown to be activated by stretch of osteoblasts (231-233) and osteocytes (234,235). However, physiological levels of stretch produce no response in osteoblasts. In contrast, physiological stretch reduces proliferation, increases bone deposition activity, increases levels of cAMP and insulin-like growth factor I (IGF-I)

in young osteocytes isolated from the same source, and these responses can be blocked by the SA ion channel blockers Gd^{3+} and amiloride (228). Similarly, physiological levels of fluid flow have been shown to cause release of PGE_2 from osteocytes but not from osteoblasts or periosteal fibroblasts (227). *In vitro* loading studies with rat vertebrae and ulnae have demonstrated the production of nitric oxide by bone cells and this has been shown to result from a transient and rapid NOS-dependent production of NO by osteoblasts and osteocytes under strain conditions associated with increased cell proliferation (202). Osteocytes, but not periosteal fibroblasts, exposed to pulsatile fluid flow rapidly, but transiently release NO. Block of this release with the NOS-inhibitor N -monomethyl-L-arginine (L-NMMA) blocks the PGE_2 release that is stimulated by flow (236). Taken together, the responsiveness of osteocytes to physiological levels of strain, their predominance relative to other cell types, and their location and morphology all point to their being responsible for detecting and transducing normal mechanical signals in bone (226).

3.7. Implications of non-cartilaginous cell loading studies for chondrocytes

Chondrocytes of articular cartilage exist in a very different environment to the cell types described above but common mechanotransduction pathways may be involved in the response to mechanical stress. In common with the EC and SMC responses adenylate cyclase (169-171,237) and membrane ion channels (238,239) have already been implicated in the chondrocyte response to mechanical stress. Chondrocytes probably do not share the pathways used by cardiac myocytes in mechanotransduction since SA ion channels (239) and the cytoskeleton (133) have been implicated in chondrocyte responses to mechanical stress but are not apparantly activated in cardiac myocytes (197).

Studies of chondrocyte membrane potential changes in response to cyclical stretch indicate that stretch induces membrane hyperpolarisation (239). The

hyperpolarisation response has been shown, by the use of blocking agents, to involve Ca^{2+}-channels, Na^{+}-channels, Ca^{2+}-activated K^{+}-channels, and SA-ion channels (238,239). Ca^{2+} entry from the extracellular medium and IP_3-mediated release from intracellular stores have both been implicated in the response (239,240). The inhibition of PLC, PKC and tyrosine-protein kinase by selective antagonists blocks the hyperpolarisation response, and activation of adenylate cyclase stimulates hyperpolarisation without mechanical stress (240). The response of chondrocytes to mechanical stress was reduced in the presence of cytochalasin D which disrupts the cytoskeleton, by RGD-peptides which block integrin-matrix interactions, and by anti-integrin antibodies (241). Thus, chondrocytes may share many of the same elements used in mechanotransduction by other cell types.

The release of one or more autocrine factors occurs in response to mechanical stress of all the cell types described. Recent experiments have shown that human chondrocytes and also immortalised chondrocyte cell lines exposed to cyclical strain release a soluble factor which induces hyperpolarisation of unstrained cells exposed to conditioned medium from cells subjected to cyclical strain (363). The hyperpolarisation response to cyclical strain was abolished by the addition of anti Il-4-receptor antibody and anti Il-4 antibody in both chondrocytes and chondrocyte cell lines (363). The conditioned medium induced hyperpolarisation was also blocked by anti Il-4 antibody in the chondrocyte cell lines. Previous studies have shown that Il-6 mRNA is upregulated by shear stress (175). There is no evidence as yet for the involvement of soluble autocrine factors in the changes in chondrocyte PG synthesis that occur in response to mechanical stress.

The study of the effects of mechanical stress in cell types other than chondrocytes, and preliminary results obtained in chondrocytes, indicate candidate signal transduction mechanisms responsible for the mechanical stress induced responses of chondrocytes (Fig. 3.11).

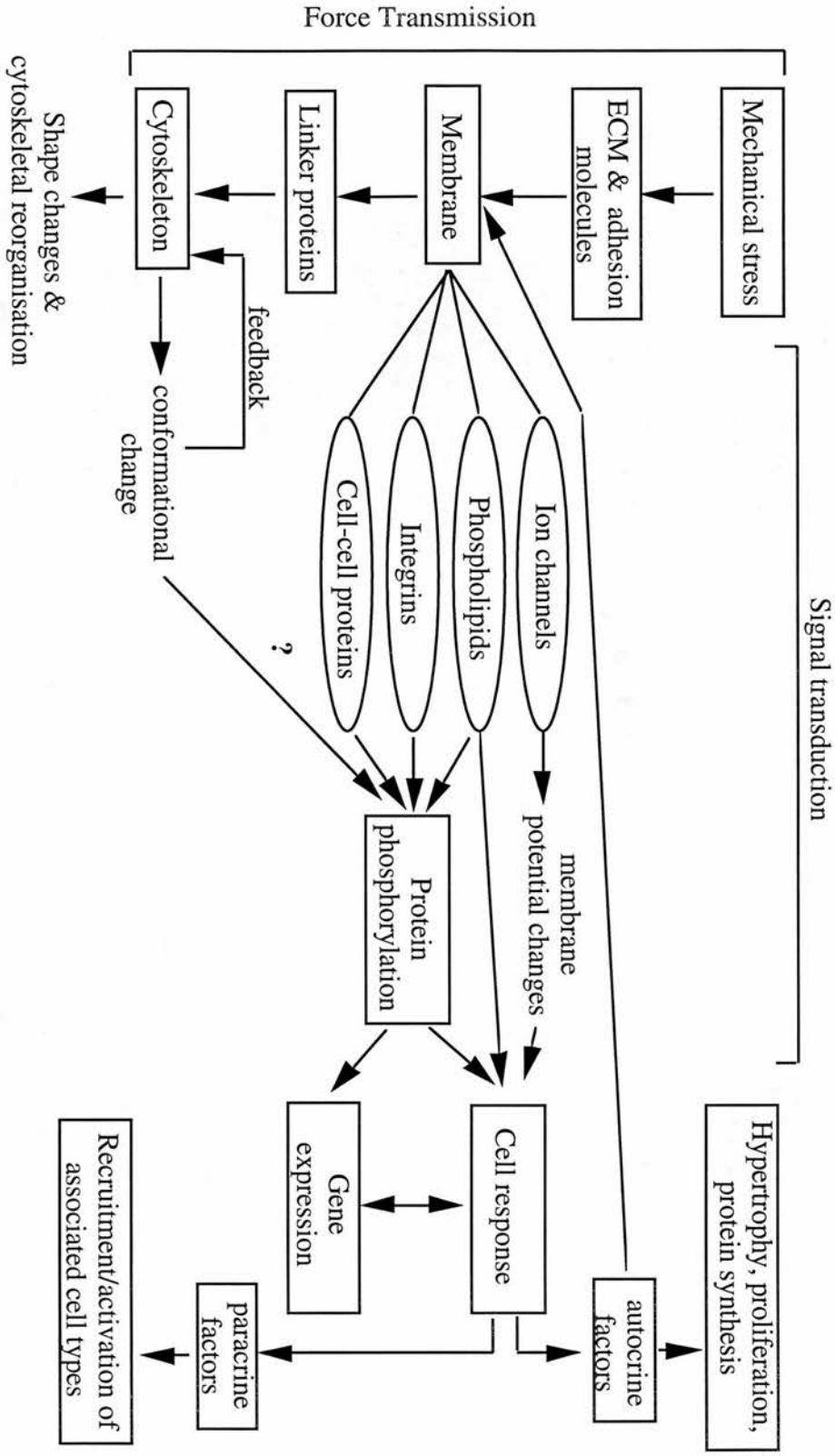


Figure 3.11 Diagram showing possible mechanisms of mechanical stress transmission and transduction in cells. ECM, extracellular matrix.

3.8. Aims and Objectives

The aim of the this study was to investigate the regulation of chondrocyte PG synthesis by mechanical forces. In particular how chondrocytes detect, transmit and transduce mechanical stimuli. In order to achieve these aims a number of objectives were identified:

(i) establish the use of pressurisation apparatus previously used in studies of chondrocyte electrophysiology to alter PG synthesis,

(ii) investigate the effect of mechanical forces on expression of specific OA-related CS epitopes,

(iii) investigate the role of stretch-activated ion channels as mechanotransducers using specific channel blockers,

(iv) investigate the role of integrin receptors as mechanotransmitters and mechanotransducers using RGD peptides and anti-integrin antibodies,

(v) investigate the possible role of cyclic nucleotides as second messengers involved in mechanical regulation of PG synthesis.

4. GENERAL METHODS

4.1. Introduction

The methods used in the majority of experimental studies are described here. Other methods are described in the chapters to which they pertain.

4.2. Materials

All chemicals and laboratory supplies were obtained from Sigma (Poole, Dorset, UK) unless otherwise stated.

All radioisotopes were obtained from Amersham Life Science (Amersham, Bucks, UK)

Human knees were obtained with relatives' consent from the Royal Infirmary of Edinburgh Hospitals Trust autopsy suite.

Hooves from steers and cows were obtained from Scottish Premier Meats Ltd. (Edinburgh).

Chondrocytes were cultured in Hams F12 medium, which was supplemented with 100 u. ml^{-1} penicillin, 100 μg. ml^{-1} streptomycin, 50 μg. ml^{-1} ascorbate, and 2mM L-glutamine, unless otherwise stated.

4.3. Chondrocyte isolation and culture

Primary cultures of human chondrocytes in monolayer were used in the majority of the experiments described in this thesis. In some experiments human chondrocytes which had been maintained in alginate-bead culture were also used. Immortalised human chondrocyte cell lines were used in a limited number of experiments.

Monolayer cultures of bovine chondrocytes were used in selected experiments when human cartilage was unavailable.

4.3.1. Human chondrocyte isolation and monolayer culture

Human knee joints were removed at autopsy from patients who had died of diseases unrelated to the locomoter system. The joint was removed without penetrating the synovial membrane, in order to preserve sterility. Knee joints from patients who had died as a result of infection such as septicaemia were not accepted. Joints were removed within two days of death. In the laboratory, the joint was opened under sterile conditions in a Class 1 biological safety cabinet. The synovial membrane was cut below the patella and along each side of the knee, using a scalpel (Swann-Morton No. 22) and forceps. The cruciate ligaments were then severed, taking care not to touch the exposed cartilage surfaces, following which the remaining synovium was cut to separate the tibial and femoral parts of the joint. The patella was then separated from the femur and, using a new scalpel handle and blade, the tibial plateaux were exposed by dissecting away the menisci. Cartilage slices were cut from the patella and tibial plateaux using a scalpel (Swann-Morton No. 22) and forceps; each knee yielded 5-10 g of tissue. The thickness of the cartilage slices varied from 1-3 mm depending on the area from which it was removed, the age and sex of the patient, and the disease state of the cartilage. Cartilage slices were cut as near to the bone as possible, in order to remove full-thickness cartilage, and placed in a sterile universal tube (Philip Harris Scientific, Glasgow, Scotland) in Hams F12 medium containing penicillin (200 u. ml^{-1}), streptomycin (200 μg. ml^{-1}), ascorbate (50 μg. ml^{-1}), L-glutamine (2 mM), and Fungizone (amphotericin b, 0.25 μg. ml^{-1}; Gibco, Paisley, Scotland).

During removal, the disease state of the cartilage was assessed and recorded along with the age, sex of the patient, and cause of death. The cartilage disease state was classified according to our own criteria based upon the macroscopic appearance and texture of the most severely affected area of the specimen:

Normal - smooth, firm texture.

Mild OA - superficial fibrillation with little softening.

Moderate OA - marked fibrillation with softening.

Severe OA - spongy tissue with complete loss of cartilage in some areas.

The method for isolation of chondrocytes from cartilage was adapted from that described by van Kampen and Veldhuijzen (242). Following removal from the knee joint, cartilage was transported to the tissue culture laboratory where it was transferred into 100 mm polystyrene petri dishes (Philip Harris Scientific, Glasgow, Scotland) and chopped into small pieces approximately 2-3 mm square. The tissue was incubated overnight in Hams F12 medium at 37 °C in a humidified 5 % CO_2 / 95 % air incubator. The following morning, cartilage fragments were divided into two approximately equal amounts and transferred into two universal tubes each containing 10 ml of sterile collagenase (900 - 1100 $i.u.ml^{-1}$, Sigma Type 1A in Hams F12 medium). The cartilage was incubated at 37 °C with vigorous shaking; this process allowed the digestion time to be reduced to only 4-6 h. The digestion was stopped when approximately 75 % of the tissue had been digested. Sequential digestion using trypsin prior to collagenase treatment was not required as the collagenase preparation used contained clostripain, which has neutral protease and tryptic activity. The digest was passed through a 40 μm cell strainer (Falcon, A & J Beveridge, Edinburgh, Scotland) to remove any tissue fragments and then centrifuged for 5 min at 250 g. The cell pellet was washed by resuspension in Ham's F12 medium and recentrifuged. After one further wash the chondrocytes were spun down and the pellet was resuspended in 1 ml of Ham's F12 medium. The total number of cells in the pellet was estimated using a Neubauer haemocytometer and viability was assessed on the basis of trypan blue exclusion. A normal knee from a

middle aged adult contained between $5x10^6$ and $10x10^6$ cells, and viability was greater than 90 %.

Chondrocytes were plated-out in 50 mm polystyrene petri dishes (Philip Harris Scientific, Glasgow, Scotland), each dish containing 5 x 10^5 cells. The volume of medium in the dishes was made up to 5 ml with Ham's F12 medium and dishes containing the cells were transferred to a humidified 5 % CO_2 / 95 % air incubator overnight. On the following day the chondrocytes were examined, using an inverted microscope (Leitz, Diavert), for the presence of infection and to ensure that they had adhered completely. The Ham's F12 medium was then additionally supplemented with 10 % foetal calf serum (F12-FCS). The cultures were maintained in Ham's F12-FCS for 7 - 14 days prior to use; during this time the cultures were inspected daily for the presence of infection and the medium was replaced with fresh Ham's F12-FCS every four days. Following experimental treatment the number of cells in a dish was assessed by measuring the DNA concentration of Guanidine extracts, this would not take account of any cell death occuring during the experiment. Initial studies indicated that cell viability, assessed by trypan blue exclusion, was not effected by any of the pressurisation protocols used.

Chondrocytes were plated out in the absence of serum because it was repeatedly observed that freshly isolated chondrocytes adhered very slowly to plastic in the presence of serum (2 days for 100 % adherence), but adhered very rapidly (10 mins - 3 h for 100 % adherence) in serum free conditions. This rapid adherence may have involved a purely electrostatic interaction between the cell and the plastic, which could be blocked by soluble proteins in serum; subsequent cell spreading was observed to be a much slower process (1 - 2 days) which was accelerated by the addition of serum. These observations suggest that isolated chondrocytes undergo a passive cell attachment step followed by an active cell spreading step which may be mediated by adhesion to endogenous and exogenous matrix proteins such as fibronectin. Chondrocytes in culture synthesise fibronectin (243) and serum contains

considerable amounts of soluble fibronectin (244). Both of these sources could coat the culture-dish plastic and lead to fibronectin-mediated attachment.

When chondrocytes are isolated from cartilage and suspended in Ham's F12 medium they are exposed to considerably lower concentrations of K^+, Ca^{2+} and sulphate, and considerably reduced osmolality. The concentration of sulphate in Ham's F12 is only 6 μM which may mean that newly synthesised PG will be under-sulphated, and $^{35}SO_4$-incorporation will vary considerably from experiment to experiment. In Hams' F12 the concentration of Ca^{2+} is only 300 μM, compared with approximately 20 mM *in vivo*, and $^{35}SO_4$-incorporation has been shown to be sensitive to small changes in the concentration of this ion (262). These problems are important when comparing different studies, but the use of paired controls in all the following experiments means that the medium is suitable for the experiments described.

4.3.2. Human chondrocyte culture in alginate-beads

Alginate is a seaweed extract which polymerises spontaneously in the presence of polyvalent cations such as calcium, to form a negatively charged matrix (36). Chondrocytes cultured in alginate maintain their shape and a more differentiated phenotype than chondrocytes in monolayer culture (35,36). This culture system has the additional advantage that the cells can be easily released from the matrix by depolymerising the alginate with a chelating agent such as EDTA and so avoids the need to use trypsin or cell scrapers which are required to release cells from monolayer culture (36). The method for alginate culture used in this study was adapted from the method of Guo (36).

A 2 % alginate solution was prepared by slowly adding alginate powder (sodium salt of alginic acid from kelp, obtained from Sigma, Poole, UK), with stirring to 0.3 M NaCl solution preheated to approximately 60 °C. Once the alginate had dissolved the solution was autoclaved. Human chondrocytes were isolated as described above

and counted. The cells were then resuspended in Ham's F12 medium, the volume of which was calculated according to the formula below, to achieve a density of chondrocytes in the alginate beads (~350 cells. mm^{-3}) significantly lower than human adult cartilage (~10,000 cells. mm^{-3});

$$\frac{\text{total cell Number}}{9.6 \text{x} 10^{6}} = \text{volume (ml) of medium required}$$

An equal volume of alginate solution was then added to the cell suspension and thoroughly mixed using a pipette. The mixture was then transferred to a disposable 20 ml syringe fitted with a 23 gauge needle. The chondrocyte suspension was slowly expressed dropwise into 20 ml of a sterile solution of 100 mM $CaCl_2$ in 0.15 M NaCl in a 100 mm polystyrene petri dish. Although beads formed instantaneously in the solution they became more resilient after incubation for 10 min at 37 °C. Approximately 120 beads formed per ml of mixture, each bead being 2-3 mm in diameter and containing about 40,000 chondrocytes. The excess $CaCl_2$/NaCl solution was then removed with a 23 gauge needle and syringe, taking care not to discard any beads, and Hams F12-FCS was then added. Chondrocyte/alginate cultures were maintained for up to 14 days, the Ham's F12-FCS medium being changed for fresh medium every 2-3 days.

Chondrocytes were released from alginate by removing the culture medium and transferring up to 200 beads into a universal tube containing 20 ml of 55 mM Na citrate in 0.15 M NaCl solution. Following gentle agitation for 10 mins at 37 °C in a shaking incubator, the beads were dissolved and chondrocytes centrifuged at 250 g for 10 min, ready for experimental use.

4.3.3. Immortalised human chondrocyte cell lines

Obtaining a reliable and adequate supply of human articular cartilage proved to be difficult. The random variation in the age and osteoarthritic disease state of the patients from which the joints were taken meant that the chondrocytes isolated from

the cartilage varied considerably in the experimental studies. Cartilage from older patients (>70 y), which was the usual source of chondrocytes for the experiments described in this thesis, yielded low numbers of cells. The numbers of chondrocytes could not be increased by prolonged culture and subculture because of phenotypic changes which occur under these conditions. To circumvent these difficulties immortalised human chondrocytes were used in some studies. Attempts to establish immortalised chondrocyte cell lines (245-248) have previously resulted in cells that express cartilage specific proteoglycans but little or no type II collagen. The cell lines used in this study have been shown to express cartilage-specific proteoglycans and collagen, and to display cartilage-specific modulation by IL-1β (249).

SV40 immortalised chondrocyte cell lines C20a4 and TC28a4 were obtained from Dr M. Goldring (Arthritis Research Laboratory, Massachusetts General Hospital, Charleston, USA). Cell stocks were stored in liquid nitrogen. Chondrocytes were grown up from frozen stock and passaged prior to experiments. One vial of cells was thawed in a water bath at 37 °C and the cells were immediately added to a prewarmed (37 °C) 225 cm^2 tissue-culture flask (Corning, McKay and Lynn, Edinburgh, Scotland) containing 100ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FCS (DMEM-FCS). The cell lines were maintained at 37 °C in a humidified 95 % air / 5 % CO_2 incubator. The cells were passaged twice weekly by adding phosphate buffered saline (PBS) containing 5 mM EDTA to the flask and shaking for about 5 mins at 37 °C. Once all the cells were seen to be free from the flask the cell suspension was centrifuged at 250 g for 5 mins. The pellet was resuspended in 20 ml of PBS and the suspension was divided into 5 aliquots. These cell aliquots were then centrifuged again under the same conditions and the resulting cell pellets were either added to a flask containing DMEM-FCS or resuspended in DMEM containing 50 % FCS and 8 % Dimethyl sulphoxide (DMSO) and transferred to a vial for freezing. For experimental studies cell lines were passaged as above and the pellet was resuspended in 1 ml of DMEM-FCS. The

cells were counted using a Neubauer haemocytometer and plated out, in DMEM-FCS, in 50 mm tissue culture treated petri dishes (Nunc, Gibco, Paisley, Scotland) to give a density of $5x10^5$ cells per dish. Both cell lines were used for experiments examining the effect of cyclical pressure-induced strain on PG synthesis. Cells were used for experimental purposes for up to three days following passage.

4.3.4. Bovine chondrocyte isolation and culture

The procedures for the removal of cartilage from bovine hooves and the subsequent isolation of chondrocytes were based on the procedures followed for human knee joints. Hooves from the forelimbs of 18 - 24 month old heifers and cows were obtained from the local abattoir immediately after the animals had been killed. They were washed with a steam hose at the abattoir to remove excess dirt, before returning to the laboratory where they were skinned using a scalpel. The hooves were then rinsed with tap water and the hoof and severed end of the limb were covered with disposable latex gloves. Hooves were sprayed with 70 % alcohol prior to their being transferred to a sterile class II biological safety cabinet for dissection. Articular cartilage was removed from the metacarpophalangeal joint using a scalpel (Swann-Morton No. 22) and collected in universal tubes containing Ham's F12 medium with penicillin (200 u. ml^{-1}), streptomycin (200µg. ml^{-1}), ascorbate (50 µg/ml), L-glutamine (2mM), and Fungizone (amphotericin b, 0.25 µg. ml^{-1}). Following removal from the joint the cartilage was maintained overnight in the universal tubes at 37 °C in a 5% CO_2/95% air incubator. Cartilage slices were removed by cutting as close to the bone as possible; the cartilage was always less than 1mm thick. Each hoof yielded 10-20 g of tissue which was digested the following day in two universal tubes each containing 10 ml of sterile collagenase (900 - 1100 i.u. ml^{-1} in Hams F12 medium). The cartilage was incubated with collagenase for approximately 3 h at 37 °C in a shaking incubator, after which it was completely digested. Bovine

chondrocyte monolayer and alginate cultures were established as previously described for human chondrocytes.

4.4. Determination of rate of proteoglycan synthesis

PG synthesis was assayed by measuring the incorporation of sodium [$^{35}SO_4$]-sulphate into GAGs. This is the method of choice for measuring total PG synthesis rates in both cartilage explant and chondrocyte cultures (250), and the labelled products are suitable for subsequent analytical procedures. The [$^{35}SO_4$]-sulphate rapidly equilibrates between the extracellular fluid and the intracellular donor, 3' phosphoadenosine - 5 - phosphosulphate (PAPS), and the intracellular specific activity of [$^{35}SO_4$]-sulphate can be taken as equal to that of the [$^{35}SO_4$]-sulphate in the extracellular fluid (251). These features mean that this technique is an accurate measure of total PG synthesis.

4.4.1. [^{35} SO_4]-Sulphate incorporation

Chondrocyte PG-synthesis was assayed using a method adapted from that of Parkinnen (158). Chondrocyte cultures were labelled with 20 μCi. ml^{-1} (740 kBq. ml^{-1}) sodium [$^{35}SO_4$]-sulphate (aqueous carrier-free solution) over a period of either three or six hours (see section 5.3., p83, for details of labelling conditions) during which the cells were exposed to mechanical or pharmacological stimulation. The label was added from a stock solution of 2 mCi. ml^{-1} (74 MBq. ml^{-1}) [$^{35}SO_4$]-sulphate to fresh culture medium which was added to the cells prior to experiments. On completion of the labelling period, the medium was collected and stored at -20 °C and the cells were then washed twice with 5 ml of PBS to remove free label. The cells were dissolved by incubation for 2h at room temperature in 2 ml of a solution containing 4 M Guanidine - HCl (GnHCl), 0.5 % CHAPS, 10 μM EDTA, 3 μM Tris (GCET) (158).

The efficiency of extraction was confirmed by comparing the [$^{35}SO_4$]-PG content of an extract with that of a sample produced by papain digestion of cells. Cell cultures which had been labelled and washed identically were treated with GCET-solution or removed from the dish for papain digestion. Chondrocytes were removed in PBS using a disposable cell scraper and spun down at 250 g for 10 min. The pellet was then digested for 2 h at 37 °C in 0.5 ml of a solution containing 0.5 mg. ml^{-1} Papain (11 i.u. ml^{-1}), 20 mM Na_2HPO_4, 1mM EDTA, 2mM Dithioerythritol. The [$^{35}SO_4$]-PG content of the samples was then measured as described below. Following a 2 h GCET extraction 645 cpm [$^{35}SO_4$]-PG were liberated compared with 425 cpm [$^{35}SO_4$]-PG following papain digestion. On completion of the 2h GCET-extraction the amount of [$^{35}SO_4$]-PG remaining unextracted was measured by using a cell scraper (Costar, Gibco, Paisley, Scotland) and a further 2 ml of GCET. This released a further 85 cpm [$^{35}SO_4$]-PG from the dish. These results indicate that GCET is an effective reagent for solubilising chondrocytes and their associated matrix and that a cell scraper should be used in order to avoid loss of labelled product. The cell extract was stored at -20 °C prior to analysis.

In order to separate labelled PGs from unincorporated [$^{35}SO_4$]-sulphate within the GCET extract, the extract was applied to pre-packed disposable Sephadex G-25 size exclusion columns (PD10; Pharmacia Biotech, St. Albans, Herts, UK). The columns were first equilibrated with 2 M Guanidine-HCl in 0.05 M Na acetate solution; this solution was also used to elute samples. The labelled PGs eluted in the void volume whereas the free label was retarded by the column. Half ml fractions of the eluent were collected and mixed with 5 ml of Cocktail T scintillation fluid (Merck, Lutterworth, Liecs, UK) before counting using a Packard Minaxi Tricarb 4000 scintillation counter. A typical elution profile of one sample is shown in Fig. 4.1.

The efficiency of separation of labelled products from free label using Sephadex G-25 columns was tested by comparing the results obtained from aliquots of four samples with those obtained by dialysis of aliquots of the same four samples for 24

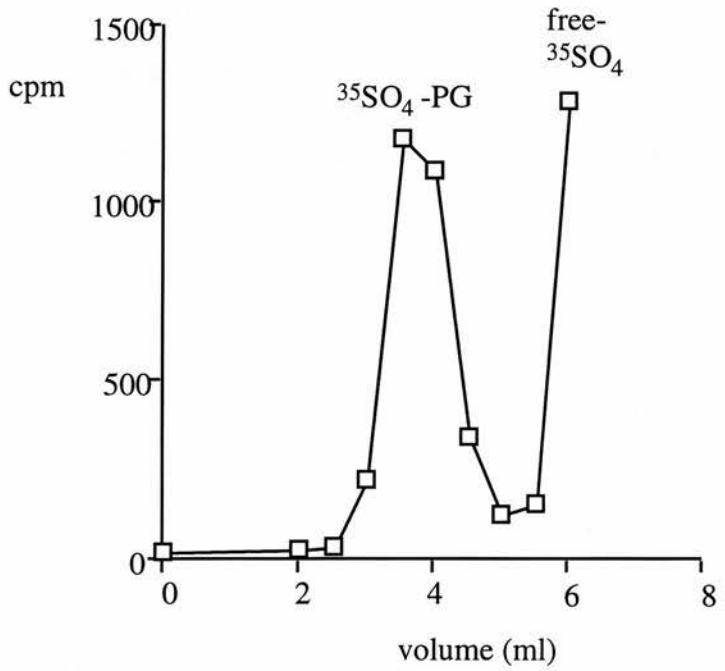


Figure 4.1 The separation of $^{35}SO_4$-labelled PGs from free label using a PD10 size-exclusion column. A typical elution profile showing the separation of labelled PGs and free label from human chondrocytes which had been labelled with $^{35}SO_4$ for 3h at 37 °C and 'dissolved' in GCET prior to application to the column.

h. Samples were split into two, half being separated by PD10 columns as above, and half being dialysed. Dialysis was performed using slidalyse (Pierce & Warriner, Chester, UK) dialyse cassettes, and samples were dialysed against three changes of 1 l of 2 M Guanidine-HCl in 0.05 M NaCl, over 24 h at 4 °C. The mean [$^{35}SO_4$]-incorporation rate for the samples was 27923 ± 5959 (SD) cpm. h^{-1} following Sephadex G-25 separation and 24809 ± 5803 cpm. h^{-1} following separation by dialysis. These results suggest that both methods are comparably efficient. Separation using Sephadex G-25 columns was however the method of choice because of the practical difficulties involved in dialysing large numbers of small samples.

The total number of counts eluted in the void volume of the Sephadex G-25 column was the total amount of [$^{35}SO_4$]-sulphate incorporated into PGs. The number of counts in each sample was corrected by subtracting the number of background counts, measured using a tube containing scintillant alone, from the cpm in each fraction counted. The rate of PG synthesis was calculated as follows;

$$\text{Rate of PG synthesis} = \left[\frac{\text{total corrected cpm}}{\text{labelling time (h)}}\right] \div \text{DNA}(\mu g)$$

Values were normalised by measuring the DNA content of cell extracts as described below. PG synthesis rates were expressed as cpm. h^{-1}. μg DNA^{-1}.

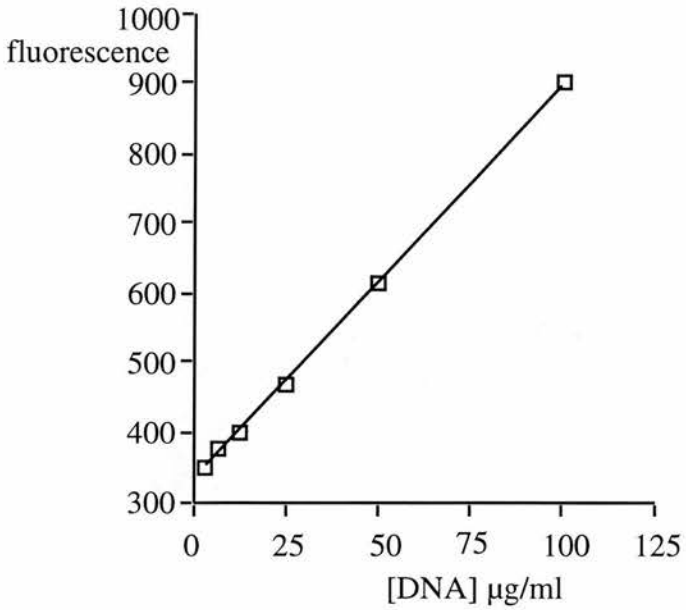
In initial experiments the rate of [$^{35}SO_4$]-sulphate incorporation was measured both in cell extracts and in media collected after incubation of chondrocytes for 6h at RT °C in air. The mean rates of [$^{35}SO_4$]-sulphate incorporation were 7(4-14(confidence limits), n = 15) cpm. h^{-1}. μg DNA^{-1} in media and 153 (87 - 268, n = 15) cpm. h^{-1}. μg DNA^{-1} in cell extracts. This indicates that [$^{35}SO_4$]-sulphate incorporation into PGs released into the medium is negligible over the time period of these experiments. The rate of [$^{35}SO_4$]-sulphate incorporation was only measured in cell extracts in all subsequent experiments.

4.4.2. Determination of DNA concentration

The DNA content of cell extracts was used as a surrogate measure of cell number. DNA concentration was assayed using the bisbenzimidazole fluorescent dye Hoechst 33258 (Calbiochem, La Jolla, CA, USA) by a method adapted from that of Lipman (252). The dye fluoresces considerably in the absence of DNA but on binding to Adenine-Thymine rich DNA regions it forms a complex that increases its innate fluorescence, and the dye-binding is dependent on DNA structure not intercalcation (253). The method was adapted to measure the DNA content of samples in GCET-solution. Highly polymerised calf thymus DNA (Sigma type 1) was dissolved, at a concentration of 1 mg. ml^{-1}, overnight in distilled water at 4°C and DNA standards ranging from 0-10 μg. ml^{-1} were prepared from this stock by dilution in GCET-solution. Cell extracts and standards were assayed in batches, using freshly prepared solutions filtered through nitro-cellulose (0.2 μm, Whatman, Maidstone, Kent, UK) to prevent drift due to particulate contamination. Samples and standards (25 μl) were assayed in triplicate in polystyrene cuvettes (Elkay Ultra-Vu, Beveridge, Edinburgh, UK) following the addition of 75 μl Tris HCl pH 8.0, 1.9 ml saline (0.154 M) / sodium citrate (0.015 M), and 1ml of 1.5 μM H33258, followed by a 10 minute incubation at room temperature in the absence of light. Fluorescence was measured using a Perkin-Elmer LS-5B luminescence spectrometer with excitation and emission wavelengths of 350 nm and 450 nm respectively and slit widths of 10 nm.

Standard curves of fluorescence against DNA concentration (μg/ml) were plotted and lines fitted using Cricket Graph V. III software (Edinburgh University Computing Service). The data was linear in the range 1 - 10 μg DNA. ml^{-1} of the standard curve and up to 80 μg. ml^{-1} (Fig. 4.2). The gradient and intercept of standard curves were sensitive to small variations in the composition of solutions, stressing the importance of assaying standard curves with every batch of samples. The DNA concentration of samples in each batch assayed was calculated using the standard curve obtained with that batch of assays.

a.



b.

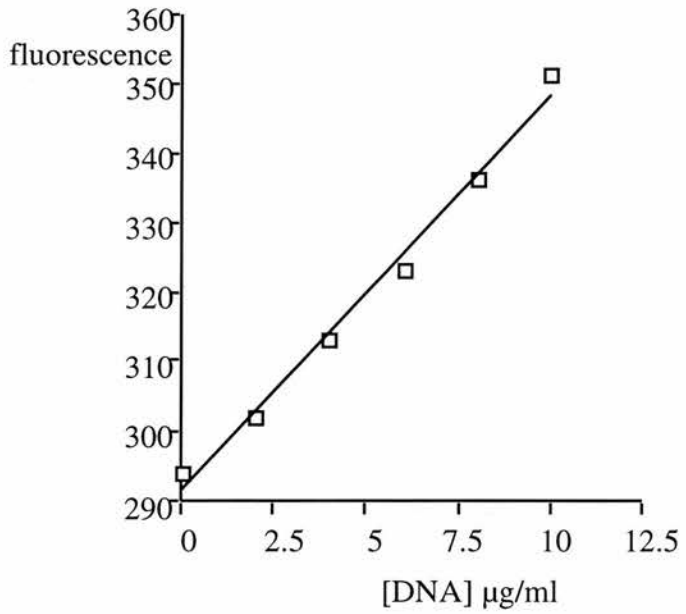


Figure 4.2 Fluorescence of DNA samples measured using the fluorimetric dye H33258. Typical standard curves for samples of highly polymerised calf thymus DNA in the range (a.) 0-100 µg/ml and (b.) 0-10 µg/ml. Lines were fitted with Cricket Graph III(v1.5) software: the equation of lines were (a) fluorescence = 5.654[DNA] + 332.478, correlation coefficient (r) = 1.000 (b) fluorescence = 5.671[DNA] + 291.476, r = 0.995.

4.5. Pressurisation apparatus

In the early studies, chondrocyte monolayer cultures were pressurised using an apparatus designed to apply cyclical increases in hydrostatic pressure (238). The initial apparatus consisted of a sealed pressure chamber with inlet and outlet ports, which was fed with nitrogen gas from a cylinder via reducing valves (Fig. 4.3). The pressure in the chamber was measured with a mercury manometer which also allowed accurate regulation of the magnitude of the pressure pulses. An electronic timer controlled the inlet and exhaust valves so allowing variations in the duration of the applied pressure and the intervals between pressure pulses. The level of pressure within the chamber was adjusted in conjunction with the manometer using the reducing valve on the cylinder head. The electronic controller had two timers which were set to control the opening of the inlet and outlet valves in a cyclical manner. The inlet valve allowed gas to flow into the chamber. It remained open for a set interval, after which the exhaust valve opened to relieve the pressure within the chamber. A single petri dish was fitted into a dish-holder within the chamber, held in a tight seal by a rubber 'o'-ring.

Initial studies using this apparatus investigated membrane potential changes in response to low levels of pressure (16 kPa) (238 - 241). The electrophysiological effects of the applied pressure were later shown to occur as a result of deformation of the base of the culture dish inducing microstrain, the degree of which was shown to be dependent on the pressure applied (239).

