CHARACTERIZATION OF PROTEOGLYCAN ACCUMULATION DURING THE FORMATION OF CARTILAGENOUS TISSUE IN VITRO

BY

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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0-612-41109-5
Characterization of Proteoglycan Accumulation during the Formation of Cartilagenous Tissue \textit{in vitro}  
Doctor of Philosophy, 1999  
Jennifer Mae Boyle  
Laboratory Medicine and Pathobiology, University of Toronto

We developed a cell culture system in which bovine articular chondrocytes grown on filter inserts accumulate extracellular matrix (ECM) and form a continuous layer of cartilagenous tissue. The aim of this study was to determine if the tissue formed \textit{in vitro} had features similar to \textit{in vivo} cartilage. In these cultures, the chondrocytes synthesize large aggregating proteoglycans (PG) and type II collagen characteristic of cartilage. Collagen and cells, in the superficial zone of the filter culture generated tissue, were oriented parallel to the surface whereas the collagen surrounded the chondrocytes in the deeper zone similar to the \textit{in vivo} organization. The PG and collagen content increased until the ECM contained threefold more collagen than PG, similar to \textit{in vivo} cartilage. After 35 days, there was no detectable increase in the amount of PG, collagen or tissue thickness. These cultures demonstrated two phases: matrix accumulation (<35 days) and then one in which the matrix is maintained (>35 days). The newly synthesized PG retained within the matrix (PG-R) and those not retained (PG-NR) were characterized. Column chromatography (Sepharose CL-2B) showed that the PG eluted as broad peaks. The average hydrodynamic size of PG-R was smaller (Kav 0.44±0.02) than PG-NR (Kav 0.23±0.02). PG-NR demonstrated a lower ability to bind to hyaluronate which may contribute to their decreased retention. The glycosaminoglycan (GAG) chain lengths of PG-R and PG-NR were similar but the hexosamine/protein content suggested a higher degree of GAG substitution in PG-NR as compared with PG-R. The core protein size(s) were determined by SDS-PAGE and autoradiography after deglycosylation. PG-R demonstrated bands at 187, 225, 300, and 500 kD while PG-NR showed one band at 500 kD. Amino acid analysis, size of peptides generated by trypsin, and internal sequencing (two peptides) of the 500 kD protein were consistent with aggrecan suggesting that the 500 kD band is likely a dimer of the 225 kD core protein. The different sizes of PG-R and PG-NR are in keeping with post-translational modifications similar to those that occur \textit{in vivo}. Based on the similarities identified to date, filter cultures provide a unique method to study chondrocyte metabolism, matrix organization, and macromolecule interactions.
Acknowledgements

I would first like to sincerely thank my thesis supervisor, Dr. Rita Kandel, for her excellent teaching and guidance over the course of my degree. I would like to thank my committee members for their helpful comments and suggestions: Dr. Tony Cruz, Dr. Fred Keeley, Dr. Doug Templeton, and Dr. Howard Tenenbaum. I also wish to thank Dr. Graeme Hunter and Dr. Marc Grynpas for being the external examiners of my thesis.

Chandra Boon and Rey Interior from the Hospital for Sick Children’s Biotechnology Service Centre were invaluable in the sequencing analysis, hexosamine and amino acid analyses. Nelson Amoranto provided me with the histological sections of the cultures. The members of our laboratory have been a great assistance and wonderful friends. I would especially like to thank Hanje Chen and Mary Speagle.

I would like to express my deepest thanks to my family for their constant love and support. They always encouraged me to pursue my goals. A special thanks to my friends Maria Mendes, Sandra Daley, Nancy Podworny, Angeline Sarabura, Gabi Wolff, Michael Pollanen, and David Swanson. Thank you for your patience, understanding, and encouragement!

Finally, I would like to acknowledge the publishers of Osteoarthritis and Cartilage for giving me permission to reproduce the article: Boyle J, Luan B, Cruz TF, and Kandel R (1995) Characterization of proteoglycan accumulation during the formation of cartilagenous tissue in vitro, Osteoarthritis and Cartilage, 3: 117-125.
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>β-GP</td>
<td>beta-glycerophosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CL</td>
<td>cross linked</td>
</tr>
<tr>
<td>cmd</td>
<td>cartilage matrix deficiency</td>
</tr>
<tr>
<td>CMP</td>
<td>cartilage matrix protein</td>
</tr>
<tr>
<td>COMP</td>
<td>cartilage oligomeric matrix protein</td>
</tr>
<tr>
<td>CS</td>
<td>chondroitin sulphate</td>
</tr>
<tr>
<td>DMMB</td>
<td>dimethylmethylen blue</td>
</tr>
<tr>
<td>DS</td>
<td>dermatan sulphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiotreitol</td>
</tr>
<tr>
<td>E:S</td>
<td>enzyme substrate ratio</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbt assay</td>
</tr>
<tr>
<td>F</td>
<td>filter</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>gal</td>
<td>galactosamine</td>
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<tr>
<td>glu</td>
<td>glucosamine</td>
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<td>GuHCl</td>
<td>guanidine hydrochloride</td>
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<td>HA</td>
<td>hyaluronic acid/ hyaluronan</td>
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<td>HABR</td>
<td>hyaluronic acid binding region</td>
</tr>
<tr>
<td>HCI</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>IGD</td>
<td>interglobular domain</td>
</tr>
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<td>Kav</td>
<td>partition coefficient</td>
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<td>kD</td>
<td>kilodalton</td>
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<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>membrane type 1-matrix metalloproteinase</td>
</tr>
<tr>
<td>Mw</td>
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</tr>
<tr>
<td>NBT/BCIP</td>
<td>nitroblue tetrazolium / 5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>NFDM</td>
<td>non fat dry milk</td>
</tr>
<tr>
<td>NR</td>
<td>non-retained</td>
</tr>
<tr>
<td>OA</td>
<td>osteoarthritis</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAPS</td>
<td>phosphoadenosine phosphosulphate</td>
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<tr>
<td>PG</td>
<td>proteoglycan</td>
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<td>PGA</td>
<td>polyglycolic acid</td>
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<td>PG-NR</td>
<td>proteoglycan not retained in the matrix</td>
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<tr>
<td>PG-R</td>
<td>proteoglycan retained in the matrix</td>
</tr>
<tr>
<td>PI</td>
<td>protease inhibitors</td>
</tr>
<tr>
<td>PNP</td>
<td>p-nitrophenol</td>
</tr>
<tr>
<td>PTH</td>
<td>phenolthiohydantoin</td>
</tr>
<tr>
<td>PTR</td>
<td>proteoglycan tandem repeat</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>R</td>
<td>retained</td>
</tr>
<tr>
<td>rpm</td>
<td>rotations per minute</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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<tr>
<td>TFMS</td>
<td>trifluoromethane sulphon acid</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)amino-methane</td>
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<td>Ala</td>
<td>A</td>
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<tr>
<td>Val</td>
<td>V</td>
</tr>
<tr>
<td>Leu</td>
<td>L</td>
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<tr>
<td>Ile</td>
<td>I</td>
</tr>
<tr>
<td>Met</td>
<td>M</td>
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<td>F</td>
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<td>Pro</td>
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<td>Thr</td>
<td>T</td>
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<td>Asp</td>
<td>D</td>
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<td>E</td>
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<tr>
<td>Lys</td>
<td>K</td>
</tr>
<tr>
<td>Arg</td>
<td>R</td>
</tr>
<tr>
<td>His</td>
<td>H</td>
</tr>
</tbody>
</table>

Gly = glycine  
Ala = alanine  
Val = valine  
Leu = leucine  
Ile = isoleucine  
Met = methionine  
Phe = phenylalanine  
Trp = tryptophan  
Pro = proline  
Ser = serine  
Thr = threonine  
Cys = cysteine  
Tyr = tyrosine  
Asn = asparagine  
Gln = glutamine  
Asp = aspartic acid  
Glu = glutamic acid  
Lys = lysine  
Arg = arginine  
His = histidine
Chapter One: Literature Review

1.1 Articular Cartilage

1.1.1 Composition, Structure and Organization of Articular Cartilage

Articular cartilage is a specialized connective tissue found at the end of articulating bones. It provides a virtually frictionless surface for articulation and functions to withstand compressive forces. It is avascular, aneural, and alymphatic. It is composed primarily of extracellular matrix elaborated and maintained by a sparse population of resident chondrocytes. The extensive extracellular matrix is composed mainly of proteoglycans interspersed in a collagen fibrillar network. Water is a major component of articular cartilage, contributing 65-80% to the tissue's wet mass. Collagens (10-30% of wet weight) and proteoglycans (5-10% wet weight) are the principle organic components of the extracellular matrix. Noncollagenous matrix proteins make up the rest of the extracellular matrix. The physical and mechanical properties of articular cartilage depend on the composition and organization of the extracellular matrix macromolecules (Hardingham, 1990). Articular cartilage is divided into four zones: 1) superficial (tangential), 2) intermediate (middle, transitional), 3) deep (radial), and 4) calcified. Calcified cartilage is the interface between articular cartilage and the underlying subchondral bone in synovial joints (Oegema and Thompson, 1992). Each zone varies in its relative depth from the surface, composition of extracellular matrix, and in its cellular morphological features.
1.1.2 Chondrocytes

Chondrocytes are derived from the mesoderm. Mesenchymal cells condense to form cartilage and terminally differentiate into chondrocytes. Chondrocytes occupy only 2-10% of the tissue's total volume (Kuettner, 1991). Superficial chondrocytes appear flattened, whereas chondrocytes within the deeper zones are rounded in morphology. Chondrocytes are surrounded by extracellular matrix and have very little if any cell-cell contact. Chondrocytes have a limited ability to replicate (Aydelotte et al, 1992). The pericellular matrix of the chondrocyte consists of hyaluronic acid, aggregcan and type VI collagen forming a basket-like structure around the cell (Hambach et al, 1998). Subpopulations of chondrocytes have been demonstrated in the different zones of articular cartilage, in terms of both their morphology and their synthetic activity (Archer et al, 1990). For example, deep chondrocytes are alkaline-phosphatase positive cells (Xu et al, 1994) and they are responsible for synthesizing the matrix that develops into the calcified cartilage. The chondrocyte is responsible for the synthesis of matrix components; the incorporation and organization of these components into the matrix; and their degradation and loss from the matrix. The balance of these biological functions results in the maintenance of the tissue, however, disruption of the balance results in the remodelling of the extracellular matrix (ECM), or degradation of that matrix (for example osteoarthritis).
Table 1.1: Extracellular Matrix of Articular Cartilage

<table>
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<th>Component</th>
<th>Molecular Weight (kD)</th>
<th>Function</th>
<th>References</th>
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<tr>
<td><strong>Large proteoglycans</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>aggreccan</td>
<td>220</td>
<td>compressive properties, resilience</td>
<td>Doege et al, 1991</td>
</tr>
<tr>
<td>versican</td>
<td>200-400</td>
<td>compressive properties, resilience</td>
<td>Grover and Roughley, 1993</td>
</tr>
<tr>
<td>perlecain</td>
<td>400</td>
<td>cell adhesion to matrix</td>
<td>SundarRaj et al, 1995</td>
</tr>
<tr>
<td><strong>Small proteoglycans</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>biglycan</td>
<td>38</td>
<td>function unknown</td>
<td>Fosang and Hardingham, 1996</td>
</tr>
<tr>
<td>decorin</td>
<td>36</td>
<td>modulates collagen fibrillogenesis</td>
<td>Kuc and Scott, 1997</td>
</tr>
<tr>
<td>fibromodulin</td>
<td>59</td>
<td>modulates collagen fibrillogenesis</td>
<td>Plaas et al, 1990</td>
</tr>
<tr>
<td>link protein</td>
<td>45</td>
<td>stabilizes interaction between aggregating proteoglycans and hyaluronan</td>
<td>Doege et al, 1986</td>
</tr>
<tr>
<td>fibronectin</td>
<td>550-dimer</td>
<td>interacts with collagen type II and CS-PG promotes cell attachment via RGD sequence</td>
<td>Johansson, 1996</td>
</tr>
<tr>
<td>tenascin</td>
<td>220 / 320</td>
<td>function unknown</td>
<td>Savarese et al, 1996</td>
</tr>
<tr>
<td>hyaluronic acid</td>
<td>1-4 x 10³</td>
<td>formation of aggregates with aggregating proteoglycans</td>
<td>Hardingham and Muir, 1972</td>
</tr>
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<td>COMP</td>
<td>36</td>
<td>localized to territorial matrix</td>
<td>Hedbom, 1992</td>
</tr>
<tr>
<td>CMP</td>
<td>148</td>
<td>interacts with aggrecan core (E2) forms filmentous network may function as matrix receptor</td>
<td>Hauser et al. 1996</td>
</tr>
<tr>
<td>thrombospondin</td>
<td>150</td>
<td>binds cells via RGD sequence</td>
<td>Miller and McDevitt, 1995</td>
</tr>
<tr>
<td>anchorin</td>
<td>34</td>
<td>binds collagen type II localized on plasma membrane may mediate anchoring of collagen to chondrocytes</td>
<td>Pfaffle et al, 1990</td>
</tr>
</tbody>
</table>
1.1.3 Extracellular Matrix of Articular Cartilage

Collagen and proteoglycans are the two principle components of articular cartilage extracellular matrix. In addition there are small amounts of other matrix proteins and lipids. Table 1.1 summarizes the major components of the extracellular matrix of articular cartilage identified to date.

1.1.3.1 Collagens of Articular Cartilage

Collagen is responsible for tensile strength and is essential for maintenance of the tissue's volume and shape. The predominant type of collagen in articular cartilage is type II and forms the basic fibrillar structure of the extracellular matrix. Up to 10% of the total collagen in articular cartilage is made up of types I, V, VI, IX, XI (Table 2) (van der Rest and Garrone, 1991; Thomas et al, 1994).

Table 1.2: Collagen types present in articular cartilage and their structure

<table>
<thead>
<tr>
<th>Collagen Type</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>$[\alpha_1(I)]_2, \alpha_2(I)$</td>
</tr>
<tr>
<td>II</td>
<td>$[\alpha_1(II)]_3$</td>
</tr>
<tr>
<td>V</td>
<td>$[\alpha_1(V)]_2\alpha_2(V)$</td>
</tr>
<tr>
<td>VI</td>
<td>$\alpha_1(VI), \alpha_2(VI), \alpha_3(VI)$</td>
</tr>
<tr>
<td>IX</td>
<td>$\alpha_1(IX), \alpha_2(IX), \alpha_3(IX)$</td>
</tr>
<tr>
<td>X</td>
<td>$[\alpha_1(X)]_3$</td>
</tr>
<tr>
<td>XI</td>
<td>$\alpha_1(XI), \alpha_2(XI), \alpha_3(XI)$</td>
</tr>
</tbody>
</table>
Type II collagen contains three identical polypeptide chains [alpha (II)_3] which are synthesized as precursors (procollagen) with nonhelical amino and carboxy extension peptides. Post-translational modification of type II collagen involves the hydroxylation of proline and lysine residues. Hydroxyproline and hydroxylysine are rarely found in proteins other than collagen. The formation of these amino acids is catalyzed by prolyl-4-hydroxylase, prolyl-3-hydroxylase, and lysyl hydroxylase. The cofactors for these enzymes are Fe^{2+} (ferrous iron) and ascorbate, and the cosubstrates are α-ketoglutarate and O_2 (Kivirikko and Myllyla, 1982). The hydroxyl group of hydroxyproline residues form interchain hydrogen bonds that help stabilize the triple helix at physiological temperatures (Rosenbloom and Harsh, 1973). The hydroxylation of certain lysine residues is crucial for the cross-linking of collagen molecules that occurs during collagen assembly. Hydroxylysine residues are also an attachment site for carbohydrate units (Kivirikko et al, 1973).

The extension peptides guide the intracellular formation of triple stranded collagen molecules and because they are removed only after secretion, they prevent the formation of collagen fibers intracellularly. In the extracellular matrix, the nonhelical extension peptides of procollagen are enzymatically removed by specific endopeptidases, procollagen N-proteinase, and the procollagen C-proteinase which are responsible for removing the NH_2 and COOH propeptides respectively (Olsen, 1991). This converts the procollagen to collagen (tropocollagen) molecules which can be incorporated into a collagen fibril or fiber. Type XI is present within the type II collagen fiber and may play
a role in regulating the size of the fiber (Mendler et al, 1989). Small diameter fibrils (10-25 nm) are formed pericellularly, and much larger diameter fibrils (up to 300 nm) are formed in the territorial and interterritorial matrix (Schenk et al, 1986).

Type IX collagen is assembled from three distinct alpha chains [α1(IX), α2(IX), α3(IX)] and is present on the surface of type II collagen fibrils in an antiparallel fashion (Wu et al, 1992). Each type IX collagen molecule is covalently bound to at least one type II collagen molecule (van der Rest and Mayne, 1988). Type IX collagen appears to be important in stabilizing the three dimensional organization of the collagen network and contributes to its tensile strength. Type IX collagen is also a minor proteoglycan since it contains a chondroitin sulphate glycosaminoglycan on the α2 chain (van der Rest and Mayne, 1987). It has been hypothesized that type IX collagen plays a role in the stabilization of the type II collagen network via its GAG chain thus contributing to the organization of the matrix and its biomechanical properties (Broom, 1988).

There are small amounts of other collagens present in articular cartilage. Type X collagen is present in the zone of calcified cartilage and may be synthesized by chondrocytes in other zones during disease (Eerola et al, 1998). Type X collagen has been shown to be involved in mineralization (Chen et al, 1992). Type VI collagen represents 1-2% of the total collagen in articular cartilage and is primarily located in the pericellular matrix of the chondrocyte (Hambach et al, 1998). Type I collagen is found in the calcified zone of articular cartilage (Wardale and Duance, 1993).
1.1.3.2 Other Matrix Proteins of Articular Cartilage

There are other extracellular matrix proteins in articular cartilage present in smaller amounts than the collagens and proteoglycans. Many of these proteins play a role in matrix assembly (co-operative interactions with other major macromolecules), interactions with chondrocytes (cell attachment, matrix maintenance), and regulation of matrix metabolism. These functions are not mutually exclusive and are interrelated in a complex manner which may enable the chondrocyte to respond to environmental changes within the matrix. Some of these molecules are well characterized. For example, link protein stabilizes the interaction between hyaluronan and aggrecan (Hardingham, 1979). Anchorin, a receptor which is present on the chondrocyte membrane, appears to be important in cell-matrix interactions by mediating the binding between type II collagen and the cell (Pfafflé et al, 1990). Thrombospondin and fibronectin are present in the matrix and may play a role in cartilage repair (Miller and McDevitt, 1995; Johansson, 1996). Other proteins such as cartilage oligomeric matrix protein (COMP) and cartilage matrix protein (CMP) have been found in the matrix but their functions are unknown (Hedborn, 1992; Heinegard and Oldberg, 1989).
1.1.4 Proteoglycans

1.1.4.1 Proteoglycan Nomenclature and Classification

Proteoglycans are complex molecules made up of a protein core onto which one or more glycosaminoglycan (GAG) chains are covalently attached. There are five main families into which proteoglycans are grouped; A) large extracellular B) small interstitial C) basement membrane heparan sulphates D) cell surface and E) intracellular (Table 1.3).

Glycosaminoglycans are unbranched chains of repeating disaccharides. There are four main groups of glycosaminoglycans (Table 1.4) all of which, with the exception of hyaluronic acid, are bound to a core protein. At neutral pH, hyaluronic acid is referred to as hyaluronan. Glycosaminoglycan chains are variably sulphated except for hyaluronic acid, which is non-sulphated. The most common sites of sulphation for chondroitin sulphate are the 4 and 6 positions of the N-acetylgalactosamine residue. Any given chain may contain stretches of disaccharides sulphated in the 4 position followed by stretches having sulphate in the 6 position. Not all disaccharides in a chain are sulphated and in some cases disulphated disaccharides are present. Keratan sulphate is normally sulphated on the 6 position of either or both sugars. The degree of N-sulphation is one major characteristic that distinguishes HS from heparin in that heparin is more highly sulphated than HS.
Table 1.3: Classification of Proteoglycans

<table>
<thead>
<tr>
<th>Proteoglycan Family</th>
<th>Examples of Members</th>
</tr>
</thead>
<tbody>
<tr>
<td>A large extracellular</td>
<td>aggrecan, versican</td>
</tr>
<tr>
<td>B small interstitial</td>
<td>biglycan, decorin, fibromodulin, lumican</td>
</tr>
<tr>
<td>C basement membrane heparan sulphates</td>
<td>perlecan</td>
</tr>
<tr>
<td>D cell surface</td>
<td>syndecan, betaglycan, thrombomodulin</td>
</tr>
<tr>
<td>E intracellular</td>
<td>serglycin, heparin</td>
</tr>
</tbody>
</table>

* Based on the classification of proteoglycans according to Kjellén and Lindahl (1991)

Table 1.4: Glycosaminoglycan Chains

<table>
<thead>
<tr>
<th>Group</th>
<th>Glycosaminoglycan</th>
<th>Repeating Disaccharide</th>
<th>Molecular Weight (kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>hyaluronic acid (HA)</td>
<td>1,4-glucuronic acid - 1,3-N-acetylglucosamine</td>
<td>4 - 800</td>
</tr>
<tr>
<td>2</td>
<td>chondroitin sulphate (CS)</td>
<td>1,4-glucuronic acid - 1,3-N-acetylglucosamine</td>
<td>5-50</td>
</tr>
<tr>
<td></td>
<td>dermatan sulphate (DS)</td>
<td>1,4-iduronic acid-1,3-N-acetylglucosamine (1,4-glucuronic acid)</td>
<td>15-40</td>
</tr>
<tr>
<td>3</td>
<td>heparan sulphate (HS)</td>
<td>1,4-iduronic acid - 1,4-N-acetylglucosamine (1,4-glucuronic acid)</td>
<td>5-12</td>
</tr>
<tr>
<td></td>
<td>heparin</td>
<td>1,4-iduronic acid- 1,4-N-acetylglucosamine (1,4-glucuronic acid)</td>
<td>6-25</td>
</tr>
<tr>
<td>4</td>
<td>keratan sulphate (KS)</td>
<td>1,3-galactose - 1,4-N-acetylglucosamine</td>
<td>4-19</td>
</tr>
</tbody>
</table>

1.1.4.2 Proteoglycans of Articular Cartilage

The large aggregating proteoglycans (aggrecan and versican) make up 50-85% of the proteoglycans present in cartilage while the large non-aggregating forms make up 10-40% of the total proteoglycan (Ratcliff and Mow, 1996). In mature cartilage the small proteoglycans (biglycan, decorin, and fibromodulin) constitute less than 10% the total amount of proteoglycans. Cell surface proteoglycans (syndecans, fibroglycan, amphiglycan, glypicans, betaglycan and CD44) are expressed by chondrocytes (Grover and Roughley, 1995). Perlecan, a heparan sulphate proteoglycan, has been demonstrated in articular cartilage (SundarRaj, 1995). The predominant proteoglycan in articular cartilage is aggrecan, and it has been studied extensively because of its role in skeletal growth and joint function, and its loss in arthritis (Poole, 1986; Heinegard and Oldberg, 1989; Hardingham and Fosang, 1992). Aggrecan is a large keratan sulphate/chondroitin sulphate proteoglycan. Its name reflects its ability to aggregate with hyaluronic acid. Both aggregating and non-aggregating large proteoglycans have been identified in articular cartilage.

1.2 Aggrecan

1.2.1 Structure of Aggrecan

Aggrecan consist of a protein core to which are attached many covalently bound glycosaminoglycan chains; over 100 chondroitin sulphate (CS) chains and approximately 20-50 keratan sulphate (KS) chains and 40 O- and N-linked oligosaccharides (Figure 1.1).
Aggrecan has a relative molecular weight exceeding $3 \times 10^6$ kD. The protein core (210-248 kD) contains three globular domains (G1-G3) and two extended regions (Hardingham, 1990).

The proteoglycan G1 domain is located at the amino terminus and can interact non-covalently with hyaluronic acid and is termed the hyaluronic acid binding region (HABR). The G1 domain contains an immunoglobulin fold making aggrecan part of the immunoglobulin superfamily (Perkins et al, 1989). The immunoglobulin fold forms two $\beta$-sheets stabilized by a disulphide bond. A short extended segment (E1) separates G1 and G2 and is referred to as the interglobular domain (IGD). The function of the G2 domain is unclear. G2 is homologous to G1, however, it cannot bind hyaluronic acid (Doege et al, 1987).

Most of the glycosaminoglycan chains are clustered between G2 and G3. The keratan sulphate rich region is located between the CS rich region and G2 globular domain of the core protein and is composed of a repeating hexapeptide motif conforming to a consensus sequence of E(E/K)PFPS (Hering et al, 1997). The keratan sulphate rich region is composed of two distinct zones (KS I and KS II) due to the sequence differences between the two segments of the KS region (Valhmu et al, 1995). The chondroitin sulphate rich region (1/2 core protein- E2) carries almost all of the CS (up to 100 chains) and about half of the KS chains and is divided into two subdomains (CS I and CS II) [Doege et al, 1991]. The CS I region has an evenly spaced serine-glycine
containing repeat whereas the CS II region contains serine-glycine sequences that occur in clusters (Doege et al, 1991).

The function of the G3 domain is unclear. It contains sequence similarities to three other protein families. It contains a complement B regulatory component (Patthy, 1987), Ca\(^{2+}\)-dependent mammalian type C lectin-like region also called carbohydrate recognition domain (Halberg et al, 1988) and epidermal growth factor (EGF)-like domains (Baldwin et al, 1989). Human aggrecan has two EGF-like domains that may be alternatively spliced (Fulop et al, 1993). The lectin-like properties of the G3 region may help anchor proteoglycan molecules within the extracellular matrix (Doege, 1988). The lectin-binding region has affinity for galactose and fucose and also binds tenascin by calcium-mediated non-lectin interactions (Halberg et al, 1988; Asberg et al, 1997). Studies of chick nanomelia have suggested that the G3 region plays an important role in the intracellular translocation of proteoglycans (Vertel et al, 1994). The G3 domain is proteolytically removed soon after synthesis and secretion. The consequence of the loss of the G3 domain is not yet understood (Hardingham et al, 1992). Since the core protein only constitutes approximately 10% of the total mass (the remainder being the carbohydrate components) there is a large potential for post-translational modifications, which can contribute to the heterogeneity of the proteoglycan population in the cartilage.
Figure 1.1: Schematic representation of the structure of aggrecan
Adapted from Hardingham et al (1994)
1.2.2 Proteoglycan Aggregates

The interactions of hyaluronan with aggrecan and link protein are well characterized (Hardingham, 1979). In this role its function is to form aggregates with aggrecan monomers that are immobilized within the collagen network. The hyaluronic acid binding region (G1) enables proteoglycan monomers (Mw 1-4 kDa) to associate noncovalently with hyaluronic acid to form aggregates (Mw $5 \times 10^4 - 5 \times 10^5$ kDa). Aggregates of aggrecan contribute to the mechanical and physicochemical properties of cartilage (Maroudas, 1968). As many as 200 (typically 20-100) monomers can bind to a single hyaluronic acid to form an aggregate (Hardingham et al, 1994). Link protein interacts with both the hyaluronic acid binding region of the core protein and the hyaluronic acid chain in order to stabilize their interaction (Mow et al, 1989). Hyaluronan levels change with age, increasing five fold from 0.5 to 2.5 μg/mg tissue wet weight between 2.5-86 years in human articular cartilage (Holmes et al, 1988). In addition, a hyaluronan receptor (CD 44) on the cell surface links the chondrocyte to the extracellular matrix (Knudson, 1993). It has been suggested that the linkage between the chondrocyte and its surrounding matrix may allow the cell to detect changes in the ECM and respond accordingly.

1.2.3 Compressive Properties of Aggrecan

Proteoglycans draw in water due to their high negative fixed charge density and are constrained by the collagen framework. This interaction provides the viscoelastic
properties and compressive strength to cartilage. Osmotic pressure increases within the matrix by the Donnan effect (Lai et al, 1991). The high negative fixed charge attracts counter ions (Na\(^+\), K\(^+\), Ca\(^{++}\)) creating an osmotic imbalance which draws water into the tissue. When cartilage is compressed, water is forced out, resulting in a higher proteoglycan concentration that in turn increases osmotic pressure. When the force is released, water flows back into the proteoglycan gel (Handley et al, 1985). In pathological states such as degenerative and inflammatory arthritis, the resiliency of the cartilage is markedly decreased and is correlated with the loss of proteoglycans from the matrix of the tissue (Kempson et al, 1976).