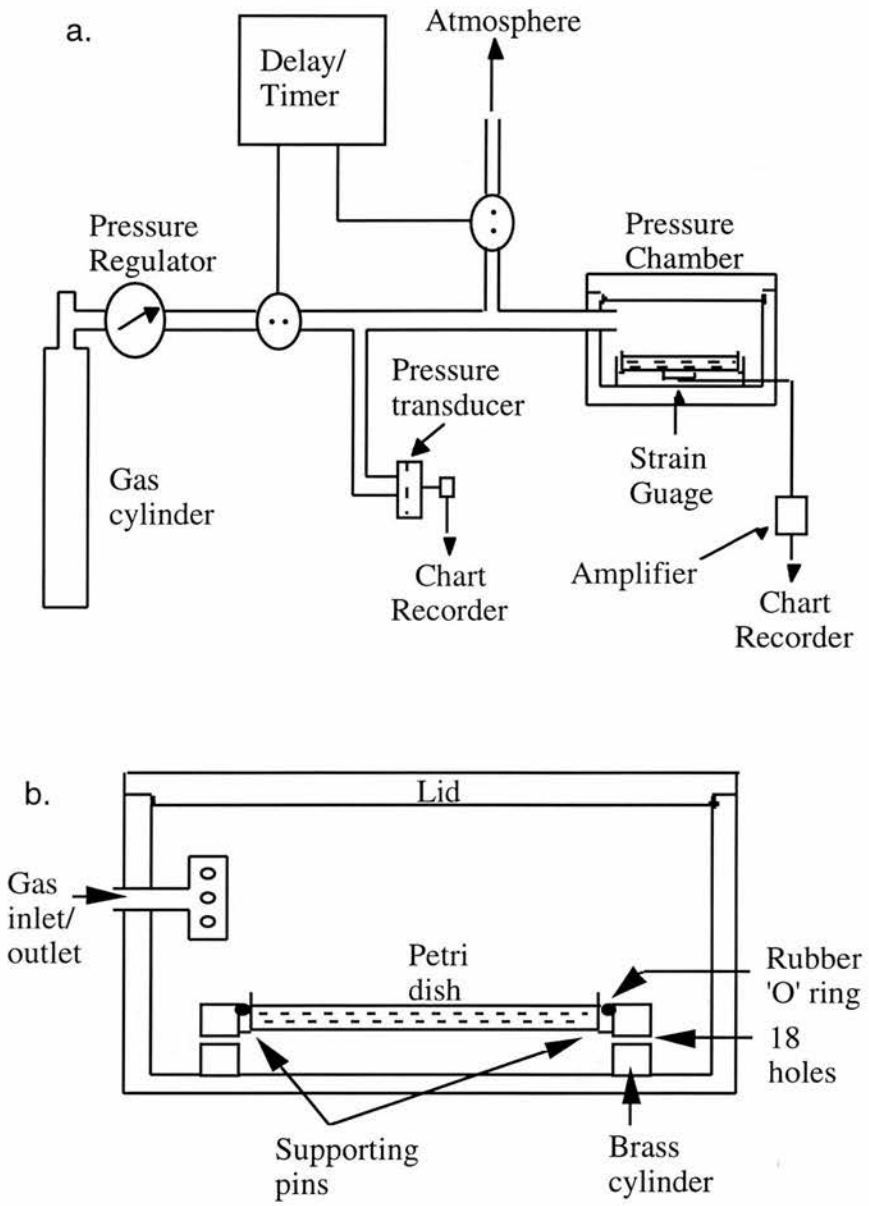


Figure 4.3 Original apparatus for application of cyclical pressure.
Shematic diagram of (a.) pressurisation apparatus and (b.) details of the pressure chamber.

4.5.1. Measurement of strain in the culture-dish base

Strain on the base of culture dishes produced following the application of cyclical pressure pulses was assessed using strain gauges (239). Mild steel rosette strain gauges consisting of two 8 mm strain gauges (Radiospares products) placed at right angles were attached (a) to the centre and (b) to the edge of the base of culture dishes identical to those used for cell culture. The output from the strain gauge was fed into a purpose built linear DC amplifier whose output was a digital display calibrated in microstrains. The apparatus was calibrated by applying a known pressure (13.3 kPa) and a fixed voltage (2 V) to the half bridge. The resulting voltage output was measured and used to calibrate the strain according the formula below(239).

$$e = \frac{2 \times v_{out}}{v_{bridge} \times K}$$

where: e = strain

v_{out} = bridge output (volts)

v_{bridge} = voltage applied to bridge (volts)

K = gauge factor (2.05).

In the original apparatus pressure pulses of between 0 and 26.7 kPa were applied in sequence at a frequency of 0.33 Hz (1 s on 2 s off). The resulting strain on the base of the culture dish at each particular pressure was recorded on a chart recorder for a minimum of 20 cycles. The results for strain were plotted against the applied pressure; a linear relationship between the degree of strain and the applied pressure was observed (Fig. 4.4). Additional strain measurements were made using the same apparatus but with a dish held in the centre of the chamber such that gas could flow freely around its base. In this situation there was negligible strain of the base of the dish (Fig. 4.4).

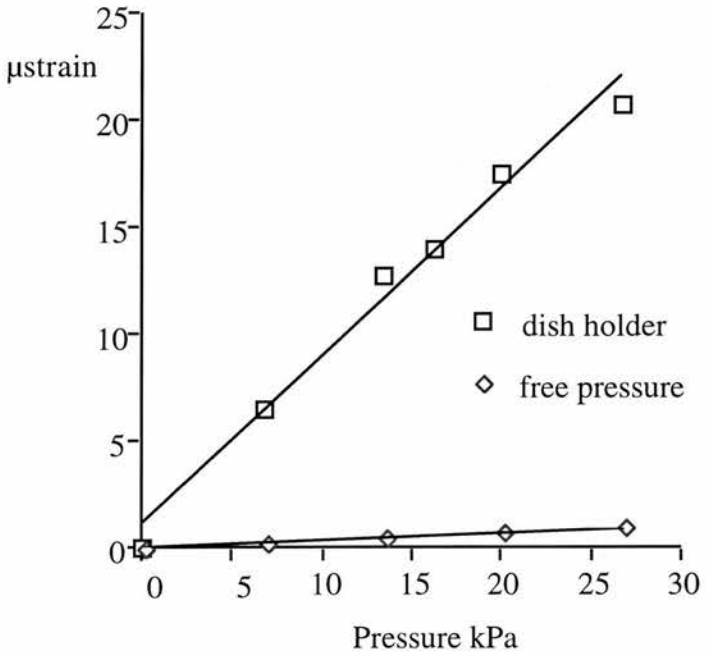


Figure 4.4 The relationship between applied pressure and resultant strain of the dish-base in the original apparatus. Strain measurements during pressurisation were made for dishes fitted in the dish holder and dishes not fitted in the dish holder (free pressure). Lines were fitted to the data using Cricket Graph III(v1.5) software. The equations of the lines are; dish-holder microstrain = 0.786(pressure) + 1.030, r = 0.992, free-pressure microstrain = 0.033(pressure) + 0.020, r = 0.998.

4.5.2. The development of a new pressurisation apparatus

In the earlier experiments described in this thesis, the original apparatus used to pressurise chondrocytes for electrophysiological studies (238,239) was used. Chondrocytes were exposed to 26.7 kPa pressure at a frequency of 0.33 Hz (2 s on 1 s off) for 6 h at RT °C. This frequency was selected following initial studies that demonstrated a maximal increase in PG synthesis in chondrocyte cultures subjected to intermittent pressurisation at 0.33 Hz (see section 5.2.). The apparatus was, however, unsuitable for extensive biochemical studies, as only one dish could be pressurised at a time and it was not possible to maintain the chamber at 37 °C.

A new apparatus was designed to accommodate duplicate dishes or 6 - well plates. It also allowed the use of much higher pressures (up to 1 MPa), and maintenance of cultures at 37 °C using an incubator. The basic components (pressure chamber, nitrogen cylinder, pressure transducer, inlet / exhaust valve controller) were similar to those of the original apparatus (Fig. 4.5). The pressure chamber and valve controller were custom made (Mr. Peter Frew, Dept. of Physiology Workshops, University of Edinburgh) and the valves (Martonair, Murex Ltd, UK) and pressure transducer (Digitron P200, Radiospares, UK) were purchased commercially. The new apparatus was built before it was appreciated that the electrophysiological and biochemical changes following pressurisation in the original apparatus resulted from pressure-induced strain on the base of the culture dish in its tight-fitting dish holder rather than cyclical increases in pressure *per se*. Consequently dish holders were not incorporated in the design of the new apparatus.

The effect of labelling cells with $^{35}SO_4$ at 37 °C instead of RT °C was established by labelling 2 identical dishes in duplicate for 6 h both at RT °C and 37 °C. Cultures labelled at RT °C had a mean incorporation rate of 97 $cpm^{-1}.hr^{-1}.\ \mu gDNA^{-1}$ compared to 632 $cpm^{-1}.\ hr^{-1}.\ \mu gDNA^{-1}$ at 37 °C. Hence, incubation at 37 °C greatly increased PG synthesis rates by chondrocyte cultures allowing measurable levels of $[^{35}SO_4]$-sulphate incorporation in a shorter period. This, combined with the need to

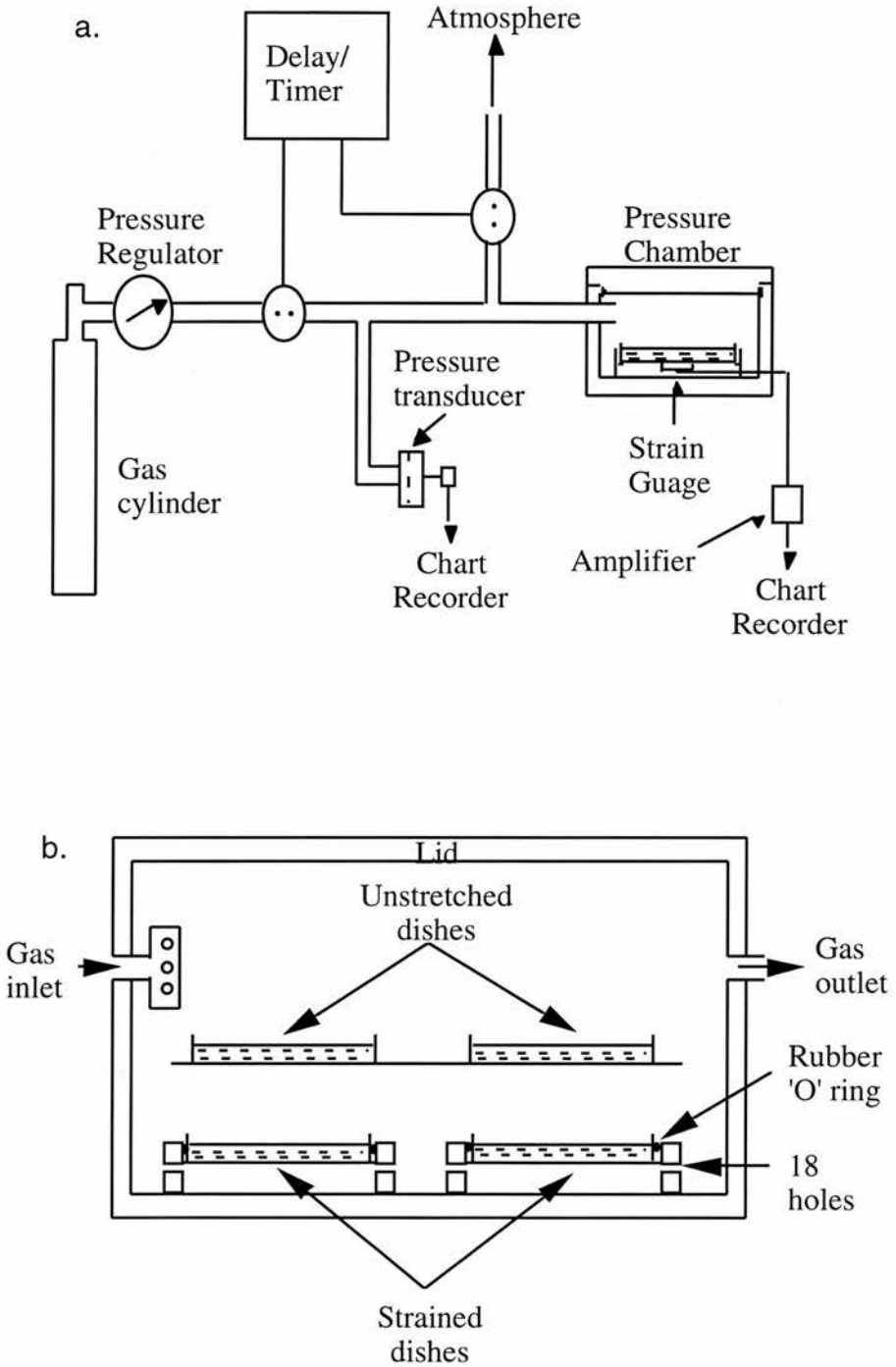


Figure 4.5 New apparatus for application of cyclical pressure.
Shematic diagram of (a.) pressurisation apparatus and (b.) details of the pressure chamber.

reduce the period of exposure to pressurisation-induced hypoxia and nitrogen narcosis suggested that the pressurisation period should be shortened to 3 h.

The new apparatus failed to elicit any change in chondrocytes' [$^{35}SO_4$] - sulphate incorporation using the same level of hydrostatic pressure and the same frequency of pressurisation as used in the original apparatus. Strain measurements were made in culture dishes in response to pressure pulses in both the new and original pressurisation apparatus. The new apparatus was shown to cause negligible deformation of the dish base at pressures that induced considerable strain in the original apparatus. When the new chamber was modified by the addition of dish - holders (Fig. 4.5) strain was produced in the dish-base proportional to the applied pressure, but only when the dishes were fitted tightly in the holders (Fig. 4.6). Strain develops in the base of the dish due to a pressure differential developing during the pressure pulse, between the large space above the dish and the small space below the dish. When the pressurising gas (oxygen-free nitrogen) flows into the chamber through the gas inlet the pressure rises rapidly in the space above the dish but access to the small space below the dish is limited, because of the resistance of the 18 small holes in the dish holder, so that the pressure rises less rapidly in the space below the dish. The resultant pressure differential is thus dependent on the pressure applied, the volumes above and below the dish, and the access to the space below the dish (239). The differential rise of pressure in the two spaces within the chamber causes distortion of the base of the dish. The larger new apparatus requires higher pressures (203 kPa) to achieve similar levels of μstrain produced in the original apparatus, due to larger volume of the new chamber (Fig. 4.6).

The large volume of the pressure chamber in the new apparatus allowed experimental protocols to be undertaken in which two petri-dishes were exposed to pressure-induced strain with simultaneous exposure of two dishes to pressurisation without strain by placing them on a platform above the strained dishes (Fig. 4.5 *(b)*). This allowed duplicate experiments with controls to be performed. The unstrained

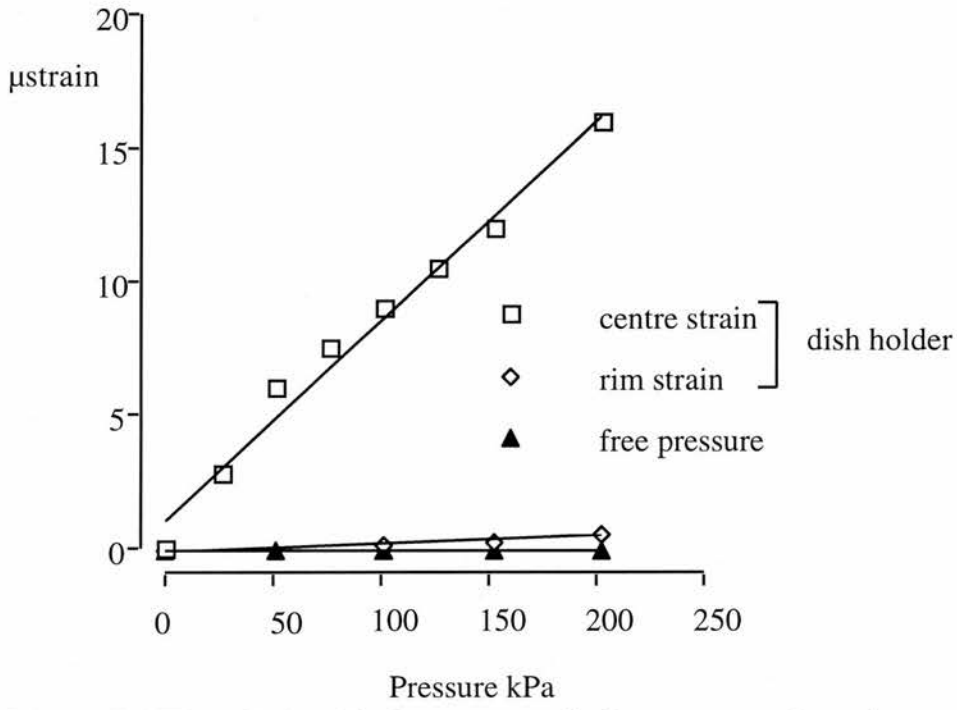


Figure 4.6 The relationship between applied pressure and resultant stretch of the dish-base in the new apparatus. Strain measurements during pressurisation were made in the centre and periphery (rim strain) of dishes fitted in the dish holder and in the centre of dishes not fitted in the dish holder (free pressure). Lines were fitted to the data using Cricket Graph III(v1.5) software. The equations of the lines are; centre microstrain = 0.075(pressure) + 1.072, r = 0.991, rim microstrain = 0.003(pressure) - 0.040 r = 0.956.

controls were exposed to identical increases in pressure and any resultant changes in the partial pressure of gases which might influence chondrocyte metabolism. Hence the effect of pressure alone could also be assessed by comparing the results obtained from cells in these dishes with those obtained from cells in dishes labelled outside the chamber over the same period.

4.6. Statistical methods

The number of experiments that could be undertaken on human chondrocytes in primary culture was relatively small and limited by the variable and uncertain availability of suitable post-mortem articular cartilage. It was also found that rates of PG synthesis varied considerably in chondrocytes from different cartilage specimens due to factors such the as age of the patient and disease state of the cartilage from which the chondrocytes were isolated. The combination of small n-numbers and the wide variance of values make assumptions regarding the distribution of data invalid. This problem can be overcome by performing a logarithmic transformation ($\log_{10}$) of data and analysing the differences between stimulated samples and paired controls under different conditions. This transformation of data allows us to use parametric test where assumptions of normality and equal variance are invalid.

[$^{35}SO_4$]-sulphate incorporation

[$^{35}SO_4$]-sulphate incorporation rates were $\log_{10}$ transformed. The number of experiments (n) was taken as the the total number of dishes exposed to a particular treatment. The differences between unstrained or strained cells and their paired controls was calculated by subtraction. These differences represented the effects of pressurisation alone and pressurisation plus strain on chondrocyte PG synthesis rates. The significance of any change between these differences was calculated by single factor ANOVA using Excel v.4 software (Edinburgh University Computing Service).

Mean values of PG synthesis were obtained by calculating the anti-log of the mean log values; the confidence intervals for these values were similarly calculated from the log confidence intervals. The use of 95 % confidence limits calculated in this way means that the confidence limits will be assymetrical. The percentage change in PG synthesis rates compared to controls was calculated from the log differences:

$$\log \text{strain} - \log \text{control} = \log\left(\frac{\text{strain}}{\text{control}}\right)$$

$$\therefore \%\ \text{change PG synthesis} = \left(10^{\log \text{strain} - \log \text{control}} - 1\right) \times 100$$

Effects of agents on the response to strain

In experiments where the effect of agents on the response to strain were being studied the log difference between strained and control cells under all treatments was compared by ANOVA using Excel v.4 software.

5. THE EFFECT OF PRESSURE-INDUCED STRAIN ON CHONDROCYTE PG SYNTHESIS

5.1. Introduction

PG is the only major component of the cartilage matrix that is relatively rapidly turned over in normal tissue (72). This and disease related changes in its synthesis, structure and function (2,43,105,111,254-256) make it a potential therapeutic target. In an attempt to understand the regulation of matrix production in normal cartilage and joint diseases, PG synthesis and turnover have been extensively investigated in response to mechanical stress, by measuring [$^{35}SO_4$]-sulphate incorporation. This technique has the advantage that the labelled products are amenable to structural analysis (257) chromatographic separation (258) or selective removal of CS and KS chains using chondroitinases and keratanases (259). More recently northern blotting and quantitative PCR techniques have been applied to measuring changes in matrix synthesis at the mRNA level. In general, the results have been similar to those obtained from [$^{35}SO_4$]-sulphate incorporation experiments (142,163,165,172).

PG synthesis and turnover are accelerated in OA cartilage (2,18,111,260), and in the early development of arthritis in experimental animal models of OA (2,5). Increased tissue hydration is also an early event in the development of OA (2,5,104,105) and probably reflects damage to the integrity of the collagen meshwork which leads to reduced tensile strength of the tissue (2,5). The PG content of cartilage does not change significantly (2,5,104) until the later stages of the disease when severe matrix damage and loss occur (2,104,105). The increase in PG synthesis is accompanied by an increase in the CS:KS ratio as CS chain length increases (2,5), and also by an increase in the level of CS-4 and foetal-type epitopes (43,254). Interestingly, these changes are the reverse of those seen in ageing cartilage where the proportion of KS and CS-6 gradually increase (2,44,260). The increase in PG synthesis in OA cartilage can be interpreted as a re-expression of a

developmental or foetal phenotype (255) and it is clearly an important early event in the development of OA.

5.1.1. The effect of mechanical compression on PG synthesis by cartilage explants and chondrocyte agarose cultures

Many studies have examined the effect of loading by compression of cartilage plugs (136-140,142-145,261) or chondrocytes cultured in a three-dimensional agarose matrix (135,141). The compression is commonly produced using a loading head that is driven either mechanically or hydraulically onto the cartilage explant held in a loading platen. The explant is maintained in culture medium during loading at 37 °C. The information required for articular cartilage to respond to load is contained within cartilage explants alone and the cellular responses are sensitive to the loading pattern (116).

Static Loading

In general, static loading leads to reduced matrix synthesis and the degree of response is related to the amplitude of the applied load (135-145,261). The effects of static compression on PG synthesis, as assessed by measurement of either [$^{35}SO_4$]-sulphate incorporation or core protein mRNA levels, are summarised in Table 5.1. It is noteworthy that Stazzone et al (142) found that core protein mRNA was elevated after 1-4 h of compression of bovine cartilage plugs and this then fell below control levels with longer periods of loading. Core protein mRNA was also elevated 1 h after a 10 min pulse of compression (142). [$^{35}SO_4$]-sulphate incorporation measured following 1 h of compression demonstrated a similar increase (142). Earlier studies with canine (139) and human (140) cartilage plugs showed reduced [$^{35}SO_4$]-sulphate incorporation following 2 h and 4 h of static compression respectively. The observed differences (139,140,142) suggest that initiation of static compression, or a short

Table 5.1 The effect of static mechanical compression on PG synthesis in cartilage explants and chondrocyte / agarose cultures.

Study	Pressure (MPa)	Compression amplitude %	Loading time (h)	Compressed /non-compressed
Jones et al. 1982 (261)	2.9		96	0.5
Palmoski and Brandt 1984 (139)	0.001		2	0.30†
	0.006		2	0.52†
	0.011		2	0.35†
Schneidermann et al. 1986 (140)	0.3		4	0.7†
	0.5		4	0.6†
	0.8		4	0.3†
Gray et al. 1988 (144)	0.07		12	1.1†
	0.25		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†
	2.75		12	0.1†
Sah et al. 1989 (136)	0.2		12	0.8†
	0.5		12	0.3†
Larsson et al. 1991 (137)	1.0		24	0.8†
	1.0		48	0.5†
	1.0		72	1.0†
Kim et al 1994 (143)		33%	12	0.88†
		50%	12	0.9†
		65%	12	0.5†
		77%	12	0.48†
		85%	12	0.18†
Buschmann et al 1995 (135)		25%	16 (2 d cultures)	1.0 ‡
		25%	16 (41 d cultures)	1.1 ‡
		35%	16 (2 d cultures)	1.0 ‡
		50%	16 (2 d cultures)	0.95 ‡
		50%	16 (41 d cultures)	0.6 ‡
		60%	16 (41 d cultures)	0.5 ‡
Burton -Wurster et al. 1995 (138)	0.025		18	0.53†
	0.5		18	0.21†
	1.2		18	0.18†
Lee and Bader 1995 (141)		15%	48	0.9‡
Stazzone et al 1996 (142)	0.05		4	2.0 §
	0.1		4	2.0 §
	0.2		4	2.0 §
	0.1		0.5	1.0 §
	0.1		1	2.0 §
	0.1		2	2.0 §
	0.1		4	2.0 §
	0.1		12	0.3 §
	0.1		24	0.3 §
Wong et al 1996 (145)		40%	23	0.7†

Results are expressed as the ratio compressed / non-compressed for total [$^{35}SO_4$}-sulphate incorporation or core protein mRNA (§) into cartilage explants (†) or chondrocyte-agarose cultures (‡).

pulse of compression, may stimulate PG synthesis for a brief period after which continued compression reduces PG synthesis. Changes of this nature would be detected more slowly at the mRNA level, so care must be taken when comparing mRNA results with [$^{35}SO_4$]-sulphate incorporation results.

Chondrocyte cultures in three-dimensional agarose gels responded to continuous compression by reducing PG synthesis (135,141) and total protein synthesis (135). The response to continuous compression was greater as the concentration of cell-derived matrix components in the agarose gel increased with time in culture, emphasising the importance of chondrocyte matrix interactions (135).

Static compression of explants reduces synthesis of fibronectin (138), core protein and link protein (142), and total protein synthesis (138,143,145). Increased osmotic pressure in the medium surrounding explants was also shown to decrease PG and protein synthesis (149,262), but the response did not appear to be mediated via effects on solute transport or concentration (149). The decrease in matrix synthesis that follows static compression was shown not be the result of limited diffusional transport (143) although loss of labelled molecules from the matrix was reduced due to tissue consolidation (155). Static compression delayed the formation of aggregates (147) without altering the synthesis of other matrix components such as HA. It has been suggested that this may have been a consequence of changes in pH of the interstitial fluid of the cartilage, that follows fluid expression (147).

Dynamic Loading

Dynamic compression of cartilage explants leads to increased matrix synthesis. The degree of response varies with the frequency of the loading cycle (135-139,141,148-153). In general, frequencies above 0.01 Hz are stimulatory to matrix synthesis (135-137,139,143,144,148-152) whereas lower frequencies are inhibitory (135-137,139,143,152). The effects of dynamic loading on cartilage explant PG synthesis are summarised in Table 5.2. In a number of studies total protein synthesis was also

Table 5.2 The effect of cyclical mechanical compression on sulphate incorporation in cartilage explants and chondrocyte-agarose cultures

Study	Pressure (MPa)	Compression amplitude %	Loading time (h)	Cycle (Hz)	Time on/off	Compressed /non-compressed
Palmoski and Brandt 1984 (139)	0.001-0.011		2	0.0083	60 s / 60s	0.5†
	0.001		2	0.067	4s / 11s	1.0†
	0.0055		2	0.067	4s / 11s	1.3†
	0.011		2	0.067	4s / 11s	0.9†
Sah et al. 1989 (136)	0.5		24		2h / 6h	1.2†
	0.5		23		2h / 2h	0.4†
	1-4		23	0.0001-0.001		1.0†
	1.1-4.5		23	0.01		1.3†
	³0.7		23	0.1-1		1.3†
Larsson et al 1991 (137)	1.0		24	0.25	2s / 2s	1.5†
	1.0		48	0.25	2s / 2s	1.7†
	1.0		72	0.25	2s / 2s	1.0†
	1.0		24	0.0083	60s / 60s	0.8†
	1.0		48	0.0083	60s / 60s	0.5†
	1.0		72	0.0083	60s / 60s	0.9†
Korver et al. 1992 (148)	0.2		168	0.33	1.5s / 1.5s	1.4 §
Parkinnen et al 1992 (152)	0.5		1.5	0.055	0.05s / 4s	1.7†
	1.0		1.5	0.055	0.05s / 4s	1.4†
	0.5		1.5	0.055	0.05s / 4s	1.2†
	1.0		1.5	0.055	0.05s / 4s	0.8†
	0.5		1.5	0.017	0.05s / 60s	1.1†
Burton-Wurster et al. 1993 (138)	0.5		120		4h / 20 h	1.14†
	1.2		120		4h / 20 h	1.12†
Visser et al. 1994 (149)	0.2		168	0.2		1.04†
Farquhar et al. 1994 (150)	0.5		1.5	0.2		1.19†
	9.0		1.5	0.2		0.8†
Kim et al. 1994 (143)	0.5		23	0.001		1.0†
	0.5		23	0.01		1.25†
	0.5		23	0.1		1.2†
Lee and Bader 1995 (141)		15%	48	0.3		0.85 ‡
		15%	48	1.0		1.45 ‡
		15%	48	3.0		1.00 ‡
Buschmann et al 1995 (135)		10%	10	0.001		1.13 ‡
		10% early	10	0.01		1.06 ‡
		10% cultures	10	0.1		1.10 ‡
		10%	10	1.0		1.13 ‡
		10%	10	0.001		0.94 ‡
		10% late	10	0.01		1.10 ‡
		10% cultures	10	0.1		1.20 ‡
		10%	10	1.0		1.35 ‡
Ostendorf et al 1995 (151)	1		72	0.2		1.66†
	1		120	0.2		2.04†
	1		168	0.2		2.10†

Results are expressed as the ratio compressed / non-compressed for total [$^{35}SO_4$]-sulphate incorporation in (†) cartilage explants, (§) sesamoid bones, (‡) chondrocyte-agarose cultures.

increased (135-137,139), and the synthesis of decorin and biglycan which is normally lost with time in culture was maintained by loading (148,149). Cartilage GAG content was not altered and there were no significant alterations in GAG composition or the size of PG's.

5.1.2. The effect of hydrostatic pressure on PG synthesis by cartilage explants and chondrocyte cultures

Cyclical hydrostatic pressurisation of cartilage or chondrocytes may stimulate chondrocyte PG synthesis whereas static pressurisation may result in reduced synthesis. It is difficult, however, to make generalisations as results have been variable and a wide variety of pressurisation techniques and tissue sources have been used.

Cyclical Hydrostatic Pressurisation

Cyclical hydrostatic pressurisation stimulates PG synthesis in cartilage explants but the response is very sensitive to the magnitude and frequency of the stimulus (Table 5.3). Physiological levels of applied pressure (2 - 20 MPa) stimulated [$^{35}SO_4$]-sulphate incorporation in bovine cartilage explants (158,161) and mouse growth plate cartilage (159) at frequencies above 0.25 Hz, and in bovine cartilage which was exposed to a short pulse of pressure followed by a long resting period (156). [$^{35}SO_4$]-sulphate incorporation in bovine articular cartilage explants (156,161) was increased by 10 MPa pressure but further elevation of the pressure reduced this response (156). Lower pressures had negligible effects in normal human cartilage (157), but were stimulatory in mouse growth plate (159) and OA human cartilage (157). Both bovine cartilage explants (156) and rabbit mandibular chondrocytes (162) are remarkably sensitive to very short periods of pressurisation followed by long periods of rest. One minute of pressurisation followed by 27 h rest reduced PG synthesis and five minutes of pressurisation followed by the same rest period increased PG synthesis

Table 5.3 The effect of cyclical hydrostatic pressurisation on sulphate incorporation by cartilage and cultured chondrocytes §.

Study	Pressure (MPa)	Loading time (h)	Cycle (Hz)	time on / off	Compressed /non-compressed
van Kampen et al. 1985 (160)	0.013	24	0.3		1.4 §
Klein-Nulend et al. 1987 (159)	0.013	120	0.3		3.4 N
Hall et al. 1991 (156)	5.0			20s / 2h	1.2 N
	7.5			20s / 2h	1.2 N
	10.0			20s / 2h	1.5 N
	15			20s / 2h	1.4 N
	20			20s / 2h	1.3 N
	50			20s / 2h	1.0 N
Lafeber et al. 1992 (157)	0.013	0	0.33	1s / 2s	1.1 N
	0.013	96	0.33	1s / 2s	1.0 N
	0.013	192	0.33	1s / 2s	0.9 N
	0.013	0	0.33	1s / 2s	0.95 OA
	0.013	96	0.33	1s / 2s	1.5 OA
	0.013	192	0.33	1s / 2s	1.55 OA
Takano-Yamamoto et al. 1991 (162)	0.0005			5min / 27h	1.0 §
	0.0025				1.1 §
	0.005				1.4 §
	0.01				1.6 §
	0.02				1.6 §
				1min / 27h	
	0.0005				1.0 §
	0.0025				0.9 §
	0.005				0.9 §
	0.01				0.9 §
	0.02				0.9 §
Parkinnen et al. 1993 (158)	5	1.5	0.0167		1.0 N
	5	1.5	0.05		1.0 N
	5	1.5	0.25		1.0 N
	5	1.5	0.5		1.17 N
	5	1.5	0.0167		1.0 §
	5	1.5	0.05		0.65 §
	5	1.5	0.25		0.55 §
	5	1.5	0.5		0.75 §
	5	20	0.0034		1.0 §
	5	20	0.0084		1.0 §
	5	20	0.0167		0.8 §
	5	20	0.05		1.0 §
	5	20	0.25		1.25 §
	5	20	0.5		1.2 §
Ellison et al. 1994 (161)	10	12	1		1.5 §
	10	24	1		1.5 §
	10	48	1		2.33 §
	10	72	1		3.00 §

Results are expressed as the ratio compressed / non-compressed for total [$^{35}SO_4$]-sulphate incorporation for normal (N) and arthritic (OA) cartilage explants and cultured chondrocytes (§).

(162). In studies using chondrocyte cultures (158,162) cyclical pressurisation could be stimulatory to [$^{35}SO_4$]-sulphate incorporation but both the frequency and duration of pressurisation were crucially important.

Cyclical pressurisation has also been shown to elevate intracellular cAMP levels (166) and to induce changes in the golgi apparatus (167) and cytoskeletal organisation (168).

Static Hydrostatic Pressurisation

In general, static hydrostatic pressures of 10 - 50 MPa reduce PG synthesis by cartilage explants (156,163,164). Static hydrostatic pressures of 1 - 10 MPa have however been associated with reduced, increased or unaltered PG synthesis (156,163,164).

Changes in hydrostatic pressure are clearly a consequence of mechanical loading of cartilage but the threshold for chondrocyte stimulation is variable, depending on the tissue source and the site from which the cartilage was taken.

Table 5.4 The effect of static hydrostatic pressure on sulphate incorporation in cartilage explants.

Study	Pressure (MPa)	Loading time (h)	Compressed /non-compressed
Lippiello et al. 1985 (164)	0.5	24	0.8
	1.0	24	0.8
	1.5	24	0.5
	2.0	24	0.5
	2.5	24	1.1
Hall et al. 1991 (156)	5.0	2	1.4
	7.5	2	1.2
	10	2	1.1
	15	2	0.9
	20	2	0.9
	30	2	0.5
	40	2	0.2
	50	2	0.2
Takahashi et al. 1996 (163)	1	2	1.06
	5	2	1.0
	10	2	0.88
	50	2	0.86

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

5.1.3. The effect of strain on matrix synthesis by cultured chondrocytes

In order to mimic the strain on chondrocytes induced by the deformation of the cartilage substrate under load *in vivo,* chondrocytes have been grown on flexible membranes or coverslips and then exposed to stretch by deformation of the culture substrate (169-171). Both intermittent and continuous stretch of cultured chondrocytes cause significant increases in sulphate incorporation, as well as an elevation of intracellular cAMP (169,171) and DNA synthesis (171). Intermittent stretch also increases type II collagen mRNA and PG core protein mRNA levels in cultured chondrocytes (172). The results are summarised in Table 5.5.

Application of continuous fluid-induced shear stress on chondrocytes, in a cone viscometer, producing a force on the cell membrane equivalent to 1.6 Pa, induced a two fold increase in [$^{35}SO_4$]-sulphate incorporation (173). Shear stress has also been shown to increase the release of PGE_2, and cause cells to elongate and align in the direction of the applied stress (4).

Chondrocytes may be exposed to shear stress *in vivo* as a result of fluid flow but the strain effects recorded result from cell deformation.