1.2.4 Synthesis of Proteoglycans

The synthesis of the proteoglycans of cartilage reflects the specific gene expression resulting from the phenotype of chondrocytes (Kimura et al, 1987). Control of synthesis and processing of a proteoglycan involves the selection and expression specific core protein(s), followed by the determination of the number, size, and type of GAG chains that are added.

1.2.4.1 Aggrecan Core Protein Gene

The aggrecan gene has been mapped to human chromosome 15q26.1 (Korenberg et al, 1993), and mouse chromosome 7 (Walcz et al, 1994). The aggrecan gene is particularly large (80 kB) and is composed of 19 exons (Doege et al, 1994; Valhmu et al,
1995). The aggrecan gene has a modular organization and is probably derived from gene duplication (Ratcliffe and Mow, 1996). Possible regulatory regions are in the promoter region 5' upstream of the transcribed regions, and the 5' (including intron 1) and 3' untranslated regions (large part of exon 19) of the mRNA. Aggrecan was the first member of the large aggregating proteoglycan gene family to be described. Several complete cDNA sequences have been reported for aggrecans from different species: human (Doege et al, 1991), rat (Doege et al, 1987), chicken (Chandrasekaran and Tanzer, 1992), mouse (Walcz et al, 1994) and bovine (Hering et al, 1997; Wiedemann et al, 1984; Oldberg et al, 1987; Antonsson et al, 1989). Partial sequences are known for pig (Barry et al, 1992). There are a number of animal mutants which are defective in this gene, such as the nanomelic chicken (Vertel et al, 1994) and the cartilage matrix deficient mouse (cmd) (Kimata et al, 1981). Altered expression of aggrecan seems to be a hallmark of osteoarthritis and other degenerative diseases of cartilage.

1.2.4.2 Synthesis of Proteoglycans

Figure 1.2 shows the synthesis pathway for aggrecan. During normal synthesis the core protein enters the endoplasmic reticulum and assembly of N-linked oligosaccarides commences. The assembly of chondroitin sulphate chains and O-linked oligosaccarides and keratan sulphate chains take place in the Golgi apparatus. Chain synthesis of glycosaminoglycans results from the successive addition of single sugars from uridine precursors by the action of specific glycosyltransferases. There is no universal consensus sequence for GAG chain attachment, however, for chondroitin sulphate the amino acids
glutamic acid or aspartic acid commonly precede the target serine followed by glycine, or alanine (Kjellén and Lindahl, 1991). Keratan sulphate chains are added to the core in a similar manner by glycosyltransferases. KS usually is initiated on asparagine, serine, or threonine residues. Sulphation of the disaccharides also occurs in the Golgi apparatus by activated sulphate transferases [3'-phosphoadenosine-5'-phosphosulphate (PAPS)].

Synthesis of complex carbohydrates is not under direct control of genes. Connective tissue polysaccharides exhibit molecular weight polydispersity and the exact mechanism for chain termination at a precise molecular weight is not known. The termination of chain elongation in chondroitin sulphate may be achieved by 4-sulphation or 6-sulphation of terminal hexosamine residues or by other unknown mechanisms (Plaas et al, 1997). Large differences in size and composition of proteoglycans can be seen due to variations of the pattern of biosynthesis of post-translational glycosylation. The length and pattern of sulphation of chondroitin sulphate chains and the proportion of keratan sulphate chains as well as their length may vary. The monomer is then exported to the cell membrane and enters into the extracellular matrix. Most newly synthesized proteoglycans appear to be secreted from the cell within 5-20 minutes (Kimura, 1979).
**Figure 1.2: Diagram of proteoglycan synthesis pathway**
Adapted from Mankin et al (1994)
1.2.5 Proteoglycan Metabolism

Chondrocytes maintain the amount of proteoglycan within the extracellular matrix at a relatively constant level under normal physiological conditions. Proteoglycans are continuously being synthesized, incorporated into the matrix and lost from the tissue. Changes in the rates of synthesis or catabolism will lead to changes in the proteoglycan content within the matrix and this ultimately alters the mechanical properties of the tissue. Aggrecan accounts for 5-10% of the total protein synthesis by articular chondrocytes, resulting in about 20 pg of proteoglycan per day which is sufficient to replace up to 3% of the total in the tissue per day (Hascall et al 1983, 1990). In order for the tissue content of proteoglycan to be maintained at a constant level the mechanisms of proteoglycan turnover must be co-ordinated. Synthesis and degradation are co-regulated by the chondrocytes via a feedback mechanism that is sensitive to the proteoglycan content of the matrix (Handley and Lowther, 1977; Lucas and Diewiatkowski, 1987). For example proteoglycan synthesis by cultured chondrocytes was shown to be depressed by extracellular concentrations of proteoglycan and partially degraded proteoglycan (concentration greater than 5 mg/ml) at the level of glycosaminoglycan synthesis (Handley and Lowther, 1977).

Depletion of proteoglycans and changes in their size and ability to form aggregates are seen with age and in pathological conditions. It is not known if this is the result of an alteration at the level of biosynthesis or whether extensive extracellular
modifications of the biosynthetic product in the older or damaged tissue are responsible (Bayliss, 1990).

A number of growth factors and regulatory peptides regulate the synthesis and catabolism of the matrix macromolecules and these factors may vary in their expression in articular cartilage. These include insulin-like growth factors, transforming growth factor β, interleukin-1 and tumor necrosis factor α (Morales and Hascall, 1989). Mechanical and humoral factors affecting matrix metabolism vary for chondrocytes from different species, site (which joint), depth in tissue (zone) and region of the joint (Bayliss, 1990). Age of the tissue, however, seems to have the most dramatic effect on the composition of proteoglycans in articular cartilage (Thonar and Kuettner, 1986).

1.2.6 Aggrecan Turnover

Aggrecan has a turnover time of 200-800 days in human articular cartilage whereas in culture the turnover time is much shorter (Hardingham and Bayliss, 1990). Campbell et al (1984) observed that proteoglycans in adult bovine articular cartilage explants labelled with $[^{35}\text{S}]$-sulphate after five days in culture and maintained in medium containing 20% fetal calf serum had longer half lives (average 11 days) compared with those maintained in medium alone (average 6 days). Proteoglycans in explant cultures of calf cartilage maintained in medium with serum demonstrated considerably longer half-lives (average 21 days) as compared to proteoglycans in adult cartilage. One study examining the half-life of newly synthesized proteoglycans in cartilage explants
demonstrated two phases of proteoglycan loss; one which is rapid (< 1 day) and a slower second phase (< 20 days)[Sandy and Plaas, 1986]. The significance of this is unknown.

1.3 Small Proteoglycans of Articular Cartilage

Three small non-aggregating proteoglycans; decorin, biglycan and fibromodulin are present in varying amounts in cartilage depending on the age and zone of the tissue. Decorin (PG-II) and biglycan (PG-I) are dermatan sulphate-containing proteoglycans. Biglycan is substituted with two GAG chains; dermatan sulphate or chondroitin sulphate, and has a molecular weight of 100 kD (core protein 38 kD). Biglycan is mainly localized to the pericellular matrix (Bianco et al, 1990). Decorin has a molecular weight of 74 kD (core protein 36 kD) and a single GAG chain, which can be either dermatan sulphate or chondroitin sulphate. Decorin is present throughout the interterritorial matrix but increased amounts are detected in the superficial layer of articular cartilage (Poole et al, 1986). In human articular cartilage there is a steady age related increase in the content of decorin up to 25 years after which it declines (Hardingham, 1990). Decorin reduces the rate of fibril growth and increases the diameter of the fibrils formed (Kuc and Scott, 1997). Fibromodulin (core protein 59 kD) differs from other small proteoglycans in that it contains several sulphated tyrosine residues in its N-terminus and the keratan sulphate chains are attached to the central domain of the molecule. Dermatan sulphate containing proteoglycans do not bind to hyaluronic acid, however, biglycan can self-associate to form relatively small unstable aggregates (Rosenberg, 1985).
1.4 Compositional Differences

Articular cartilage is heterogeneous. On the basis of differences in chondrocyte morphology, collagen fiber orientation, and glycosaminoglycan concentration, articular cartilage has been divided into superficial, middle, deep, and calcified layers (Meachim and Stockwell, 1979)[Figure 1.3]. The architecture and biochemical composition varies in the different zones of the cartilage. Ultrastructural examination shows that within a zone the matrix can be subdivided into three concentric zones or matrices; the pericellular, territorial and interterritorial zones (Stockwell et al, 1983; Poole et al, 1984).

The superficial zone consists of a dense network of small diameter collagen fibres oriented parallel to the articular surface (Buckwalter et al, 1994; Clark, 1991)[Figure 1.3]. In the intermediate zone, collagen fibrils are larger in diameter, random in arrangement, with pericellular organization. The deep zone collagen fibrils are of the largest diameter and organized perpendicularly to the subchondral bone (Clark, 1991). Collagen bundles cross the tidemark and insert into the calcified cartilage, which anchors the tissue to the underlying subchondral bone (Oegema and Thompson, 1992).
Figure 1.3: Organization of articular cartilage
Compositional analysis of articular cartilage shows that collagen content decreases by approximately 15% from the superficial to the middle and deep zones (constant in lower two zones per wet weight)[Muir et al, 1970]. The proteoglycan content is lowest in the superficial zone and increases steadily through the middle and deep zones by as much as 50% (Brocklehurst et al, 1984). Water content is highest in the superficial zone (~80%) and decreases to approximately 65% in the middle and deep zones (Torzilli et al, 1982).

Specific types of proteoglycan, collagen and other minor components are found in the different locations in the tissue. Type II and type IX collagens are distributed throughout the tissue (Wardale and Duance, 1993). Type I and type VI collagen have been differentially localized at the surface of the cartilage (Wardale and Duance, 1993) and pericellular collagens include types II, VI, IX, and XI (Poole et al, 1992; Wardale and Duance, 1993). Type I and X collagens have been identified in the calcified cartilage (Wardale and Duance, 1993).

Aggrecan is located throughout cartilage (Poole et al, 1982; Ratcliffe et al, 1984) but is at its highest concentration in the middle zone, and immediately surrounding the chondrocyte. In the pericellular matrix, aggrecan is 2 fold higher when compared to the interterritorial region (Ratcliffe et al, 1984). Link protein, which is involved in the stabilization of aggrecan aggregates, mirrors aggrecan distribution in cartilage however there is some evidence of a differential distribution (Poole et al, 1982). Increased amounts of decorin are detected in the superficial zone in adult cartilage (Poole et al,
Chondroitin sulphate (CS) and keratan sulphate (KS) glycosaminoglycans are distributed heterogeneously throughout the tissue. CS concentration is highest in the middle zone, while KS concentration increases with the distance from the surface (Bayliss et al, 1983). The sizes of the aggrecan aggregate also vary with depth in the tissue. Smaller aggregates are found predominantly in superficial cartilage while the middle and deep zones have larger aggregates (Muller et al, 1989). These differences appear to be due to the number of aggrecan molecules bound to hyaluronan, and to the degree of link stabilization (Pita et al, 1992). Compositional and organizational differences of cartilage through its depth, and the complex interactions of molecules, result in differences in the material properties of the tissue with depth (Akizuki et al, 1986; 1987). Chondrocytes are responsible for the synthesis and maintenance of this complex organization, and their activities also differ with depth (Archer, 1990). Phenotypically, superficial chondrocytes synthesize less collagen and proteoglycan than cells in the deep zone and they degrade proteoglycans more rapidly (Aydelotte et al, 1992).

1.5 Proteoglycan Retention and Accumulation

1.5.1 Matrix Assembly

Regulation of assembly of the extracellular matrix and chondrocyte-matrix interactions are key elements in cartilage matrix homeostasis. Initial matrix assembly occurs within an area immediately adjacent to the chondrocyte cell surface termed the pericellular matrix (Knudson, 1993). Cell surface receptor-matrix interactions would
promote macromolecular assembly and may be involved in feedback regulation. Receptors such as CD44, or anchorin could activate signal transduction mechanisms that regulate biosynthesis and/or turnover. The pericellular matrix, once assembled, may then serve as a nucleating template for territorial or interterritorial matrix organization (Maleski and Knudson, 1996).

Aggrecan, link protein, and hyaluronic acid are secreted independently from the cell, and then assembled into aggregates in the extracellular matrix. The G1 domain of the proteoglycan may not be functional on secretion and may only become functional after extracellular processing or a maturation process involving a change in conformation of the G1 domain (Bayliss and Roughley, 1985; Sandy et al, 1989). This delayed aggregation allows most of the proteoglycan to move away from the chondrocyte before forming aggregates (Zhu et al, 1994). This allows for newly synthesized proteoglycans to be incorporated into both the territorial and the interterritorial matrix.

The assembly of collagen into the extracellular matrix follows a distinct sequence of events. At the time of secretion the non-helical regions of collagen molecules are enzymatically removed and the tropocollagen molecules then become organized in quarter staggered array to form fibrils. Fibrillogenesis is the process of aggregation of the fibrils both in the lateral direction and end to end. Fibril/fiber diameter, orientation, network formation, and ultrastructural organization differ depending on the zone, site and types of cartilage. Variations in collagen fibril/fiber size are also seen with age, disease, and repair. Large and small proteoglycans and other types of collagen (types IX and XI
collagen) contribute to fibril formation (Scott, 1990). However, the precise mechanisms that control fibril formation remain to be elucidated (Vogel et al, 1984; Scott, 1990, 1992). Covalent crosslinks between collagen molecules are a major contributor to the structural organization of collagen and the tensile strength of the tissue.

Extracellular matrix proteoglycans bind to other matrix molecules. The most common method of binding is through the interaction of the glycosaminoglycan component with a matrix protein. For example, decorin has been demonstrated to bind collagen and fibronectin through its core protein (Vogel, 1986; Lewandowska et al 1987; Schmidt et al, 1987). Proteoglycans may help assemble and hold together the matrix components in the extracellular matrix by linking together several extracellular matrix components. Ruoslahti and Pierschbacher (1987) showed that proteoglycans and in particular glycosaminoglycans could enhance the interaction between fibronectin and collagen. It is possible that cell surface proteoglycans act as receptors to help regulate matrix assembly (Ruoslahti, 1988).

Studies on proteoglycan mutations and their subsequent effect on cartilage formation support the importance of proteoglycans in matrix assembly. Cartilage matrix deficiency (cmd/cmd) is an autosomal recessive lethal mutation in mice resulting in a syndrome including disproportionate dwarfism, short snout, and cleft palate (Rittenhouse et al, 1978). These abnormalities have been shown to result from the failure in the synthesis of aggrecan core protein (Kimata et al, 1981). Other matrix components, such as type II collagen, small proteoglycans, hyaluronic acid, and link protein are synthesized at nearly
normal rates (Kimata et al, 1981; Kimata et al, 1983). Takeda et al (1986) demonstrated that the abnormal matrix formed by cmd/cmd chondrocytes in culture could be corrected by the addition of exogenous cartilage proteoglycan. Assembly or processing of collagen by chondrocytes may be influenced by a failure of the cells to produce aggrecan. Therefore, proteoglycans themselves may play a regulatory role in the assembly of the matrix macromolecules.

1.5.2 Mechanisms of Proteoglycan Retention

The principal mechanism of proteoglycan retention within the tissue is by the interaction of aggrecan with hyaluronic acid. This interaction is stabilized by link protein. The hyaluronic acid binding region (HABR) has been shown to undergo a maturation process in which the affinity of this region for hyaluronic acid goes from low to high (Plaas and Sandy, 1986). A conformational change in the G1 domain via the formation of the disulphide bonds is thought to be the mechanism by which the maturation process occurs (Sandy and Plaas, 1989).

Collagen also plays a role in proteoglycan retention. Electron microscopy has demonstrated that at the subfibril level the proteoglycans condense in a regular registry in specific regions of collagen fibrils (Scott, 1990, 1992). Proteoglycans become trapped within the collagen network either physically or as a result of interactions with collagen fibers (Figure 1.4). Glycosaminoglycan chains have been shown to interact electrostatically with the basic amino acids within collagen (Oegema, 1975). The core
protein of the small proteoglycan decorin has been demonstrated to bind to collagen fibres within the extracellular matrix. The core protein of aggregcan has not been shown to interact directly with the collagen fibers but aggregcan becomes trapped within the network of collagen fibers because of the large size of these molecules. Collagen concentration within the tissue is critical in creating entanglement properties for other proteins (Zhu, 1997). The collagen fibers in cartilage are oriented in such a way that reduces the loss of proteoglycans from the matrix (Buckwalter and Mankin, 1998).
Figure 1.4: Interactions of proteoglycans and the collagen network
1.5.3 Factors Influencing Retention and Accumulation

Many factors are involved in regulating the retention and accumulation of the proteoglycans within articular cartilage. Factors affecting aggregation such as the amount of hyaluronic acid (HA) and link protein, and the absence of the hyaluronic acid binding region (HABR) on the proteoglycan would all have a major effect on the amount of proteoglycan retained within the tissue. The amount of link protein and HA synthesized is dependent on the concentration of proteoglycan within the tissue (Lucas and Dziewiatkowski, 1987). The size of the proteoglycan synthesized may also influence its ability to be incorporated into the tissue. Conditions affecting the maturation of the HABR, such as compression and the pH of the tissue fluid, would influence the retention of proteoglycans as they would have an effect on PG aggregation (Plaas and Sandy, 1986; Sandy et al, 1990). Factors or diseases that destroy the collagen network would also influence PG retention.

1.6 Proteolytic Degradation of Cartilage

A central role has been established for matrix metalloproteinases (MMP) in pathologic cartilage degradation but little is known about their role in physiological turnover. Four types of proteases are present in the extracellular matrix: interstitial collagenases (MMP-1, MMP-8), gelatinases (type IV collagenases, MMP-2, MMP-9), membrane bound MMPs, and stromelysin (MMP-3, MMP-7) (Murphy, 1990). Collagenases are substrate-specific and cleave the native helix of fibrillar collagens at a
single locus. The gelatinases degrade collagen types IV, V and VII and may work in conjunction with collagenases in the degradation of collagen since they have the ability to degrade denatured forms of collagen (gelatins). Stromelysin (1 and 2) and matrilysin (MMP-7) (stromelysin-3) have a broader substrate specificity and are able to degrade many extracellular matrix proteins. Matrix proteins involved in matrix-matrix and cell-matrix interaction are also susceptible to the action of stromelysins (ex. fibronectin, type IX collagen etc.). Stromelysin also activates collagenase (Murphy, 1989). Another method of regulation of metalloproteinase activities occurs by the action of tissue inhibitors of metalloproteinases (TIMP 1 and 2) which are synthesized by chondrocytes. One study in a rabbit model of osteoarthritis suggested that metalloproteinase activity increases when inhibitor concentrations are reduced relative to that of the enzyme (Tanaka et al, 1998).

During the processes of normal maintenance of cartilage (synthesis, repair, and degradation) the proteoglycans are continually being broken down and lost from the matrix. Metalloproteinases and aggrecanase, another type of enzyme, are involved in normal proteoglycan turnover. Two major and specific sites of proteolytic cleavage have been identified on aggrecan in the interglobular domain. Members of the matrix metalloproteinases cleave at the site Asn$^{341}$-Phe$^{342}$ of the interglobular domain (IGD) (Fosang et al, 1991; Flannery et al, 1992; Fosang et al, 1993). Specifically, proteolysis of proteoglycan subunits results in shorter core proteins which lack the C-terminal portion so that hyaluronan binding regions (N-terminal) accumulate. Cleavage at this site has significant functional consequence because it uncouples the G1 domain from the GAG
rich domains which are no longer anchored within the tissue and hence may be lost from the tissue. The Asn$^{341}$-Phe$^{342}$ site has been shown to be cleaved in vitro by a number of matrix metalloproteinases (MMPs) (MMP-1,-2,-3,-7,-8,-9,-13) [Fosang et al, 1994, 1996, 1993, 1992).

A second distinct cleavage site at Glu$^{373}$-Ala$^{374}$ involves an enzyme termed "aggrecanase" (Sandy et al 1991; Ilic et al, 1992; Loulakis et al, 1992). Aggrecanase activity has prompted several investigations into the enzyme’s identity. MMP-8 (neutrophil collagenase) exhibits aggrecanase activity in vitro and has been shown to be expressed by articular chondrocytes (Fosang et al, 1994; Cole and Kuettner, 1995). Membrane type 1 matrix metalloproteinase (MT1-MMP) expression was detected in human articular cartilage (Buttner et al, 1997). Buttner et al (1998) demonstrated that MMP-8 had weak aggrecanase activity and MT1-MMP had strong aggrecanase activity. Therefore, MT1-MMP may be responsible for aggrecanase activity in vivo. Aggrecanase cleavage sites have also been identified at four other sites within the CS domain of the aggrecan core protein. Studies have suggested that the proteolytic activity of aggrecanase has a specificity for Glu-X peptide bonds where X is Ala, Gly, or Leu within aggrecan (Lohmander et al, 1993; Sandy et al, 1991a, 1991b).

Other processes are involved in cartilage destruction. Proteinases such as cathepsin (B, D, G, L), elastase and plasmin are involved in the proteolytic degradation. Reactive oxygen metabolites may also contribute to proteoglycan degradation (Panasyuk et al, 1994).
1.6.1 Alterations of Proteoglycan Retention in Disease

Proteoglycan loss is an integral part of the many diseases that affect the joint. There are many theories as to the mechanism by which proteoglycans are lost from the tissue. An imbalance between degradative enzymes and their inhibitors, loss of collagen integrity, and altered synthesis of proteoglycans are proposed reasons for proteoglycan loss. It is unlikely, however, that these mechanisms are mutually exclusive. In disease states where proteoglycan concentrations decrease markedly, the chondrocyte cannot replace the proteoglycans lost from the tissue rapidly enough to prevent further damage to the tissue. The loss of proteoglycan not only changes the composition of the matrix, it also exposes collagen fibers to proteolytic enzymes which may have increased activity (Poole, 1982).

1.7 Proteoglycans: Age and Disease

1.7.1 Age

Biochemical and biophysical studies have demonstrated age-related changes in the size and composition of articular cartilage proteoglycan monomers. With increasing age, there is a decrease in the size and number of chondroitin sulphate chains, and increase in the 6-sulphation relative to the 4-sulphation, and an increase in the size and number of keratan sulphate chains (Roughley, 1987). Keratan sulphate-rich monomers may increase with age in order to maintain the fixed charge density, viscoelastic properties and the osmotic swelling pressure of the cartilage. The glucosamine/galactosamine ratio of the proteoglycans increases with age and supports the hypothesis
that keratan sulphate content of proteoglycan monomers increases with age. Hyaluronate content increases with age in normal tissue, particularly in regions of maximum weight bearing (Thonar et al, 1978).

Heterogeneity in the proteoglycan monomer populations has been demonstrated by gel chromatography and composite agarose/polyacrylamide gel electrophoresis. Fetal and newborn cartilage proteoglycans migrate as one band whereas proteoglycans extracted from immature and adult cartilage separate into two and three bands respectively (Roughley and White, 1980). The differences in migration are attributed to differences in keratan sulphate, chondroitin sulphate, and in the size of their core proteins. Glycosaminoglycan chain length and extent of sulphation (and position) can be modified by changes in the activity of post-translational enzymes which can contribute to the heterogeneity in proteoglycan structure during aging. Proteolysis of the core protein may also contribute to the multiple proteoglycan species observed.

1.7.2 Alterations in Proteoglycans with Osteoarthritis

The mechanisms responsible for accelerated cartilage degradation in diseases of the joint remains unclear. Indirect evidence for the involvement of proteinases derived from invading inflammatory cells, synovial fibroblasts, and from chondrocytes themselves exists. Metalloproteinases, cysteine proteinases, serine proteinases, and aggreganase have been implicated in contributing to cartilage degradation. In degenerative joint diseases such as osteoarthritis, the rate of loss of proteoglycans from
the matrix eventually exceeds the rate of deposition of newly synthesized molecules (Mankin et al, 1986). The progressive loss of proteoglycans from the tissue results in diminished function and may exacerbate the disease process by exposing the underlying bone to increased mechanical loading.

Osteoarthritis (OA) is a disease of joints that involves alterations in the integrity and biomechanical function of cartilage and bone. It is generally agreed that an important early event in OA is a loss of proteoglycans from the cartilage matrix (Mankin et al, 1986). There have been many studies of aggrecan synthesis and turnover in osteoarthritic articular cartilage which have produced contradictory results. It has been shown that the induction of experimental OA is accompanied by an increase in proteoglycan and collagen synthesis (Eyre et al, 1980, Sandy et al, 1984). Synthesis of aggrecan is increased in the early degenerative phase and later decreases as cartilage is further destroyed (Sandy et al, 1987). Carney et al (1984) showed that in experimental osteoarthritis, cartilage has a diminished capacity to retain new molecules in the tissue. Aggregation with hyaluronic acid has been shown to decrease or not change in OA (Heinegard et al, 1987; Carney et al, 1985; Santer et al, 1981). Delayed aggregation due to low affinity of the HABR for hyaluronic acid, resulting in susceptibility of the proteoglycan to stromelysin degradation has been reported (Bayliss et al, 1988). The size of aggrecan has been demonstrated to increase, decrease, or remain unchanged depending on the study (Carney et al, 1985; Rizkalla et al, 1992; Santer et al, 1981). Core protein size and carbohydrate epitope changes are detected in diseased cartilage. A reduction in keratan sulphate relative to chondroitin sulphate has been observed (Sweet and Thonar,
It has been suggested that there is more than one phase in the progression of OA and this may explain some of the contradictions in the proteoglycan changes detected in OA cartilage. Rizkalla et al (1992) described two phases in OA: an early predominantly degenerate phase I followed by a reparative phase II. The proteoglycans synthesized during these two phases differed in their size and degree of retention.

Pathological conditions are believed to cause changes in proteoglycan synthesis. Altered proteoglycans could have an effect on their ability to be incorporated into the tissue. Rizkalla et al (1992) noted the appearance of proteoglycans with a larger hydrodynamic size in the reparative phase of osteoarthritis which are eventually lost from the tissue. These proteoglycans also expressed a fetal epitope (846). Caterson et al (1990) reported an increase in the chondroitin sulphate epitope 3B3 in osteoarthritic cartilage. Bayliss (1986) noted that the structure of the proteoglycan in osteoarthritis is more comparable to the juvenile than the adult phenotype but the significance of this is not clear as cartilage becomes thicker during development and growth. Other types of proteoglycans may be synthesized in OA. Versican, a large aggregating proteoglycan, is not present in normal adult articular cartilage. However, versican levels are very high during chondrogenesis and in disease versican is once again synthesized by chondrocytes (Nishada et al, 1994). The significance of these changes remains to be elucidated.

1.7.3 Repair and Regeneration

Articular cartilage has a limited capacity to repair itself. Defects greater than 3mm cannot be repaired (Newman, 1998). Disease states (rheumatoid arthritis, osteoarthritis),
infection, trauma, or immobilization stimulate repair processes to different extents. The lack of blood supply to articular cartilage results in 1) poor or nonexistent immune response thus damaged cartilage components cannot be removed 2) undifferentiated cells cannot be recruited to the injured site and 3) cell migration within the damaged tissue is limited (Newman, 1998). Repair in terms of replacement of proteoglycans can occur providing the collagen framework has not been destroyed (Jubb and Fell, 1980). Normal cartilage architecture is lost when proteoglycan loss exceeds the chondrocyte’s ability to replace the matrix (Buckwalter et al, 1992).

Injury to articular cartilage stimulates a repair process largely dependent on the type of damage involved. Superficial defects that do not reach bone and do not initiate an inflammatory response due to the absence of blood vessels result in minimal repair of the defects. Deep lesions extending into the subchondral bone are strikingly different as a result of the exposure of blood vessels and bone marrow. The defect fills with reparative granulation tissue and ingrowth of fibrocartilage which bonds to the cartilage surfaces surrounding the injury. Loss of proteoglycans leads to an increase in their synthesis and collagen synthesis may also be stimulated. Cell proliferation adjacent to injured surfaces may occur. However, this tissue does not have the appropriate composition and organization so it cannot replace articular cartilage function and over time undergoes degeneration.

The age of the tissue also may have an effect on repair. Mature chondrocytes have a decreased ability to proliferate and therefore have a limited ability to increase
extracellular matrix synthesis (Buckwalter et al, 1988). The number of chondrocytes decreases with age perhaps further limiting the ability of mature articular cartilage to repair itself (Mankin, 1968).