Table 5.5 The effect of stretch on sulphate incorporation in cultured chondrocytes

Study	Strain or pressure	Loading time (h)	Cycle (Hz)	stretched/ unstretched
Lee et al. 1982 (170)	10 %	8	1	2.6
de Witt et al. 1984 (171)	5.5 %	24	0.2	1.4
Uchida et al. 1988 (169)	1.32	24	0	1.9
Holmvall et el. 1995 (172)	24%	1 3	0.5	3.5§ 3.6§

Results are expressed as the ratio stretched / unstretched for total [$^{35}SO_4$]-sulphate incorporation, and the ratio stretched / unstretched of mRNA levels (§).

5.2 Summary

A large number of studies have been performed in which the effect of mechanical stress on cartilage and chondrocytes have been examined. Taken as a whole, the results often appear to be conflicting and confusing, and to date have provided little insight into the mechanisms involved in any of the responses. The studies described in this thesis set out to examine how mechanical force is detected by chondrocytes and how the mechanical stimuli provoke the biochemical responses that have been described.

The experiments described in this chapter chronicle the development of the pressurisation apparatus for use in biochemical studies, and confirmation that the apparatus acts via chondrocyte deformation. The technique had previously been shown to be effective in producing chondrocyte membrane hyperpolarisation in response to cyclical pressurisation (238). The first objective was to examine the effect of cyclical pressurisation on chondrocyte PG synthesis, as membrane hyperpolarisation was not known to influence chondrocyte metabolism.

5.3. Methods

The rate of PG synthesis by cultured human and bovine chondrocytes was assayed by [$^{35}SO_4$]-sulphate incorporation using the methods already described in section 4.4. (p58) PG synthesis rates during cyclical pressurisation were measured under a variety of different conditions.

The influence of the age and the disease state of the cartilage from which chondrocytes were isolated, on rates of PG synthesis were assessed by multiple regression analysis using Uniststat software on an IBM-compatible microcomputer (Dell Dimension P75). The influence of the time that chondrocytes were held in culture on the rate of PG synthesis was assessed by multiple regression analysis using Unistat software, in both human and bovine chondrocytes.

5.3.1. Investigation of the effect of chondrocyte pressurisation on PG synthesis rates in the original apparatus

Previous studies of the effect of mechanical stress on chondrocyte [$^{35}SO_4$]-sulphate incorporation had shown that cyclical hydrostatic pressurisation for 1.5 h (158) and cyclical stretching for 8 h (169) could elicit change, in [$^{35}SO_4$]-sulphate incorporation rates. Initially the rate of sulphate incorporation was measured over a 6h period at room temperature (RT °C, 23-26 °C) in the absence of load to establish that measurable levels of [$^{35}SO_4$]-sulphate incorporation would take place under these conditions. The experiments were performed at RT °C because the original pressurisation apparatus was not designed to allow for temperature control and a suitable incubator was unavailable. Six hours proved to be an adequate and convenient labelling period for initial loading studies. The original pressure apparatus could sustain pressures up to 26.7 kPa (200 mm Hg) and this pressure was used for loading experiments, although it was several orders of magnitude below physiological levels of pressure that have been recorded *in vivo* (118). Since previous studies of chondrocyte membrane potential changes in response to cyclical pressurisation using this apparatus (238) had been undertaken at a frequency of 0.33 Hz, and since a number of studies had shown that low levels of cyclical hydrostatic pressure at 0.3 Hz stimulated PG synthesis (157,159,160) chondrocytes were exposed to a loading regime of 26.7 kPa above atmospheric pressure at a frequency of 0.33 Hz for 6 h at RT °C.

[$^{35}SO_4$]-sulphate incorporation rates by cultured human chondrocytes were determined in unpressurised chondrocytes and in cells exposed to 26.7 kPa, 0.33 Hz for 6h in the absence of strain and in the presence of strain (20 μstrain) at RT °C.

An identical loading regime was also used in experiments in which the same apparatus was used to examine the role of stretch-activated ion channels in the response to cyclical pressure-induced strain (PIS) (see section 6.4.1., p120).

5.3.2. Investigation of the effect of chondrocyte pressurisation on PG synthesis rates in the new apparatus

As previously described in section 4.5. (p64) the original pressurisation apparatus was unsuitable for extensive biochemical studies of chondrocyte metabolism because only one dish could be pressurised at any one time. New apparatus was designed, therefore, which would accommodate up to four dishes, which would withstand higher pressures, and which could be maintained at 37 °C by placing it in an incubator. After initial studies indicated that [$^{35}SO_4$]-sulphate incorporation rates were higher at 37 °C compared to RT °C (23-26 °C), experiments were undertaken in which chondrocytes were loaded for only 3 h. Measurements of PIS were made in the new apparatus to establish the pressure-strain relationship. It was found that approximately 10 fold higher pressures (202.6 kPa) were required in the new apparatus to achieve similar levels of μstrain as had been induced by 26.7 kPa in the original apparatus (20 μstrain). The new apparatus could safely sustain up to 203 kPa (2 bar) which induced 16 μstrain on the base of the petri dish and this pressure was selected as the standard experimental pressure pulse. Dishes pressurised in the chamber but not fitted into the dish holder were unstrained. This allowed the simultaneous measurement of [$^{35}SO_4$]-sulphate incorporation in cells subjected to PIS and pressure in the absence of strain.

[$^{35}SO_4$]-sulphate incorporation rates by cultured human chondrocytes were measured in the new apparatus in (a) controls labelled in air for 3 h at 37 °C (b) cells exposed to 203 kPa, 0.33 Hz for 3h at 37 °C in the presence and absence of cyclical PIS (16 μstrain). In separate experiments, chondrocytes were exposed to nitrogen gas at atmospheric pressure over 3 h at 37 °C, in order to assess the effect of the pressurising gas in the absence of pressure and strain. The effects of exposure of chondrocytes to 26.7 kPa pressure in the absence of strain was measured over 6 h at RT °C and over 3h at 37 °C and the results were compared to [$^{35}SO_4$]-sulphate incorporation by paired controls labelled in air.

[$^{35}SO_4$]-sulphate incorporation rates by cultured bovine chondrocytes were measured in cells pressurised (203 kPa, 0.33 Hz) in the new apparatus for 1, 2, 3, 4, and 6 h in the absence of strain. The effect of cyclical pressurisation (3 h at 37 °C), in the presence and absence of strain (16 μstrain), on [$^{35}SO_4$]-sulphate incorporation rates was measured in cultured bovine chondrocytes.

[$^{35}SO_4$]-sulphate incorporation rates (3 h at 37 °C) were also measured in the immortalised human chondrocyte cell lines C20a4 and TC28a4 and the effect of 3 h cyclical pressurisation at 37 °C, in the presence and absence of strain (16 μstrain), on [$^{35}SO_4$]-sulphate incorporation rates was also measured.

5.3.3. Identification of GAGs labelled by [$^{35}SO_4$]-sulphate incorporation

The [$^{35}SO_4$]-sulphate incorporation assay is well established as a method for measuring PG synthesis but it does not provide information with regard to the identity of the synthesised product. Proteoglycans are sensitive to digestion by chondroitinases and keratanases which act on CS and KS respectively to yield disaccharide subunits of the GAGs and core protein (259). The products of such a digestion can easily be separated by size exclusion chromatography to quantify the amount of GAG present in the nascent PG. Studies with chondroitinases and keratanases were therefore undertaken to identify the GAGs which were labelled by the [$^{35}SO_4$]-sulphate incorporation protocol in both human and bovine chondrocytes.

Cell samples, prepared with GCET-solution as described in section 4.4. (p58), were dialysed using slidalyse (Pierce & Warriner, Chester, UK) dialysis cassettes. One ml of each cell sample was injected into a separate dialysis cassette using a 2 ml disposable syringe (Becton-Dickinson Plastipak; Lothian Supply Centre, Edinburgh, Scotland) and a 21 gauge needle (Becton-Dickinson Microlance 3; Lothian Supply Centre), in accordance with the manufacturers instructions. The samples were dialysed against three changes of one litre of deionised distilled water over a period

of 48 h. The samples were removed from the cassettes, using a syringe and 21 gauge needle as before, and placed in 1.5 ml microfuge tubes. The samples were then dried using a DNA Speed Vac (Savant, DNA110). The dried samples were reconstituted in 100 µl of distilled water; the GAG-content of a 10 µl aliquot of each sample was assayed using the dimethylmethylene blue dye-binding assay (263).

The concentration of GAGs in a solution can be measured using the dye dimethylmethylene blue (DMMB) which undergoes a colour change when it binds to sulphated GAGs (263). GAG standards in the range 0 - 5 µg. ml^{-1} were prepared in 10 mM Tris HCl pH 8.0. The dye solution contained 46 mM DMMB in 40.5 mM Na Cl, 40.5 mM Glycine, and 10 mM HCl. This solution could be stored for up to one month in the absence of light. As crystallisation started to occur within this period, the dye solution was filtered through Whatman No.1 filter paper prior to use. The GAG assay was carried out in a flat-bottomed 96-well ELISA plate (Dynatech, Billinghurst, UK) by adding 190 µl of dye solution to 10 µl of sample and immediately reading the absorbance of the samples at 490 nm using a Dynatech MR580 plate reader (Dynatech, Billinghurst, UK). The standard curve was plotted using Cricket Graph III (v.1.5) software on a Macintosh Performa 475 microcomputer. This was used to calculate the concentration of GAGs in the unknown samples.

An aliquot of each sample was digested with:

(a) chondroitinase ABC - 30 µl of each sample was digested overnight at 37 °C with chondroitinase ABC (0.1 unit. ml^{-1}) diluted in 0.1 M Tris HCl pH 8.0 to give a final concentration in the digest of 0.01 units. $\mu gGAG^{-1}$.

(b) keratanase and keratanase II - 30 µl of each sample was digested for 24 h at 37 °C with keratanase (0.01 units. μl^{-1}, ICN, Thame, UK) and keratanase II (0.001 units. μl^{-1}, Seikagaku Corp.) which had been diluted 1:100 in 50 mM acetate buffer pH7.4. The enzymes were added together in four equal aliquots

at approximately 6 h intervals, to give a final concentration of 0.0004 units. μg GAG^{-1} of keratanase and 0.00004 units. μg GAG^{-1} of keratanase II.

(c) all three enzymes - 30 μl of each sample was digested sequentially with both keratanases followed by chondroitinase ABC, following the protocols described for the individual enzymes.

Following digestion, samples were separated using a Sephadex G50 size exclusion column (1.5 cm x 25 cm) and eluted with 0.1M Tris HCl pH7. Resulting 1 ml aliquots were mixed with 5 ml Cocktail T scintillation fluid and counted. The counts in each sample were plotted against volume eluted; undigested protein-associated $[^{35}SO_4]$-sulphate eluted in the void volume and labelled disaccharides resulting from digestion eluted in the included volume.

Samples from bovine chondrocytes were digested with chondroitinase ABC or keratanase plus keratanase II as described above.

5.4. Results

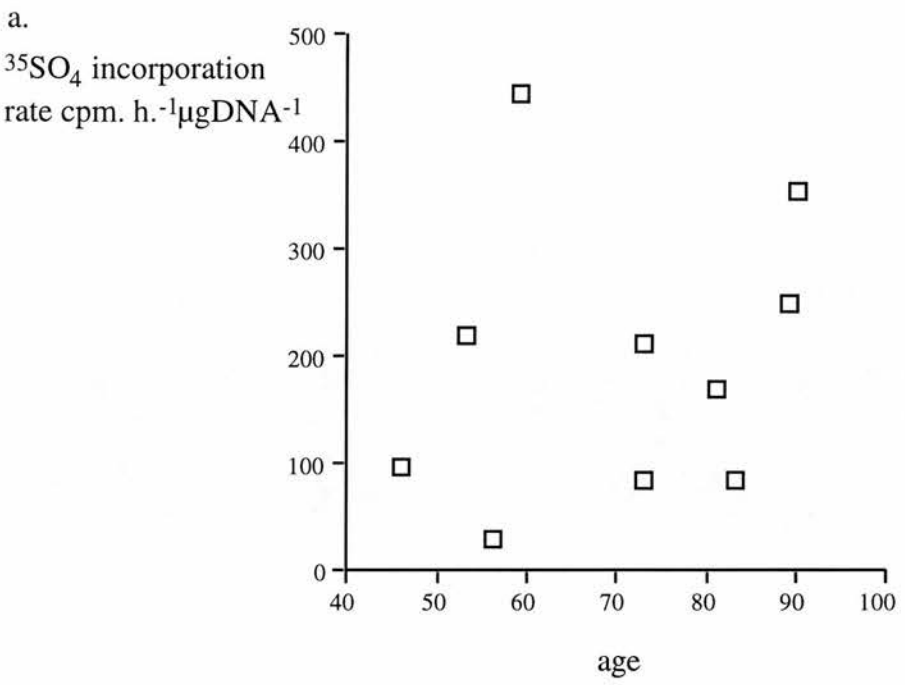
5.4.1.The effect of age and disease state on PG synthesis rates

There was no significant correlation between age (Fig. 5.1) or disease state of the articular cartilage from which the chondrocytes were derived (Fig 5.2) and the rates of PG synthesis measured for 3 h at 37 °C or for 6 h at RT °C.

Mean $[^{35}SO_4]$-sulphate incorporation measured in air was 153 (87 - 268, n = 15) cpm. h^{-1}. $\mu gDNA^{-1}$ [mean (95 % confidence intervals)] over 6 h at RT °C, and 102 (70 - 148, n = 37) cpm. h^{-1}. $\mu gDNA^{-1}$ over 3 h at 37 °C.

The mean rates of $[^{35}SO_4]$-sulphate incorporation in chondrocyte cell lines C20a4 and TC28a4, measured in air over 3 h at 37 °C, were 60 (18 - 203, n = 5) cpm. h^{-1}. $\mu gDNA^{-1}$ and 100 (29 - 337, n = 4) respectively.

The mean rate of $[^{35}SO_4]$-sulphate incorporation in bovine chondrocytes measured in air over 3 h at 37 °C was 1585 (981 - 2562) cpm. h^{-1}. $\mu gDNA^{-1}$.



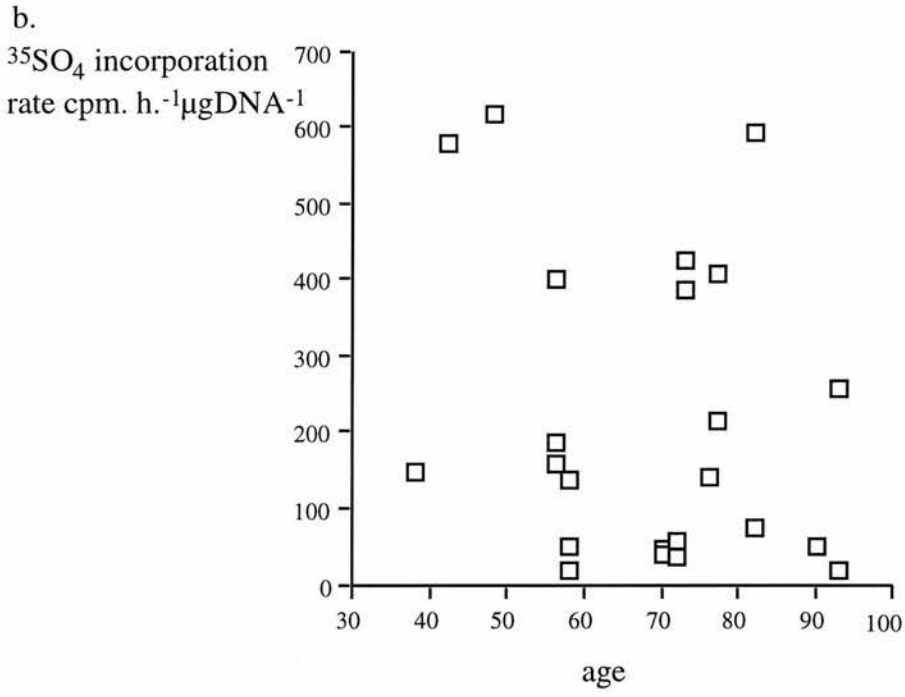


Fig 5.1 the effect of age on PG synthesis rates.
$^{35}SO_4$-incorporation was assayed (a.) over 6h at RT °C, or (b.) over 3h at 37°C. Multile regression analysis showed no correlation between age and the rate of PG synthesis.

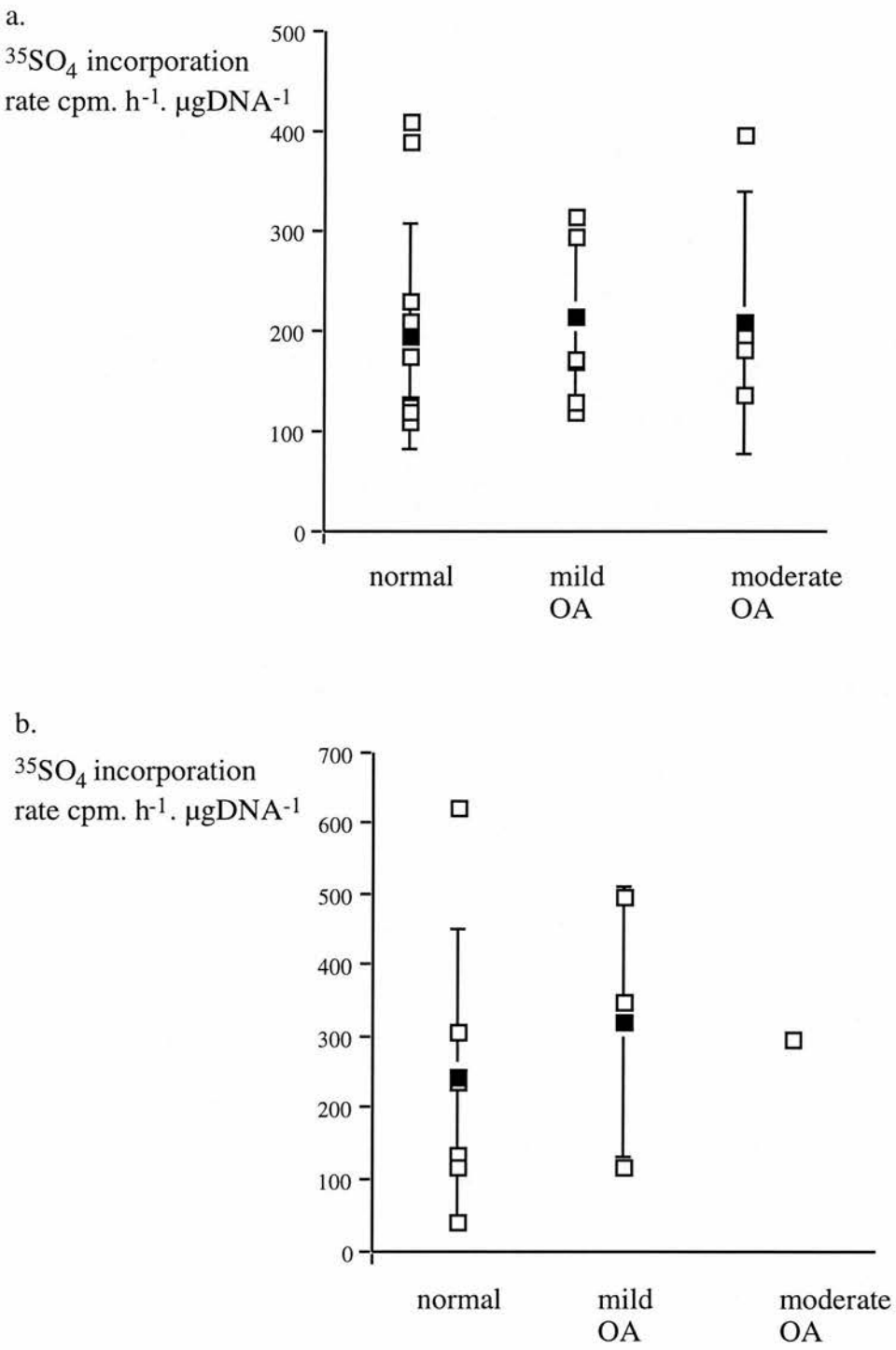


Figure 5.2 The effect of disease state on baseline PG synthesis.
$^{35}SO_4$-incorporation was assayed (a.) over 6h at RT °C, or (b.) over 3h at 37 °C. Open symbols represent incorporation rates of individual samples, filled symbols represent means ± standard deviation.

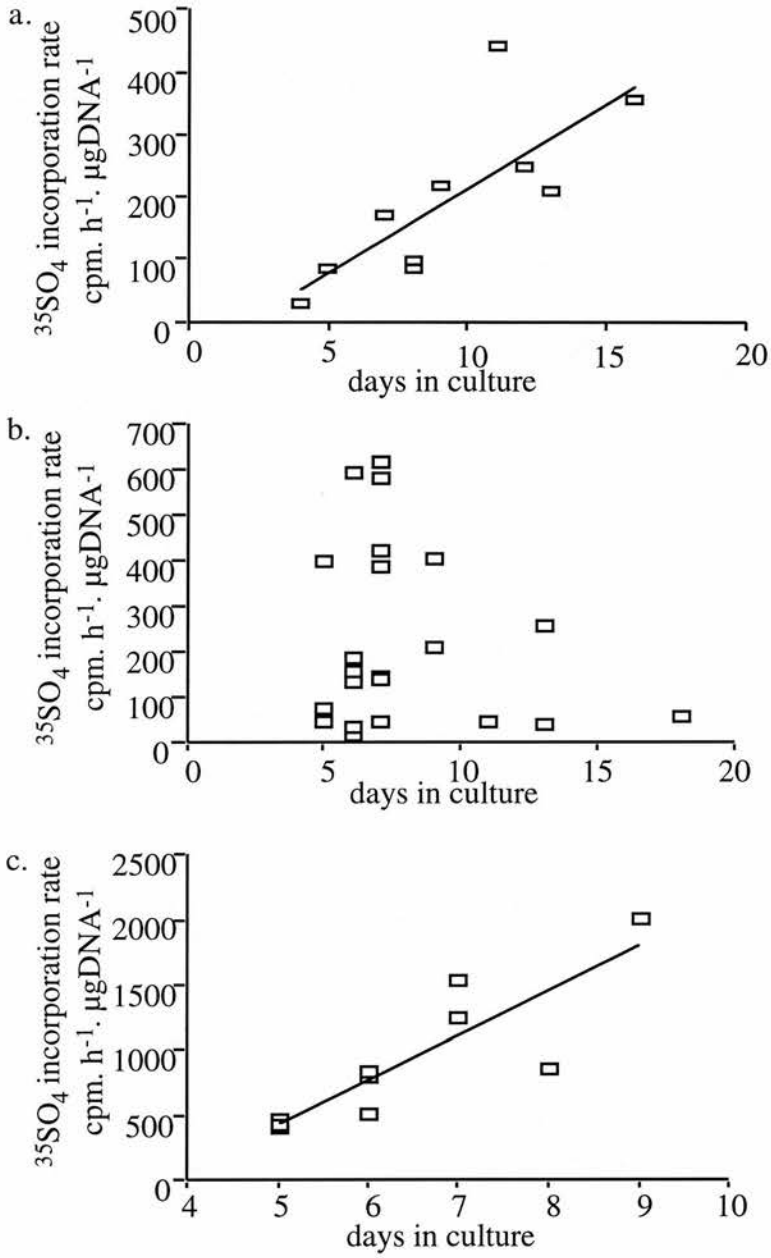


Figure 5.3 The effect of time in culture on PG synthesis rates. $^{35}SO_4$ incorporation was assayed (a.) over 6h at RT °C in human chondrocytes (b.) over 3h at 37 °C in human chondrocytes (c.) over 3 h at 37 °C in bovine chondrocytes . Multiple regression analysis demonstrated a positive correlation between the rate of PG synthesis measured over 6 h in human chondrocytes and days in culture (a.) r = 0.83. There was no correlation between PG synthesis rates measured over 3 h in human chondrocytes and days in culture. There was a positive correlation between PG synthesis rates in bovine cultures and days in culture (c.) r = 0.90.

5.4.2. The effect of time in culture on PG synthesis rates

PG synthesis rates were estimated between 5 and 18 days after isolation and initiation of primary chondrocyte cultures. There was a positive correlation (r = 0.83) between the rate of PG synthesis measured over 6 h at RT °C and the period cells had been cultured before assay (Fig. 5.3*a.*). This correlation was not seen when PG synthesis was measured over 3 h at 37 °C in human chondrocytes (Fig 5.3*b.*). In bovine chondrocytes, however, there was a positive correlation between the rate of PG synthesis measured over 3 h at 37 °C and time in culture.(5 - 10 days, r = 0.90, Fig 5.3*c.*).

5.4.3. The identification of labelled GAGs by chondroitinase and keratanase digestion

Characteristic elution profiles of PG samples from human chondrocytes prior to digestion and following treatment with ABC chondroitinase, keratanase and keratanase II, and all three enzymes are shown in Fig. 5.4 The amount of GAG present is calculated from the total counts in the disaccharide fraction (included volume) expressed as a percentage of the total number of counts eluted. Digestion with chondroitinase ABC removed 53±6.3 (s.d.)% (n = 6) of incorporated [$^{35}SO_4$]-sulphate from labelled PGs indicating that 53 % of the labelled GAG was CS. Keratanases removed 19.5±6.3 % (n = 6) of incorporated [$^{35}SO_4$]-sulphate from labelled PGs indicating that 20% of labelled GAG was KS. Treatment with all enzymes removed 86.3±2.5 % (n = 6) of incorporated [$^{35}SO_4$]-sulphate from labelled PGs.

Digestion of PG samples from bovine chondrocytes demonstrated that 87.5±4.4 % (n = 3) of incorporated [$^{35}SO_4$]-sulphate was chondroitinase ABC sensitive and 5.8±1.0 % (n = 4) was keratanase sensitive, indicating that 88 % of labelled GAGs were CS and 6% were KS.

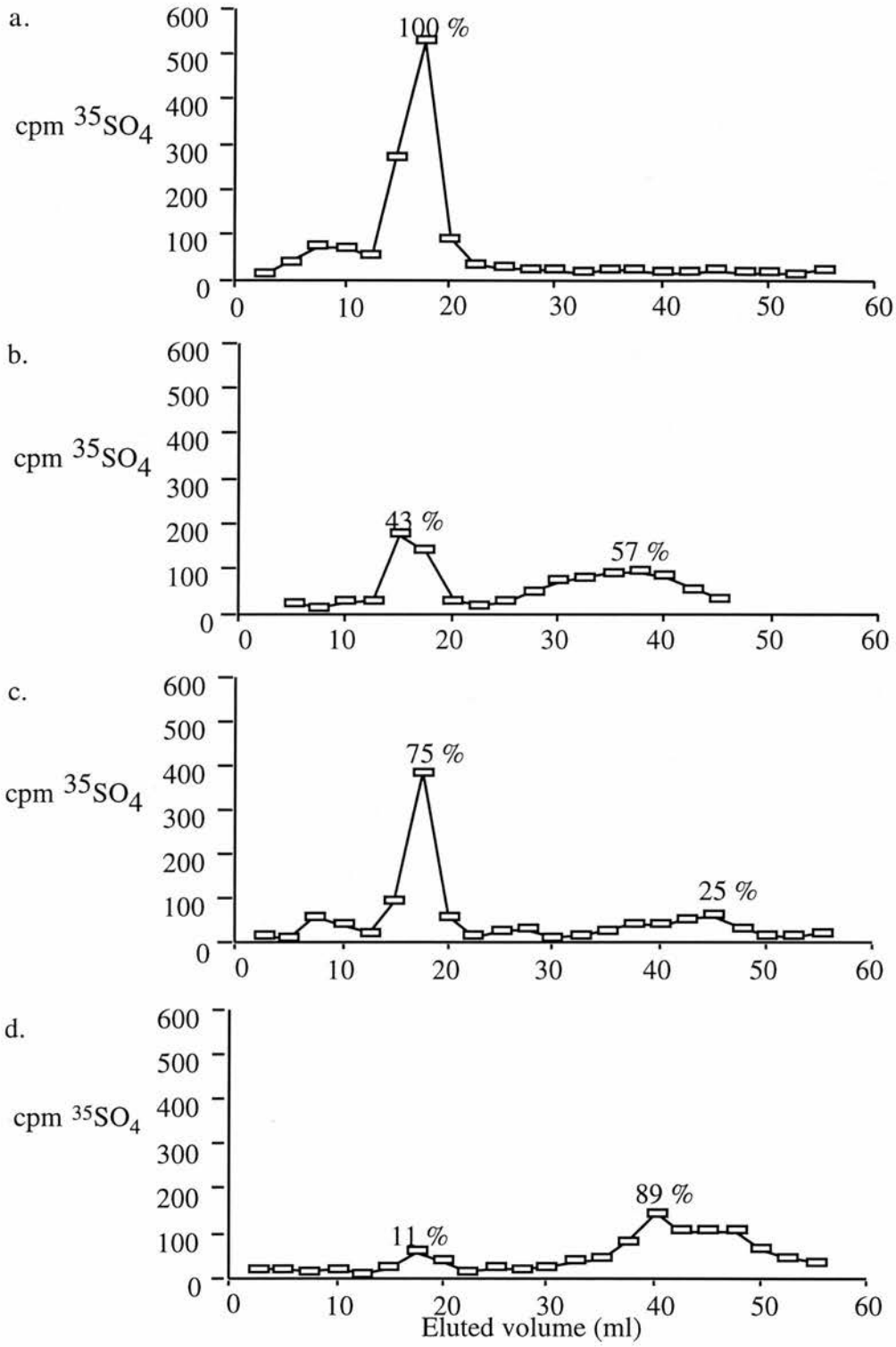


Figure 5.4 PGs from human chondrocytes separated from disaacharide digestion products by size exclusion chromatography. Figures show elution profiles of: (a) undigested sample (b) ABC chondroitinase digest (c) keratanase and keratanase II digest (d) digest with all three enzymes, using sephadex G50 size-exclusion columns. Percentages represent the proportion of total cpm present in peak.

5.4.4. The effect of cyclical pressurisation on PG synthesis rates

Human chondrocytes

Pressurisation in the absence of strain had variable effects on PG synthesis. There was no significant change in [$^{35}SO_4$]-sulphate incorporation (mean ratio unstrained / control = 0.95 (0.82 - 1.09)[95% confidence interval], n=7, not significant [ns]) in human chondrocytes exposed to 26.7 kPa pressurisation over 6h at RT °C compared to controls in air (Fig. 5.5).

Table 5.6 The effect of cyclical pressurisation on PG synthesis rates

	mean [$^{35}SO_4$]-sulphate incorporation cpm. h^{-1}. $\mu gDNA^{-1}$ (95 % confidence interval)		mean ratio pressure/control (95 % confidence interval)
	control	pressure	ratio
26.7 kPa (6 h RT _C, n = 7)	128 (87 - 186)	121 (87 - 168)	0.95 (0.82 - 1.09)
0 kPa (3 h 37 _C, n=6)	16 (14.6 - 17.4,)	15 (11.8 - 18.5)	0.93 (0.71-1.22)
26.7 kPa (3 h 37 _C, n=37)	102 (70 - 148)	69 (48 - 98)	0.67 (0.56 - 0.80)
203 kPa (3 h 37 _C, n=6)	154 (57 - 154)	104 (33 - 326)	0.67 (0.46 - 0.99)

The table shows mean PG synthesis rates calculated from log_{10}-transformed data.

Human chondrocytes similarly exposed to 26.7 kPa but over 3 h at 37 °C showed a 33 % decrease in [$^{35}SO_4$]-sulphate incorporation compared to controls in air (ratio = 0.67 (0.56 - 0.80), n=37, P<0.001, Table 5.6). In separate experiments chondrocytes exposed to nitrogen gas at atmospheric pressure over 3 h at 37 °C demonstrated a non-significant decrease in [$^{35}SO_4$]-sulphate incorporation compared to controls in air (ratio = 0.93 (0.71 - 1.22), n = 6, ns). Chondrocytes exposed to 203 kPa pressurisation at 37 °C over a 3h period showed a 33 % reduction in PG synthesis (ratio = 0.67 (0.46 - 0.99), n=6, ns) compared to controls in air.

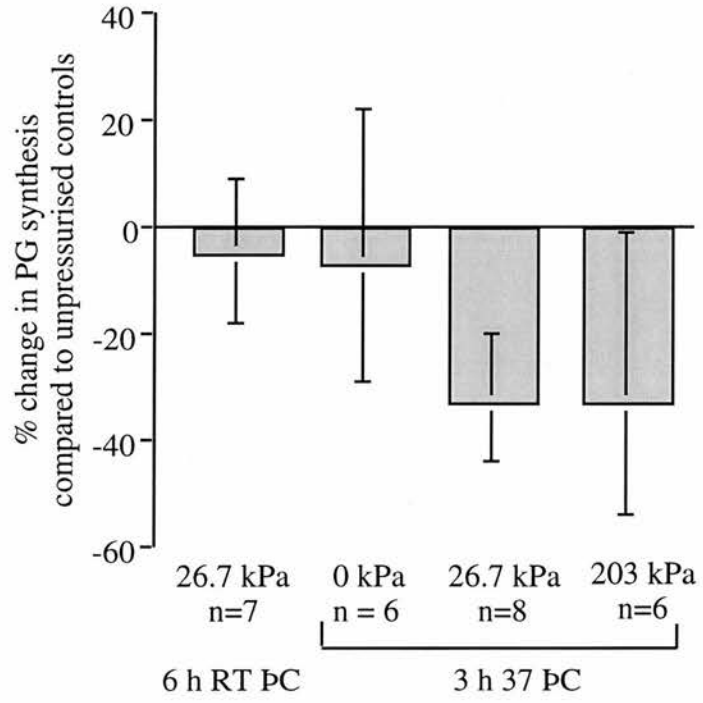


Figure 5.5 The effect of cyclical pressurisation on chondrocyte PG synthesis. Human chondrocytes were subjected to intermittent (0.33 Hz) pressurisation at the pressures shown, and paired controls were unpressurised in air under the same conditions. The figure shows the % change in PG synthesis dusing pressurisation compared with paired unpressurised controls. Error bars show 95 % confidence limits.

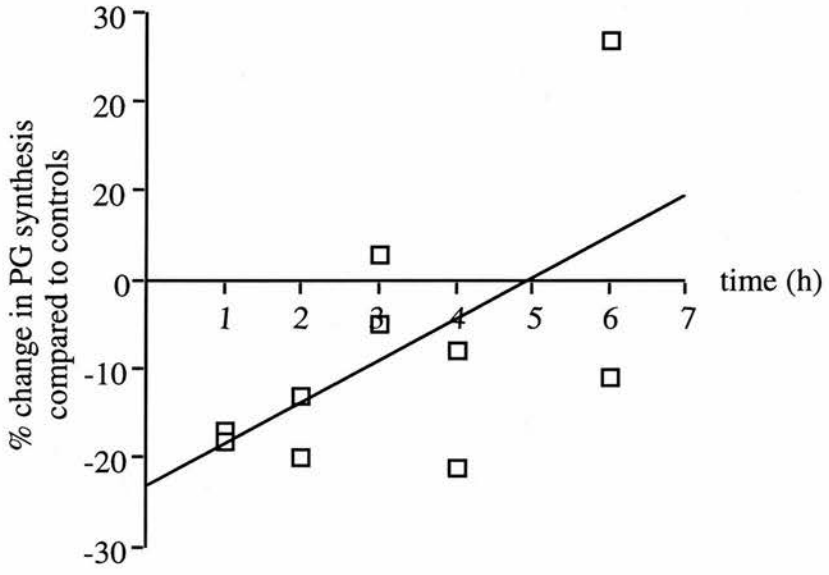


Figure 5.6 The effect of pressurisation on PG synthesis rates in bovine chondrocytes. The cells were exposed to cyclical pressurisation (203 kPa, 0.33 Hz) at 37 °C for the times shown. Multiple regression analysis indicated a positive correlation between the % change in PG synthesis and the pressurisation period.

Bovine chondrocytes

Bovine chondrocytes exposed to cyclical pressurisation for between1 and 6 h at 37 °C PG synthesis rates were decreased compared to unpressurised controls (Fig.5.6). The extent of the decrease in PG synthesis was reduced as the pressurisation time increased. There was a significant positive correlation between the pressurisation time and the degree of response ($r = 0.75$, $p<0.05$). Bovine chondrocytes exposed to 203 kPa for 3 h at 37 °C decreased PG synthesis by 28 % compared to controls in air (ratio = 0.72 (0.52 - 1.00), n = 5, ns), cells pressurised under the same conditions but at RT °C for 3 h decreased PG synthesis by 22 % compared to controls in air (ratio = 0.78 (0.64 - 0.95), n = 3, ns).