1.8 Summary

Articular cartilage has a highly organized and complex structure. Chondrocytes are responsible for the development and maintenance of normal cartilage. Collagens, proteoglycans, and noncollagenous proteins are important components of the extracellular matrix and give the tissue its biomechanical properties. The structure and composition of articular cartilage varies according to the distance from the surface and in relation to the distance from the cells. Chondrocytes have a limited ability to repair articular cartilage that has been damaged by injury or disease.

1.9 Research Summary

Hypothesis

The cartilagenous tissue generated in chondrocyte filter culture is similar to the in vivo tissue and should be suitable as a model for the study of cartilage regeneration.

In our laboratory, we have developed a culture system in which chondrocytes form cartilagenous tissue. This culture method will be further studied in order to characterize matrix accumulation, maintenance and turnover under controlled conditions.
The development of a culture method in which isolated chondrocytes are induced to reconstitute their matrix would provide information that is important for elucidating the pathway(s) of normal cartilage formation. Manipulating or inducing recapitulation of cartilage formation may provide insight into the prevention of pathological degeneration of cartilage that occurs in disease.
Chapter Two: Characterization of Chondrocyte Filter Cultures

2.1 Introduction

Articular cartilage differs from other tissues in that it is avascular so that all repair of cartilage defects or cartilage loss must be accomplished by the chondrocytes (Kuettner et al, 1991; Heinegard and Oldberg, 1989). Yet chondrocytes only comprise 2% of the tissue and each cell is surrounded by abundant extracellular matrix, which shows a very specific organization. The chondrocyte is a functional unit with little if any interactions between cells. Although it responds to structural damage with cell proliferation and matrix synthesis, its response is usually insufficient to fill defects and restore function. Therefore cartilage tissue has only a limited capacity for regeneration in vivo when it is damaged particularly in the presence of continued weight bearing.

The accumulation and organization of cartilage matrix is a continuous process as there is turnover of matrix macromolecules in cartilage. The half-life of proteoglycans in vitro is variable depending on the source of cartilage, and can range up to 30 days (Hascall et al, 1991; Hascall et al, 1983). Collagen is more stable and has a much longer half-life ranging from 300 days to years (Prockop et al, 1992). How the chondrocyte "senses" that synthesis of matrix macromolecules is necessary and how they are retained and organized into this well-ordered structure is complex and difficult to examine. There is abundant information about chondrocyte biosynthesis of proteoglycans in the presence of growth factors and cytokines and during chondrogenesis in developing limbs. However, the
process of matrix accumulation by articular chondrocytes has not been studied as extensively (Hascall et al, 1983; Hauselmann et al, 1992; Cheung, 1985; Kimura et al., 1984). Understanding these processes may identify molecules or the sequence of events regulating cartilage formation and maintenance. Ultimately, the conditions that favour regeneration of cartilage, that has been lost due to disease or injury, may be identified.

Culturing chondrocytes in vitro began in the 1960s (Holtzer, 1960). Until recently chondrocytes were most commonly grown in monolayer culture. However, chondrocytes in monolayer culture can lose their phenotype and dedifferentiate into fibroblast-like cells in that they express type I collagen (Holtzer, 1960; Mark et al, 1977). They also synthesize less proteoglycans, which are smaller in size than aggrecan (Benya and Shaffer, 1982). Suspension culture (agitated or spinner) was used to grow chondrocytes in order to preserve phenotypic differentiation; however, this method requires a large number of cells. Cell proliferation and extracellular matrix deposition are initially rapid then decline. Neither monolayer or suspension cultures accumulate sufficient extracellular matrix to form a continuous layer of tissue (Benya, 1990; Bassleer et al, 1986; Horwitz and Dorfman, 1970). Chondrocytes have been grown in alginate beads (Hauselmann et al, 1992), on agarose (Aulthouse et al, 1989; Delbruck et al, 1986) or on hydroxyapatite beads (Cheung, 1985) and under these conditions the cells remain spherical and accumulate type II collagen and cartilage-type proteoglycans but do not form tissue. Chondrocytes embedded in three-dimensional cultures (for example with collagen or polymer scaffolds) produce cartilagenous tissue (Kimura et al, 1984; Freed et al, 1993).
However, by providing an exogenous scaffold to support tissue formation, the organization of the matrix is affected.

In this chapter a culture system is characterized in which bovine articular chondrocytes, plated on Millipore CM filter inserts accumulate extracellular matrix to form a continuous layer of cartilage-like tissue. While several alternative methods of chondrocyte culture \textit{in vitro} exist, maintaining chondrocytes on filter inserts has advantages over these other culture methods in that a continuous layer of tissue can be obtained which is amenable to histological and biochemical assessment. Also it has been shown that the environment in which chondrocytes are cultured can affect their synthetic response. Therefore studying chondrocyte metabolism in a tissue may be more physiological and would minimize culture artifacts.

\subsection*{2.1.1 Objectives}

The main objectives of these experiments were to characterize the cartilagenous tissue and examine chondrocyte phenotype in the filter culture system. Specifically this involved:

1) Determining whether chondrocytes were synthesizing cartilage type macromolecules such as type II collagen and large proteoglycans (keratan sulphate containing) to ensure that the chondrocytes maintained their phenotype under the culture conditions.

2) Examining proteoglycan synthesis by chondrocytes in filter cultures.
3) Studying the accumulation of newly synthesized proteoglycans over time.

4) Quantifying the collagen and proteoglycan content over time in culture.

5) Determining the DNA content of the tissue over time.

6) Quantifying the changes in cartilagenous tissue thickness over time.
2.2 Materials and Methods

2.2.1 Chondrocyte Cultures

Chondrocytes were obtained from calf articular cartilage removed from the metacarpophalangeal joints as described previously (Kandel 1990). Briefly, the metacarpophalangeal cartilage is exposed by cutting the skin and joint capsule under sterile conditions in a laminar flow hood. The cartilage is then removed with a scalpel and placed in Ham’s F12 medium with 1% antibiotics. Sequential enzymatic digestion is used to release the cells from their surrounding matrix. Protease (0.5% Type XIV: bacterial from streptomyces griseus, Sigma) is added to the cartilage and incubated for one hour at 37°C. The protease is then removed and the cartilage pieces are washed three times with sterile Ham's F12 medium. Collagenase A (0.1%) is added and incubated overnight at 37°C. The result is digestion of the extracellular matrix releasing cells into the medium. The cells are filtered and washed extensively before culture. The Millipore CM® filter inserts are coated with type II collagen in sterile 0.1N acetic acid and allowed to dry. Ultraviolet light (20 minutes) was used to sterilize the filter inserts before use. The inserts are washed with sterile Ham's F12 to remove any residual acetic acid. The chondrocytes were plated on the filter inserts at a density of 2.5 x 10^6 cells/cm^2 and maintained in Ham’s F12 with 5% fetal bovine serum (FBS). The serum concentration was increased from 5% to 20% FBS on day 5. On day 7, ascorbic acid (final concentration: 100 μg/ml) was added to the medium of the cultures. The cells were grown at 37°C in a humidified atmosphere supplemented with 5% CO₂. Medium was changed every two days and fresh ascorbic acid was added with each change of medium.
2.2.2 Analysis of Collagen

The cultures were labeled with $[^{14}C]$-proline (4 μCi per well) for 24 hours and then digested with pepsin (100 μg/ml in 0.5M acetic acid) for 48 hours at 4°C. The digestion was stopped by the addition of 4 x Laemmli's buffer. The pepsin digest was separated on a 7% SDS-polyacrylamide gel and either prepared for autoradiography or transferred to nitrocellulose for Western blot analysis. The presence of type II or type I collagen was determined by immunoblot analysis using antibody reactive to type II collagen (dilution 1/100) or type I collagen (dilution 1/100) [Southern Biotechnology Associates Inc.]. The blots were washed three times and reactivity detected using anti-goat IgG antibody conjugated to alkaline phosphatase. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) were added as substrate for the alkaline phosphatase activity of the secondary antibody resulting in a colour reaction.

2.2.3 Analysis of Proteoglycans

2.2.3.1 Radiolabelling of Cultures

Cultures were labelled to analyze proteoglycan size and biosynthesis. The medium of the cultures was replaced prior to radiolabelling. Cultures were incubated with $[^{35}S]$-sulphate (4 μCi per well) which was added to the inner well of the filter insert for 24 hours prior to harvesting. Matrix proteoglycans were extracted with 4M guanidine HCl in 50 mM sodium acetate, pH 5.8 containing 0.1 M 6-amino-hexanoic acid, 50 mM benzamidine HCl, 10 mM EDTA and 5 mM N-ethylmaleimide for 24 hours at 4°C. The proteoglycans were precipitated by addition of three volumes of cold ethanol. After 24
hours at 4°C the pellets were collected by centrifugation at 16,000 g for 30 minutes, washed three times with 70% ethanol (Hoffman et al, 1993). The pellets were either dissolved in 8 M urea for agarose gel electrophoresis or 4 M guanidinium hydrochloride for quantifying [$^{35}$S]-sulphate incorporation using a scintillation counter. Bovine cartilage ex vivo cultures that had been incubated with [$^{35}$S]-sulphate for 24 hours served as controls. The proteoglycans from the ex vivo cultures were extracted identically to the filter cultures.

2.2.3.2 Agarose Gel Electrophoresis of Proteoglycans

Prior to loading on the gel, the [$^{35}$S]-sulphate matrix proteoglycan extracts were resuspended in 8 M urea, heated to 60°C for 1 hour and an equal volume of 2 x loading buffer added (60% glycerol, 0.04 M TRIS acetate, pH 6.3, 1 mM sodium sulphate and 0.05% bromphenol blue). The proteoglycans were separated on 0.8% submerged horizontal agarose gels. The gels were subsequently prepared for autoradiography or Western blotting.

2.2.3.3 Western Blot Analysis of Proteoglycans

Selected gels were transferred to nitrocellulose for Western blot analysis. The blots were blocked with 5% bovine serum albumin and then incubated with a monoclonal antibody reactive with keratan sulphate (D1B2, generous gift from Dr. M. Adams, University of Calgary, Canada). Reactivity was detected using affinity-purified rabbit anti-mouse IgG antibody conjugated to alkaline phosphatase. NBT/BCIP were added for substrate and colour reaction.
2.2.4 Histological Examination

The chondrocyte filter cultures were harvested on days 3, 6, 10, 21, 35, and 49. The tissue was fixed in 10% buffered formalin and paraffin-embedded. Tissue sections (5 μm) were cut and stained with toluidine blue which stains sulphated proteoglycans.

2.2.5 Proteoglycan Quantification

Tissue was digested with papain (10 μg/ml) in 20 mM ammonium acetate, 1 mM EDTA and 2 mM dithiotreitol (DTT) for at least 18 hours at 65°C. Proteoglycan content was determined by measuring the amount of glycosaminoglycan in the papain digest of each culture using the dimethylmethylene blue (DMMB) dye binding assay and spectrophotometry as described by Farndale et al (1986) and modified by Goldberg and Kolibas (1990). Chondroitin sulphate was used as a standard.

2.2.6 Collagen Quantification

An aliquot of the papain digest was hydrolyzed using 12 N hydrochloric acid overnight at 110°C. The amount of collagen in the extracellular matrix was determined by measuring hydroxyproline using high pressure liquid chromatography (HPLC) on a C18 reverse column and a Waters PicoTag amino acid analysis system.
2.2.7 DNA Quantification

The cultures at different time points were digested with papain (10 μg/ml in 20 mM ammonium acetate, 1 mM EDTA and 2 mM DTT) for at least 18 hours at 65°C. The DNA content was measured using Hoescht 33258 dye and fluorometry (emission wavelength 365 nm and excitation wavelength 458 nm) as described by Kim et al (1988). Calf thymus DNA was used to generate the standard curve.

2.2.8 Culture Thickness

Using light microscopy, the culture thickness was determined by measuring ten separate points in each histological section of the cartilagenous tissue. Three sections per culture were examined. The value for each time point was calculated by determining the mean value of triplicate cultures.
2.3 Results

2.3.1 Morphological Appearance of Cultures

The chondrocyte cultures were examined histologically at 3, 6, 10, 21, 35, and 49 days after initiation of the cultures in order to assess the morphological appearance of the tissue. Figure 2.1 shows toluidine-blue stained tissue from filter cultures at days 6, 21, and 49. On days 3 and 6 the cells were multilayered and surrounded by small amounts of proteoglycans. By day 10, three days after ascorbic acid addition, there was a significant increase in the amount of matrix accumulated such that a continuous layer of cartilagenous tissue had formed. The tissue became thicker over time. The cells are surrounded by extracellular matrix.
**Figure 2.1: Cartilagenous tissue formed over time**
Photomicrographs of tissue from chondrocyte filter cultures. The cultures were formalin fixed, paraffin embedded, and 5 μm sections stained with toluidine blue. The cells were plated at $2.5 \times 10^6$ cells/cm² and harvested at (A) 6 days, (B) 21 days (C) 49 days. These photographs demonstrate the accumulation of extracellular matrix with time. The filters are indicated with an F (original magnification x 400).
2.3.2 Maintenance of Chondrocyte Phenotype: Analysis of Collagen and Proteoglycan

2.3.2.1 Collagen Type

To ensure that the chondrocytes maintained their phenotype under the culture conditions, we examined whether chondrocytes were synthesizing cartilage type macromolecules such as type II collagen and large proteoglycans. Analysis of the [$^{14}$C]-proline labeled pepsin extracts of the matrix of 21 day old cultures by gel electrophoresis and autoradiography showed the presence of a single major band with a mobility similar to type II collagen (Figure 2.2A). By Western blot analysis, this band was identified as type II collagen (Figure 2.2B). No type I collagen was detected in the pepsin extracts by either autoradiography or western blot analysis using an antibody reactive with type I collagen (Figure 2.2C).
Figure 2.2: Analysis of collagen synthesized by chondrocytes in filter culture

[A] Representative autoradiogram of [14C]-proline-labeled pepsin-digested collagen from the extracellular matrix formed by chondrocytes in culture for 3 weeks. The location of the αI(II) chain is indicated by the arrowhead. [B] Western blot analysis of the pepsin extract (E) of the chondrocyte cultures. The blots have been incubated either with antibody reactive with type II collagen (II) or type I collagen (I) as described under the Methods. Only type II collagen was detected. No type I collagen was detected as demonstrated by the absence of any reactivity in the extract (E). The appropriate collagens, Type I and Type II, were run as standards (S).
2.3.2.2 Proteoglycan Monomer

In order to determine if the filter cultures were synthesizing large proteoglycans that are characteristic of articular cartilage, proteoglycans from the matrix of filter cultures were extracted and examined by agarose gel electrophoresis. Newly synthesized proteoglycans extracted from ex vivo cartilage explants served as a control. By agarose gel electrophoresis, the proteoglycan monomers migrated similarly to proteoglycans extracted from the cartilage explants although some of the proteoglycans ran slightly faster. The chondrocytes in filter culture were synthesizing large proteoglycans but there were also proteoglycans similar but not identical in size to those from the explants (Figure 2.3A). Aggrecan, the principal proteoglycan in articular cartilage, contains keratan sulphate. Western blot analysis using an anti-keratan sulphate antibody demonstrated the presence of keratan sulphate in filter culture proteoglycans (Figure 2.3B). Proteoglycan monomer heterogeneity, when examined by agarose gel electrophoresis, did not appear to vary over the 49 days (data not shown).
Figure 2.3: Analysis of proteoglycans extracted from the matrix

[A] Autoradiogram of an agarose gel showing $[^{35}\text{S}]$-sulphate labeled proteoglycan monomers extracted from chondrocyte filter culture (F) at 3 weeks or chondrocyte ex vivo culture (E). [B] Western blot of proteoglycans from filter and explant cultures using an antibody reactive with keratan sulphate.
2.3.3 Proteoglycan and Collagen Accumulation in the Matrix

The proteoglycan (Figure 2.4A) and collagen (Figure 2.4B) content in the extracellular matrix of these cultures was quantified over 49 days. Only small amounts of proteoglycans and collagen were present in six-day cultures. The proteoglycan and collagen content in the matrix increased linearly up to 35 days and then plateaued. There was no further increase detected at day 49. At 35 days the tissue contained approximately 280 μg collagen and 95 μg of glycosaminoglycans, representing a collagen to proteoglycan ratio of approximately 3:1. The amount of collagen was calculated from the hydroxyproline content, as hydroxyproline comprises approximately 10% of the weight of fibrillar collagens (Berg, 1982).
**Figure 2.4: Collagen and proteoglycan content over time**

Chondrocyte filter cultures were harvested at varying times and digested with papain to quantitate the amount of glycosaminoglycan (A) and OH-proline (B) in the matrix. OH-proline was quantitated by HPLC. Glycosaminoglycan content was determined by dimethylmethylen blue dye binding assay and spectrophotometry as described under the Methods. The results are expressed as the mean ± SEM of triplicate cultures from one representative experiment.
2.3.4 Proteoglycan Synthesis

Proteoglycan synthesis was determined by quantifying the total amount of $[^{35}\text{S}]$-sulphate incorporated into proteoglycans in the matrix and supernatant normalized to DNA content of the cultures (Figure 2.5). Proteoglycans synthesis increased linearly between 6 and 21 days and remained high up to day 35. By 49 days, proteoglycan synthesis declined to levels similar to those observed in 6 day old cultures. At 63 days there was a further drop in proteoglycan synthesis (data not shown).

2.3.4.1 Proteoglycan Distribution

Figure 2.6A demonstrates that the amount of newly synthesized proteoglycans retained in the matrix or secreted into the medium varied during the culture period. The amount of newly synthesized proteoglycans incorporated into the matrix increases up to 35 days, whereas the amount secreted into the supernatant declined approximately fourfold between days 10 and 49. An increase in the percentage of newly synthesized proteoglycans was observed between 6 and 35 days (Figure 2.6B). The increase in percentage of newly synthesized proteoglycans retained in the matrix paralleled the proteoglycan content in the extracellular matrix since no further increase in proteoglycan (GAG) content was observed after 35 days in culture.
Figure 2.5: Proteoglycan synthesis in filter cultures
Proteoglycan biosynthesis from days 6 to 49 was determined by quantifying $[^{35}\text{S}]$-sulphate incorporation into proteoglycans by counting the total amount of radioactivity in the supernatant and matrix as described under the Materials and Methods. This graph is from one representative experiment and is expressed as cpm/µg DNA (mean ± SEM) of quintuplet cultures. The experiment was repeated four times.
Figure 2.6: Distribution of newly synthesized proteoglycans and percent retained within the matrix

[A] The amount of radioactivity in the matrix (○) and supernatant (●) at various times was quantified as described under the Materials and Methods. The experiment was repeated four times and the results are expressed as the mean ± SEM. [B] The percentage of newly synthesized proteoglycans retained in the matrix at different times was calculated.
2.3.5 Quantification of DNA Content of Filter Cultures

DNA content of the tissue was determined over time in culture. There was no significant change (ANOVA, $p>0.05$) in the DNA content during the 49 days of culture (Figure 2.7). The average DNA content of the cultures was $33\pm5$ μg (mean ± SEM).

2.3.6 Quantification of Matrix Thickness of Filter Cultures

To quantify matrix accumulation histologically, the matrix thickness over time in cultures was determined for the chondrocyte cultures. As shown in Figure 2.8 the tissue increased in thickness up to 35 days in culture after which no further increase in thickness was detected.
Figure 2.7: DNA content of chondrocyte filter cultures over time
DNA was quantified in filter cultures at different time points. The tissue was digested with papain and the DNA content was measured using Hoescht 33258 dye and fluorometry. The results are expressed as the mean ± SEM of one representative experiment which was repeated three times.
Figure 2.8: Thickness of chondrocyte cultures
The thickness of cartilagenous tissue formed by chondrocytes in filter culture was examined. Chondrocytes were plated at 2.5 x 10⁶ cells/cm² on filter inserts and harvested on days 6, 10, 21, 35, and 49. The cultures were fixed in formalin and the thickness of the cultures measured as described under the Materials and Methods. The results are from one representative experiment performed in triplicate and expressed as mean ± SEM. The experiment was repeated three times.
2.4 Discussion

During the period of growth, articular cartilage becomes thicker suggesting that chondrocytes can synthesize and accumulate sufficient amounts of matrix macromolecules to increase the amount of cartilage under normal physiological conditions. Yet chondrocytes can not adequately accumulate and replace the matrix, which has been lost due to trauma or disease (Poole, 1992). In the present study, we have optimized an articular chondrocyte culture system in which isolated chondrocytes placed on filter inserts accumulate extracellular matrix and form cartilagenous tissue. This system provides an *in vitro* model system to investigate the mechanism(s) regulating proteoglycan and collagen retention that results in matrix accumulation. This culture method should allow us to identify ways to induce chondrocytes to accumulate sufficient matrix to replace cartilage that has been lost due to trauma or disease.

While many methods for culturing chondrocytes have been developed, maintaining chondrocytes on filter inserts has advantages over these other culture methods in that a continuous layer of tissue can be obtained in a reasonable amount of time which is amenable to histological and biochemical assessment.

Under many of the conditions developed previously to culture chondrocytes *in vitro*, the cells dedifferentiate. Therefore, it was necessary to confirm that chondrocytes in filter culture maintained their phenotype. Determining whether the chondrocytes synthesized proteoglycans and type II collagen was used to assess phenotypic stability. The large proteoglycans of cartilage contain keratan sulphate/chondroitin sulphate
(Hardingham, 1979). The proteoglycans in filter cultures were large and contained keratan sulphate. The average size of the proteoglycan monomers synthesized and incorporated into the matrix did not change over the time examined. Our studies demonstrated that the cells produced type II collagen. No type I collagen, which would have indicated chondrocyte dedifferentiation, was detected by autoradiography and Western blot analysis. It is possible that synthesis of small amounts of type I collagen might not have been detected using these methods. Under these culture conditions the chondrocytes are maintaining their phenotype.

The proteoglycan and collagen content of articular cartilage are important for it to be able to withstand compressive and tensile forces. The extracellular matrix contains approximately three fold more collagen than proteoglycan by 35 days. This ratio is similar to in vivo cartilage (Hardingham, 1990). The cultures reach a point where there is no further change in proteoglycan and collagen content in the extracellular matrix. These data indicate that the cultures go through two phases, one of matrix accumulation that occurs less than 35 days and one where the matrix is being maintained which occurs after 35 days. These data suggest that if the cultures are to be used to study matrix accumulation, it should be done during the first 35 days of culture.

Under optimal conditions, the cartilaginous tissue attains a thickness of 110 \( \mu \text{m} \) by 35 days and does not show any further increase. It should be noted that the tissue thickness obtained using this methodology is not the true thickness of the tissue. During the processing for paraffin embedding the tissue undergoes dehydration (80% of the
water removed) and as cartilage contains abundant water, the thickness measured histologically is underestimated.

The tissue thickness and content of proteoglycan and collagen in the extracellular matrix increased in parallel. There is no obvious explanation as to why matrix accumulation in cartilagenous tissue from filter cultures levels off by 35 days. It may be related to limited nutrition as the diffusion of nutrients through the tissue may be affected by the thickness of the cartilagenous tissue. This explanation seems unlikely as the cartilagenous tissue thickness can be modified by other factors. For example, cell plating density can influence the thickness of the tissue obtained. Cells plated at low density (1 x 10^6 cells/cm^2) formed tissue that by day 35 measured 34±2 μm (data not shown). When cells were plated at a higher density (2.5 x10^6 cells/cm^2) a thickness of 110 ±7 μm was obtained. The data suggest that there may be regulatory mechanism(s) involved in controlling cartilage thickness.

The amount of [35S]-sulphate incorporated into proteoglycans increased during tissue formation and decreased during the period of tissue maintenance. When these studies were repeated with [3H]-glucosamine instead of [35S]-sulphate (data not shown), similar changes in synthesis occurred suggesting that [35S]-sulphate incorporation reflects the changes in proteoglycan synthesis and not differences in proteoglycan sulphation. These data demonstrate that chondrocyte synthesis of proteoglycans is greater when matrix is accumulating. The environmental conditions or matrix molecules responsible for regulating proteoglycan synthesis by chondrocytes have yet to be elucidated. It has been shown that
chondrocyte synthesis of proteoglycans can be either stimulated or inhibited by extracellular proteoglycans or hyaluronate (Takeda et al, 1986; Lucas and Dziewiatkowski, 1987; Solursh et al, 1980; Bansal et al, 1986; Brown et al, 1990). It may be that the amount of proteoglycans synthesized in filter culture is being regulated by the concentration of macromolecules in the pericellular matrix during tissue formation and maintenance.

In this culture system, the percent of newly synthesized proteoglycans incorporated in the extracellular matrix increased during the period of matrix accumulation and maximal retention occurred during tissue maintenance. It is not evident why proteoglycan retention changes over time but it may be due to the developing tissue organization and/or the increasing amount of collagen in the matrix. The latter explanation seems most likely as the collagen and proteoglycan content in the matrix of these cultures increase in parallel. This hypothesis is supported by other experimental work which showed when chondrocytes in culture are grown in the presence of ascorbate, which induces accumulation of collagen in the pericellular region, there is increased proteoglycan retention (Levenson, 1970; Jouis et al, 1985). Fibroblast cultures supplemented with L-ascorbic acid 2-phosphate, a long acting ascorbic acid derivative, will form a three dimensional tissue (Hata and Senoo, 1989). Alternatively, as proteoglycan retention in cartilage has been attributed to proteoglycan aggregation with hyaluronate and its stabilization by link protein (Melching and Roughley, 1985), it may be that link protein synthesis by chondrocytes in filter culture changes during the culture period. Plaas et al. (1993), using long term rabbit chondrocyte cultures, has demonstrated a decrease in the amount of link protein synthesized with time in culture which would
alter proteoglycan aggregation and its retention. Aggregation of the proteoglycans synthesized in filter culture is discussed further in Chapter 4. Nevertheless, the mechanism(s) regulating proteoglycan retention in the matrix requires further investigation.

In summary, the chondrocytes are maintaining their phenotype under these culture conditions. Interestingly, as the chondrocytes reconstitute their extracellular matrix there is a period of matrix accumulation and then the cells “switch” and there is no further detectable accumulation. The cells then maintain the extracellular matrix components similar to the in vivo tissue.
Chapter Three: Similarity of cartilagenous tissue formed in filter culture to in vivo articular cartilage

3.1 Introduction

Articular cartilage grossly appears homogeneous. However, morphological and biochemical studies have shown that it has a very specific organization and composition. The organization of the collagen network is critical for the tensile strength of the tissue and for the entrapment of proteoglycans within the matrix. The superficial zone consists of a network of collagen fibres oriented parallel to the articular surface (Weiss et al, 1968; Clarke, 1971). The collagen fibrils in the intermediate zone are random in arrangement and surround the chondrocytes. The deep zone collagen fibrils are organized so that they are to be perpendicular to the subchondral bone.

The types and/or amounts of proteoglycan, collagen and other minor components have been shown to vary depending on the depth of the tissue. Compositional analysis of articular cartilage shows that collagen content decreases by approximately 15% from the superficial to the middle and deep zones (constant in lower two zones per wet weight). Proteoglycan content is lowest in the superficial zone and increases steadily through the middle and deep zones by as much as 50% (Brookehurst et al, 1984). Water content is highest in the superficial zone (~80%) and decreases to approximately 65% in the middle and deep zones (Torzilli et al, 1982).

Type II and type IX collagens are distributed throughout the tissue (Wardale and
Duance, 1993). Type VI collagen is confined in the pericellular region (Poole et al, 1992; Wardale and Duance, 1993). Aggrecan is present throughout cartilage (Poole et al, 1982; Ratcliffe et al, 1984) but is at its highest concentration in the middle zone, and immediately surrounding the chondrocyte. Aggrecan aggregate size also varies with depth in the tissue. Smaller aggregates are predominant in superficial cartilage while the middle and deep have larger aggregates (Muller et al, 1989). Chondroitin sulphate (CS) and keratan sulphate (KS) are distributed heterogeneously throughout the tissue. CS is highest in the middle zone (Bayliss, 1983). While KS levels increase with distance from the surface (Bayliss et al, 1983). Decorin is present primarily in the superficial zone of adult cartilage (Poole et al, 1986).