5.4.5. The effect of cyclical strain on PG synthesis rates

Human chondrocytes

Chondrocyte cultures exposed to cyclical PIS showed a significant increase in [$^{35}SO_4$]-sulphate incorporation indicating increased PG synthesis (Fig. 5.7). Cells subjected to cyclical PIS of 20 μstrain (0.33 Hz) for 6h at RT °C increased PG synthesis by 89 % ($p<0.0001$) whilst unstrained cells pressurised under the same conditions showed no significant change in PG synthesis. Cells subjected to only 3 μstrain over 6 h at RT °C showed a 13 % (ns) increase in PG synthesis compared with unpressurised control cells, indicating that the degree of response may be related to the level of strain applied.

Chondrocytes subjected to cyclical PIS of 20 μstrain (26.7 kPa) for 3 h at 37 °C increased PG synthesis by 25 % ($p=0.003$) compared with paired controls in air (Table 5.7). Cells subjected to cyclical PIS of 16 μstrain (203 kPa) for 3 h at 37 °C decreased PG synthesis by 18 % when compared to paired controls in air (ns), but showed an increase of 24 % when compared to paired unstrained cells ($p<0.03$).

Table 5.7 The effect of cyclical PIS on PG synthesis rates in human chondrocytes

Conditions		mean [$^{35}SO_4$]-sulphate incorporation cpm. h^{-1}. $\mu gDNA^{-1}$ (95 % confidence interval)			mean ratio (95 % confidence interval)	
		control	pressure	strain	pressure /control	strain /control
6 h	3 μs (n=7)	128 (87 - 186)	121 (87 - 168)	145 (107 - 196)	0.95 (0.82 - 1.09)	1.13 (0.89 - 1.45)
RT _C	20 μs (n=15)	153 (87 - 268)		289 (184 - 451)		1.89 (1.53 - 2.32)
3 h 37 _C	20 μs (n=37)	102 (70 - 148)	69 (48 - 98)		0.67 (0.56 - 0.80)	
	20 μs (n=8)	52 (25 - 108)		65 (34 - 125)		1.25 (1.07 - 1.45)
	16 μs (n=6)	154 (57 - 411)	104 (33 - 326)	125 (36 - 428)	0.67 (0.46 - 0.99)	0.82 (0.53 - 1.27)
	16 μs (n=13)		219 (114 - 424)	272 (135 - 548)		1.24* (1.12 - 1.45)

The table shows mean PG synthesis rates calculated from log_{10}-transformed data.
* = strain/pressure

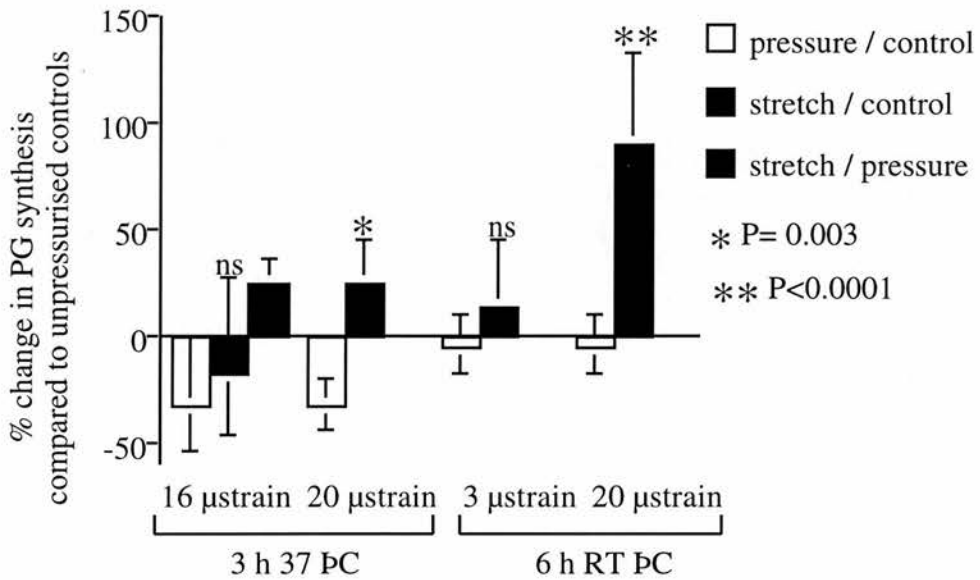


Figure 5.7 The effect of cyclical PIS on human chondrocyte PG synthesis. Chondrocytes were exposed to cyclical pressurisation under the conditions shown in the presence or absence of strain. Diagrams show percentage change in $^{35}SO_4$-incorporation compared to paired unpressurised controls. Error bars represent 95 % confidence interval. Values for n are given in Table 5.7.

Chondrocyte cell lines

Chondrocyte cell lines C20a4 and TC28a4 subjected to cyclical PIS (16 μstrain) for 3 h at 37 °C decreased PG synthesis by 13 % (ns) and 14 % (ns) respectively compared to paired controls in air (Fig. 5.8). Compared to paired unstrained cells these cells increased PG synthesis by 115 % ($p<0.005$, n = 5) and 159 % ($p<0.007$, n = 4) respectively (Table 5.8).

Table 5.8 The effect of cyclical PIS on PG synthesis rates in immortalised human chondrocytes

	mean [$^{35}SO_4$]-sulphate incorporation cpm. h^{-1}. $\mu gDNA^{-1}$ (95 % confidence interval)			mean ratio (95 % confidence interval)		
Cell line	control	pressure	strain	pressure/ control	strain/ control	strain/ pressure
C20a4 n = 5	60 (18 - 203)	24 (7 - 82)	52 (12 - 222)	0.41 (0.25 - 0.66)	0.87 (0.58 - 1.31)	2.15 (1.06 - 4.36)
TC28a4 n = 4	100 (29 - 337)	33 (8 - 129)	86 (32 - 232)	0.33 (0.28 - 0.39)	0.86 (0.56 - 1.12)	2.59 (1.56 - 4.29)

The table shows mean PG synthesis rates calculated from log_{10}-transformed data.

Bovine chondrocytes

Bovine chondrocytes increased PG synthesis from a mean unstrained value of 643(410 - 1007) cpm. h^{-1}. $\mu gDNA^{-1}$ to 974 (637 - 1490) cpm. h^{-1}. $\mu gDNA^{-1}$ following exposure to 16 μstrain for 3 h at 37 °C. This was a non-significant rise of 52 % compared to paired unstretched cells pressurised under the same conditions (mean ratio strain / unstrained = 1.52 (1.38 - 1.67), n = 13, ns).

5.5. Discussion

The results obtained in this study indicate that [$^{35}SO_4$]-sulphate incorporation can be used to measure PG synthesis in cultured chondrocytes. Treatment of the labelled product with chondroitinase ABC and keratanase/keratanase II demonstrated that the main GAGs produced under the culture conditions are KS and CS.

The composition of the GAGs present in human articular cartilage varies with age. Infant articular cartilage is almost entirely composed of CS with virtually no KS present (44). The proportion of CS in human articular cartilage decreases with increasing age, reaching 70 - 80 % of the total GAG at 70 years of age (44). Simultaneously the proportion of KS rises, reaching 12-15 % of total GAG at 70 years (44). The samples of articular cartilage from which the chondrocytes were prepared were obtained from an 85 year old female with normal cartilage. This may, in part, explain the relatively low proportion of CS (53 %) and the relatively high proportion of KS (19.5 %), although no figures are available from the literature for cartilage of this age. The ratio of [$^{35}SO_4$]-sulphate incorporation into CS and KS is broadly consistent with that seen in aggrecan, the predominant PG in articular cartilage (2,258,259). It would be of interest to carry out an extensive analysis of the relative amounts of CS and KS before and after pressurisation, since changes in the CS : KS ratio occur in ageing and joint disease (2).

Treatment of samples in this study with a combination of chondroitinase and keratanases indicated that 86 % of sulphated GAGs were CS and KS. The portion of incorporated [$^{35}SO_4$]-sulphate that is insensitive to digestion may correspond to the reducing end of CS chains since chondroitinase ABC does not remove the sulphated disaccharide adjacent to the linkage region of the GAG (258). It is also possible that a small proportion of the label is incorporated into minor PG's such as biglycan or other sulphated glycoproteins such as fibronectin which are not digested by chondroitinases or keratanases. Digestion of samples with chondroitinase alone (53 %) added to digestion with keratanases alone (19.5 %) did not remove the same total amount of incorporated [$^{35}SO_4$]-sulphate as a combined digestion with all three enzymes (86%). These results indicate that the digestion with individual enzymes was incomplete, possibly due to an inhibitory effect of non-substrate GAG which was successfully removed in the sequential digestion.

The bovine samples were composed of a high percentage of CS (85 %) and a low percentage of KS (5 %) compared with the human samples. The bovine chondrocytes were from cartilage from an immature animal (aged 12 months) which would be expected to have high CS and low KS content. The differences in the maturity of the cartilage samples may also explain differences in the CS:KS ratios in the human and bovine samples. Overall the PG-digestion analysis suggests that the proportion of CS and KS produced by the cultured human chondrocytes is similar to those measured in cartilage, when one bears in mind the age of the subject from whom the cartilage was obtained and some degree of incomplete digestion. A more complete analysis of the structure of PG samples from different age groups would be required to confirm this conclusively.

PG synthesis rates have previously been reported to increase with age (2,260) and also in OA (2,18,111,260). The rates of PG synthesis in unstimulated cultured chondrocytes were independent of both of these variables in this study. Previous studies which have examined the relationship between PG synthesis and ageing or disease have used cartilage explants (111,260) and it was demonstrated that disease related increases in synthesis was lost after three to four days in culture (111). This would suggest that it would be unlikely that isolated chondrocytes cultured for varying periods, up to 14 days, would maintain any disease or age related metabolic differences, especially as the rate of synthesis was shown to be dependent on the time in culture prior to assay.

The increase in PG synthesis rates with time in culture, seen in human chondrocytes assayed over 6 h at RT °C and in bovine cells assayed over 3 h at 37 °C, could reflect a phenotypic change in the chondrocytes with time in culture. However, chondrocytes which had been cultured for comparable periods consistently stained positive for KS (chapter 8.) suggesting that a chondrocyte phenotype was still being expressed. The range of PG synthesis rates assayed for 3 h at 37 °C in chondrocytes from separate human cartilage samples, which had been in culture for

the same number of days, was very wide. This inter-individual variation obscured any correlation that there might have been between the rate of synthesis and time in culture. To confirm this relationship PG synthesis rates in cells from one individual would need to be followed over a 0 - 14 day period in culture.

PG synthesis rates in controls in air assayed for 6 h at RT °C and for 3 h at 37 °C were not significantly different. This was surprising as initial studies had indicated that synthesis rates are much higher at 37 °C (section 4.5.2.) when compared to paired samples labelled at RT °C. Absolute PG synthesis rates varied widely in controls. This may have been the result of a number of factors such as time in culture, time after death before cartilage removal, age, disease state, as well as the possible influence of other unrelated diseases. This variability makes data analysis difficult, especially when n is unavoidably small due to limited tissue availability. The use of different experimental temperatures and times in the original and the new apparatus mean that care must be exercised when comparing data from different groups since chondrocyte metabolism and ion channel function will be different at different temperatures. However, the use of paired controls in all studies allowed the effects of stimulation by pressure or strain to be effectively assayed. Differences in the degree of response to the stimulus under different experimental conditions provided some measure of the effect of those experimental conditions.

Measurement of [$^{35}SO_4$]-sulphate incorporation rates in unstrained human and bovine chondrocytes exposed to intermittent pressurisation in nitrogen indicated that pressurisation decreases PG synthesis. The decrease in the rate of [$^{35}SO_4$]-sulphate incorporation was significant in cells pressurised for 3 h but the effect was lost when the pressurisation period was increased to 6 h. The decrease in [$^{35}SO_4$]-sulphate incorporation was seen in bovine cells pressurised for 3 h at both 37 °C and RT °C. Human chondrocytes were also exposed to the pressurising gas (Nitrogen) at atmospheric pressure over 3h at 37 °C, in an attempt to measure any effects that may result from hypoxia, but no change was seen in these circumstances. It appears,

therefore, that exposure to pressure for short periods of less than 6 h reduces [$^{35}SO_4$]-sulphate incorporation in a temperature independent manner. Pressurisation with N_2 will result in reduction of the partial pressure of oxygen and $C0_2$ in the medium and a considerable increase in the partial pressure of N_2. Since chondrocyte metabolism is predominantly by anaerobic glycolysis, even at high oxygen levels (38,264), and *in vivo* measurements of the partial pressure of O_2 in canine intervertebral discs have shown readings of 0.3 -5 % (38), hypoxia would not be expected to alter chondrocyte metabolism over the short time periods under investigation in this study. Changes in the pH of the medium that resulted from a reduced partial pressure of CO_2 were effectively buffered with HEPES buffer.

Parkinnen *et al.* obtained a 45 % decrease in [$^{35}SO_4$]-sulphate incorporation in bovine chondrocytes exposed to 5 MPa cyclical pressurisation (0.25 Hz) for 1.5 h (158). Pressurisation under the same conditions for 20 h, however, produced a 25 % increase in [$^{35}SO_4$]-sulphate incorporation. Parkinnen *et al.* (158) concluded that hydrostatic pressure influences different steps in the synthesis of PGs in different ways. Translation of PG core protein takes approximately 120 mins and the post-translational steps, including GAG chain elongation and sulphation, take a further 15 mins (82). Thus pressurisation may act to reduce PG synthesis in the short term by inhibiting translational or post-translational steps, whereas more prolonged pressurisation may increase PG synthesis by increasing gene expression. The results of this study are consistent with this hypothesis and indicate that the inhibitory effect of pressurisation are subsequently overcome by its stimulatory effects. The design of the new apparatus allowed chondrocytes to be pressurised simultaneously with and without strain. This allowed the effects of pressure and strain to be distinguished in a way not possible in the original apparatus.

The studies have demonstrated accelerated PG synthesis in response to cyclical pressure-induced strain under a variety of different conditions in human chondrocytes, immortalised human chondrocyte cell-lines, and bovine chondrocytes.

The increase in PG synthesis was greatest in chondrocyte cell lines but reached significance in both human and bovine chondrocytes. The increase in PG synthesis induced by pressure induced strain was dependent upon the degree of strain applied, but was of similar magnitude to those reported by others in chondrocytes stimulated by cyclical stretch (170,171). Pressure induced strain of 20 μstrain is associated with a membrane deformation of only 0.002 % (239) compared with cell deformations of 10 % and 5.5 % in the studies reported by Lee *et al* (170) and de Witt *et al* (171) who demonstrated increases in PG synthesis in cultured chondrocytes of 110 % and 40 % respectively. The level of deformation used in this study is far less than that which can occur in cartilage *in vivo* where peak contact pressures of approximately 20 MPa (118) can result in an average compression amplitude of over 13 % (121). The use of confocal microscopy to measure cell shape and volume during cartilage compression has demonstrated that 15 % compression of articular cartilage and subchondral bone explants leads to 19 % reduction in cell height and a 16 % reduction in cell volume (124). The present studies demonstrate that cultured chondrocytes are able to respond to levels of strain which are orders of magnitude lower than peak strains induced in cartilage *in vivo*.

The design of the apparatus used in this study meant that monolayer cultures of chondrocytes had to be used if PIS was to be studied. Chondrocytes in monolayer culture are not surrounded by matrix as they would be in cartilage or alginate cultures and in adhesion to the culture plate they adopt a completely different cell morphology. Chondrocytes in monolayer culture are polyhedral with large actin stress fibres (168) whereas chondrocytes in cartilage are spheroidal and the actin is distributed cortically with focal points from which spikes extend inwards (265). Thus chondrocytes in monolayer culture are characterised by differences in shape and cell-matrix interactions compared to chondrocytes in cartilage, which may influence the mechanical responsiveness of the cells (266).

The experimental system used in these experiments has several drawbacks for studies of chondrocyte matrix metabolism. The use of pressure to induce strain in the base of culture dishes is inefficient and induces changes in the partial pressures of dissolved gases in the medium. The decreased partial pressure of oxygen and CO_2 and the elevated partial pressure of N_2 that result from pressurisation may produce changes in chondrocyte metabolism which would prevent the apparatus being used for studies of long term mechanical stress. As previously discussed the apparatus produces very low levels of strain when compared to those seen *in vivo* and in other experimental studies. This makes it difficult to relate the results obtained with physiological regulation in chondrocytes. The apparatus could be adapted to pressurise the cells with a mixture of gases which would achieve partial pressures similar to those seen *in vivo* . This would require a regulator of the type used by divers in which the proportion of oxygen and nitrogen is controlled in the breathing mix and which can be adjusted manually or automatically as the depth increases. Such a system could be complicated (and expensive) and would only increase the strain level in the apparatus if higher pressures were used. Physiological strain levels without the problems of pressurisation can be achieved using a commercially available Flexercell apparatus (Flexcell Corp., McKeesport, PA, USA) which applies suction to the base of culture dishes which have a flexible-membrane. This type of apparatus would lend itself to the type of experiments that have been undertaken.

6. MECHANOTRANSDUCTION MECHANISMS IN ARTICULAR CHONDROCYTES

6.1. Introduction

"For cells to respond to physical stimuli with alterations in their biology and biochemistry, it is essential that the mechanical stimulus that initiates the biological response be converted into the dialect of the cell, a biochemical signal (mechanotransduction)" (267, p2013). Although the importance of mechanical stress in regulating cartilage function and its importance in achieving a greater understanding disease processes, such as OA is well established, knowledge of mechanotransduction mechanisms in chondrocytes is negligible. The extensive study of mechanical stress responses in other cell types (referred to in section 3.6 of this thesis, p39) may provide insights into potential mechanotransduction mechanisms in chondrocytes and point to areas for future study.

6.1.1. Mechanocoupling

The conversion of physical stimuli into a biochemical signal requires a transducer that is physically connected to the stimulus and that can regulate biochemical signalling elements (268). The transmission of the signal to the transducer, or mechanocoupling, can be viewed simply as the requirement for mechanical forces to be transmitted across structural elements that are physically connected (269). Transmission of mechanical signals is achieved in many instances by the linkage of the cell cytoskeleton (CSK) to the extracellular matrix (ECM) through cell surface adhesion molecules, primarily the integrins (267,269,270). Mechanocoupling can either be centralised so that forces are transmitted to transducers at the cell surface, or it can be decentralised so that the CSK 'distributes' the stimulus to transducers located throughout the cell (Fig. 6.1*a.*) (177). Mechanotransducers limited to the cell surface could elicit their response through a signal transduction cascade to the rest of

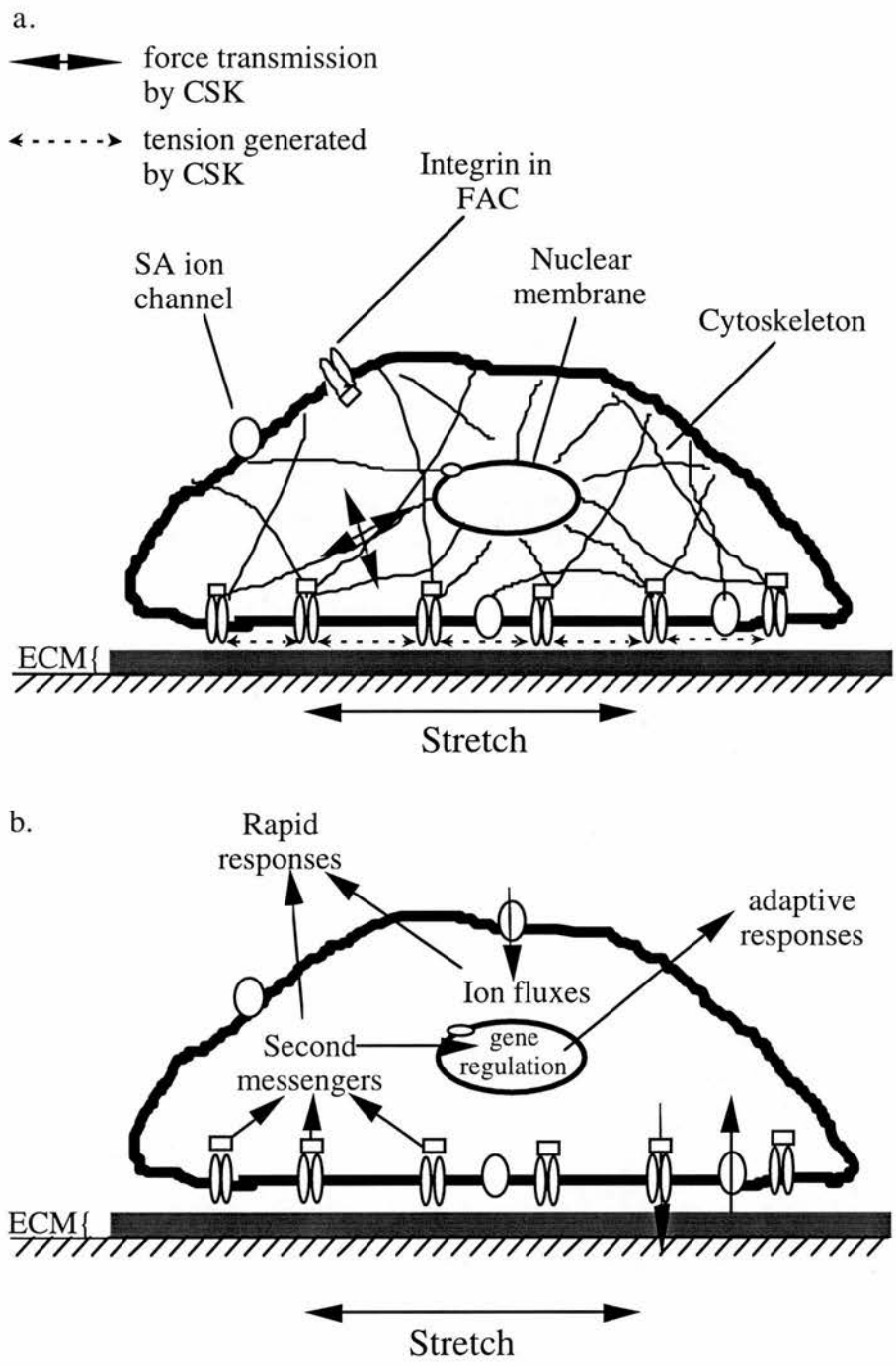


Figure 6.1 (a.) Force transmission (b.) Force transduction. Schematic representation of possible pathways of mechanocoupling and mechanotransduction. ECM - extracellular matrix, FAC - focal adhesion complex. Adapted from Davies (177).

the cell, whereas mechanotransducers distributed throughout the cell may not require such pathways (Fig. 6.1*b.*). The response of a cell to a given stimulus depends on the precise architecture of the signal transmission apparatus and this will determine which transducers will be activated.

The integrin family of adhesion molecules consists of a large number of heterodimers which interact with specific ECM proteins and have highly regulated cytoskeletal linkages (271). ECM proteins can act as ligands for several integrin heterodimers (271). This may allow signals transmitted through the same ECM proteins to elicit different responses at the cell surface as a result of their linkage to different integrins (269). Signal regulation of this type would suggest that the cell could influence its response to the mechanical stimuli to which it is exposed by altering the structure of its mechanocoupling elements. Thus, mechanical stimuli can regulate cell function (outside-in signalling), and the cell can regulate its exposure to mechanical stimuli (inside-out signalling) (269).

Studies of many cell types have shown that cell attachment and spreading is required for cell growth and proliferation (270). These events lead to the formation of actin stress fibres within the cell which are linked via integrins to the attachment substrate. Attachment occurs at specific sites where CSK-integrin-ECM linkages form focal adhesion complexes (FAC, Fig. 6.2) (272). The FAC connects CSK tension elements via linker proteins to the integrins which are bound to the ECM (272). The CSK consists of microtubules which form rigid "struts", actin microfibrils which are the tension elements, and intermediate filaments which act as tensile stiffeners (270). Together these components form a structure which regulates cell shape and movement by maintaining a balance between the tension-generating elements and the rigid elements. This includes the ECM to which the cell is attached via FACs (273). Such a structure determines that when a cell detaches from its substrate it will round-up as FACs and stress fibres disappear and actin redistributes from the stress fibres to the cell surface (225). The organisation of the CSK is vital to

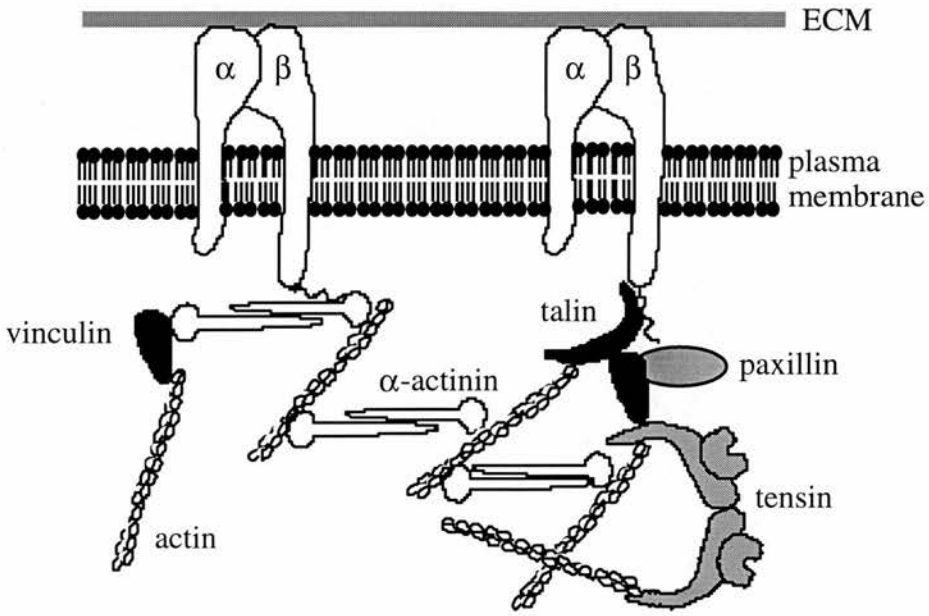


Figure 6.2 The focal adhesion complex. Shematic representation of interaction between cytoskeletal proteins that localise to the FAC and link F-actin to transmembrane integrin receptors which bind to extracellular matrix (ECM) proteins. Adapted from Plopper *et al.* (272).

regulation both of the cell's ability to respond to mechanical stress and of the nature of the response.

Mechanocoupling in Endothelial cells and Smooth Muscle cells

Endothelial cells exposed to shear stress and stretch undergo shape changes and CSK reorganisation such that cells become elongated in the direction of fluid flow or perpendicular to the direction of stretch, and form large central stress fibres (179). Hydrostatic pressure leads to the rearrangement of ECM components and FACs (179,181), and shear stress modulates adhesion molecule expression in ECs (182). Mechanical twisting of EC integrins by the use of RGD peptide coated magnetic beads results in CSK-stiffening, and this can be abolished by disruption of the cytoskeleton with cytochalasin D (274). Thus the organisation of EC adhesion molecules, CSK, and ECM is altered by mechanical stress, as a consequence of outside-in and inside-out mechanical signalling. The mitogenic response of vascular SMCs to stretch has been found to be dependent on the nature of ECM coating proteins and was abolished by RGD-peptide (67) and anti-integrin antibodies, both of which block the interaction between integrins and their ligands (191). These results demonstrate that mechanical force transmission and transduction occurs through integrins in SMCs and provide firm evidence for ECM-integrin-CSK mechanocoupling in the vascular endothelium.

Mechanocoupling in other cell types

Fibroblasts grown on collagen matrices generate tension in the underlying matrix and there are accompanying changes in the organisation of the CSK to form stress-fibres and FACs (225). When the collagen matrix is dislodged from the culture dish, so that the stress generated within the fibroblast is relaxed, the stress-fibres disappear and actin and β1-integrin containing vesicles are formed (225). This demonstrates

the existence of ECM-integrin-CSK structures in fibroblasts which have been maintained in a particular mechanical environment.

In cardiac myocytes, there is no evidence for mechanocoupling via the ECM-integrin-CSK pathway. Stretch-induced IEG expression is not altered by treatment of cells with colchicine, which disrupts microtubules, or cytochalasin D which depolymerises actin microfilaments (205).

Bone cells exposed to cyclical pressure-induced strain, using the same apparatus as that described in this thesis, undergo membrane hyperpolarisation which can be blocked by RGD peptides, anti-α_V and anti-β_1 integrin antibodies, and by disrupting the cytoskeleton with cytochalasin D (275). These responses are similar to those observed in human chondrocytes which hyperpolarise in response to pressure-induced strain, a response which can also be blocked by RGD peptides, anti-α_5 and anti-β_1 integrin antibodies, and by treatment of the chondrocytes with cytochalasin D (276). Thus in both bone cells and chondrocytes mechanocoupling may occur via the ECM-integrin-CSK pathway.

The existence of a mechanocoupling pathway involving the ECM-integrins-CSK linkage is implicated in many cells including chondrocytes. As has been discussed, this pathway regulates the structure of the CSK and its association with the ECM through integrins. Mechanically induced changes in integrins and the CSK may precede the biochemical events in the cell that lead to alterations in metabolism in response to mechanical stress. It is likely that integrins have a role in both mechanocoupling and mechanotransduction (124,171).

6.1.2. Mechanotransduction

The investigation of mechanocoupling pathways has highlighted the importance of integrins in responses to mechanical stress. The integrins are ideally placed, not only to transmit physical stimuli by reorganising the CSK and regulating cell shape, but

also to transduce mechanical stimuli into biochemical signals (267,269,270,272,277,278).

Other candidate mechanotransducers which have been identified in many cell types are the stretch-activated ion channels (279,280).

There must remain the possibility of other, as yet unidentified, types of mechanoreceptor located in the plasma membrane, the cytoplasm or the nuclear membrane which are physically connected by cytoskeletal linkages to the ECM.

Integrins as mechanoreceptors

Integrins within the FAC have been shown to activate signalling pathways which are shared by multiple receptor systems (268,272,277,278). Integrins have short cytoplasmic domains (β-subunit ~60 a.a., α-subunit ~40 a.a.) which usually interact with CSK actin via a number of linker proteins, to assemble the FAC (278,281) (Fig. 6.2) although α_2 integrin cytoplasmic domain peptides have been shown to interact directly with F-actin (282). The recruitment of α-actinin, talin, vinculin, paxillin and β1-integrin to the FAC can be visualised immunohistochemically in the 15 min following ligand binding in fibroblasts (272,283). Soluble ligand does not trigger these events, but immobilised ligand does, indicating that both receptor occupancy and aggregation are required for the formation of a FAC (283) (Fig. 6.3*a.*). Signalling proteins have also been shown to associate with the FAC following ligand binding by integrins. These include tyrosine kinases, serine/threonine kinases, G-proteins, phospholipase C (PLC), phospholipase A (PLA), and also Ca^{2+} channels, and the Na^+/H^+ antiporter (272).

Focal adhesion kinase (FAK) and Src (Rous sarcoma virus oncogene) are the best characterised of the tyrosine kinases that localise at the FAC (272). FAK may be able to bind directly to the β-integrin cytoplasmic domains and may also bind to sites on paxillin and tensin (278). PKC and MAPK are the serine/threonine kinases that are associated with ligand binding. Tyrosine kinase and PKC inhibitors block FAC

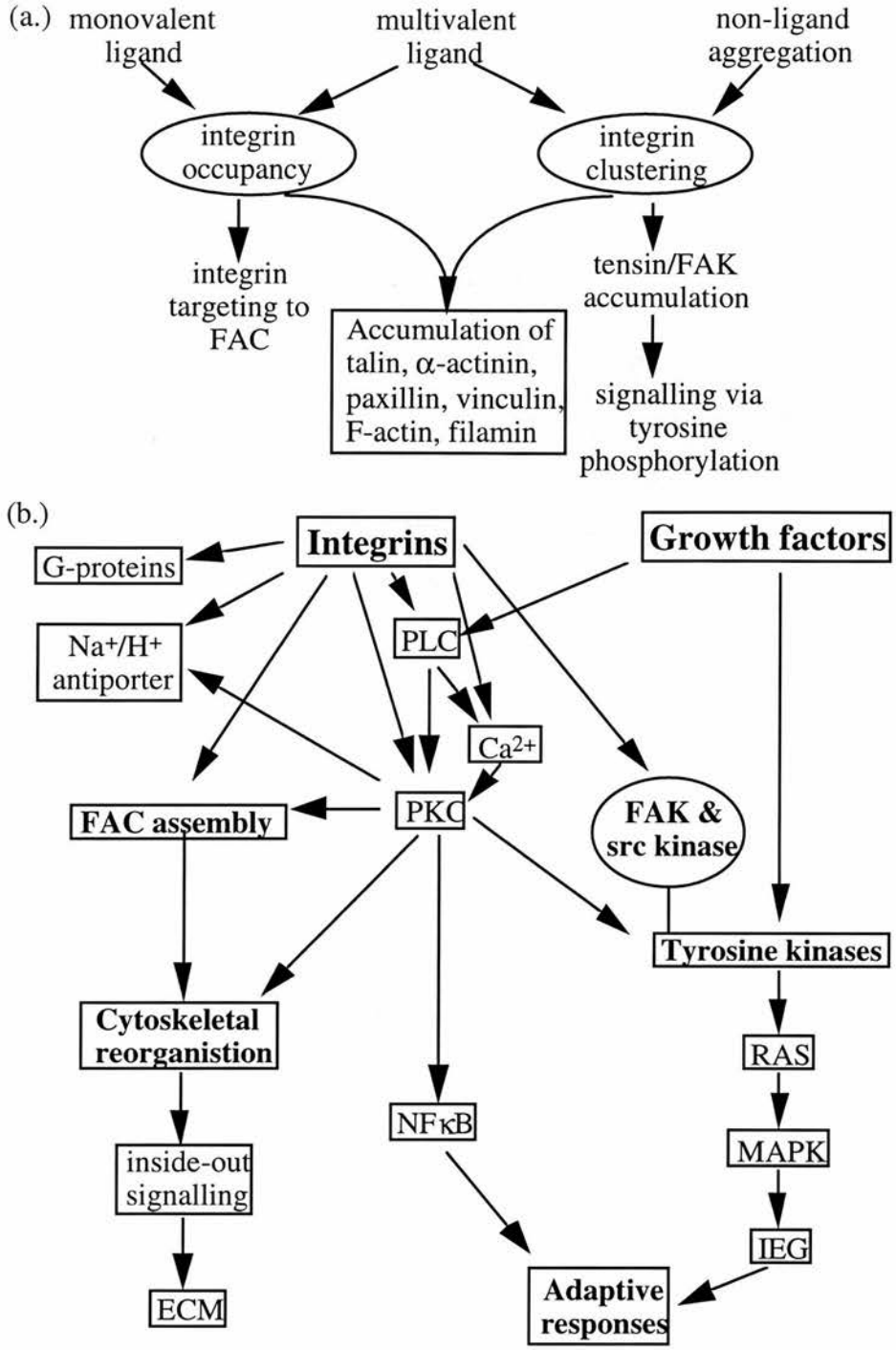


Figure 6.3 Diagramatic representation of (a.) the role of integrin occupancy and aggregation in transmembrane function (b.) signalling mechanisms activated following integrin activation and growth-factor receptor activation.

formation indicating that they may regulate these events (272,278). The binding of Grb2 (growth-factor-receptor-bound protein 2) to FAK acts as an adapter protein to allow SOS (*Drosphila* gene product 'son of sevenless') binding (278). This implicates the involvment of the Ras (Rat sarcoma virus) signalling pathway in response to integrin activation (278). SOS acts as a guanine nucleotide exchange factor (GEF), which converts Ras-GDP to active Ras-GTP; and the resulting phosphorylation cascade activates transcription factors which alter gene expression(284). The signalling elements that have been identified as associating with integrins at the FAC are also common to many growth factor signalling pathways. Structural interaction between growth factor receptors and FACs (272), and glycophosphatidylinositol (GPI)-linked receptors and integrins has also been demonstrated (277). The evidence points, therefore, to the FAC being a site for convergence and integration of many signal inputs. It is proposed that the FAC provide a scaffold which orientates and co-ordinates signalling elements, so that membrane signalling occurs rapidly in the solid state (272).

A number of integrin-mediated signal transduction events have been observed on integrin activation by ligand binding (Fig. 6.3*b.*). FAK tyrosine residue phosphorylation has been observed on binding of fibroblasts to fibronectin, leading to the SRC-mediated binding of Grb2 which could lead to Ras activation (285). In ECs and osteoclasts, integrin activation has been shown to elevate intracellular Ca^{2+} concentration (286,287). Mechanical stimulation of both osteoclasts and osteoblast-like cells has been shown to promote release of Ca^{2+}.from intracellular stores (233,234) and to stimulate integrin-dependent cell proliferation (288). Cyclical stretch applied to osteoblast cultures increases cAMP, inositol 1,4,5-trisphosphate (IP_3) and PKC activity, and causes redistribution of FAC proteins and PKC (230). This signalling response is further enhanced by parathyroid hormone (PTH) providing evidence for the integration of growth factor and mechanical signalling in the FAC in osteoblasts (230). Mechanical stimulation of EC by exposure to fluid-

flow induced shear stress is reported to cause increased paxillin tyrosine residue phosphorylation and PKC-dependent phosphorylation of MAP (mitogen activated protein)-kinases (177) indicating the possibility of Ras pathway activation. The mitogenic response in SMC that follows intermittent strain can be blocked by RGD peptides and anti-β_3 or anti-$\alpha_V\beta_5$ integrin antibodies.These agents also prevent strain-induced expression and secretion of PDGF but do not alter the response to exogenous PDGF (191).

In all the cell types which have been studied, signalling mechanisms such as PKC, PLC, and MAPK, which can be regulated by integrins, have been shown to be activated by mechanical stress but in most cases direct evidence for the role of integrins as the mechanotransducers producing these signals is lacking. The data on mechanocoupling via integrins combined with the activation of signalling pathways provides circumstantial evidence for the involvement of integrins in mechanotransduction.

Stretch-activated ion channels as mechanoreceptors

The application of patch clamp technology has revealed the presence of mechanosensitive (MS) ion channels across the evolutionary tree (279). This family of channels include both stretch-activated (SA) and stretch-inactivated (SI) ion channels (279). Unfortunately, the lack of a high affinity ligand for these channels has hampered the identification of their protein structure and genes encoding these channels. Consequently the gating mechanisms for these channels remain theoretical (279). A protein with SA channel activity has been cloned in *E. Coli* and should yield considerable structural and functional information (289).

It has been proposed that the gating of MS ion channels occurs either via an intrinsic or an extrinsic mechanism or through a combination of both. The intrinsic mechanism proposed suggests that tension in the lipid bilayer acts to produce a conformational change in MS ion channels which leads to the opening of the channel

(289). Pore-opening could occur as a result of reorganisation of subunit packing in a barrel-stave type oligomeric channel. The maintenance of SA channel function in reconstituted liposomes supports the possibility of an intrinsic gating mechanism (290). Extrinsic gating could be achieved by connection to an ECM or CSK component which transmits mechanical forces to the ion channel to produce the conformational changes that lead to channel opening. This model is supported by the results of experiments in cells in which the CSK and ECM have been disrupted. Under these circumstances the MS ion channels fail to open in response to stretch (291,292). There is evidence, therefore, for the existence of two gating mechanisms both of which are dependent on tension within the membrane and not upon pressure gradients across the membrane (289).

SA ion channels have been identified by patch clamp studies in many mammalian cell types including rat cardiac myocytes (205), porcine EC (293), human fibroblasts (222), and osteoblast-like cells (231). These ion channels have been shown to be cation selective (SACat) channels in all cell types. A K^+-specific high conductance channel has been identified in EC (195) and in osteoblast-like osteosarcoma cells (231). Two low conductance SACat channels were also identified in these osteoblast-like cells (231).

The investigations of the role of MS ion channels in cellular physiology has been advanced by the discovery that Gadolinium (Gd^{3+}) can block SA ion channels. The effect was first demonstrated in *Xenopus* oocytes, using the patch clamp technique, where it was shown that 10 μM Gd^{3+} blocked MS ion channel opening (294). SA ion channels can also be blocked by Amiloride and Hexamethylene Amiloride (295), but the specificity of all of these blockers is poor (295-297). They remain however, the only available tool for functional studies. Gd^{3+} has been shown to block increased intracellular Ca^{2+} and cell proliferation that result from mechanical stress in lung cells, indicating the presence of a Ca^{2+}-permeable SA channel in these cells (214,217).

Mechanical stress-induced Ca^{2+} transients have been measured in fibroblasts (199), osteoclasts (234), osteoblasts (233), and endothelial cells (195). In all these cell types, the increase in intracellular calcium resulted from release Ca^{2+} from intracellular stores. Gd^{3+} had no effect on mechanical stress-induced calcium responses that occurred in osteoclasts (234). Gd^{3+} did, however, block the strain-induced increase in whole cell conductance that occurs in osteoblasts following chronic strain (232) implicating ion entry through SA ion channels. The SACat which are present in cardiac myocytes have been shown, using the patch clamp technique, to be blocked by Gd^{3+}, but these channels do not appear to be involved in stretch-induced IEG expression or increased protein synthesis (205). The evidence for the involvement of MS ion channels in cellular responses to mechanical stimuli is at present limited. Nevertheless the evidence for their existence in non-excitable cells together with preliminary data from the cell types reviewed above suggests that evidence may emerge to implicate them as important mechanotransducers.

6.2. Mechanocoupling and mechanotransduction in chondrocytes

Integrins

Articular chondrocytes have been shown, in immunohistochemical studies, to express α_5 and β_1 integrins strongly and α_1, α_2, α_3, α_v, $\alpha_v\beta_1$ and $\alpha_v\beta_3$ integrins less strongly (282,298,299). The fibronectin receptor, $\alpha_5\beta_1$-integrin, is the predominant integrin dimer in cartilage and its ligand is assumed to be fibronectin which is present in the pericellular matrix, although it could bind other matrix components such as collagen and PG (69). Fibronectin forms a fibrillar matrix which facilitates cell adhesion (67,244) by its interaction with other matrix components such as chondroitin sulphate (300) and collagen (301). Cell spreading on fibronectin is mediated by PKC (302) which also modulates the assembly of fibronectin matrices (303). The fibronectin receptor is linked to the cytoskeleton through talin (304) and

receptor clustering following fibronectin binding activates the Na^+/H^+ antiporter (305). Attachment to fibronectin is permissive for Il-1 responses in fibroblasts (306), supporting the hypothesis that there is an integration of adhesive signalling pathways and those activated by soluble factors (272). Fibronectin accumulates in OA cartilage (307) and may be important in the development or progression of the disease (70).

Immunofluorescence microscopy of the chondrocyte cytoskeleton in cartilage slices has shown that actin forms a cortical shell with spikes extending inwards from focal points at the surface of cells (265). This structure could be responsible for the compression of the nucleus that follows cartilage compression and which is abolished following disruption of actin filaments by cytochalasin D (133). The chondrocyte cytoskeleton also contains microtubules radiating from a point near the nucleus, vinculin which colocalises with actin, and vimentin which forms a network around the nucleus and underlying the cell membrane (265). As in ECs, chondrocyte β_1 integrin has been shown to localise in FACs. Chondrocytes also elongate perpendicular to the direction of shear stress in much the same way as ECs (173). There is therefore some limited evidence in chondrocytes for the existence of ECM-integrin and integrin-CSK linkages of the type described in other cell types.

Chondrocyte binding of RGD peptides (which bind to the fibronectin receptor) stimulates stromelysin, collagenase, and gelatinase production and the response is further enhanced by treatment with Il-1 (308). Il-1 and inflammatory synovial fluid have also been shown to modulate chondrocyte integrin expression (243,308). TGF-β alters attachment of chondrocytes to both fibronectin and collagen (309). Cyclical pressurisation of chondrocytes stimulates matrix metalloproteinase-3 (MMP-3) production, a response which is further enhanced by the addition of Il-1 (310). Cyclical stretch stimulates α_5 integrin expression in chondrosarcoma cells adherent upon plastic and α_2 integrin expression when adherent to collagen type II (172). These experiments suggest that chondrocyte activity may be regulated by attachment mediated pathways which transmit signals following stimulation both by

growth factors, cytokines, and mechanical forces. The results of these experiments also indicate that the presence or absence of serum, which contains growth factors and cytokines, will be important in experiments designed to study mechanical stimulation of chondrocytes.

Static pressurisation results in golgi compaction and reduced PG synthesis in chondrocytes, and the response can be blocked by treatment with nocodazole, an agent known to disrupt microtubules (167). The increase in PG synthesis which follows cyclical pressurisation was also blocked by nocodazole (311). Chondrocyte hyperpolarisation which follows exposure to cyclical strain was blocked by anti-α_5 and anti-β_1 integrin antibodies and RGD peptide, and reduced by cytochalasin D (312). These experiments support a mechanocoupling process in chondrocytes which proceeds through an ECM-integrin-CSK pathway. They also point to the $\alpha_5\beta_1$ integrin dimer as a possible mechanotransduction agent.

SA ion channels

Patch-clamp studies have demonstrated the presence of Ca^{2+}-permeable SA ion channels in chondrocytes (313). Calcium transients within chondrocytes have been visualised, using Fura-2, spreading from the site of membrane deformation in bovine chondrocytes. These Ca^{2+}-transients were blocked by treatment with Gd^{3+} but were not affected by disruption of the actin cytoskeleton with cytochalasin D (134).

Chondrocyte membrane hyperpolarisation following cyclical strain was blocked by Gd^{3+}, amiloride, and hexamethylene amiloride (HMA) (239). Thus SACat channels are also implicated as mechanoreceptors in chondrocyte responses to mechanical stress. The failure of cytochalasin D to block the opening of these channels (134) suggests they may be gated by a mechanism not involving the actin component of the cytoskeleton.

6.3. Objectives

The objective of the present study was to investigate the possible role of integrins and SA ion channels in the mechanocoupling/mechanotransduction events that lead to increased PG synthesis during cyclical PIS.

6.4. Methods

Human articular chondrocytes and bovine articular chondrocyts used for these experiments were prepared and cultured in monolayer as described in chapter 4 (p51). The rate of PG synthesis by cultured human and bovine chondrocytes was assayed by [$^{35}SO_4$]-sulphate incorporation using the methods which have been previously described in section 4.4. (p58). PG synthesis rates during cyclical pressurisation were measured under a variety of different experimental conditions, as described below.

6.4.1. Blockade of SA ion channels

A stock aqueous solution of 5 mM Gd^{3+} was prepared by first dissolving Gd_2O_3 in a few drops of concentrated nitric acid, due to low solubility in water. A stock solution of 16 mM HMA in water was stored in the absence of light at 4 °C.

[$^{35}SO_4$]-sulphate incorporation was assayed in the presence and absence of 10 μM Gd^{3+} or 100 μM HMA. These concentrations had been previously demonstrated to be effective in blocking chondrocyte electrophysiological responses to cyclical strain (239). Chondrocytes were then labelled with [$^{35}SO_4$]-sulphate for 6 h at RT °C either in air at atmospheric pressure or during exposure to cyclical PIS (0.33 Hz, 26.7 kPa, 20 μstrain). Following pressurisation cells were washed and treated with GCET as previously described (section 4.4., p58), and [$^{35}SO_4$]-sulphate incorporation rates were determined.

6.4.2. The treatment of bovine chondrocytes with RGD-peptides

The ability of fibronectin to bind to cells may reside in the RGD peptide sequence which has been shown to be involved in integrin binding of fibronectin and at least five other proteins. RGD-peptides are short (4-6 amino acids) synthetic peptides which duplicate the cell attachment activity of fibronectin (67). In these experiments GRGDSP and an inactive control peptide, GRADSP (both obtained from Sigma, Poole, Dorset, UK) were used.

[$^{35}SO_4$]-sulphate incorporation was assayed in the presence and absence of RGD peptide and control peptide. Bovine chondrocytes were utilised in these studies due to the limited availability of human cells. Aliquots of stock solutions containing 1 mg. ml^{-1} GRGDSP or 1 mg. ml^{-1} GRADSP were added to the labelling medium (F12, serum free) to give final concentrations of 10 μg. ml^{-1}. The experiments were performed in serum free medium in order to avoid potential interference from fibronectin which is present in serum in considerable amounts and could interfere with the action of the peptides. Bovine chondrocytes were then exposed to cyclical pressurisation (0.33 Hz, 203 kPa) with and without PIS (16 μstrain) for 3 h at 37 °C. Following pressurisation labelled cells were treated as previously described (section 4.4) and [$^{35}SO_4$]-sulphate incorporation rates were determined.

6.4.3. Treatment of human chondrocytes with anti-integrin antibodies

[$^{35}SO_4$]-sulphate incorporation was measured in human chondrocytes in the presence and absence of monoclonal antibodies raised against human integrin subunits. The antibodies used were TS2/16 (anti-β_1 integrin; obtained from Dr. D.M. Salter, Department of Pathology, University of Edinburgh), P_4C_{10} (anti-β_1 integrin; Gibco, Paisley, Scotland), P1D6 (anti-α_5 integrin; Chemicon International, Harrow, England), LM609 (anti-$\alpha_v\beta_3$ integrin; Chemicon International, Harrow, England).

P1D6 and P_4C_{10} were supplied as ascites fluid and were used at a final dilution of 1:1000. TS2/16 and LM609 were supplied as purified 1 mg. ml^{-1} solutions which were used at a final concentration of 1 μg. ml^{-1}. The antibodies were added to the labelling medium immediately prior to pressurisation. Both TS2/16 and P_4C_{10} were initially tested in the presence of serum. These two antibodies, and P1D6 and LM609 were then tested in serum-free conditions. Chondrocytes were labelled for 3 h at 37 °C either in air or during exposure to cyclical pressurisation (0.33 Hz, 203 kPa) with and without PIS (16 μstrain). Following each procedure labelled cells were treated as previously described (section 4.4., p58) and [$^{35}SO_4$]-sulphate incorporation rates were determined.

6.5. Results

The rate of PG synthesis measured in chondrocytes covered a large range, as can be seen from the 95 % confidence intervals of mean values. Results are therefore also shown as the mean % change in PG synthesis relative to controls as this was felt to be a better indicator of response.

In studies on the effect of loading on chondrocyte PG synthesis data is commonly presented as the ratio of loaded synthesis rates compared with unloaded synthesis rates. Absolute values of $^{35}SO_4$-incorporation are expressed as $mmol^{-1}$. $(10^6 \text{ cells})^{-1}$. h^{-1} or $mmol^{-1}$. (mg dry weight)$^{-1}$. h^{-1} for explant studies. In the studies described here it was not possible to express results in this way as the low concentration of sulphate in Ham's F12 makes it very difficult to translate cpm $^{35}SO_4$ incorporated into mmol. It is difficult, therefore, to compare the incorporation rates obtained in these experiments with other studies.

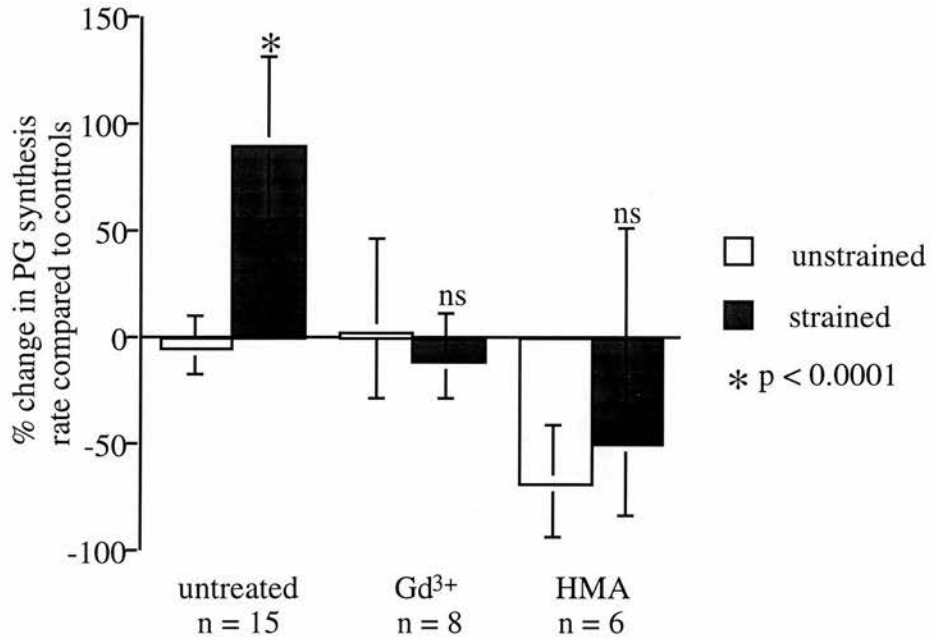


Figure 6.4 The effect of SA ion channel blockers on the response to cyclical PIS. The figure represents the % change in the rate of PG synthesis of cells were exposed to cyclical PIS (20 μstrain, 6 h, RT °C) compared to unpressurised controls. Cells were also loaded in the presence of SA ion channels blockers Gd3+ (10 μM) and HMA (100 μM). Error bars are 95 % confidence limits.

6.5.1. The effect of SA ion channel blockers on PG synthesis and the response to cyclical PIS

[$^{35}SO_4$]-sulphate incorporation increased from a mean value of 153 cpm. h^{-1}. $\mu gDNA^{-1}$ (87-268 [95% confidence interval]) to 289 cpm. h^{-1}. $\mu gDNA^{-1}$ (18- 451) in human chondrocytes exposed to 6 h cyclical PIS (20 μstrain) at room temperature (Fig 6.4). [$^{35}SO_4$]-sulphate incorporation in unstrained chondrocytes in the presence of 10 μM Gd^{3+} (303 cpm. h^{-1}. $\mu gDNA^{-1}$, 167 - 530) was not significantly different from paired controls (273 cpm. h^{-1}. $\mu gDNA^{-1}$, 181 -413). Exposure of chondrocytes to 10 μM Gd^{3+} during 6 h cyclical PIS (20 μstrain) completely abolished the increase in [$^{35}SO_4$]-sulphate incorporation induced by PIS. The addition of 100 μM HMA to control chondrocytes in culture reduced [$^{35}SO_4$]-sulphate incorporation from a mean rate of 153 cpm. h^{-1}. $\mu gDNA^{-1}$ (87 - 268) to 22 cpm. h^{-1}. $\mu gDNA^{-1}$ (9 - 49). During the application of cyclical PIS (20 μstrain, 6 h) in the presence of 100 μM HMA there was a non-significant rise in [$^{35}SO_4$]-sulphate incorporation to 35 cpm. h^{-1}. $\mu gDNA^{-1}$ (10 - 125). Cell viability, as assessed by the exclusion of trypan blue, was not affected by exposure to 100 μM HMA for 6 h (>99 %).

6.5.2. The effect of RGD-peptides on PG synthesis in bovine chondrocytes and the response to cyclical PIS

[$^{35}SO_4$]-sulphate incorporation rates increased from a mean unstrained value of 643 cpm. h^{-1}. $\mu gDNA^{-1}$ (410 - 1007) to 974 cpm. h^{-1}. $\mu gDNA^{-1}$ (637 - 1490) in bovine chondrocytes following exposure to 3 h cyclical PIS (16 μstrain) at 37 °C (Fig. 6.5, n = 13, not significant). [$^{35}SO_4$]-sulphate incorporation rates in unstrained chondrocytes were not significantly altered by exposure to 10 μg. ml^{-1} GRADSP or 10 μg. ml^{-1} GRGDSP. [$^{35}SO_4$]-sulphate incorporation in the presence of GRADSP increased from a mean unstrained value of 653 cpm. h^{-1}. $\mu gDNA^{-1}$ (412 - 1036) in unstrained cells to 1017 cpm. h^{-1}. $\mu gDNA^{-1}$ (695- 1488) in strained cells (n = 10, ns). In the presence of GRGDSP the rate of PG synthesis increased from

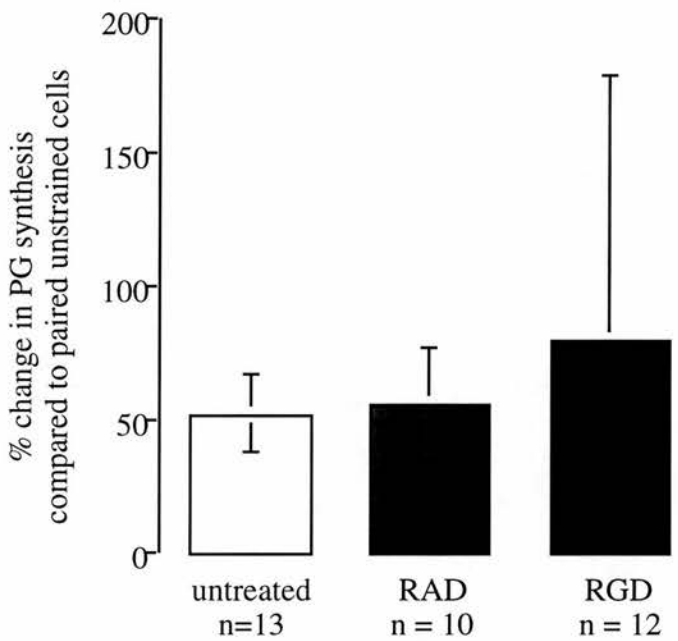


Figure 6.5 The effect of cyclical PIS on PG synthesis in the presence of RGD-peptide. The figure shows the % change in PG synthesis compared to paired unstrained cells. Cells were exposed to cyclical pressurisation (203 kPa, 0.33 Hz, 3 h 37 °C) with and without strain (16 μstrain), in the presence of control peptide (GRADSP), RGD-peptide (GRGDSP), and the absence of peptide. Error bars are 95 % confidence limits.

534 cpm. h^{-1}. $\mu gDNA^{-1}$ (246 - 1158) to 959 cpm. h^{-1}. $\mu gDNA^{-1}$ (590 - 1561) during cyclical PIS (n = 12, ns). Thus, exposure of bovine chondrocytes to the control peptide and RGD-peptide did not significantly alter the response to cyclical PIS.

PG synthesis rates in untreated cells increased by 52 % (38 - 67 %, n = 13) during cyclical strain compared to paired unstrained values (Fig. 6.5). Following the addition of control peptide PG synthesis rates increased by 56 % (36 - 77 %, n = 10) during strain, whereas in the presence of RGD-peptide rates in strained cells increased by 80 % (16 - 179 %, n =12). None of these results differed significantly from each other .

6.5.3. The effect of anti-integrin antibodies on PG synthesis and the response to cyclical PIS

Experiments in the presence of serum

[$^{35}SO_4$]-sulphate incorporation increased from a mean value of 81 cpm. h^{-1}. $\mu gDNA^{-1}$ (34 - 191 [95 % confidence interval) in unstrained human chondrocytes to 102 cpm. h^{-1}. $\mu gDNA^{-1}$ (43 - 237) in chondrocytes exposed to cyclical PIS for 3 h at 37 °C (n = 10, not significant). In the presence of the anti-β_1-integrin antibody P_4C_{10} PG synthesis rose from 70 cpm. h^{-1}. $\mu gDNA^{-1}$ (33 -150) in unstrained cells to 103 cpm. h^{-1}. $\mu gDNA^{-1}$ (51 - 204) in strained cells (n = 9, ns). In the presence of TS2/16 PG synthesis increased from 84 cpm. h^{-1}. $\mu gDNA^{-1}$ (40 - 174) in unstrained cells to 141 cpm. h^{-1}. $\mu gDNA^{-1}$ (67 - 301) in strained cells (n = 10, ns).

PG synthesis rates in chondrocytes stimulated with PIS, but not exposed to antibody, increased by 26 % (11 - 43 %) compared to paired unstrained cells (Fig. 6.6). In the presence of the anti-β_1-integrin antibodies P_4C_{10} and TS2/16 PG synthesis rates increased by 47 % (11-95 %) and 69 % (22-132 %). respectively following cyclical PIS. None of these results differed significantly from each other.

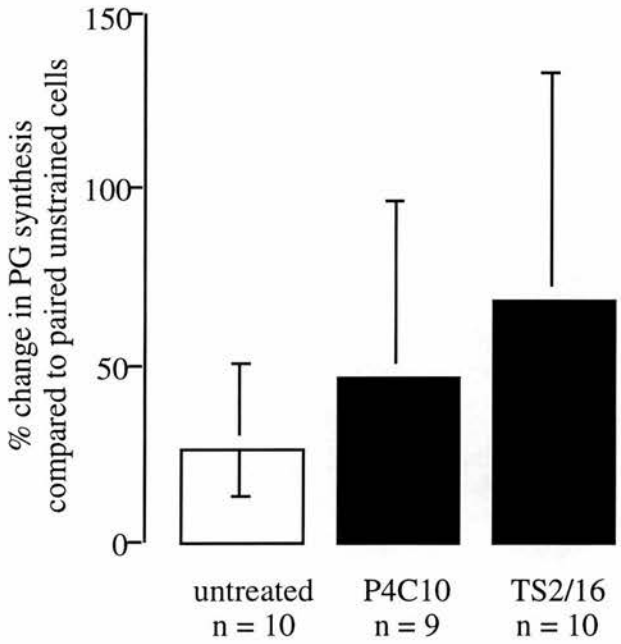


Figure 6.6 The effect of anti-β1 integrin antibodies on the response to cylical PIS in the presence of serum. The figure shows the % change in PG synthesis during pressurisation compared to paired unstrained cells. Cells were exposed to cyclical pressurisation (203 kPa, 0.33 Hz, 3 h, 37 °C) with and without strain (16 μstrain), in the presence and absence of anti-integrin antibodies. Error bars are 95 % confidence limits.

Experiments in the absence of serum

The effect of anti-integrin antibodies on mean PG synthesis rates in response to cyclical PIS is shown in table 6.1.

[$^{35}SO_4$]-sulphate incorporation rates in chondrocytes stimulated with PIS in the absence of serum increased by 21 % (7 - 37 % [95 % confidence interval]) when compared to paired unstretched cells (Fig. 6.7). In the presence of anti-β_1-integrin antibody P_4C_{10} the rate of PG synthesis was reduced by 1 % (-23 - +28 %) and in the presence of anti-β_1-integrin antibody TS2/16 the rate increased by 30 % (14 - 49 %) following cyclical PIS (16 μstrain, 3 h, 37 °C). The anti-α_5-integrin antibody P1D6 reduced the acceleration in the rate of PG synthesis following cyclical PIS to 12 % (-23 - +65 %). In the presence of the anti-$\alpha_V\beta_3$-integrin antibody LM609 PG synthesis rates decreased by 31 % (n = 2) in response to cyclical PIS. None of these results differed significantly from each other.

Table 6.1 The effect of anti-integrin antibodies on PG synthesis rates in response to cyclical PIS in serum-free conditions

	mean [$^{35}SO_4$]-sulphate incorporation cpm. h^{-1}. $\mu gDNA^{-1}$ (95 % confidence interval)				
	untreated (n = 8)	**P_4C_{10}** anti-β_1 (n = 6)	**TS2/16** anti-β_1 (n = 6)	**P1D6** anti-α_5 (n = 4)	**LM609** anti-$\alpha_v\beta_3$ (n = 2)
control	125 (101-156)			87 (49 - 129)	268
unstrained	128 (76 -215)	178 (110 - 288)	175 (113 - 272)	96 (65 - 144)	132
strained	155 (90 - 267)	176 (110 - 282)	229 (131 - 400)	108 (56 - 209)	90

The table shows mean PG synthesis rates calculated from $\log_{10}$-transformed data.

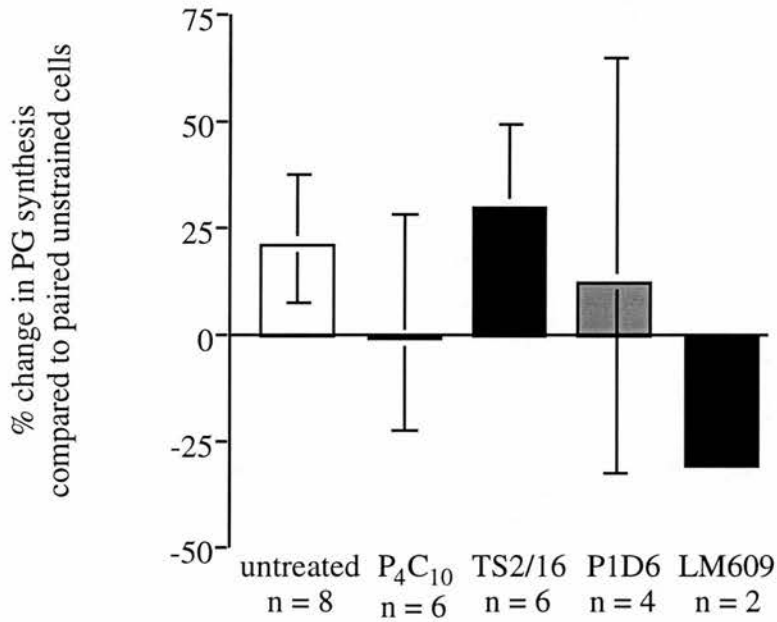


Figure 6.7 The effect of anti-β1 integrin antibodies on the response to cyclical PIS in serum-free conditions. The figure shows the % change in PG synthesis during pressurisation compared to paired unstrained cells. Cells were exposed to cyclical pressurisation (203 kPa, 0.33 Hz, 3 h, 37 °C) with and without strain (16 μstrain), in the presence and absence of anti-integrin antibodies. Error bars are 95 % confidence intervals.

6.6. Discussion

The results presented in this study indicate that SA ion channels are involved in the events that lead to increased PG synthesis following cyclical PIS. Calcium ion influx through SA ion channels in response to chondrocyte membrane deformation has been previously demonstrated (313). Increases in intracellular calcium from intracellular stores and/or the extracellular fluid are associated with the chondrocyte hyperpolarisation that follows cyclical PIS (239). The influx of calcium through SA-ion channels may be one of the primary events that occurs in response to PIS leading to increased PG synthesis. This possibility could be further investigated by measuring PG synthesis after exposing chondrocytes to cylical PIS in Ca^{2+}-free conditions or following incubation with membrane permeable Ca^{2+}-chelators.

Mechanical deformation of many cell types results in increases in intracellular [Ca^{2+}] (199,233,234,314-319). These responses have been shown to involve the opening of SA ion channels in ECs, SMCs, and osteoclasts (314,315,318,319). Ca^{2+} can be released from intracellular stores by the action of IP_3 which is produced by the action of PLC (320). The hyperpolarisation response of chondrocytes to cyclical PIS has been shown to involve both PLC and intracellular calcium stores (239). The hyperpolarisation response of chondrocytes to cyclical PIS was also inhibited by the calmodulin inhibitor, W7, the tyrosine kinase inhibitor, genistein, and the protein kinase inhibitors calphostin-C, H7 and staurosporine (M 240). Ca^{2+}-activated K^+-channels and L-type Ca^{2+} channels are involved in the hyperpolarisation of chondrocytes (239). This suggests that increases in intracellular Ca^{2+} may be important in the regulation of chondrocyte activity following mechanical stimulation. PG and collagen synthesis have been shown to be inhibited by thapsigargin, which acts to mobilise Ca^{2+} from intracellular IP_3-releasable stores. The effect was reported to be due to store deletion and not the alteration in intracellular Ca^{2+} concentration (321,322).

Increases in intracellular Ca^{2+} concentration regulate the function of adenylyl cyclase (AC) which is responsible for the production of cAMP from ATP (323). Several families of adenylyl cyclase exist, some of which are stimulated by Ca^{2+} and some of which are inhibited by Ca^{2+} (323). Immediate early gene (c-fos) expression can be increased by Ca^{2+} influx which stimulates phosphorylation of the transcription factor, cAMP response element-binding protein (CREB) by Ca^{2+} - activated kinase (324). However, transcriptional activation also requires the activity of cAMP-dependent protein kinase (PKA) (324). The type of adenylyl cyclase present in chondrocytes is not known but cAMP has previously been shown to be increased in response to cyclical stretch (169,171) and cyclical hydrostatic pressurisation (166) of chondrocytes. In contrast, continuous hydrostatic pressurisation of chick epiphyseal cartilage cells leads to inhibition of cAMP accumulation by a mechanism that involves increased intracellular Ca^{2+}, indicating that Ca^{2+}-inhibited ACs may be present in chondrocytes (325). Ca^{2+}-responses in articular chondrocytes have been shown to be potentiated by cAMP levels indicating the existence of a positive feedback mechanism between Ca^{2+} and cAMP (326). Thus, the increase of intracellular calcium in chondrocytes which results from the opening of SA ion channels could be responsible for increased PG synthesis by a mechanism involving cAMP. Investigation of the possible role for cAMP in the increase in PG synthesis is described in Chapter 7 (p135) of this thesis.

The role of integrin-ECM linkage was investigated by using RGD peptides which block the interaction between $\alpha 5\beta 1$ integrin and fibronectin, as well as many other ECM ligands (67). The control peptide (GRADSP) and the RGD-peptide (GRGDSP) had no significant effect on the PG synthesis rates or the response to cyclical PIS. These preliminary results suggest that integrin mediated adhesion to ECM proteins is not involved in the signal transduction pathway that leads to accelerated PG synthesis following cyclical PIS. In these experiments only one dose of peptide, selected on the basis of experiments investigating the

electrophysiological response of chondrocytes to strain, was tested. In order to be certain that RGD-peptide has no effect on PG synthesis rates a dose response curve should be performed. The chondrocytes should also be pre-incubated with RGD peptide prior to pressurisation to ensure sufficient time for binding to integrins.

The response of human chondrocytes to cyclical PIS was not modulated by anti-integrin antibodies to a statistically significant degree. The short loading time used makes the response to PIS small and means that the effects of modulating antibodies are correspondingly small. In serum-free conditions the increase in PG synthesis rates during PIS was reduced and not statistically significant. Despite these sub-optimal experimental conditions the data shows that adhesion-enhancing anti-β_1 antibody TS2/16 increased the response to PIS both in the presence and absence of serum. The adhesion inhibiting anti-β_1 antibody P_4C_{10} produced a non-significant increase in the response to PIS in the presence of serum but completely inhibited the response in the absence of serum. Taken together, these results suggest that β_1-integrins are involved in the mechanocoupling or mechanotransduction mechanisms that lead to increased PG synthesis in response to cyclical PIS. P_4C_{10} and TS2/16 have both been shown to bind to a specific 'hinge' region adjacent to the ligand binding site of the β_1-integrin and it is proposed that they act by stabilising conformations of the 'hinge' which either activate or deactivate the integrin (327). The ligand binding function of $\alpha_5\beta_1$ -integrin has been shown to be regulated by the β-subunit (328) so that activation by TS2/16 could lead to both increased ligand binding and mechanical induced signalling. The results with anti-α_5 antibody P1D6 and the anti $\alpha_V\beta_3$-antibody LM609 are incomplete. P1D6 is an adhesion blocking antibody. A reduction in PG synthesis in response to strain in the presence of P1D6 would be compatible with the hypothesised role for $\alpha_5\beta_1$ integrin (the fibronectin receptor and predominant integrin dimer in cartilage(282,329)) in the mechanotransduction mechanism. Chondrocytes express the $\alpha_V\beta_3$ integrin dimer much less strongly than the $\alpha_5\beta_1$ integrin dimer (298). The effect of LM609 on PG

synthesis rates in response to strain is potentially interesting but more experiments are needed to confirm the result. In general more experiments with the anti-integrin antibodies described should be performed in order to confirm or negate the results which have been obtained, for at present these results are not statistically significant. The chondrocytes should, in future, be pre-incubated with the antibodies to ensure that their effect on integrin function is present from the outset of pressurisation regime.

Integrins have been shown to mediate increases in intracellular Ca^{2+} following antibody binding (286), RGD peptide binding (287), and mechanical stress (330). It was shown that α_V integrins and not $\alpha5\beta1$ integrins mediate the rise in Ca^{2+} during the spreading of endothelial cells, even though substrate binding was predominantly mediated by $\alpha5\beta1$ integrins (286). This demonstrates that adhesion and signalling processes may be mediated by different integrins and that calcium signalling by integrins is mediated by the α subunit.

The results obtained in the present study demonstrate that mechanotransduction leading to increased PG synthesis in chondrocytes is dependent on SA ion channels. These channels may mediate increases in PG synthesis via increased intracellular Ca^{2+}.

The precise role of integrins in the response to cyclical PIS leading to increased PG sythesis remains to be elucidated, but they do appear to be implicated in the mechanotransduction pathway.

7. THE EFFECT OF PRESSURE-INDUCED STRAIN ON CHONDROCYTE CYCLIC-NUCLEOTIDE LEVELS

7.1 Introduction

Cyclic adenosine monophosphate is a ubiquitous signalling molecule which introduced the concept of signalling by second messengers (331). It was originally discovered as a second messenger produced in response to receptor activation by adrenaline, but it has been found to be involved in the action of many other hormones (331,332). It regulates many cellular cascades through the activation of cAMP-dependent protein kinase (PKA) (332-334). The integral-membrane adenylyl cyclases convert ATP to cAMP (323). These are large polypeptides which have 12 transmembrane spanning domains arranged in a 'barrel-stave' structure similar to that of ion channels or transporter molecules (323). The cytoplasmic domains of the enzyme include the site of ATP binding and sites for interaction with guanine nucleotide-binding proteins (G-proteins) (323). There are six families of adenylyl cyclases which are regulated by a number of mechanisms (Fig. 7.1) including G-protein α-subunit, G-protein $\beta\gamma$-subunit, Ca^{2+}, and PKC (323). These regulators can be both stimulatory and inhibitory but the precise nature of their interactions is unknown (323). Cyclic AMP is rapidly broken down by the action of cytosolic phosphodiesterases (PD) (331).