Chondrocytes are responsible for the synthesis and maintenance of this complex organization. Chondrocytes themselves also show an organization as the superficial zone cells are flattened and oriented parallel to the surface whereas the deeper cells are more spherical. The cells are more numerous in deeper tissue. In keeping with the complex structure of cartilage, chondrocyte metabolism differs with depth (Archer, 1990). In addition to synthesizing different types and/or amounts of macromolecules they can have different activities. For example, deep chondrocytes have alkaline phosphatase activity (Xu et al, 1994) and are responsible for synthesizing the matrix that develops into the calcified cartilage (calcified cartilage allows for the attachment of articular cartilage to the underlying subchondral bone).

Biosynthetic and proteolytic processes contribute to the structure of the
macromolecules that are incorporated into cartilagenous tissue. While the macromolecules reside in the matrix they can be proteolytically modified. Proteoglycans for example have been shown to decrease in size over time in the extracellular matrix (Campbell et al, 1989; Ilic et al, 1992, Ilic et al, 1995). If the cartilagenous tissue formed in filter culture is to serve as a model for in vivo formed articular cartilage it is necessary to demonstrate the similarity between the two tissues.

3.1.1 Objectives

The objective of these experiments was to characterize the in vitro cartilagenous tissue to determine its similarity to the in vivo tissue. Specifically this involved:

1) Examining the chondrocyte filter cultures histologically in order to assess the organization of the cellular and matrix components of the tissue formed.

2) Determining water content of the cartilagenous tissue formed in filter culture.

3) Determining whether chondrocytes isolated from the different zones of cartilage will maintain their in vivo characteristics while in filter culture.

4) Determining the size of the proteoglycans synthesized by chondrocytes in filter culture and compare them to those synthesized by articular chondrocytes in ex vivo cartilage cultures and high density monolayer cultures.

5) Determining whether proteoglycan size decreases over time in the matrix.
3.2 Materials and Methods

3.2.1 Chondrocyte Cultures

Chondrocytes were obtained from calf articular cartilage removed from the metacarpophalangeal joints. Chondrocytes were obtained from either the upper 2/3 (superficial/intermediate zones), the deep zone, or the full thickness of the cartilage. The cell isolation on filter inserts was done as described in Chapter 2. The cell densities at the time the culture was initiated are as follows: full thickness $2.5 \times 10^6$ cells/cm$^2$, deep zone $1.6 \times 10^6$ cells/cm$^2$ and superficial/intermediate zone $2 \times 10^6$ cells/cm$^2$.

To ensure that we were isolating chondrocytes from the deep zone of articular cartilage we utilized the finding that articular chondrocytes in or just above the calcified cartilage have detectable alkaline phosphatase activity (Xu et al, 1994). Therefore the amount of enzyme activity of the isolated cells was determined to assess whether the cells were enriched with chondrocytes from deep cartilage. Chondrocyte preparations with an alkaline phosphatase activity of at least $2 \mu$M PNP/h.$\mu$g DNA were used to establish deep cultures. This value represented approximately a four fold increase in alkaline phosphatase activity when compared to the same number of cells obtained from the entire thickness of cartilage (Kandel et al, 1997).

The culture conditions for the deep zone and superficial/intermediate zone chondrocytes were different from the culture conditions of cell from the full thickness of cartilage. Cultures of the deep zone cells and selected cultures of superficial/intermediate
chondrocytes the medium were maintained in Dulbecco's modified Eagle's medium (DMEM) with 20% FBS and ascorbic acid (100 μg/ml, final concentration) after 5 days. Furthermore, after two weeks in culture, β-glycerophosphate [β-GP] (10 mM, final concentration) was added to induce mineral formation in the tissue.

Monolayer cultures and ex vivo articular cartilage cultures were also established. Monolayer cultures were established from chondrocytes from the full thickness of cartilage and plated at a density of 2.5 × 10^6 cells/cm^2 on tissue culture plastic. This high density helps maintain chondrocyte phenotype. Ex vivo (explant) cultures are slices of full thickness articular cartilage. Monolayer and ex vivo cultures were cultured in Ham's F12 with 20% FBS. Monolayer cultures were harvested after seven days in culture and ex vivo cultures were harvested after one day in culture.

3.2.1.1 Histological Assessment

Full thickness, superficial/intermediate, and deep chondrocyte culture tissues were examined histologically. The cultures were harvested at various times after plating, fixed in 10% buffered formalin and paraffin-embedded. Histological sections (5 μm) were examined by light microscopy following staining with hematoxylin and eosin, toluidine blue (sulphated proteoglycans) or picrosirius red. Picrosirius red stains collagen in the extracellular matrix and can be visualized under polarized light. Von Kossa staining was used to stain the mineral deposits.
3.2.1.2 Measurement of Wet Weight / Dry Weight of the Cartilaginous Tissue

The difference between the wet weight and dry weight of the cultures was determined in order to estimate the water content of the tissue. The tissue was harvested at 42 days, blotted dry, and weighed. The tissue was lyophilized for at least 12 hours and weighed immediately. Previous studies showed that this was a sufficient period of lyophilization as the dry weight remained stable (did not change) with longer periods of lyophilization.

3.2.2 Proteoglycan Isolation

3.2.2.1 Radiolabelling of Cultures

The culture medium was replaced prior to labelling. To analyze proteoglycan size the cultures were incubated with $[^{35}\text{S}]$-sulphate (4 μCi / per well). The radioisotope was added to the inner well of the filter insert and incubated for 24 hours prior to harvesting. To examine proteoglycan degradation over time in culture a pulse chase design was used. Cultures were incubated with $[^{35}\text{S}]$-sulphate (30 μCi / per well) for 6 hours. The cultures were then washed extensively and the medium was replaced with 20% FBS in Ham's F12. Cultures were harvested 1, 7, and 14 days after labelling.
3.2.2.2 Proteoglycan Extraction

Matrix proteoglycans were extracted with 4 M guanidine hydrochloride in 50 mM sodium acetate, pH 5.8 containing 0.1 M 6-amino-hexanoic acid, 50 mM benzamidine HCl, 10 mM EDTA and 5 mM N-ethylmaleimide for 24 hours at 4°C. The proteoglycans from the matrix and supernatant were precipitated by the addition of three volumes ice cold ethanol. After 24 hours at 4°C the precipitate was collected by centrifugation at 14,000 rpm for 30 minutes and washed three times with 70% ethanol. The pellets were resuspended in 4 M guanidium HCl with protease inhibitors for gel filtration chromatography. Bovine cartilage explant or monolayer cultures labelled for 24 hours with $[^{35}\text{S}]$-sulphate served as controls. Proteoglycans from these cultures were extracted identically to the filter cultures.

3.2.3 Gel Filtration Chromatography

3.2.3.1 Gel Filtration Chromatography of Proteoglycan Monomers

The proteoglycans extracted from the matrix and supernatant of 21 day old (accumulation phase) or 49 day old (maintenance phase) cultures were analyzed by gel filtration chromatography under dissociative conditions in order to examine proteoglycan monomer size. Proteoglycans extracted from tissue generated by deep zone chondrocytes in filter cultures were analyzed after receiving four weeks or six weeks of $\beta$-glycerophosphate. Proteoglycans extracted from monolayer cultures and ex vivo cultures were examined by chromatography after seven days and one day in culture respectively.
In the pulse chase experiments, matrix proteoglycan size was examined 1, 7, and 14 days after labelling. The same number of cpm of $^{35}$S-sulphate labelled proteoglycans was loaded on the column for each of these time points.

Aliquots of the guanidinium hydrochloride extracts of the proteoglycans were applied to an analytical Sepharose CL-2B column (100 x 1 cm). Any residual mineral was removed by centrifugation for 5 minutes at 4$^\circ$C before being applied to the column. Columns were eluted (6 ml/hr) with 4 M GuHCl in 0.05 M sodium acetate, pH 5.8 containing 0.1 M 6-amino-hexanoic acid, 0.05 M benzamidine HCl, 0.01 M EDTA and 0.05 mM N-ethylmaleimide at 4$^\circ$C. Fractions (2ml) were collected. The elution profile was analyzed for its partition coeffient $Kav$ [$Kav = (Ve-Vo)/Vt-Vo$], where $Vo$=void volume, $Vt$=total volume, $Ve$=elution volume. $Vt$ was determined using $^{35}$S-sulphate and $Vo$ was determined using blue dextran 2000.

3.2.3.2 Gel Filtration Chromatography of Endogenous Proteoglycans

Endogenous proteoglycans from the matrix at days 10, 21 and 49 were chromatographed as described above and detected in the eluted fractions using the dimethylmethylene blue dye binding (DMMB) assay. Proteoglycans from each fraction were precipitated with ethanol and digested with papain and quantified as described in Chapter 2 (part 2.2.3). Elution profiles were generated from the values of the DMMB dye binding assay.
3.3 Results

3.3.1 Morphological Appearance of Cultures

At day 6 no cellular organization was evident (Figure 3.1A). By 21 days (Figure 3.1B) the chondrocytes of the superficial layer were elongated and oriented parallel to the surface of the tissue. The deeper cells were spherical in shape. At 49 days the cellular and matrix organization was maintained (Figure 3.1C). Picrosirius red staining showed that at day 21 the collagen fibrils in the superficial aspect were oriented parallel to the surface whereas in the deeper zone they surrounded the chondrocytes (Figure 3.2).
Figure 3.1: Morphological appearance of tissue from full thickness chondrocyte cultures
Photomicrographs of tissue from chondrocyte filter cultures. The cultures were formalin-fixed, paraffin-embedded, and 5 μm thick sections stained with hematoxylin and eosin. The tissue was harvested at (A) 6 days, (B) 21 days, and (C) 49 days. These photomicrographs demonstrate the morphology of the chondrocytes in filter culture with time (magnification x 400, F = filter).
Figure 3.2: Collagen orientation in cartilagenous tissue
Photomicrograph of tissue from chondrocyte filter culture harvested at 21 days. The cultures were formalin-fixed, paraffin-embedded, and sections stained with picrosirius red. The tissue was examined under polarized light. The photomicrograph demonstrates the collagen orientation within the extracellular matrix. (magnification x 700, F = filter)
3.3.2 Histological Assessment of Filter Cultures

Chondrocyte cultures established from cells isolated either from superficial/intermediate or deep zones of articular cartilage were maintained in the presence of β-glycerophosphate for eight weeks. The tissue was harvested and examined histologically following staining with either toluidine blue or Von Kossa. Cells from both zones accumulated extracellular matrix and formed cartilagenous tissue. Toluidine blue staining demonstrated that a large amount of proteoglycans accumulated. The tissue formed by the deep zone chondrocytes appeared thicker than the tissue formed by superficial chondrocytes at the same time point (eight weeks) [Figure 3.3]. The cartilagenous tissue formed by superficial cells did not demonstrate the ability to form mineral deposits (Figure 3.4A). Only the tissue generated by deep chondrocytes underwent calcification, which can be seen as black mineral deposits within the matrix (Figure 3.4 B). After eight weeks, two zones were formed in deep zone chondrocyte cultures: zone of mineralization formed near the filter and a non-mineralized zone formed above the mineralization. Other studies in our laboratory have identified the mineral as hydroxyapatite (Kandel et al, 1997).
Figure 3.3: Cartilagenous tissue formed in filter culture by superficial and deep zone chondrocytes

Tissue generated in filter culture by superficial [A] and deep [B] zone chondrocytes after eight weeks in culture. The cultures were formalin-fixed, paraffin-embedded, and sections stained with toluidine blue. At eight weeks the cartilagenous tissue formed by superficial chondrocytes is thinner than the tissue generated by deep zone chondrocytes. (magnification x 400, F = filter)
Figure 3.4: Histology of superficial and deep zone chondrocyte filter cultures

[A] Tissue formed by superficial and [B] deep chondrocytes while in filter culture have been formalin-fixed, paraffin-embedded. Tissue was harvested after eight weeks in culture. At eight weeks the cartilagenous tissue formed by superficial chondrocytes does not show calcification whereas the deep chondrocyte cultures show a zone of mineralization. (magnification x 400, Von kossa with basic fuchsin counterstain)
3.3.3 *Cartilagenous Tissue Water Content*

The difference between the wet weight and dry weight of the cartilagenous tissue generated in filter culture was determined in order to estimate the water content of the tissue. Full thickness chondrocyte cultures demonstrated a water content of 80.6 ± 2.2% on day 42.

3.3.4 *Proteoglycan Monomer Size from Full Thickness Cultures*

When examined using gel filtration chromatography under dissociative conditions the newly synthesized proteoglycans retained in the matrix (PG-R) in cultures of chondrocytes obtained from full thickness cartilage were large and heterogeneous with a Kav of 0.44±0.02 (n=10). At 49 days, the newly synthesized proteoglycans in the matrix remained at the same size as day 21 cultures (Kav 0.44±0.02, n=5) [Figure 3.5].

3.3.5 *Endogenous Proteoglycan Size*

Analysis of the same fractions for unlabelled sulphated GAG (S-GAG) extracted from non-mineralizing chondrocyte (obtained from full thickness cartilage) cultures using the dimethylmethylene dye binding assay showed elution profiles with a peak that was similar or slightly shifted to the right compared to the newly synthesized proteoglycans (Figure 3.6). This indicates that the total proteoglycan population present in the filter cultures was of a similar size or slightly smaller than the newly synthesized aggrecan population. The range of partition coefficients (Kav) of the endogenous proteoglycans was 0.44-0.54.
Figure 3.5: Proteoglycans from the matrix of chondrocyte filter cultures

Gel chromatography of proteoglycans extracted from the matrix of 21 (---) and 49 (—) day old filter cultures. Proteoglycan hydrodynamic size was determined by gel filtration chromatography (Sepharose CL-2B) under dissociative conditions (4M GuHCl). These are representative elution profiles from one experiment.
Figure 3.6: Endogenous proteoglycans from full thickness chondrocyte cultures

Gel chromatography of proteoglycans retained and not retained within the matrix extracted from 21 day old cultures. Proteoglycan hydrodynamic size was determined by Sepharose CL-2B chromatography under dissociative conditions (4M GuHCl) using the DMMB assay to detect the sulphated glycosaminoglycans (o). Newly synthesized proteoglycans (●) are shown for comparison. This is a representative elution profile from one experiment, which has been repeated 4 times.
3.3.6 Proteoglycan Size Over Time

Proteoglycans synthesized in the filter cultures were labelled with $[^{35}S]$-sulphate using a pulse chase design and chromatographed. Cultures were pulsed with $[^{35}S]$-sulphate for 6 hours and then chased for 1, 7, and 14 days after labelling. Proteoglycans were extracted from the matrix and applied to Sepharose CL-2B columns under dissociative conditions. This is a representative elution profile from one experiment, which has been repeated three times. The elution profiles shifted to the right with time after labelling, indicating that the proteoglycans became progressively smaller while residing in the matrix. This is demonstrated in Figure 3.7 which shows the elution profiles of proteoglycans harvested after 24 hours, one, and two weeks after labelling with $K_{av}$s of 0.4, 0.59, and 0.74 respectively.
Figure 3.7: Proteoglycan hydrodynamic size over time in the matrix of full thickness cultures
Cultures were pulsed with $[^{35}\text{S}]-\text{sulphate}$ for 6 hours and then chased for 1 (•), 7 (○), and 14 (●) days after labelling. Proteoglycans were extracted from the matrix and applied to Sepharose CL-2B columns under dissociative conditions. This is a representative elution profile from one experiment which has been repeated three times.
3.3.7 Analysis of Proteoglycans Released into the Supernatant of Filter Cultures

The newly synthesized proteoglycans in the supernatant (non retained [PG-NR]) during the accumulation phase were larger in size than the proteoglycans retained within the ECM with a Kav = 0.23±0.01 (n=10) [Figure 3.8A]. The difference in hydrodynamic size of the newly synthesized proteoglycans retained within the matrix (PG-R) and PG-NR is significant (Student’s t-test, p<0.01). In the maintenance phase of full thickness filter cultures, the proteoglycans not retained were smaller in size (Kav 0.54±0.05, n=5) than the proteoglycans retained within the matrix [Figure 3.8B].