The production of cAMP leads to the activation of PKA by binding to its regulatory subunit (334,335). This leads to the phosphorylation of specific target proteins, which include the cAMP response element-binding protein (CREB) (336) and the cAMP response element modulator protein (CREM) (337). These transcription factors bind to the cAMP response element within gene promoters (338,339) including the immediate early gene c-fos (324,336,337). CREB acts as an

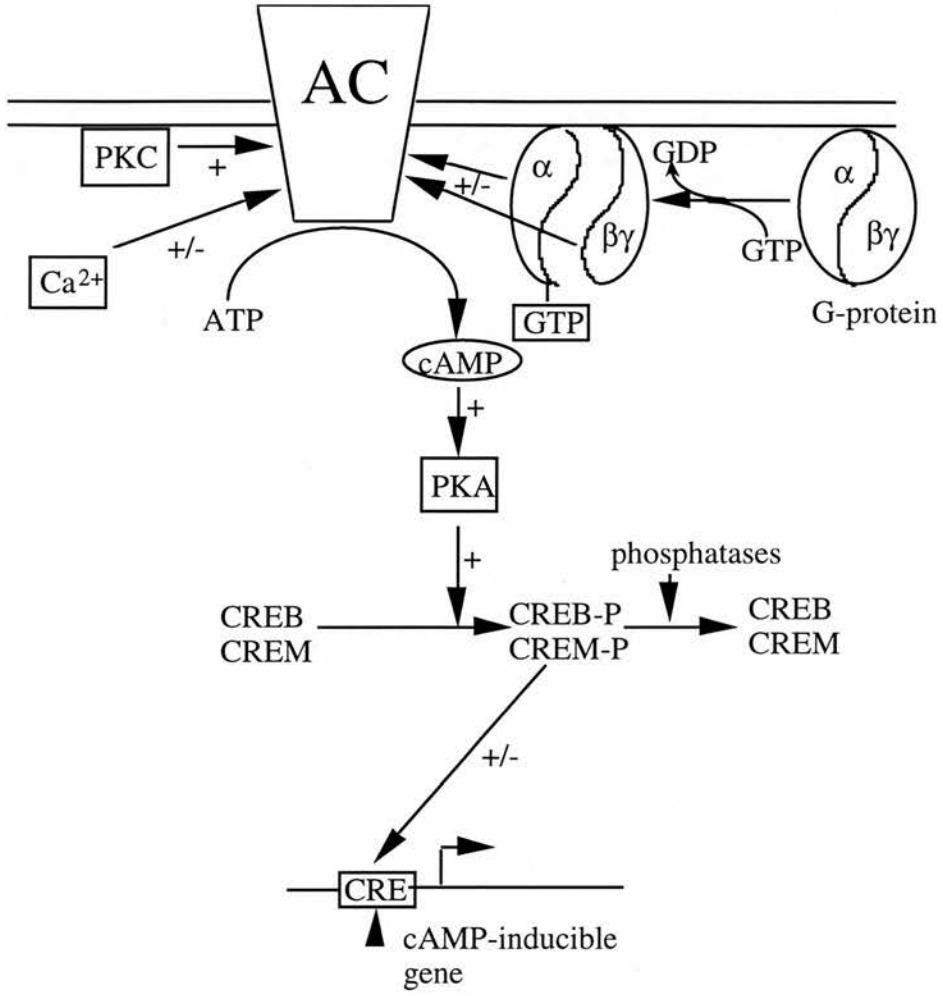


Figure 7.1 The regulation of Adenylyl cyclase and gene regulation by cAMP. A schematic representaion of the regulation of adenylyl cyclase (AC) production of cAMP and gene regulation by cAMP via PKA. Abbreviations:

ATP - adenosine triphosphate
GTP- Guanosine triphosphate
GDP - Guanosine diphosphate
PKC - protein kinase C
PKA - protein kinase A, cAMP-dependent protein kinase
CRE - cAMP response element
CREB - CRE binding protein
CREM - CRE modulator

transcriptional agonist (340) whereas CREM acts as a transcriptional antagonist (336,337).

The role of cAMP in responses to mechanical stress have been studied in many non-cartilaginous cell types. Fluid shear-stress of osteoblasts (341) and cyclical stretch of osteoblasts in the presence of parathyroid hormone (PTH) (230), for periods of up to 15 min, results in increased intracellular cAMP, and also increased IP_3 and PKC activity (230).The increase in intracellular cAMP in response to shear stress was mediated by the release and autocrine action of prostaglandins (341). Physiological levels of cyclical stretch in the presence of PTH, increased cAMP levels in young osteocytes but not in osteoblasts (228). In bone cells, an increase in cAMP following mechanical stress may be the result of the action of autocrine factors rapidly released following the application of stress.

Ten minutes after stress relaxation of fibroblasts there was a 10 - 20 fold increase in levels of cAMP which was dependent on Ca^{2+} influx into the cell and the release of arachidonic acid (224). This response was mimicked by forskolin, an activator of adenylyl cyclase, which produced a 3 fold activation of PKA (224). In fibroblasts Il-1 and tumour necrosis factor (TNF) also induced a rapid increase in intrecellular cAMP levels which resulted in the PKA-mediated expression of Il-6 mRNA (342). Il-1 induces a similar increase in intracellular cAMP in lymphocytes (343).

Cyclical stretch of ECs for 5 min induced a 2 fold increase in cAMP which was reduced by further stretch (196). A 70 % increase in intracellular cAMP levels and the release of PGI_2 was seen following 15 min of cyclical stretch of lung cells (215). In lymphoma cells hyposmotic swelling induced an increase in cAMP which was partially reduced in a Ca^{2+}-free medium (344).

Mechanical stress may cause elevation of cAMP by its action on intracellular Ca^{2+}, but in some cell types the release of autocrine factors is responsible for the response. The possibility of integration of mechanically induced signalling

mechanisms with hormonal, cytokine or growth factor signalling pathways by cAMP is an attractive one, but there is no evidence for such a mechanism as yet.

Cyclic guanosine monophosphate (cGMP) was discovered shortly after the discovery of cAMP (331) but, so far, only a few cell types have been identified, where it functions as a second messenger (345). It is produced by two families of enzymes; the particulate guanylyl cyclases (GC) (346) and the soluble guanylyl cyclases (347). The particulate guanylyl cyclase receptors , such as GCA (which is the receptor for atrial natriuretic peptide), are a group of transmembrane receptors with GC present in their intracellular domain (346). The soluble GCs are heterodimers about which little is known, but which are activated by NO (347). Cyclic GMP activates the cGMP protein kinases (G kinases) (345), which can regulate the gating of some ion channels, most notably in the retinal rods (348), and also regulate PD function (345). This latter function is the mechanism for possible interaction with cAMP mediated responses.

There is no published evidence of cGMP being produced in response to mechanical stress. Nitric oxide, however, has shown to be produced in response to shear stress in endothelial cells (194), bone cells (202), and articular chondrocytes (349) and it is thus possible that it could mediate an increase in cGMP within these cells. Nitric oxide mediates the inhibition of PG synthesis in chondrocytes following exposure to Il-1 and TNF (319,350). Thus, NO mediated activation of soluble GC is a potential mechanism for the regulation of PG synthesis.

7.2 The role of cyclic nucleotides in the regulation of chondrocyte metabolism

Activation of chondrocyte adenylyl cyclases with forskolin and treatment of chondrocytes with cAMP analogues for 24 h has been shown to increase PG synthesis (171,351). This increase was further enhanced by the blocking of PD activity with isobutylmethylxanthine (IBMX) (351). The synthesis of KS has also

been shown to involve the long term elevation of cAMP (352). Thus, the activation of chondrocyte PG synthesis may involve cAMP.

Calcium ionophores such as A23187 cause release of PGE_2 from chondrocytes and a simultaneous rise in cAMP (353). Calcium influx and mobilisation of calcium from intracellular stores of chondrocytes is potentiated by cAMP (326). There may be a close relationship between Ca^{2+} regulation and cAMP production. In proliferative epiphyseal cartilage cells a reduction in cAMP accumulation following exposure to continuous hydrostatic pressure is mediated via increased calcium uptake (325). Chick epiphyseal cartilage proliferative cells have been exposed to 15 min cyclical hydrostatic pressure (166) and 24 h cyclical stretch (171); both stimuli produced an increase in cAMP and increased PG synthesis.In rat cartilage growth cells stimulated by continuous stretch there was a peak in cAMP levels after 3 min which returned to baseline within 30 min (169).

Mechanical stress of chondrocytes under different experimental conditions increases cAMP and this may be involved in the increase in PG synthesis which also occurs. In all of these studies however, either proliferative or growth cartilage cells were used, so that extrapolation of these results to adult chondrocytes may not be justified.

In the present study, we have shown increased PG synthesis during cyclical strain and reduced synthesis during cyclical pressurisation. The purpose of experiments to be described in this chapter was to investigate the effect of these stimuli on the levels of cAMP and cGMP within human articular chondrocytes.

7.3. Methods

Human articular chondrocytes in monolayer culture were prepared as described in Chapter 4 (p.51) of this thesis. The cells were cultured in petri dishes for up to 2 weeks prior to use.

7.3.1. Radioimmunoassay of cyclic AMP and cyclic GMP

The intracellular content and culture medium concentration of cyclic AMP (cAMP) and cyclic GMP (cGMP) were measured using an in house radioimmunoassay (RIA) adapted from that of Brooker *et al* (354). The assay is a competitive radioimmunoassay in which specific anti-cyclic nucleotide antibodies are incubated with a mixture of a known amount of ^{125}I-cyclic nucleotide ligand and the unknown nonradioactive cyclic nucleotide. There is competition between radioactive and nonradioactive ligand for the limited antibody sites so that the amount of radioactivity bound to the antibody varies inversely with the concentration of the competing ligand (354).

The assay was performed using anti-cyclic nucleotide antibodies prepared according to the method of Brooker and co-workers (354) and generously donated by Dr. I. Gow (Dept. of Physiology, University of Edinburgh).

7.3.2. Preparation of Radioligands

Due to the relatively short half life of [^{125} I]-iodine (58.6 days), fresh radio-labelled cyclic nucleotides were prepared at 2 to 3 monthly intervals. The label was prepared from monosuccinyl adenosine/guanosine 3':5'-cyclic monophosphoric acid tyrosine methyl ester and high-concentration reductant-free Na^{125}I (Amersham Life Science, Amersham, Bucks, UK) taking particular care to observe the safety measures as described below. All procedures involving ^{125}I-labelled reagents were performed strictly in accordance with Local Radiation Rules (Western General Hospital, Edinburgh) after receiving approval from the Radiation Protection Advisor. The procedure for the preparation of both labels was identical so ^{125}I-cAMP and ^{125}I-cGMP were prepared simultaneously in different reaction tubes.

Stock solutions of 60 μM succinyl cyclic nucleotide esters were prepared in 0.5 M phosphate buffer, pH 7.5, and divided into 25 μl aliquots for storage at -20 °C. Solutions of chloramine T (1 mg. ml^{-1}) and sodium metabisulphite (12 mg. ml^{-1}) both

in 0.5 M phosphate buffer, pH7.5, were prepared in separate tubes immediately prior to use.

Whatman 31ET (Whatman International Ltd, Maidstone, Kent) paper used for chromatographic separation of product from reactants was soaked over night in a solution of butan-1-ol (AnalR grade; Merck Ltd): glacial acetic acid (AnalR grade ; Merck Ltd): water (12:3:5) and was then dried before use.

The iodination of the succinyl cyclic nucleotide ester was performed by mixing 5 µl of $Na^{125}I$ with 10 µl of 0.5 µ M phosphate buffer, pH 7.5, followed by 20 µl of stock succinyl cyclic nucleotide ester, in a microfuge tube. The reaction was started by adding 10 µl of chloramine T, mixed by pipetting only, and left for 1 min at which time the reaction was stopped by the addition of 50 µl of sodium metabisulphite solution. The reaction mixture was then applied in a narrow band to the pre-prepared strip (40 cm by 2 cm) of Whatman 31 ET chromatography paper and separated by descending chromatography using a butan-1-ol: glacial acetic acid: water (12:3:5) mixture in a saturated atmosphere in a large tank. When the solvent front was near the bottom of the strip (usually 3-4 h after the chromatographic sparation was commenced) the strip was dried using warm air from a hair dryer and then wrapped in cling film. The strip was used to expose X-ray film for 5 mins, in the X-Ray department of the Western General Hospital (Edinburgh). The exposed film was developed using the automated developing equipment available in the X-ray department. On examination of the developed radiograph three peaks were normally seen for both cAMP and cGMP, the middle peak being the iodinated product (R_f cAMP about 0.6 - 0.7, cGMP about 0.5 - 0.6). The first peak corresponded to the di-iodinated product and the last peak corresponded to free I^{125}. The portion of the strip corresponding to the middle peak was cut from the strip and eluted with 2 ml of a solution of propan-1-ol (AnalR grade; Merck Ltd): 5 mM sodium acetate buffer (1:1).

The above procedures were carried out in a fume hood wearing an appropriate laboratory coat and two pairs of disposable gloves. Lead bricks and pots were used to providing shielding in all procedures. All contaminated waste was collected and sealed in a plastic container and disposal took place in accordance with local regulations. Prior to and on completion of all procedures the equipment used and surfaces were monitored for contamination using a hand-held Geiger-Muller mini-monitor.

7.3.3. Cyclic nucleotide measurement

Antibody dilution curve

Prior to the experimental use of a newly prepared cyclic nucleotide label for the assay of unknown samples, the label was tested by performing an antibody dilution curve in the absence of any competing unlabelled ligand. Antibody dilutions in the range 1:5000 up to 1:40,000 were prepared by diluting stock antibody (1:1000) with 0.05 M acetate buffer, pH 4.8. The labelled cyclic nucleotide was diluted using the same buffer (approximately 1:5000) to produce 400 cpm μl^{-1}, measured using a multi-channel gamma-counter (LKB, Wallac 1260 Multigamma).

50 µl of assay buffer was then mixed with 100 µl of antibody and 150 µl of cyclic nucleotide label in disposable polystyrene tubes (12 mm x 75 mm, Phillip Harris) and incubated overnight at 4 °C. The following day 0.5 ml of cold (4 °C) activated charcoal suspension (0.5 M phosphate buffer, pH 6.3, 6 g/l charcoal, T70 dextran, gelatin) was added to each tube. The tubes were centrifuged at 250 g for 30 min at 4 °C, and the supernatant removed by suction and discarded. The radioactivity in the pellet was measured using a gamma counter (LKB Wallac 1260 Multigamma). The cpm per tube were plotted against antibody concentration and the dilution that bound approximately 50 % of the total cyclic nucleotide label (usually approximately 1:40,000) was selected to perform a standard curve.

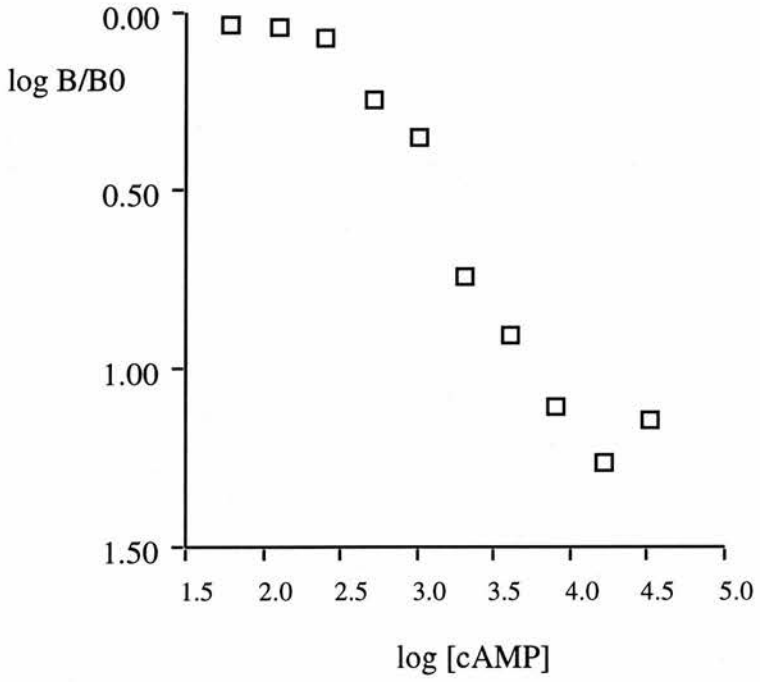


Figure 7.2 cAMP RIA standard curve.
Sample standard curve prepared by incubating acetylated standards and 125I-cAMP with R1B6 diluted 1:20000. A microcomputer connected to the gamma counter automatically calculates the equation describing the relationship between unlabeled ligand concentration and bound label (354);

$$\log (B \div B0) = a. \log(L + b) + c$$

B - Bound label (total cpm - sample cpm)
B0 - Bound label with no competing ligand
a - constant
L - Ligand concentration
b - constant
c - constant

Standard curve

Unlabelled cyclic nucleotide standards in the range 0.06 - 32 nM were prepared in 0.05 M acetate buffer from stock cyclic nucleotides (32 nM) which had been prepared previously. 500 µl of standard was then acidified in neutral borosilicate glass tubes (12 mm x 75 mm; Lab Sales, Rochdale, UK) by the addition of 5 µl of 20 % acetic acid and acetylated by the addition 15 µl of triethylamine: acetic anhydride (2: 1). The acetylation mixture was freshly prepared and run down the side of the tube onto the surface of the solution in the tube before vortexing to ensure instantaneous mixing.

Cyclic nucleotide standards (50 µl) were incubated overnight at 4 °C with 100 µl of the selected antibody dilution and 150 µl of label diluted to give 400 cpm μl^{-1}. In addition, a tube containing 150 µl of cyclic nucleotide label alone, was prepared and set aside to provide the total counts. The following day activated charcoal suspension was added to the tubes and the mixture was centrifuged as described above. The supernatant was discarded and the radioactivity in the remaining pellets was counted using a gamma counter (LKB Wallac 1260 Multigamma). The gamma counter was connected to a pre-programmed WYSE microcomputer that calculated and plotted the standard curve (354) (Fig. 7.2). A standard curve which demonstrated sensitivity to every concentration of cyclic nucleotide standard was accepted, otherwise the antibody dilution was adjusted and the above procedure was repeated until the desired sensitivity was achieved.

The concentration of cyclic nucleotide in the unknown samples from cell extracts and media was measured by incubating both samples and standards as described. The standard curve was plotted and using this the computer automatically calculated the concentration of cyclic nucleotides in the unknown samples, from their respective counts.

Each cyclic nucleotide label had a working life of 2 -3 months after which its sensitivity was considerably reduced. As a label decayed, the total number of cpm

per tube was reduced to maintain approximately the same proportion of labelled and unlabelled ligand. For example, freshly prepared label was diluted to 400 cpm. μl^{-1} and 2-3 month old label was diluted to 300 cpm. μl^{-1}.

7.3.4. Pressurisation of cells and preparation of cell extracts

Chondrocyte cultures in Ham's F12 medium containing 10 % FCS were exposed to cyclical pressurisation (26.7 kPa, 0.33 Hz), in the original apparatus for time periods ranging from 0 to 60 min.

Chondrocyte cultures in serum free Ham's F12 supplemented with IBMX (100 μM) were pressurised (203 kPa, 0.33 Hz) in the new apparatus with and without PIS (16 μstrain), for periods ranging from 0 - 60 min. The IBMX stock solution (10 mM) was prepared by dissolving 11.11 g of IBMX in 0.5 ml of DMSO and diluting the solution 1:10 with distilled water.

Chondrocyte cultures in serum free Ham's F12 containing 100 μM IBMX were also stimulated for 10 min by the addition of a 1 mM stock solution of the AC activator forskolin to the culture medium give a final concentration of 10 μM (351). This provided a positive control for the assay. The stock forskolin solution(1 mM) was prepared by dissolving 5 mg of Forskolin in 1 ml of DMSO and diluting 1:10 with distilled water.

The concentration of cyclic nucleotides was measured in both cell extracts and medium collected from cells.

Following pressurisation, the culture medium was collected in a universal tube (Phillip Harris) and the chondrocytes were lysed by the addition of 3 ml of 70 % ethanol (v/v in water). The ethanol functioned both to extract the soluble components of the cytoplasm and to precipitate the protein. The ethanol extract was then centrifuged at 250 g for 30 min to remove the precipitate. The supernatant from this centrifugation and the culture medium were transferred to neutral borosilicate glass tubes (12 mm x 75 mm; Lab Sales, Rochdale, UK) and dried in a hot block (Techne

DB5; Techne, Cambridge, UK) at 60 °C under a stream of nitrogen (Oxygen free, BOC) for approximately 30 min until dry. The dried samples were reconstituted in 0.5 ml of 0.05 M acetate buffer pH 4.8 containing 0.1 % bovine serum albumin (Fraction V; Sigma, Poole, Dorset, UK), and maintained thereafter at 4 °C. Samples were acidified with 5 µl of 20 % acetic acid (v/v in water) and mixed using a vortex. The samples were acetylated to enhance the sensitivity of the RIA (354) by the addition of 15 µl of a 2:1 mixture of triethylamine (Fluka Chemicals, Gillingham, Dorset, UK) and acetic anhydride (AnalR grade, Merck Ltd (BDH), Lutterworth,Leicestershire, UK). The acetylation mixture was freshly prepared and alowed to run down the side of the tube onto the surface of the sample to ensure instantaneous mixing. These acidification and acetylation steps were performed in the fume cupboard.The samples were stored at -20 °C prior to assay as described.

The mean concentration of cAMP in samples prepared in the same way from several experiments was expressed as the mean ± standard error of mean (SEM). The statistical significance of the effect of any treatment on cAMP concentrations was assessed using Student's paired T-test.

7.3.5. Investigation of the effect of forskolin on chondrocyte PG synthesis

Forskolin, an activator of adenylyl cyclases, was prepared as described above (7.3.4.). Forskolin (10 µM) was added to human chondrocyte cultures in Ham's F12 medium containing 10 % FCS, and the cells were labelled with 20 µCi. ml^{-1} of [$^{35}SO_4$]-sulphate for 3 h at 37 °C in air. Chondrocytes were incubated with 10 µM forskolin for 3 h in order to establish whether raising intracellular cAMP for the same period as the pressurisation studies would induce increases in sulphate incorporation comparable to PIS. Controls were labelled for the same period without forskolin.

Exposure to forskolin for 24 h has previously been shown to stimulate rabbit chondrocyte PG synthesis (351). In an attempt to duplicate this experiment 10 μM forskolin was also added to human chondrocytes in Ham's F12 containing 100 μM IBMX, and the cells were labelled with 10 μCi. ml^{-1} [$^{35}SO_4$]-sulphate for 24 h at 37 °C in air. Cells exposed to 100 μM IBMX alone acted as controls.

Following the labelling period the culture media were collected and stored. Cell associated PGs were extracted as described in Chapter 4 (p.51) of this thesis. The rate of [$^{35}SO_4$]-sulphate incorporation into cell associated PG was measured in samples from cells labelled for 3 h. The rate of [$^{35}SO_4$]-sulphate in corporation was also measured in cell associated PG and PG released into the medium by cells labelled for 24 h in the presence of 10 μM forskolin.

7.4. Results

7.4.1. Cyclic nucleotide levels in chondrocytes

On assay, cyclic GMP levels were found to be below the range of the assay (0.06 - 32 nM) under all experimental conditions examined i.e. in the presence of 100 μM IBMX, 10 μM forskolin, and following pressure-induced strain .

Negligible amounts of cAMP were detected in culture media collected from unstimulated cells, in the absence of 100 μM IBMX, after incubation periods of up to 1 h. However, in the presence of IBMX, the mean concentration of cAMP in culture media from unstimulated cells was 1.37 nM (± [SEM] 0.08, n = 4), and no significant change occured (1.34 nM ± 0.18, n = 3) following the incubation of cells with 10 μM forskolin for 10 min.

The concentration of cAMP in samples extracted from unstimulated cells in the presence of IBMX (2.88 nM ± 1.01, n = 10) was not siginificantly different to the concentration of cAMP in the absence of IBMX (2.48 nM ± 1.37, n = 10). Following the incubation of cells with 10 μM forskolin for 10 min, in the presence of IBMX,

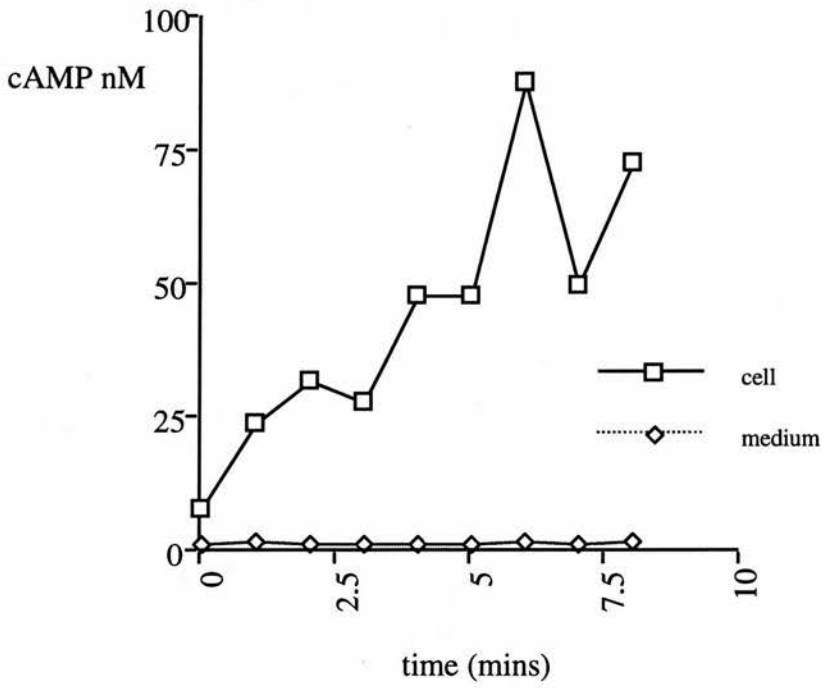


Figure 7.3 Cyclic AMP levels in chondrocytes exposed to 10 μM forskolin. The concentration of cAMP was measured by RIA in extracts of chondrocytes and media from chondrocytes which had been exposed to 10 μM forskolin, for the times shown, in the presence of 100 μM IBMX.

the concentration of cAMP in unstimulated cells increased to 113 nM (± 60, n = 5, $p<0.02$).

The time-course study (Fig 7.3) revealed that exposure of chondrocytes to forskolin for just 1 min increases intracellular cAMP levels approximately two fold. Further exposure to forskolin continued to increase intracellular cAMP for a further 5 mins. In experiments assaying cAMP, forskolin-treated cells were used as a positive control.

7.4.2. The effect of cyclical pressurisation and cyclical strain on intracellular cAMP levels

The intracellular concentration of cAMP in chondrocyte extracts was reduced following cyclical pressure induced strain (20 μstrain) for times ranging from 5 - 60 min (Figs. 7.4 & 7.5).

In the absence of IBMX the mean concentration of cAMP in cell extracts decreased from 0.38 nM (± 0.28, n = 5) to 0.13 nM (± 0.03, n = 5) following 30 min PIS (Fig. 7.4). The concentration of cAMP in extracts from cells which were exposed to cyclical pressurisation in the absence of both PIS and IBMX, was not significantly altered. The mean cAMP concentrations in the extracts from control cells were below the mean value in section 7.4.1. as these samples are a subpopulation of those described above. The difference does not invalidate the conclusions of this section as paired control and pressurised samples were compared.

In the presence of IBMX, the mean concentration of cAMP decreased from 3.56 nM (± 1.66, n = 6) to 0.24 nM (± 0.10, n = 4,) following 30 min PIS (Fig 7.5). Similar decreases in the intracellular concentration of cAMP were seen in chondrocytes exposed to cyclical pressurisation in the absence of strain. These changes failed to reach significance.

The rapid response of the cells to forskolin (Fig 7.3) suggested that cellular cAMP levels may alter more rapidly than the 0 - 60 minute time course examined in

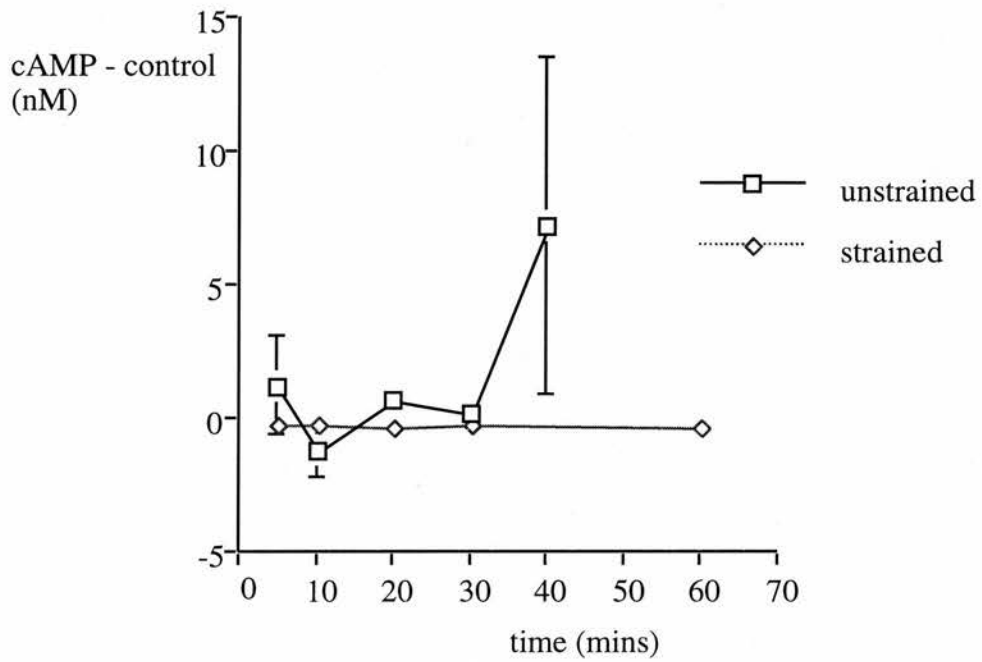


Figure 7.4 Cyclic AMP levels in chondrocytes exposed cylical presurisation and cyclical pressure-induced strain. The diagram shows the mean concentration of cAMP in pressurised samples minus the cAMP concentration in the paired unpressurised samples. The concentration of cAMP was measured by RIA of extracts of chondrocytes which had been exposed to cyclical pressurisation (203 kPa, 0.33 Hz) with (n = 5) and without (n = 6) strain (20 μstrain), for the times shown, in the absence IBMX.

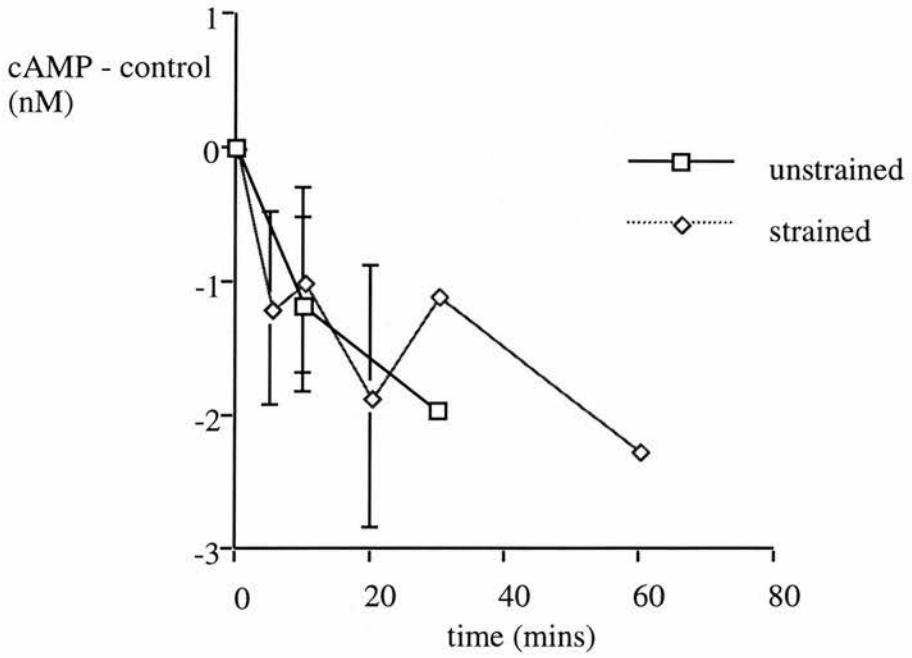


Figure 7.5 Cyclic AMP levels in chondrocytes exposed to cyclical presurisation and cyclical pressure-induced strain. The diagram shows the mean of concentration of cAMP in pressurised samples minus the cAMP concentration in paired unpressurised samples. The concentration of cAMP was measured by RIA of extracts of chondrocytes which had been exposed to cyclical pressurisation (203 kPa, 0.33 Hz) with (n = 6) and without (n = 6) strain (20 μstrain), for the times shown, in the presence of 100 μM IBMX.

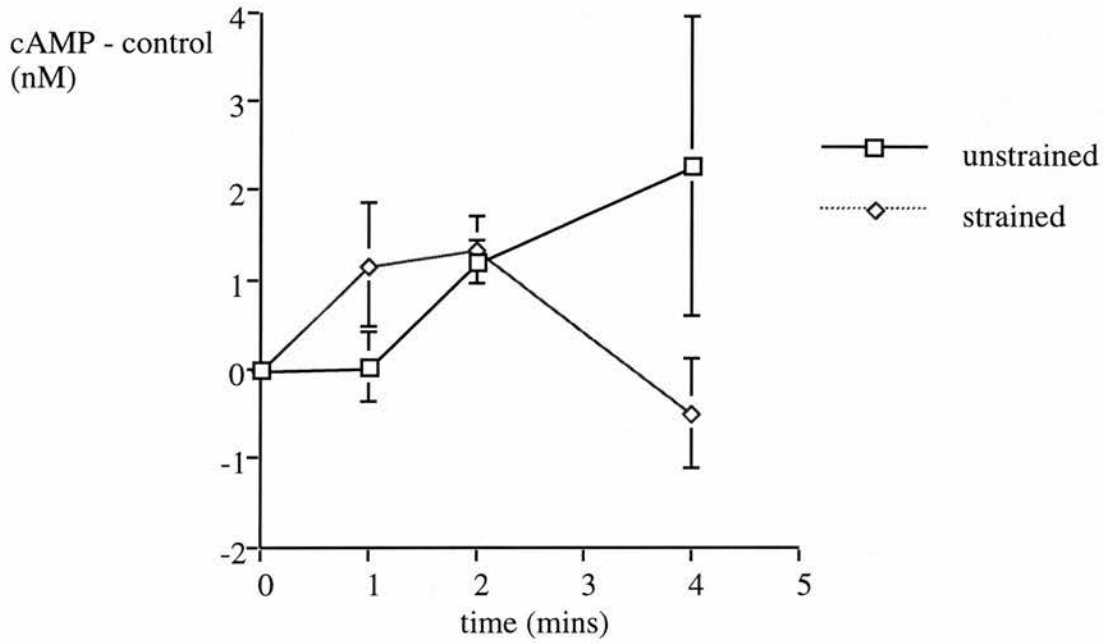


Figure 7.6 Cyclic AMP levels in chondrocytes exposed to cyclical presurisation and cyclical pressure-induced strain. The diagram shows the mean of concentration of cAMP in pressurised samples minus the cAMP concentration in paired unpressurised samples. The concentration of cAMP was measured by RIA of extracts of chondrocytes which had been exposed to cyclical pressurisation (203 kPa, 0.33 Hz) with (n = 4) and without (n = 4) strain (20 μstrain), for the times shown, in the presence of 100 μM IBMX.

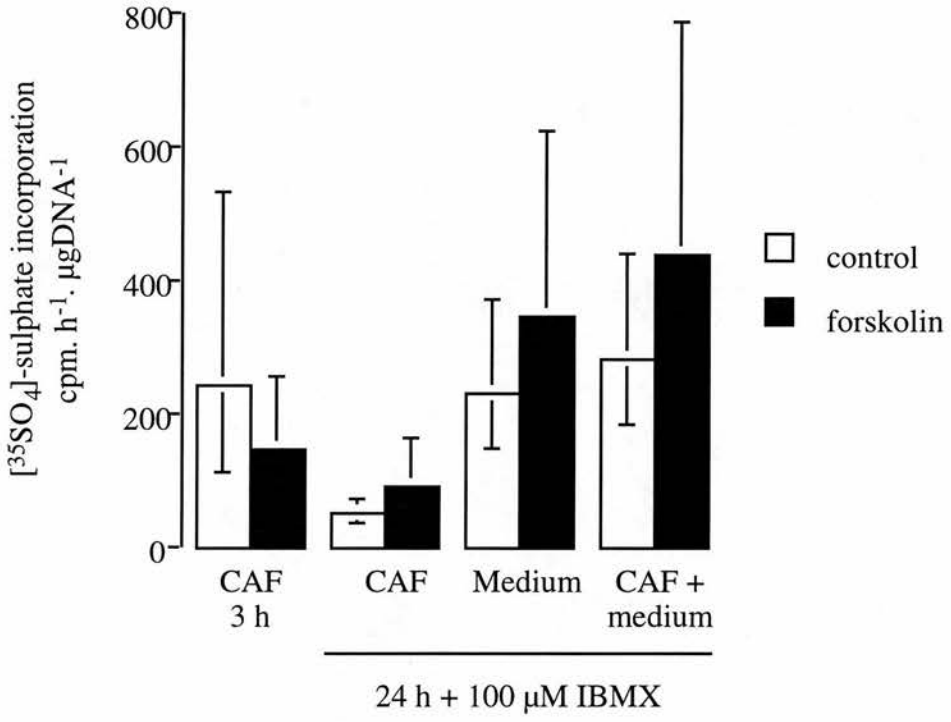


Figure 7.7 The effect of forskolin on chondrocyte PG synthesis. Chondrocytes were exposed to 10 µM forskolin for the times shown at 37 °C. Forskolin was ommited from controls. The phosphodiesterase inhibitor IBMX (100 µM) was added to 24 h studies prior to labelling. Results show mean $^{35}SO_4$-sulphate incorporation rates ± 95% confidence intervals (n=3). CAF = cell-associated fraction. The error bars in the figures appear assymetrical as they are calculated by taking the antilog_{10} of the confidence intervals of the $\log_{10}$ transformed data.

the experiments described above. The concentration of cAMP in cell extracts increased significantly above the control level following 2 min ($p<0.02$) cyclical PIS (16 μstrain), and fell to values below the initial levels after 5 min PIS (Fig. 7.6). The levels of cAMP in cells exposed to cyclical pressurisation without strain also increased significantly after 2 min of stimulation ($p<0.01$).

7.4.3. The effect of forskolin on PG synthesis

Exposure of human chondrocytes to 10 μM forskolin for 3 h at 37 °C produced a non-significant decrease (Fig 7.7) in PG synthesis rates from 244 cpm. h^{-1}. $\mu gDNA^{-1}$ (95 % confidence limits, 112-530) to 146 cpm. h^{-1}. $\mu gDNA^{-1}$ (84 - 253).

PG synthesis rates increased in chondrocytes exposed to 10 μM forskolin for 24 h in the presence of IBMX. None of the increases was statistically significant.

7.5 Discussion

These experiments have demonstrated that exposure of chondrocytes to cyclical pressurisation and cyclical strain, in the presence of PD inhibitor, leads to an increase in intracellular cAMP after 2 min. Further exposure to cyclical strain for up to 1 h leads to a decrease in intracellular cAMP. No consistent change in cAMP is seen in response to cyclical pressurisation for longer periods. In the absence of PD inhibitor there was a non significant reduction in cAMP accumulation in cells exposed to cyclical strain and no consistent response to cyclical pressure. This suggests that PD inactivation may alter the mechanisms for the regulation of intracellular levels of cAMP leading to reduced or no cAMP production. The presence of IBMX during pressurisation experiments creates an artificial situation where normal cAMP regulation clearly does not occur. IBMX should only be added to cell extracts after loading so that any effects on cell function are avoided.

Results in the present study suggest that cGMP, if present in chondrocytes, is present at very low levels, below the range of the assay used.

Previous studies have demonstrated that there is an increase in the accumulation of cAMP in proliferative epiphyseal chondrocytes following cyclical stretch (171) and also following cyclical hydrostatic pressurisation (166). An increase in cAMP lasting 3 min was also demonstrated in growth plate chondrocytes exposed to continuous stretch (169). These studies did not use IBMX or other phosphodiesterase inhibitors, but demonstrated a 2 fold increase in the accumulation of cAMP after 30 min (166) and 24 h (171). The experiments performed in the present study showed a short term increase followed by a decrease in cAMP levels in the presence of IBMX. The short term increase seen in our study may be comparable to that seen in growth plate chondrocytes (169) but care must be taken when comparing responses obtained in adult articular chondrocytes with those obtained in proliferative chondrocytes. Proliferative chondrocytes express different matrix molecules (27) but there is no evidence as yet that they respond differently to mechanical loading.

The experiments performed in this study demonstrated an 84 % increase in [$^{35}SO_4$]-sulphate incorporation in chondrocytes exposed to forskolin for 24 h, and no change in [$^{35}SO_4$]-sulphate incorporation in chondrocytes exposed to forskolin for 3 h. These results did not reach significance since only three experiments were performed, but a similar increase was seen in the synthesis rates of both cell associated PG and PG released into the medium. Activation of chondrocyte adenylyl cyclases with forskolin and treatment of chondrocytes with cAMP analogues has been shown to stimulate PG synthesis in epiphyseal proliferative chondrocytes after a 48 h exposure (171), and in mature rabbit articular chondrocytes after 24 h (351) and 48 h (352) exposure. Forskolin treatment increased [$^{35}SO_4$]-sulphate incorporation by 50 -150 % in the presence of IBMX and by 16 - 30 % in the absence of IBMX (351). As discussed above, performing experiments in the presence of IBMX may lead to non-physiological changes in the regulation of cAMP. Results obtained in the presence of phosphodiesterase inhibitors must be treated with caution.

Exposure of rabbit chondrocytes to cAMP analogues for 1 h had no effect on KS secretion but further exposure for 48 h caused a significant increase in KS secretion (352). The experiments reported here confirm the findings of the studies described above and indicate that cAMP must be elevated for long periods e.g. for 24 h, to activate PG synthesis. It is possible that long periods of elevated cAMP levels are required to accumulate sufficient kinase or transcription factor activity required to activate PG synthesis. Further studies are required to establish the time course of the stimulation of PG synthesis by cAMP. PG synthesis should be measured in chondrocytes exposed to forskolin in the presence and absence of PD inhibitors for times from 0 to 24 h.

In separate experiments, the action of histamine H_2 receptor agonists, PTH, and PGE_2 on chondrocytes has been shown to cause a short term (peak after 10 mins) increase in intracellular cAMP which was followed by a decrease in PG synthesis rates measured over the next 48 h (352). It appears that cAMP may mediate different responses in chondrocytes depending on the period of time examined. In the present studies PG synthesis was increased following exposure to cyclical pressure-induced strain for 3 h (section 5.4.5., p97) as compared to chondrocytes pressurised without strain. This may be a reflection of the decrease in cAMP measured after 1 h cyclical PIS.

In order to elucidate the relationship between mechanical stress, cAMP, and PG synthesis further experiments must be performed in which cAMP and PG synthesis are measured in cells that have been exposed to stimulation for the same time period. Experiments should also be performed to differentiate the effects of pressure and strain on chondrocyte cAMP levels. The radioimmunoassay described in these studies is a reliable and sensitive technique for measuring the concentration of cAMP in cell extracts. In the studies described no effort was made to normalise the cAMP concentration in a cell extract using some measure of cell number. It was assumed that every sample in an experiment contained the same number of cells. In order to

avoid the inaccuracies that such an assumption makes future studies should measure the total protein content of cell extracts and express cAMP concentration in terms of total protein.

8.THE EFFECT OF PRESSURE-INDUCED STRAIN ON THE EXPRESSION OF CHONDROITIN SULPHATE EPITOPES

8.1.Introduction

One of the early events observed in experimental osteoarthritis, induced by section of the cruciate ligament, is an increase in PG synthesis (2). This is followed by changes in the PG structure which include elongation of the CS chains, an increase in the concentration of CS-4, and a decrease in KS (2,43). Monoclonal antibodies have been developed which detect specific epitopes within the CS chains expressed in developmental and OA cartilage (355,356). Two of these antibodies (3B3 and 7D4) have emerged as candidate markers for the detection of early changes in osteoarthritis when gross changes such as the loss of PG are not detectable (357).

The monoclonal antibody 3B3 was raised against PG which had been subjected to chondroitinase digestion. This antibody was shown to detect delta-unsaturated or saturated tetrasaccharides derived from chondroitin or CS-6 (355) (Fig. 8.1). The epitope recognised by 3B3 was shown to be absent or only weakly expressed in normal cartilage, but was expressed as a native epitope in pathological OA specimens and in developmental tissue (356,358,359). The enzyme generated epitope is designated as 3B3(+) and the native epitope as 3B3(-). Immunohistochemistry has demonstrated increased staining with 3B3(-) in specimens of human arthritic cartilage (356) and also in specimens of osteoarthritic cartilage from Cynomolgus Macaques (360). Articular cartilage removed from dogs after the anterior cruciate ligament of the knee joint had been sectioned, stained more strongly for 3B3(-) in the superficial and upper middle zones (357). Mechanical compression of intact bovine sesamoid bones for three days was demonstrated to increase the expression of 3B3(-) following 3 days of loading and the increased expression of 3B3(-) was maintained for up to 3 weeks (151).

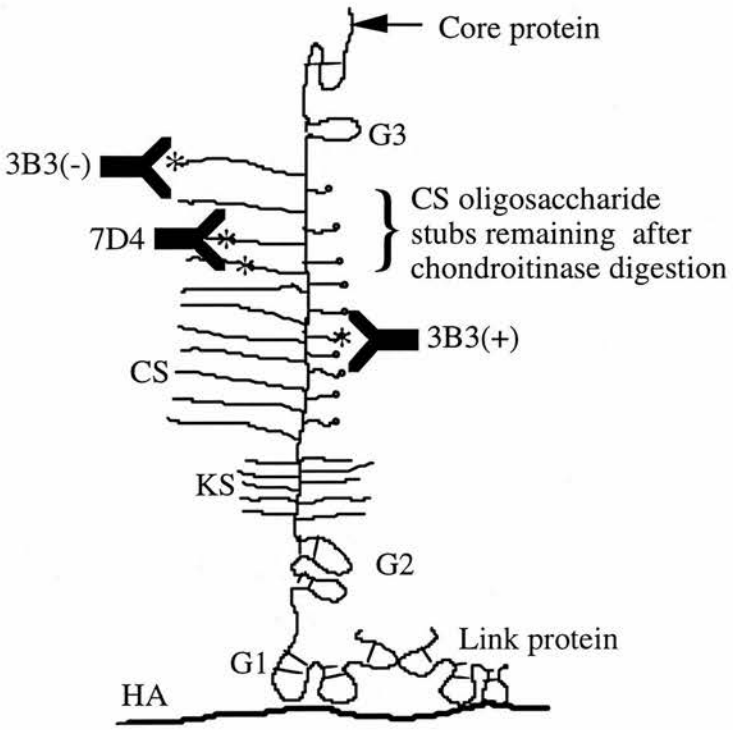


Figure 8.1 A proteoglycan monomer and the binding sites of the monoclonal antibodies used in this study. Schematic diagram of a proteoglycan monomer showing the epitopes recognised by the monoclonal antibodies 3B3 and 7D4. To the left of the core protein intact CS chains before chondroitinase digestion and the sites of the 3B3(-) mimotope and 7D4 epitope. To the right of the core protein CS oligosaccharide stubs containing the 3B3(+) epitope produced by chondroitinase digestion. G1 -3 = three globular domains of core protein. Adapted from Slater *et al.* (356).

The monoclonal antibody 7D4 raised against intact CS chains recognises an epitope in a domain of CS chains in close proximity to the core protein (361) (Fig. 8.1). The epitope recognised by 7D4 was shown to be weakly expressed in normal cartilage (255). Expression of the 7D4 epitope has been shown to be increased in specimens of human arthritic cartilage (356), osteoarthritic cartilage from Cynomolgus Macaques (360), cartilage from dogs whose anterior cruciate ligament had been sectioned (357), and in specimens of synovial fluid aspirated from knees of patients who had sustained traumatic cruciate ligament and/or meniscus damage (362).

The increased expression of these epitopes following cartilage compression and also following abnormal joint loading caused by injury or surgical intervention suggests that they can be used as a marker of the loading-induced changes that may lead to the development of osteoarthritis. The aim of the experiments described in this chapter was to develop the use of the monoclonal antibodies 3B3 and 7D4 to measure the expression of CS epitopes produced by cultured chondrocytes using the technique of flow cytometry, and also to investigate possible changes in the level of CS epitope expression before and after exposure of cultured chondrocytes to cyclical PIS.

8.2. Methods

The expression of CS epitopes recognised by the monoclonal antibodies 3B3 and 7D4 was examined by use of these antibodies to immunostain freshly isolated bovine chondrocytes, bovine and human articular chondrocytes cultured in monolayer for periods up to 14 days, and bovine and human chondrocytes cultured in alginate beads. The effect of exposure to pressure-induced strain on the expression of these epitopes was assessed in bovine chondrocytes, but not in human chondrocytes due to the limited availability of human articular cartilage specimens.

8.2.1. Preparation of cells for immunostaining

Bovine and human articular chondrocytes were released from monolayer culture using PBS containing 5 mM EDTA (PBS-EDTA). The culture medium was removed from culture dishes, the cells washed with PBS-EDTA and then shaken vigorously with PBS-EDTA for 30 min at 37 °C using a mechanical shaker in an incubator. Cells remaining adherent after this incubation period were removed from the dish by scraping the culture dish with a cell scraper (Costar, High Wycombe, Bucks, UK).

Bovine and human articular chondrocytes were released from culture in alginate beads as described in section 4.3.2. (p55).

Cell suspensions freshly isolated from articular cartilage and from chondrocyte monlayer and alginate cultures were centrifuged at 250 g for 10 min, washed in 10 ml of PBS containing 0.1 % sodium azide (to prevent bacterial infection) and 0.2 % BSA (PBS-BSA), and recentrifuged. The pellet was resuspended in 1 ml of PBS-BSA and the number of cells in the suspension was counted using a haemocytometer. The cell suspension was then divided into aliquots and placed in capped polystyrene tubes (Falcon, 12mm x 75 mm; Fred Baker Scientific, Runcorn, Cheshire, UK), so that each tube contained a minimum of 100,000 cells.

8.2.2. Immunostaining of chondrocytes for flow cytometry

The cell surface and pericellular expression of GAGs by human and bovine articular chondrocytes was examined by indirect immunofluorescence with unconjugated mouse anti-human monoclonal antibodies and a phycoerythrin conjugated polyclonal goat anti-mouse-IgG (Sigma, Poole, Dorset, UK). Chondrocytes were also stained with anti-human-CS monoclonal antibodies 3B3 and 7D4 which had not been previously shown to cross-react with bovine cartilage. The anti-KS antibody MZ15 was used as positive control, for it had previously been shown to be expressed strongly in monolayer cultures of human articular chondrocytes (243). These

antibodies were the generous gift of Dr. M. Bayliss (Kennedy Institute for Rheumatology, London, UK).

Monoclonal antibodies against α-fetoprotein (Prof. V. van Heyningen, MRC, Edinburgh, Scotland) and mouse IgG (Sigma, Poole, Dorset, UK) acted as negative controls in experiments staining human and bovine chondrocytes respectively.

In an initial experiment the antibodies 3B3, 7D4, and MZ15 were diluted 1:5, 1:50, 1:500 to determine the optimal dilution for the experiments. The resulting immunofluorescence was measured by flow cytometry, and the maximum dilution which gave significant fluorescence was selected. Following the preliminary experiments, these antibodies were subsequently used at a dilution of 1:50 in PBS containing 0.1 % azide. The negative control antibodies were diluted 1:5 in PBS with 0.1 % azide.

All procedures were performed on ice. Cells prepared in tubes as described above were centrifuged at 250 g and the supernatant discarded by inverting the tubes and blotting onto tissue paper. Non-specific binding was blocked by adding 10 µl of heat-inactivated normal human serum, diluted 1:5 in PBS, to the pellet of cells and mixing by vortexing. The chondrocytes were incubated with normal human serum for 10 min after which 0.5 ml of PBS-BSA solution was added and mixed by vortexing. The cells were then centrifuged at 250 g for 10 min and the supernatant discarded. This spin-wash procedure was repeated and the cells were then centrifuged for 5 min at 250 g and the supernatant was discarded as described above. Ten µl of diluted primary antibody (negative control, MZ15, 3B3, 7D4) was added to the cell pellet and mixed by vortexing. The cells were incubated with the primary antibody for 45 min. The cells were spin-washed twice in 0.5 ml of PBS-BSA as described above, to remove any unbound antibody. The cells were centrifuged for 5 min at 250 g and the supernatant discarded as described above. Ten µl of phycoerythrin-conjugated goat-anti-mouse secondary antibody, diluted 1:4 in PBS, was added to the cell pellet and mixed by vortexing. The chondrocytes were

incubated with the secondary antibody for 45 min. Finally the cells were spin-washed twice as described above to remove any unbound label. The cell suspension was centrifuged for 5 min at 250 g and the supernatant discarded as described above. The cell pellet was fixed in 500 µl of PBS containing 1 % paraformaldehyde. The tubes containing the stained cells were stored at 4 °C in the absence of light until flow cytometry could be carried out (up to 48 h later).

8.2.3. Assessment of baseline immunostaining

In initial studies, human chondrocytes were used to establish the use of flow cytometry as a technique for measuring the expression of specific GAG epitopes. Articular chondrocytes, isolated from cartilage taken from individuals with normal cartilage and mildly arthritic cartilage (see section 4.3.1., p52) were cultured in monolayer for 12 days and in alginate beads for 12 days prior staining with primary antibodies as described above.

All subsequent experiments were performed with bovine chondrocytes. Baseline staining was measured in samples of bovine chondrocytes which were freshly isolated or had been cultured for periods up to 14 days. In addition, cells which had been cultured in alginate beads were immunostained in the same way, in order to establish the effect of the artificial culture matrix on cell-surface PG expression.

Ham's F12 medium is deficient in SO_4 which may effect GAG epitope structure and induce the expression of the 3B3(-) and 7D4 epitopes. To investigate the possible effect of SO_4 deficiency on CS-epitope expression bovine chondrocytes were cultured in Ham's F12 medium for 3 days as described and in Dulbecco's modified Eagle's medium (DMEM) for the same period. DMEM contains non-limiting amounts of SO_4. Chondrocytes were then passaged and immunostained as described above.

8.2.4. Pressurisation and immunostaining of bovine chondrocytes

Duplicate dishes of bovine chondrocytes in monolayer culture were exposed to cyclical pressurisation (203 kPa, 0.33 Hz) with and without PIS (16 μstrain) for 3 h at 37 °C. Unpressurised controls were incubated in air for 3 h at 37 °C. In one experiment bovine chondrocytes were additionally exposed to cyclical pressurisation with and without PIS for 5 h and 6 h.

It was not possible to pressurise chondrocytes for long periods because of the hypoxic conditions in the pressure chamber. Cells were pressurised for 3 h and incubated for a further 24 h period in order to investigate the possibility that changes in CS epitope expression require longer than the 3 h pressurisation period to become apparent. In these experiments bovine chondrocytes were exposed to cyclical pressure and cyclical strain under the same conditions for 3 h. The cells were then passaged immediately as described in section 8.2.2. or cultured for a further 24 h at 37 °C in 5 % CO_2/95 % air incubator, prior to passage.

In all experiments two tubes of unpressurised cells were digested with 10 μl of chondroitinase ABC (0.1 unit. ml^{-1}) in 0.1 M Tris HCl pH8 for 1 h at 37 °C. The cells were immunostained as previously described prior to assessment of staining by flow cytometry.

8.2.5. Assessment of Immunofluorescence by flow cytometry

Flow cytometry is a technique for making rapid measurement on individual particles or cells as they flow in a fluid stream through a laser beam. The intensity of the resulting fluorescence is determined by the amount of fluorescent label bound to the cell, and the scatter of the fluorescence is determined by the size (side scatter) and granularity (forward scatter) of the cells. The intensity and scatter are measured by detectors and the data for every fluorescent event is collected by a computer. The sensitivity of the detectors (acquisition parameters) can be adjusted in order to detect

the level of intensity and scatter obtained from one particular cell type. This is necessary since all cells are of different size and have different degrees of granularity. Having acquired the data for a sample of cells it is analysed separately by microcomputer. The analysis software creates dot plots of the forward scatter ('x'-axis) and side scatter ('y)-axis) of cells such that a homogenous sample of cells will produce a uniform population of the dots. Cell debris is also plotted but can be 'gated out' on the basis of its different forward-scatter and side-scatter characteristics. The software allows selection of the population of cells of interest, and plots a histogram of the fluorescence intensity of that population and provides statistical analysis for the fluorescence results. The mean fluorescence intensity is a measure of the amount of label bound to the cells, and hence of the amount of ligand present.

A wide range of fluorescent probes are available for directly estimating cellular parameters such as the degree of expression of surface molecules. Conjugation of fluorescent dyes to ligands and to polyclonal and monoclonal antibodies enables the density and distribution of cell surface and cytoplasmic determinants and receptors to be studied and functional sub-populations of cells to be identified with respect to a particular measured variable.

Human and bovine chondrocytes were prepared to examine cell surface and pericellular GAG expression by indirect immunofluorescence with unconjugated monoclonal antibodies and a phycoerythrin conjugated polyclonal goat anti-mouse IgG secondary antibody as described above. Immunofluorescence was assessed on 20,000 events using a FACScan (Fluorescence Activated Cell Sorter; Becton Dickinson, Mountain View, California). Acquisition parameters were determined in initial experiments, for both bovine and human chondrocytes, which were unaltered during subsequent experiments. The staining was analysed on dot plots using Lysis II software (Becton Dickinson, Mountain View, California).

8.3. Results

8.3.1. Staining of human articular chondrocytes

Chondrocytes isolated from normal cartilage of a 43 year old female and from mildly OA cartilage of an 80 year old male, were cultured for 12 days in monolayer and alginate bead culture respectively, prior to immunostaining with a range of primary antibody dilutions. Duplicate tubes were stained with each antibody at each concentration used. The histograms shown in figures 8.2 and 8.3 show the optimal staining obtained with the range of dilutions used, as described above. The dilutions and the acquisition parameters established in these experiments were used with all subsequent experiments. These preliminary studies with human chondrocytes indicate that flow cytometry can be used to detect GAG epitope expression in cultured chondrocytes.

The apparent bimodal distribution of the immunofluorescence data presented in the histograms from MZ15 and 3B3 indicate that there may be two populations of cells; one staining for these KS and CS epitopes at low level and one at high levels. This suggests that there may be two populations of cells expressing these epitopes at different levels. The bimodal distribution is not so marked in the OA chondrocytes cultured in alginate (Fig 8.2). Low levels of staining for 3B3(-) and no significant staining for 7D4 was seen in normal cartilage, but both epitopes were expressed in OA chondrocytes cultured in alginate (Table 8.1). It may be of value to use the data analysis software to calculate the level of expression in both sub-populations where a bimodal distribution exists.

Table 8.1 The expression of CS epitopes by normal and OA human chondrocytes

	Mean Fluorescence intensity			
Antibody	α-fetoprotein	MZ15	3B3(-)	7D4
Normal (Monolayer)	2.37	399.30	16.22	4.23
Mild OA (alginate)	4.04	355.21	31.54	49.3

Cells were taken from a single normal and a single mild-OA human knee joint. Mean fluorescence intensity of was calculated using Lysis II software. The cell population was selected on the basis of forward scatter and side scatter from 20,000 events.

a)

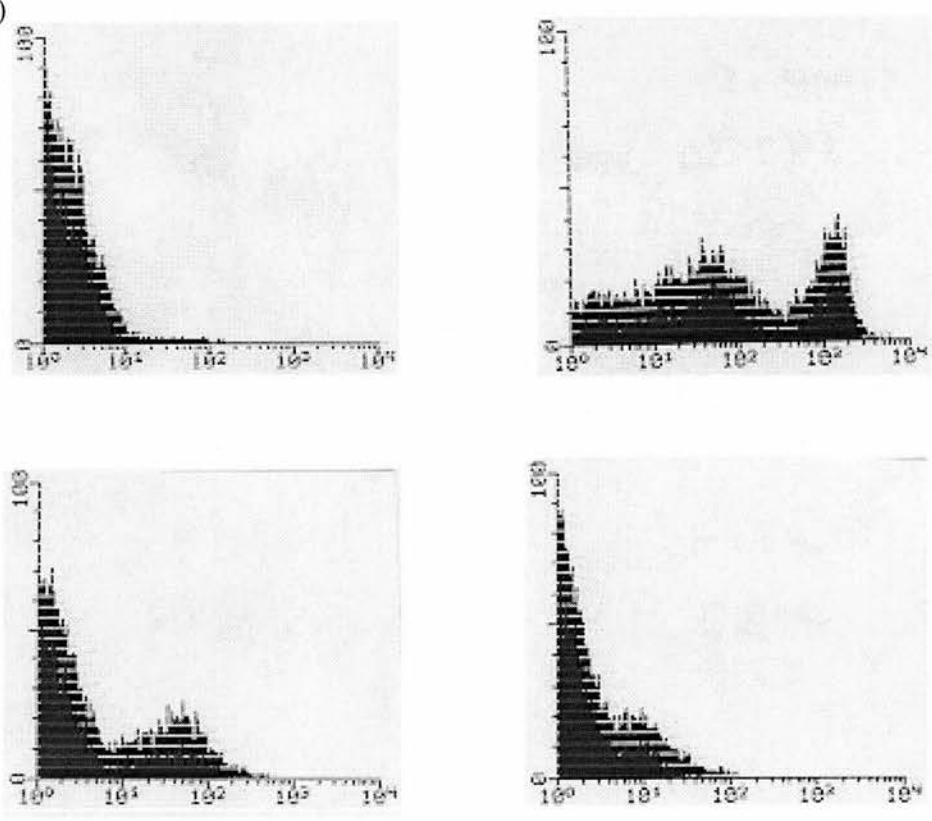


Figure 8.2 Immunofluoresence of human chondrocytes. Chondrocytes isolated from normal cartilage taken from a 43 year old female. In each histogram the x-axis represents the fluoresence intensity and the y-axis represents the number of events. Cells were cultured in monolayer for 12 days prior to staining with (a.) a-feto protein diluted 1:5 (b.) MZ15 - anti KS, diluted 1:50 (c.) 3B3(-) - anti CS, diluted 1:50 (d.) 7D4 - anti CS, diluted 1:50.

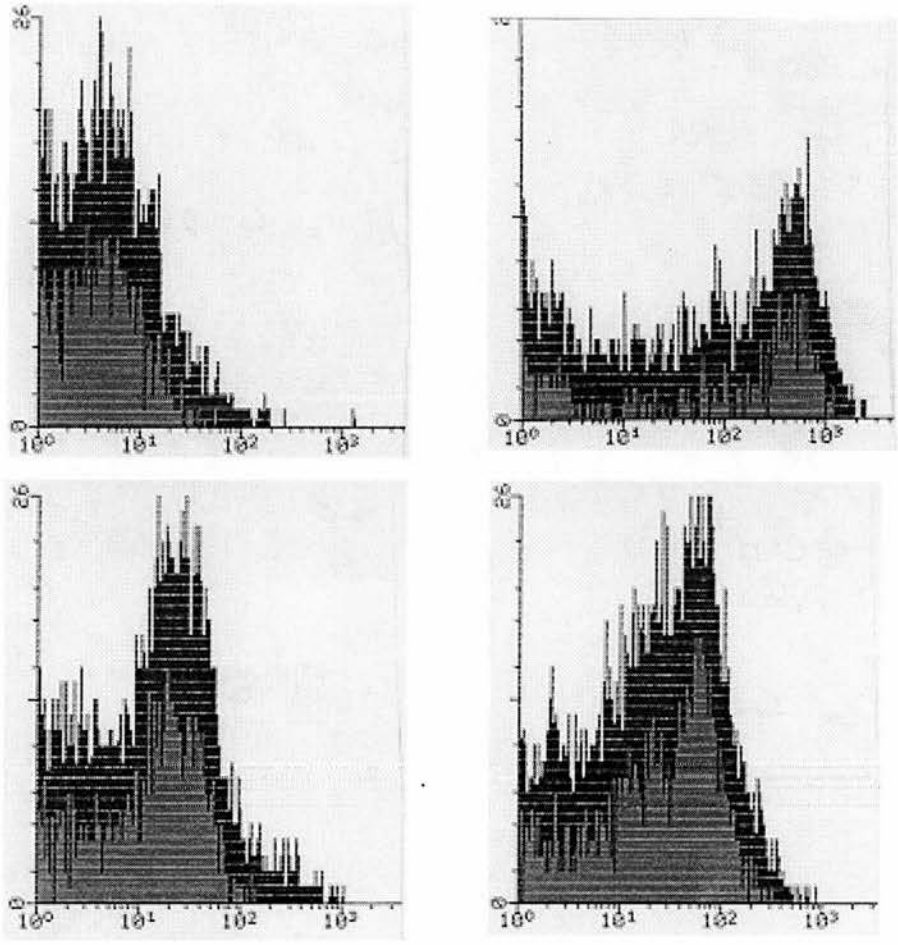


Figure 8.3 Immunofluoresence of human chondrocytes. Chondrocytes isolated from mildly OA cartilage taken from a 80 year old male. In each histogram the x-axis represents the fluoresence intensity and the y-axis represents the number of events. Cells were cultured in alginate beads for 12 days prior to staining with (a.) a-feto protein diluted 1:5 (b.) MZ15 - anti KS, diluted 1:50 (c.) 3B3(-) - anti CS, diluted 1:50 (d.) 7D4 - anti CS, diluted 1:50.

8.3.2. Staining of Bovine chondrocytes

Chondrocytes from normal bovine articular cartilage were stained after isolation and after varying periods in monolayer and alginate culture (Table 8.2). It was found that 3B3(-) epitope was expressed at low levels in cells cultured in monolayer for up to 4 days, and at higher levels in cells cultures in alginate beads for 4 days. The epitope 7D4 was expressed in freshly isolated cells but not in cultured cells. The histograms of immunofluorescence in figure 8.4 - 8.6 show staining of freshly isolated bovine chondrocytes and chondrocytes which had been cultured in monolayer for 3 days and in alginate for 4 days, respectively. The increased fluorescence intensity of cells stained with 3B3 after chondroitinase ABC digestion indicates the presence of the 3B3(+) enzyme-generated epitope. The histograms of the 3B3(+) data display a bimodal distribution similar to that seen with MZ15 and 3B3(-) in human chondrocytes (Fig. 8.1). Once again this indicates there are two populations of cells expressing this epitope at different levels following enzymatic treatment. The levels of immunostaining obtained with 7D4 remained unchanged following the treatment of cells with chondroitinase ABC.

Table 8.2 The expression of CS epitopes by normal bovine chondrocytes

	Mean Fluorescence intensity			
Days in culture	mouse-IgG	MZ15	3B3(-)	7D4
0	1.33	80.49	2.33	11.50
2	1.89	56.72	5.29	5.87
3	1.78	156.12	9.58	4.41
4	7.31	98.98	27.10	14.6
14	11.12	154.21	9.79	4.41
4 (alginate)	3.67	554.59	95.95	3.72

Mean fluoresence intensity was calculated using Lysis II software. The cell population was selected on the basis of forward scatter and side scatter from 20,000 events.

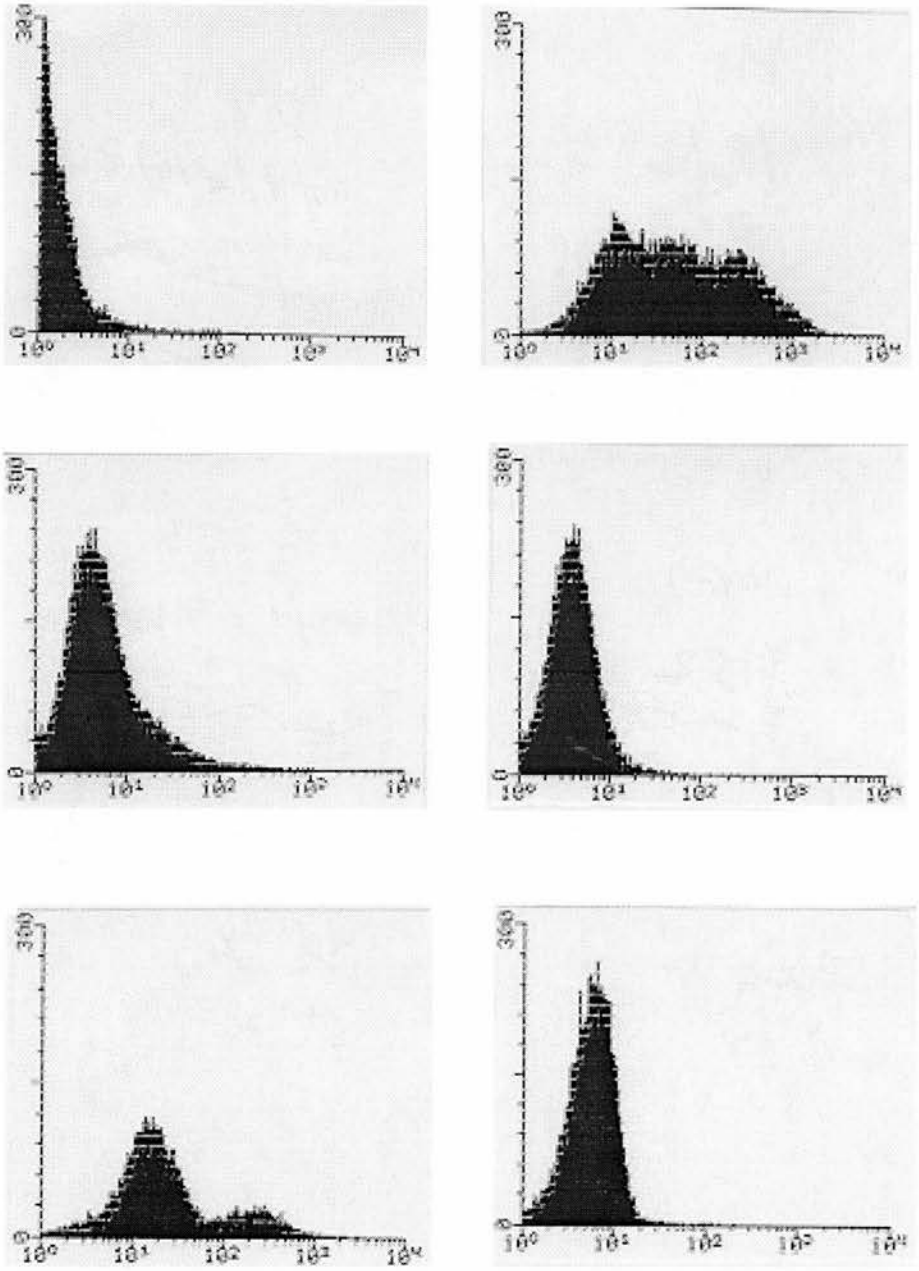


Figure 8.4 Immunofluoresence of freshly isolated bovine chondrocytes.
Chondrocytes were isolated from normal cartilage taken from a 12 month old heifer. In each histogram the x-axis represents the fluoresence intensity and the y-axis represents the number of events. Cells were stained with (a.) anti-mouse IgG, diluted 1:5 (b.) MZ15 - anti KS, diluted 1:50 (c.) 3B3(-) - anti CS, diluted 1:50 (d.) 7D4(-) - anti CS, diluted 1:50 (e.) 3B3(+) - anti CS, diluted 1;50 (f.) 7D4(+) - anti CS, diluted 1:50.

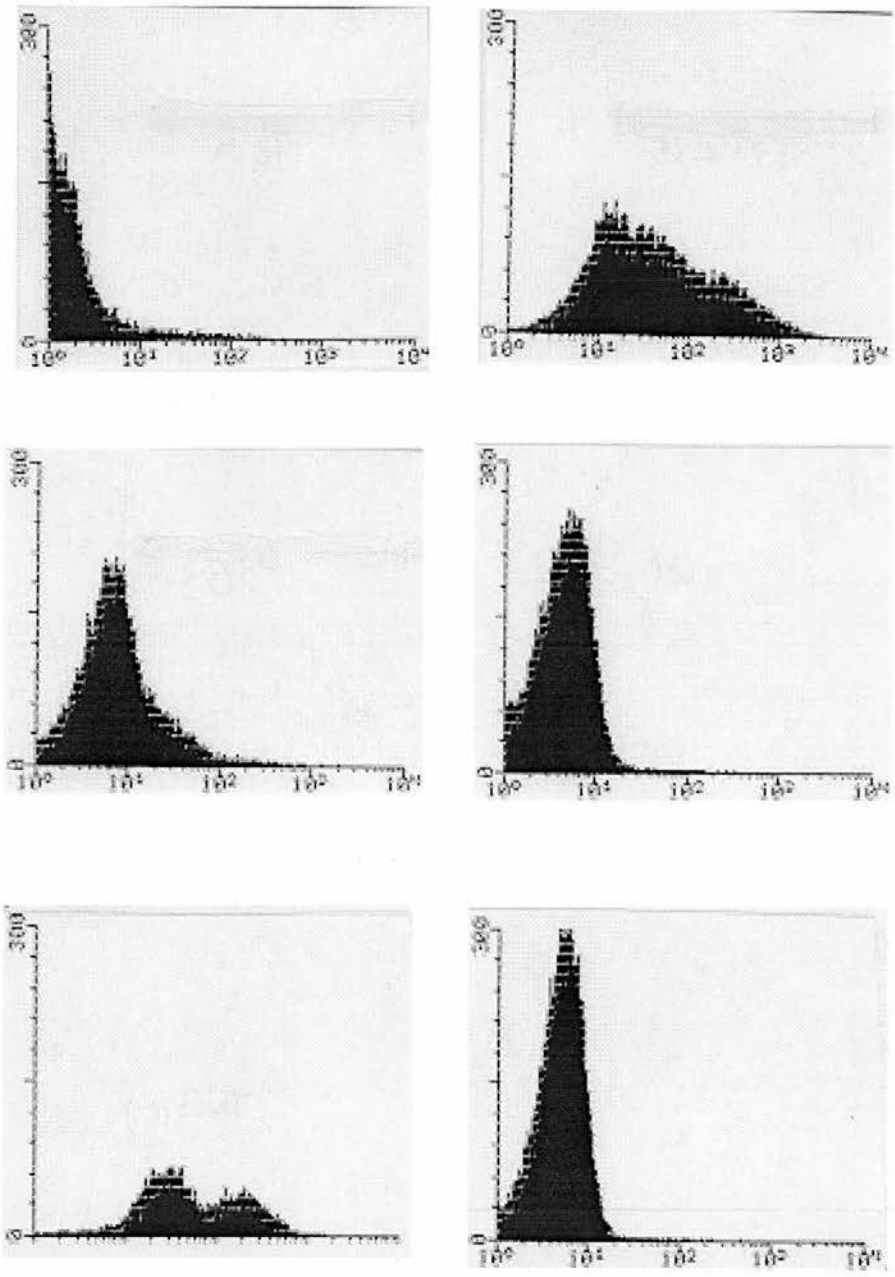


Figure 8.4 Immunofluoresence of monolayer cultured bovine chondrocytes. Chondrocytes were isolated from normal cartilage taken from a 12 month old heifer. Cells were cultured in monolayer for 3 days prior to staining. In each histogram the x-axis represents the fluoresence intensity and the y-axis represents the number of events. Cells were stained with (a.) anti-mouse IgG, diluted 1:5 (b.) MZ15 - anti KS, diluted 1:50 (c.) 3B3(-) - anti CS, diluted 1:50 (d.) 7D4(-) - anti CS, diluted 1:50 (e.) 3B3(+) - anti CS, diluted 1;50 (f.) 7D4(+) - anti CS, diluted 1:50.

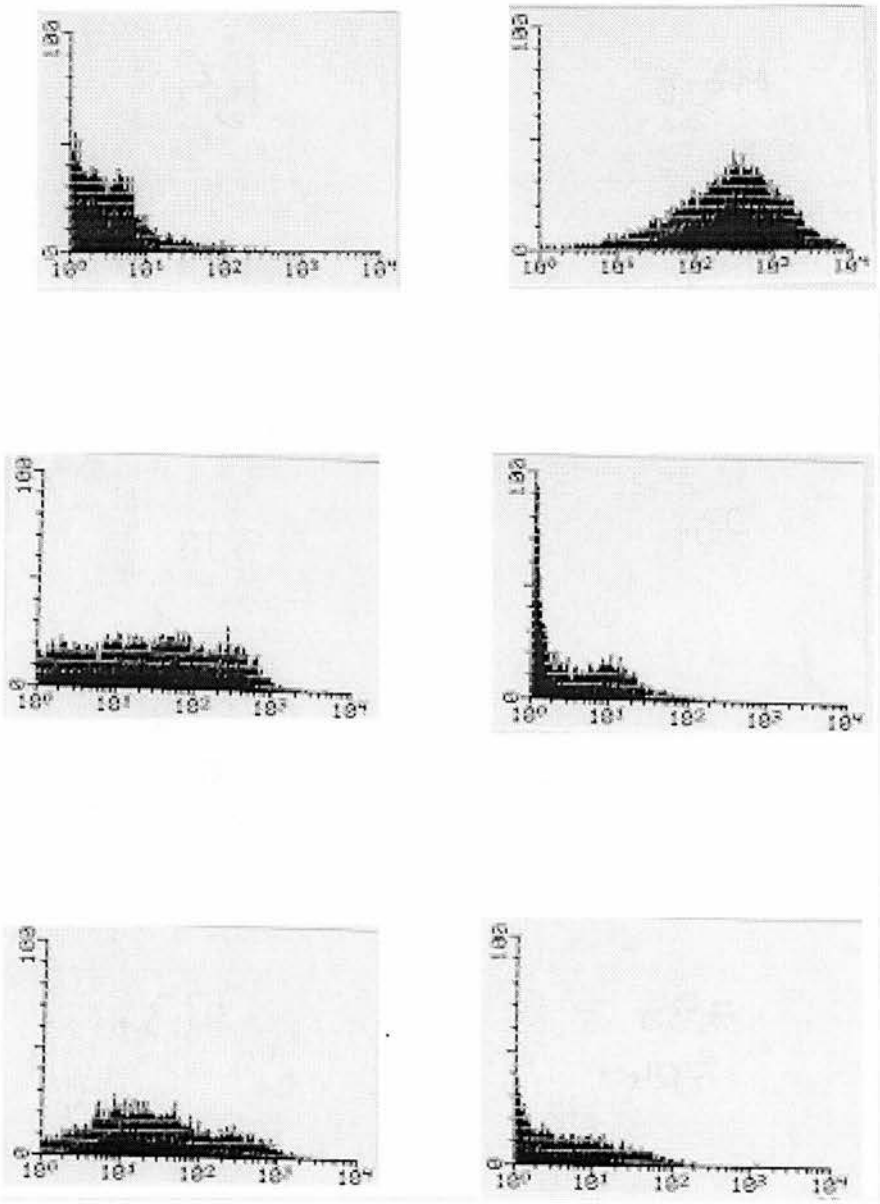


Figure 8.6 Immunofluoresence of alginate cultured bovine chondrocytes. Chondrocytes were isolated from normal cartilage taken from a 12 month old heifer. Cells were cultured in alginate beads for 4 days prior to staining. In each histogram the x-axis represents the fluoresence intensity and the y-axis represents the number of events. Cells were stained with (a.) anti-mouse IgG, diluted 1:5 (b.) MZ15 - anti KS, diluted 1:50 (c.) 3B3(-) - anti CS, diluted 1:50 (d.) 7D4(-) - anti CS, diluted 1:50 (e.) 3B3(+) - anti CS, diluted 1;50 (f.) 7D4(+) - anti CS, diluted 1:50.

8.3.3. The effect of cyclical pressure-induced strain on CS-epitope expression in bovine chondrocytes

Bovine chondrocytes subjected to cyclical pressurisation in the presence and absence of strain as described in section 8.2.3 and stained with 3B3(-) and 7D4 antibodies showed no significant change in the level of mean fluoresence intensity when compared to unpressurised control cells (Table 8.3).

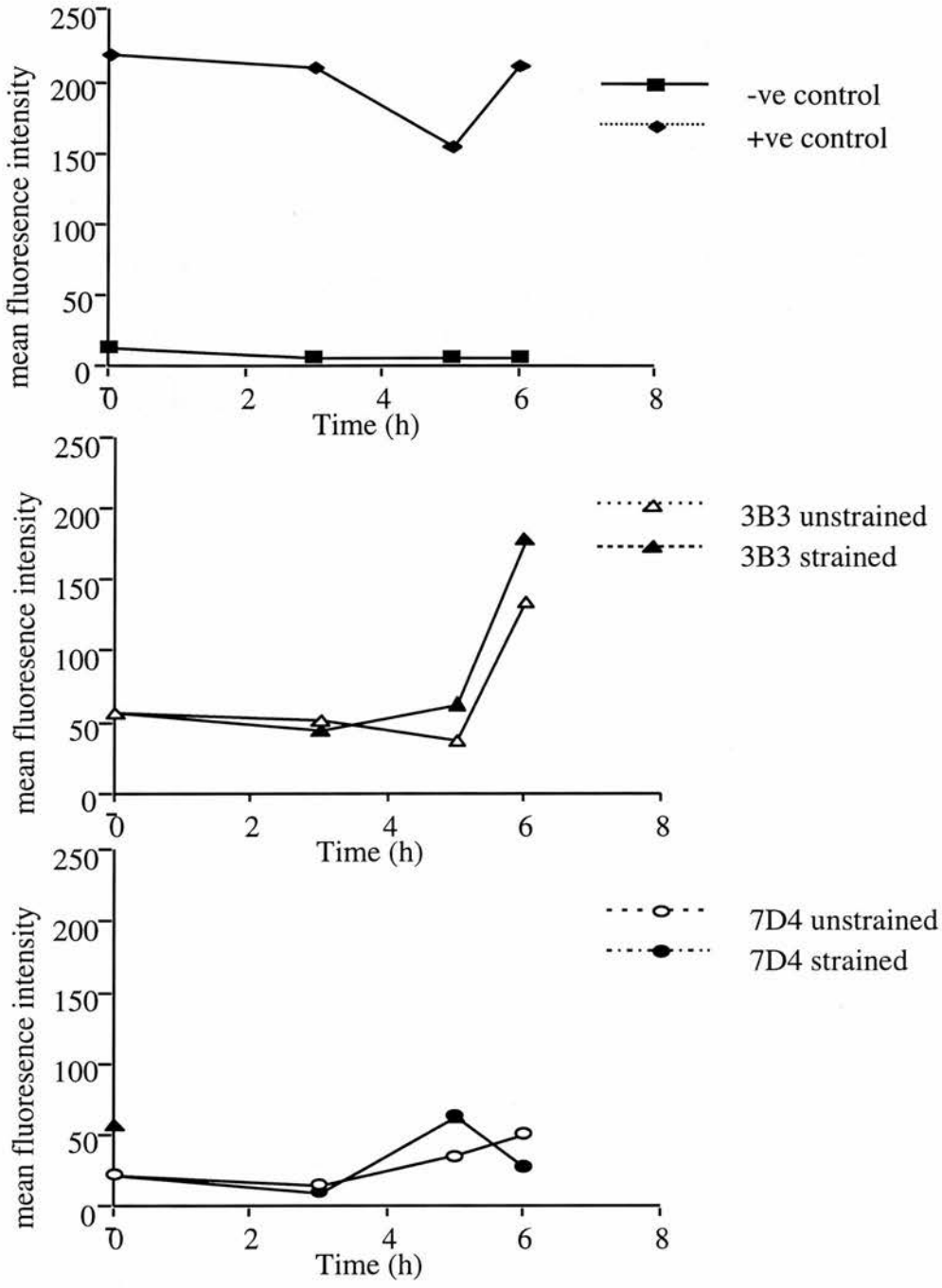
Cells exposed to cyclical PIS and pressurisation for 3, 5 and 6 h showed no consistent response to either stimulus (Fig. 8.7). The expression of the 3B3(-) epitope appears to rise after 6 h of pressurisation although these results only represent one duplicate experiment.

No significant change in the expression of 3B3(-) or 7D4 were seen in cells exposed to either 3 h of cyclical pressurisation or cyclical strain and stained after a 24 h rest period (Table 8.3).

Table 8.3 The expression of CS epitopes by normal bovine chondrocytes before and after cyclical pressurisation with and without strain

	Mean Fluorescence intensity ± SEM			
Antibody	control	3 h unstrained	3 h strained	3 h strained + 24 h
negative	5.48±2.72	ND	3.13±0.977	3.60±1.16
positive	127.30±67.7	ND	220.94±34.0	197.71±29.6
3B3(-)	38.61±6.40	44.71±7.18	34.65±5.84	42.29±3.20
7D4	7.45±4.69	11.75±2.38	8.15±2.41	13.86±6.60

Values are the means (± standard error) of mean fluorescence intensity measured in 4 experiments. ND = not done.



Fiigure 8.7 The effect of cyclical pressurisation and cyclical pressure-induced strain for 0 - 6 h on CS epitope expression. The figures shows the mean fluoresence intensity of individual samples of cells exposed to cyclical pressurisation (open symbols) and cyclical strain (filled symbols) for the times shown. The cells were from one animal and had all been cultured in monlayer for the same time period.

8.4. Discussion

The results of experiments reported in this chapter have demonstrated that indirect immunofluorescence measured by flow cytometry can be used to detect the presence of KS and CS epitopes in isolated human and bovine chondrocytes which have been cultured in monolayer for up to 2 weeks. This is the first time that chondrocyte expression of CS epitopes has been assessed by flow cytometry using these antibodies. Development of this technique in the future may be of value in studies of chondrocyte phenotype and function. In this study the cells were fixed prior to staining but staining of unfixed cells under sterile conditions would allow particular populations of cells to be isolated by using the cell sorting capability of the FACScan. Thus phenotypically distinct subpopulations of chondrocytes could be prepared and cultured for subsequent study. The technique has limited value in detecting changes in the levels of expression of a marker is limited as it measures expression in a large population. This limits its usefulness for measuring small changes in expression, such as might be expected following stimulation of cells with mechanical load. In order to accurately quantify the level of GAG epitopes expressed by chondrocytes a sensitive ELISA technique has been developed and has been applied to measuring 3B3(-) levels in PGs extracted from bovine sesamoid bones exposed to mechanical load (151). This technique could be applied to measure the level of the 3B3(-) and 7D4 epitope in PG extracts of chondrocytes pressurised in the apparatus used in this study.

Keratan sulphate is only expressed in corneal and articular cartilage (12) and hence can be used as a phenotypic marker for adult articular chondrocytes. Its expression in bovine and human chondrocytes cultured in monolayer for 2 weeks and 12 days respectively confirms that these cells have maintained at least some features of their chondrocyte phenotype.

Human chondrocytes from normal cartilage stained positively for 3B3 but not 7D4, whereas chondrocytes from OA cartilage cultured in alginate beads

demonstrated increased staining for both 3B3 and 7D4. These are preliminary results from a small number of samples but may indicate that flow cytometry can be used to measure the expression of epitopes which are markers for OA, in cultured cells and that the expression of these epitopes is maintained in culture. It is also possible that the increased level of expression in the OA cells resulted from culture in alginate beads. Increased staining in the OA cells may simply be due to the presence of a larger pericellular matrix in alginate-cultured cells. Bovine chondrocytes cultured in alginate beads also showed high levels of staining for 3B3(-) but not for 7D4. The positive staining for 3B3(-) epitope in chondrocytes from macroscopically normal cartilage could occur because (a) damage to CS structure occurred during the isolation and culture procedures, (b) early disease changes were present in the cartilage which were not yet visible as macroscopic tissue damage (360), (c) the epitopes are expressed at low levels in some individuals (356). Further study is required to confirm these findings and to assess the expression of these epitopes in greater detail in chondrocytes from diseased cartilage. The level of expression of these epitopes in freshly isolated and cultured chondrocytes derived from cartilage from individuals in a range of age groups and disease states should be measured and correlated to cartilage histology. In the future it will be important to standardise culture conditions as the period in culture may have an important influence on the level of expression.

The CS epitopes were not expressed in freshly isolated bovine articular chondrocytes. Positive staining for 3B3 and 7D4 epitopes was detected at low levels after 3 and 4 days in culture, suggesting that the epitopes may not be present after isolation with collagenase (section 4.3., p51) but may be re-expressed in culture. The use of chondroitinase to produce the 3B3(+) epitope provides another positive control for the cross-reactivity of these antibodies which were raised against human CS-epitopes. It should be noted that the bovine chondrocytes were isolated from cartilage taken from animals which were not fully mature and so the chondrocyte

phenotype may be different from that in mature cartilage (2). The expression of these epitopes has previously been reported in developmental tissue (255) so the expression of the epitopes in bovine chondrocytes in this study may occur for the same reason. The precise age of animals from which cartilage was taken was not known but may be important to future studies.

In the present study no changes in CS-epitope expression were detectable in bovine chondrocytes following 3 h pressurisation or PIS. It is possible that small changes in CS epitope expression occurred but were not detectable using the flow cytometry technique, which is at best semi-quantitative. A comprehensive time-course of pressurisation for much longer periods up to 72 h, and larger numbers of experiments are required before firm conclusions can be drawn. It may be that long periods of loading and much higher pressures are required to produce changes that may be detected by flow cytometry. Expression of 3B3(-) was previously demonstrated following 3 days of cartilage compression (151) but the apparatus used in the present series of experiments does not lend itself to such a study because it uses nitrogen as the pressurising gas and subjects cell cultures to anoxia for long time periods. In order to study the effect of strain on the expression of these epitopes in cultured chondrocytes further apparatus will be required which only exposes cells to altered strain.

The development of indirect immunofluorescence to study the expression of CS epitopes on cultured chondrocytes is, however, potentially useful as a tool for future cartilage research. It provides a relatively simple semi-quantitative method of assessing the phenotype of cultured chondrocytes, and allows the sorting of positively stained cells from negative cells. This could be used to identify other phenotypic changes in chondrocytes which accompany the expression of either 3B3 or 7D4.

9. GENERAL DISCUSSION

Proteoglycan metabolism in articular cartilage is critically dependent on mechanical stimulation of chondrocytes.

This thesis set out to investigate how chondrocytes detect, transmit and transduce mechanical forces to regulate matrix synthesis.

In order to achieve these objectives methods were developed which allowed the examination of the effects of cyclical pressurisation and pressure-induced strain on rates of PG synthesis by chondrocytes in culture. Preliminary evidence was obtained which suggests that gadolinium sensitive, stretch-activated ion channels and β_1 integrins are critically involved in the signal transduction processes.

9.1. Summary of results

9.1.1. The use of radiolabelling to measure PG synthesis in chondrocyte cultures

$[^{35}SO_4]$-sulphate incorporation was used to measure PG synthesis in primary cultures of human and bovine articular chondrocytes, and in immortalised human chondrocyte cell lines. The variability in $[^{35}SO_4]$-sulphate incorporation rates, however, made statistical analyses difficult. This variability may be due to a combination of many factors including the age and disease state of the individual from which the chondrocytes were isolated, and the zonal location of the cartilage from which the chondrocytes were isolated. Significant variability was also seen in $[^{35}SO_4]$-sulphate incorporation rates measured in immortalised human chondrocyte cell lines. This would suggest that the variability in rates of PG synthesis originates in small changes in culture conditions, such as the concentration of growth factors from serum that are altered over time in culture. The correlation between time in culture and the rate of synthesis seen in both human and bovine chondrocytes would appear to confirm this conclusion. In practice the wide range of PG synthesis rates

obtained with cultured articular chondrocytes is a largely unavoidable problem which can be best overcome by using large sample numbers to facilitate a more meaningful statistical analysis.

The use of different labelling regimes in this study made the analysis of results more difficult than would otherwise have been necessary. This problem arose when it became apparent that the original pressurisation apparatus was not suitable for extensive biochemical studies since only one dish could be pressurised at a given time. In designing a new apparatus it seemed logical to try and use more physiological conditions and for this reason the pressure conditions and temperature were altered. Mechanical stimulation time were also subsequently changed to reduce possible deleterious effect of exposure to pure nitrogen at higher pressures. Thus the pressurisation studies provide $[^{35}SO_4]$-sulphate incorporation data for chondrocytes labelled under different time and temperature conditions. All labelling regimes were equally successful but the use of different experimental conditions in the original and the new apparatus mean that care must be exercised when comparing data from different groups since chondrocyte metabolism and ion channel function may be different at different temperatures and under different partial pressures of nitrogen.

Treatment of the labelled product with chondroitinase ABC and keratanase/keratanase II demonstrated that the main GAGs being labelled under the culture conditions used were KS and CS. The immunostaining of chondrocytes for flow cytometry confirmed the presence of both these GAGs in the cell associated matrix of cultured human and bovine chondrocytes. The ratio of $[^{35}SO_4]$-sulphate incorporation into CS and KS in both human and bovine chondrocyte cultures was broadly consistent with that seen in aggrecan, the predominant PG in articular cartilage (2,258,259). It would be of interest to carry out an extensive analysis of the relative amounts of CS and KS before and after pressurisation since changes in the CS : KS ratio occur in ageing and joint disease (2).

9.1.2. The effects of cyclical pressurisation and strain on PG synthesis rates

Cyclical pressurisation in the pressurisation apparatus was initially thought to influence chondrocyte metabolism by the action of pressure alone. It subsequently became clear that the apparatus subjected chondrocytes to pressure-induced-strain and it was thought that PIS alone was responsible for the upregulation of PG synthesis. By using a variety of different loading conditions it was eventually demonstrated that both pressure and strain alter chondrocyte PG synthesis.

Measurement of [$^{35}SO_4$]-sulphate incorporation rates in unstrained human and bovine chondrocytes exposed to intermittent pressurisation in nitrogen indicated that pressurisation decreases PG synthesis. This effect was only seen, however, following exposure to pressure pulses for periods of less than 6 h. It seems unlikely that this response to short term cyclical pressurisation was due to changes in metabolism resulting from exposure to altered partial pressures of nitrogen, oxygen, or carbon dioxide. Cyclical hydrostatic pressurisation acts to alter different steps in the synthesis of PGs in different ways. Short term pressurisation may decrease PG synthesis by inhibiting translational or post-translational steps, whereas more prolonged pressurisation may increase PG synthesis by increasing gene expression (158).

The studies have demonstrated accelerated PG synthesis in response to cyclical pressure-induced strain under a variety of different conditions in human chondrocytes, immortalised human chondrocyte cell-lines, and bovine chondrocytes. The increase in PG synthesis was greatest in chondrocyte cell lines but reached significance in human chondrocytes and bovine chondrocytes. The magnitude of the deformation and strain induced in these experiments was far less than those which can occur in cartilage *in vivo* (121) but the experiments demonstrate that cultured chondrocytes can respond to levels of strain several orders of magnitude lower than the peak strains induced in cartilage *in vivo*.

9.1.3. The identification of potential mechanotransduction mechanisms in chondrocytes

The results presented in this study indicate that SA ion channels are involved in the events that lead to increased PG synthesis following cyclical PIS. Calcium ion influx through SA ion channels in response to chondrocyte membrane deformation has been previously demonstrated(134,313). The influx of calcium through SA-ion channels 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. The application of calcium ionophores such as A23187 and calcium chelators such as EDTA and BAPTA-TM to chondrocyte cultures during pressurisation would provide information regarding the role of calcium influx in the events that lead to increased PG synthesis. Further investigation of the role of stretch-activated ion channels in chondrocyte mechanotransduction will require patch-clamp technology.

The role of integrin-ECM linkage in the mechanotransduction processes that leads to accelerated PG synthesis was investigated by using RGD peptides and anti-integrin antibodies. These preliminary studies demonstrated that increased PG synthesis by chondrocytes in response to cyclical PIS was unaltered by RGD peptides and was not modulated by anti-integrin antibodies to a statistically significant degree. However the data indicated that the adhesion-enhancing anti-β_1 antibody TS2/16 may enhance the response to PIS both in the presence and absence of serum. The adhesion inhibiting anti-β_1 antibody P_4C_{10} produced conflicting results in the presence and absence of serum. Taken together these results do suggest that β_1-integrins may be involved in the mechanocoupling or mechanotransduction mechanisms that lead to increased PG synthesis in response to cyclical PIS.

A limited number of experiments were performed using the anti-integrin antibodies but the results are sufficiently interesting to merit further study. In future

studies the effect of these antibodies should be optimised by pre-incubating chondrocytes with the antibody for approximately one hour prior to pressurisation. It would also be of interest to examine the effects of an agent such as cytochalasin D to disrupt the chondrocyte cytoskeleton in order to fully investigate the role of the ECM-integrin-CSK pathway in the mechanotransduction / transmission pathways leading to PG synthesis. In recent studies magnetic beads coated with RGD-peptides have been used to stimulate cultured fibroblasts directly (274). This technique could provide a method for exposing chondrocytes to a defined mechanical stimulus via their integrin receptors and so provide direct evidence for a role for integrins in chondrocyte mechanotransduction.

9.1.4. The effect of cyclical pressurisation and strain on chondrocyte cAMP levels

An in house radioimmunoassay was successfully used to measure intracellular cAMP levels in cultured chondrocytes. These levels increased approximately ten fold following exposure to the adenylyl cyclase activator forskolin. It was not however possible to measure significant amounts of cGMP in chondrocytes, suggesting that cGMP, if present at all in chondrocytes, is present at levels below the range of the assay used (0.06 - 32 nM)

The measurement of cAMP in chondrocytes exposed to cyclical pressurisation and PIS demonstrated that exposure of chondrocytes to cyclical pressurisation and cyclical strain, in the presence of PD inhibitor (IBMX), lead to an increase in intracellular cAMP after 2 min. Further exposure to cyclical strain for up to 1 h lead to a decrease in intracellular cAMP. Previous studies have demonstrated similar increases in the intracellular concentration of cAMP in chondrocytes (166,169,171). These studies did not use IBMX or other phosphodiesterase inhibitors. In order to investigate further the possible role of cAMP in the events that lead to accelerated PG synthesis in this study further experiments should be performed in the absence of

PD inhibitors. The break down of cyclic nucleotides prior to assay can be avoided by using serum free media since serum contains significant amounts of PDs.

The effect of elevated intracellular cAMP levels on [$^{35}SO_4$]-sulphate incorporation in chondrocytes was briefly investigated by exposure to forskolin for 3 h and 24 h. No change in [$^{35}SO_4$]-sulphate incorporation was seen following the shorter incubation period but there was a non-significant rise of approximately 50 % after 24 h stimulation. These results did not reach significance as only three experiments were performed, but similar increases have been previously reported (351). This would seem to indicate that cAMP can activate chondrocyte PG synthesis but its role in the normal regulation of PG synthesis is unknown.

9.1.5. The measurement of specific GAG epitopes by immunostaining and flow cytometry

The use of immunostaining and flow cytometry was successfully developed in this study to measure the expression of KS and CS epitopes in isolated human and bovine chondrocytes which have been cultured in monolayer or alginate. This is the first time that that these antibodies have been used in conjunction with flow cytometry. Development of this technique in the future may be of value in studies of chondrocyte phenotype and function.

The method is not suitable for measuring small changes in expression of specific epitopes, but would be sufficiently sensitive to measure an increases of the order of 1 to 2 fold . In order to quantify the level of GAG epitopes expressed by chondrocytes more accurately, a sensitive ELISA technique has been developed and has been used to measure 3B3(-) levels in PGs extracted from bovine sesamoid bones exposed to mechanical load (151). This technique would have been the method of choice for measuring the level of the 3B3(-) and 7D4 epitope in PG extracts of chondrocytes following cylical pressurisation and PIS.

As the expression of keratan sulphate is limited to the cornea and articular cartilage (12), this can be used as a phenotypic marker for adult articular chondrocytes. Its expression in bovine and human chondrocytes cultured in monolayer for 2 weeks and 12 days respectively indicates some maintenance of the chondrocyte phenotype.

Human chondrocytes from normal cartilage stained positively for 3B3 but not 7D4, whereas chondrocytes from OA cartilage cultured in alginate beads demonstrated increased staining for both 3B3 and 7D4. These are preliminary results from a small number of samples but do suggest that flow cytometry can be used to measure the expression of epitopes that are markers for OA in cultured cells.

The CS epitopes were not expressed in freshly isolated bovine articular chondrocytes. Positive staining for 3B3 and 7D4 epitopes was detected at low levels after 3 and 4 days in culture, suggesting that the epitopes may not be present after isolation with collagenase (section 4.3) but may be re-expressed in culture. Bovine chondrocytes cultured in alginate beads also showed high levels of staining for 3B3(-) but not for 7D4.

No change in CS-epitope expression was induced by 3 h pressurisation or strain of bovine chondrocytes. A comprehensive time-course of pressurisation for much longer periods up to 72 h, and larger numbers of experiments would be required before firm conclusions could be drawn. It seams very likely that long periods (days) of loading would be required to produce changes that may be detected by flow cytometry.

9.2. General conclusions

In these studies it has been demonstrated that an apparatus which uses pressure to induce strain on a monolayer of cultured chondrocytes stimulates PG synthesis during cyclical pressurisation. This was observed in primary cultures of human and bovine articular chondrocytes and in immortalised human costal cartilage

chondrocyte cell lines. Increases in PG synthesis during cyclical strain were abolished by blocking stretch-activated ion channels with 10 μM gadolinium. These experiments provided the first evidence that stretch-activated ion channels are involved in the signal transduction processes that lead to accelerated PG synthesis following cyclical deformation of chondrocytes.

Preliminary experiments indicated that the reponse to cyclical strain could also be modulated with anti-integrin antibodies . Chondrocytes treated with adhesion-blocking anti-α5 and anti-β1 integrin antibodies showed a reduced response to strain, whereas those treated with an adhesion enhancing anti-β1 integrin antibody resulted in an increased response. These results implicate α5β1 integrin, which is the cell surface receptor for fibronectin, as a mechanotransducer in chondrocytes.

Stimulation of cultured human chondrocytes produced a short term increase in chondrocyte cAMP levels but no consistent long term changes. The intracellular concentration of cAMP in chondrocytes was increased 10 fold following treatment of chondrocytes with the adenylate cyclase activator forskolin but this was not influenced by PIS. Treatment of chondrocytes with Forskolin over a 24 h period increased PG synthesis. These results suggest that cAMP might be involved in activating PG synthesis but is probably not involved in the increase in PG synthesis that occurs during cyclical PIS.

Enzymatic analyses of the GAGs produced by cultured human chondrocytes demonstrated similar ratios of KS and CS as those of aggrecan, the predominant PG present in articular cartilage. The presence of these GAGs was confirmed by immunostaining and flow cytometry. The disease-related CS epitopes 3B3(-) and 7D4 were successfully measured by flow cytometry but were unaltered by cyclical pressurisation or strain under the experimental conditions used in the studies described in this thesis.

The results of these studies lead to a hypothesis for the mechanisms involved in the modulation of PG synthesis in chondrocytes following mechanical stimulation.

Mechanical forces acting on the cell surface are transmitted by cell surface receptors such as integrins to effector molecules within the cell (274). These effector molecules will include the stretch activated ion channels which open in response to mechanical stimulation to allow the influx of calcium ions (313). The elevation of intracellular calcium will activate a number of phosphorylation pathways in the cell (320,323) which ultimately lead to the activation of nuclear transcription factors and increased proteoglycan expression. The cell surface integrin receptors may in addition directly activate signalling pathways by regulating kinase activity at the cell surface (277) . Integrin activated pathways may interact with pathways triggered by other signal transdcution molecules (268). This hypothetical model is consistent with the signal transduction pathways that have been implicated in the electrophysiological response of human chondrocytes to cyclical stretch (238-241,276).

9.3. Future prospects

Further studies of the regulation of chondrocyte PG synthesis by mechanical forces will be greatly facilitated by the use of experimental apparatus which exposes cells to physiological strain without accompanying changes in pressure and dissolved gases.

The role of stretch-activated ion channels and calcium activated potassium channels, in the regulation of chondocyte metabolism by mechanical load should be investigated. This can be done quite simply in the first instance by the use of commercially available ion channel blockers, ionophores, and chelators and studies of this type would be directly comparable to the well established elctrophysiological responses to cyclical strain (238-240). In order to make investigations of the regulation of PG synthesis comparable to previous electrophysiological studies (238-

240) it would be necessary to load cells for the same time period (20 min) as that used in those studies.

Recent studies have shown that chondrocytes release Il-4 in response to a short period of cyclical strain and that this mediates the membrane hyperpolarisation that follows cyclical strain (363). This creates the exciting possibility that the release of an autocrine factor may be a central part of the chondrocyte response to mechanical stimulation. The role of Il-4 in regulation of PG synthesis should be an area for urgent investigation.

Areas of investigation which will be important to our gaining fuller understanding of mechanical regulation of chondocyte metabolism in the future should include:

(a) signal transmission via adhesion molecules such as integrins, and the cytoskeleton

(b) signal transduction by SA ion channels, adhesion molecules, and possible intracellular mechanoreceptors

(c) the role of autocrine factors released by mechanical stress

(d) the identification of phosphorylation cascades such as that described for Endothelial cells.

(e) the role of immediate early genes.

(f) the identification of possible mechanical stress response elements in the promoters of genes involved in chondrocyte metabolic responses.

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APPENDIX 1

Publications

Published papers

Effects of intermittent pressure induced strain on the electrophysiology of cultured human chondrocytes: evidence for the presence of stertch-activated ion channels . Wright MO, Jobanputra P, Bavington C, Salter DM, Nuki G. Clinical Science (1996) **90**, 61-71. ISSN 0143-5221.

Modulation of human chondrocyte integrins by inflammatory synovial fluid. Jobanputra P, Lin H, Jenkins K, Bavington C, Brennan FR, Nuki G, Salter DM, Godolphin JL. Arthritis & Rheumatism (1996) **39**, 1430 - 1434. ISSN 0004-3591.

Published Abstracts

Accelerated proteoglycan synthesis in human chondrocytes following cyclical pressure is mediated by β1 integrins and stretch-activated ion channels. Bavington C, Wright MO, Jobanputra P, Lin H, Morrison PT, Brennan FR, Salter DM, Nuki G. BJR (1996) **35**(supp), 110(No.2 11).

Interactions of soluble fibronectin with isolated human articular chondrocytes. Jobanputra P, Lin H, Brennan FR, Pinder S, Bavington C, Salter DM, Nuki G. BJR (1996) **35**(supp), 62(No.117).

Accelerated proteoglycan synthesis in human articular chondrocytes following pressure induced mechanical strain; regulation by Gd^{3+}-sensitive stretch-activated ion channels. Bavington C, Wright MO, Jobanputra P, Brennan FR, Salter DM, Nuki G. Bone (1995) **17**(supp.), 592 (No.133). ISSN 8756-3282.

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A role for $\alpha 5\beta 1$ integrin in the signal transduction pathways in human chondrocytes activated by cyclical pressure-induced strain. Salter DM, Wright MO, Dunne E, Godolphin JL, Bavington C, Jobanputra P, Nuki G. Bone (1995) **17**(supp.), 593 (No.138).

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Oral presentations

Gadolinium blockade of membrane hyperpolarisation and accelerated proteoglycan synthesis following intermittent pressurisation of human chondrocytes: evidence for the involvement of stretch activated ion channels. Scottish Membrane Transport Group, April 1995, Dundee.

Accelerated proteoglycan synthesis in human chondrocytes following cyclical pressure is mediated by β1 integrins and stretch-activated ion channels. Bavington C,

Wright MO, Jobanputra P, Lin H, Morrison PT, Brennan FR, Salter DM, Nuki G. British Society for Rheumatology, May 1996, Brighton.

Submissions

Stretch-activated ion channels regulate accelerated proteoglycan synthesis in human chondrocytes following cyclical pressure induced strain. Bavington C, Wright M, Jobanputra P, Salter D, Nuki G. Submitted to Arthritis & Rheumatism (1997).

Expression of integrins, CD44 isoforms and cell associated fibronectin isoforms in osteoarthritis. Jobanputra P, Lin H, Pinder S, Bavington C, Salter DM, Nuki G. Submitted to Arthritis and Rheumatism (1997).

Binding of soluble plasma fibronectin to isolated human articular chondrocytes. Lin H, Jobanputra P, Bavington C, Pinder S, Salter DM, Nuki K. Submitted to British Journal of Rheumatology (1997).

Hyperpolarisation of cultured human chondrocytes following cyclical pressure-induced strain: evidence for a role for $\alpha_5\beta_1$ integrin as a chondrocyte mechanoreceptor. Wright MO, Bavington C, Godolphin JL, Dunne E, Walmsley S, Jobanputra P, Nuki G, Salter D. Submitted to J. Orthop. Res. (1996).