In order to determine if the larger hydrodynamic size of non-retained proteoglycans in full thickness cultures was an artifact of the culture conditions, deep zone chondrocyte cultures were initiated. The size of the proteoglycans that were retained and not retained within the ECM was examined. In the deep zone chondrocyte cultures, the mean Kav of the supernatant proteoglycans was 0.29±0.01 (n=4) and matrix proteoglycans was 0.26±0.03 (n=5) (Figure 3.9). There was no significant difference in hydrodynamic size between the proteoglycans retained and not retained within the ECM in deep zone filter cultures suggesting that the hydrodynamic size difference observed in the full thickness cultures between PG-R and PG-NR was not an artifact of filter culture.
Figure 3.8: Newly synthesized proteoglycans retained and not retained in full thickness chondrocyte cultures

Gel chromatography of proteoglycans retained (●) and not retained (○) within the matrix extracted from (A) 21 and (B) 49 day old cultures. Proteoglycan hydrodynamic size was determined by Sepharose CL-2B chromatography under dissociative conditions (4M GuHCl). These are representative elution profiles from one experiment.
Figure 3.9: Proteoglycans synthesized in filter cultures established using deep zone chondrocytes

Gel chromatography of proteoglycans retained (●) and not retained (○) within the matrix extracted from deep chondrocyte filter cultures during mineralization (6 weeks). Proteoglycan hydrodynamic size was determined by Sepharose CL-2B chromatography under dissociative conditions (4M GuHCl). These are representative elution profiles from one experiment, which has been repeated 4 times.
3.3.8 Analysis of Proteoglycans from ex vivo and Monolayer Cultures

Proteoglycans synthesized by differentiated chondrocytes were examined under two conditions: in cultures accumulating matrix (monolayer culture) and in cultures in which there is abundant matrix (ex vivo) and the chondrocytes are replacing proteoglycan that are lost during acclimatization to culture conditions. The newly synthesized proteoglycans extracted from the matrix and supernatant of ex vivo cultures eluted as large peaks with partition coefficients of $K_{av} = 0.34 \pm 0.02$ (matrix) and $K_{av} = 0.42 \pm 0.01$ (supernatant) [Figure 3.10A]. Proteoglycans from the cell layer matrix of monolayer cultures demonstrated a peak with a $K_{av}$ of $0.25 \pm 0.01$ and those released into the supernatant had a $K_{av}$ of $0.32 \pm 0.03$ [Figure 3.10B]. Chondrocytes in filter culture can produce large proteoglycans as in ex vivo and monolayer cultures. In ex vivo and monolayer cultures, the proteoglycans not retained in the matrix have a smaller hydrodynamic size than those retained within the matrix similar to the maintenance phase of the filter cultures.
**Figure 3.10: Proteoglycans synthesized in explant and monolayer cultures**

Gel chromatography of proteoglycans retained (*) and not retained (o) within the matrix extracted from (A) explant and (B) monolayer cultures. Proteoglycan hydrodynamic size was determined by Sepharose CL-2B chromatography under dissociative conditions (4M GuHCl). These are representative elution profiles from one experiment.
3.4 Discussion

These studies demonstrate that for the features examined the cartilagenous tissue generated by chondrocytes in filter culture is similar to the in vivo articular cartilage. This was shown by the organization of the cells and matrix macromolecules such as collagen. Chondrocytes in the superficial layer were flattened and were oriented parallel to the surface. Chondrocytes deeper within the tissue were round and more numerous. Collagen in the superficial zone of the filter culture tissue was oriented parallel to the surface whereas the collagen surrounded the chondrocytes in the deeper zone which is comparable to the orientation of collagen in vivo. The tissue consists of approximately 80% water which is in the range described for articular cartilage (Torzilli, 1985).

We utilized the fact that chondrocytes from the different zones of articular cartilage show different phenotypic and/or metabolic characteristics to confirm that growing chondrocytes in filter culture allows the cells to maintain their phenotype in long term culture. Chondrocytes isolated from the superficial zone of cartilage form tissue, which is thinner. This is in keeping with the finding that chondrocytes from the superficial zone of cartilage synthesize smaller amounts of proteoglycans (Korver et al, 1990). The cells from the deep zone of articular cartilage have been shown to have alkaline phosphatase activity and the ability to produce a matrix that can mineralize (Xu et al, 1994; Kandel et al, 1997). Cells were isolated from the deep zone of articular cartilage and grown in filter culture under conditions that favour mineralization (the
presence of β-glycerophosphate). These cells formed cartilagenous tissue that had a zone of mineralization in the deep aspect of the tissue similar to the in vivo cartilage.

The size of the proteoglycans synthesized by the chondrocytes in vitro was examined in order to determine whether it was similar to the in vivo tissue. The chondrocyte synthesized a population of large proteoglycans whose size (Kav=0.23±0.01) was similar to that synthesized by chondrocytes in ex vivo cultures and chondrocytes grown in monolayer culture under conditions that favour maintenance of their phenotype. Several studies have reported that differentiated human and bovine chondrocytes synthesize this size of proteoglycan (Martel-Pelletier et al, 1987; Sweet et al, 1978; Loulakis et al, 1992). However, Axelsson and Bjelle (1979) have shown that proteoglycan monomers in calf cartilage have a partition coefficient of 0.42 (18 month old calves) which is in keeping with the size of PG retained in the matrix in filter culture. Interestingly, the size of the newly synthesized proteoglycans (PG-R) retained in the extracellular matrix was smaller than the proteoglycans not retained in the extracellular matrix (PG-NR) in these cultures (accumulation phase). This hydrodynamic size difference is not an artifact of culture as it was not detected in deep zone filter cultures and other culture types such as monolayer and ex vivo articular cartilage. This will be further investigated in the following chapter.

The proteoglycans synthesized in vitro undergo further modification as their size decreases with time as shown by the pulse chase experiments and confirmed by the analysis of the size of the endogenous proteoglycans, which were not labelled with $^{35}$S-
sulphate. This is similar to what occurs \textit{in vivo} (Sandy et al, 1989). Studies on the catabolism of $^{35}$S-sulphate labelled newly synthesized aggregan in explant bovine cartilage cultures have shown that aggregan within the matrix decreases in size with time in culture (Campbell et al, 1989; Ilic et al, 1992, Ilic et al, 1995). The smaller size proteoglycan probably results from extracellular N-terminal and/or C-terminal degradation by proteases while residing in the matrix (Roughley et al, 1991; Hardingham et al, 1991).

Proteoglycans synthesized by chondrocytes in the deep zone of cartilage have been shown to be larger than proteoglycans synthesized from the other zones (Mitrovic and Darmon, 1994; Franzen et al, 1981). In our study we were able to show that the newly synthesized proteoglycans retained in the matrix in deep chondrocyte cultures were larger than those found in the cartilagenous tissue generated by chondrocytes isolated from the full thickness of cartilage. In addition, proteoglycans synthesized in mineralizing cultures increased in hydrodynamic size on Sephose CL-2B columns with time in culture (4 weeks, $K_a = 0.35 \pm 0.02$; 6 weeks, $K_a = 0.26 \pm 0.03$). This increase in size is in keeping with findings in the \textit{in vivo} tissue as Korver et al (1990) showed that glycosaminoglycan chains of proteoglycans within the hypertrophic zone of cartilage are longer than in other zones of epiphyseal plate plate cartilage.
Chapter Four: Characterization of the Large Proteoglycans Synthesized During the Formation of Cartilagenous Tissue \textit{in vitro}

4.1 Introduction

In order to study proteoglycan synthesized during matrix accumulation, we optimized a chondrocyte cell culture system in which isolated bovine articular chondrocytes accumulate extracellular matrix and form a continuous layer of cartilagenous tissue (Boyle et al, 1995). Proteoglycans synthesized before day 35 (representing accumulation phase) and after day 35 (representing maintenance phase) in culture were analyzed. We showed that during matrix accumulation, two populations of proteoglycans are present which demonstrate differential retention in the matrix. The population of proteoglycans that is not retained in the matrix (PG-NR) is characterized by a larger hydrodynamic size than those retained within the matrix (PG-R). The difference in the hydrodynamic size of proteoglycan retained in the matrix as compared to those not incorporated into the matrix can be explained in two ways. It is possible that 1) different populations of proteoglycans are being synthesized and only specific types can be retained in the matrix or 2) one population of proteoglycans is being synthesized but while retained in the matrix it undergoes proteolytic cleavage. There is experimental data published by others to support both of these possibilities. Understanding the basis for the structural differences of the proteoglycan populations synthesized in this culture system may provide insight into the role of proteoglycans in influencing matrix accumulation.
Aggrecan usually appears polydisperse with a continuous range of sizes and composition when fractionated by gel chromatography. The heterogeneity in the monomer population can be attributed to 1) variation of the core protein gene due to alternative splicing [Fulop et al, 1996; Ito et al, 1995] 2) variation in posttranslational glycosylation and sulphation, and 3) proteolytic modification of the core protein. Biosynthetic variations and proteolytic modifications of proteoglycans that result in size changes are observed normally in articular cartilage.

Many studies have demonstrated that osteoarthritic cartilage has the capacity to synthesize new aggrecan molecules to replace those lost due to disease. Rizkalla et al (1992) postulated that the aggrecan molecules synthesized are different from the proteoglycan population present in normal tissue and only specific types are retained in the matrix. The authors define two phases of proteoglycan change in osteoarthritis (OA): an initial predominantly degenerate phase I followed by a reparative phase II accompanied by net loss of these molecules. The proteoglycans synthesized in the reparative phase have an increased hydrodynamic size, as determined by column chromatography and expressed a fetal epitope detected by the antibody 846. The increased hydrodynamic size was not due to an increase in chondroitin sulphate (CS) chain length. The authors suggested that the larger molecules contained core protein(s) that were less degraded. Glycosaminoglycan substitution may also have contributed to the larger size of these molecules however this was not addressed. The larger proteoglycans synthesized in phase II are not retained in the tissue.
Caterson et al (1990) observed an increase in the content of another fetal CS epitope recognized by the antibody 3B3 in experimental OA in dogs. These data suggest that precursor or fetal forms of aggrecan may be present in OA cartilage. Whether these findings relate to non-diseased cartilage that is accumulating ECM is not known.

An alternative explanation for the differences in proteoglycan size in filter culture is that one population of proteoglycans is being synthesized but while retained in the matrix it undergoes proteolytic cleavage. Studies using rotary shadowing and peptide analysis of extracted bovine aggrecan demonstrated that only 30-50% of the tissue molecules carries the G3 domain as compared to proteoglycans that are not retained (Flannery et al, 1992). These authors observed that calf chondrocytes grown in high-density monolayer secrete a higher percentage of aggrecan into the medium with a G3 domain (larger proteoglycan) than the aggrecan retained in the tissue. This suggests that the retention in the cell layer may be due to rapid proteolytic removal of the G3 domain and that molecules which diffuse into the medium avoid the proteolytic processing.

The integration of proteoglycans into the extracellular matrix is important for the physical and mechanical properties of articular cartilage. There is continual turnover of proteoglycans so the retention and accumulation of newly synthesized proteoglycans are critical to maintaining cartilage integrity (Hauselmann et al, 1992). The factors influencing the retention and accumulation of proteoglycans are of interest since the loss of proteoglycans from the tissue together with an inability to accumulate sufficient
amounts of newly synthesized proteoglycans may lead to the destruction of the articular surface (Poole, 1986).

The G1 region is involved in the noncovalent binding of aggregan to hyaluronan and link protein. This interaction is believed to be one of the principal mechanisms of immobilizing this proteoglycan within the tissue (Hardingham, 1981). The hyaluronic acid binding region (HABR) is believed to undergo a maturation process; changing from low affinity to high affinity for hyaluronic acid (Sandy et al, 1989). The mechanism of the increase in binding affinity is unknown. However, it is thought to be due to the formation of disulphide bonds in the HABR (Sandy et al, 1989).

4.1.1 Objectives

The specific aims of this study were to characterize the glycosaminoglycan chains, the core protein and the aggregability of the proteoglycans not retained within the matrix (PG-NR) and compare it to proteoglycans retained in the matrix (PG-R) during the period of proteoglycan accumulation by chondrocytes in filter culture. Specifically this involved:

1) Determining the ability of the endogenous and newly synthesized proteoglycans to aggregate with hyaluronic acid.

2) Determining if the proteoglycans not retained have a decreased affinity for hyaluronic acid.
3) Determining the composition and size of the glycosaminoglycan chains of the newly synthesized proteoglycans.

4) Determining the sizes of the core proteins of PG-R and PG-NR.

5) Examining the amino acid composition of PG-R and PG-NR and comparing them with the amino acid composition of aggrecan and versican.

6) Sequencing an internal peptide from the core protein of PG-NR in order to determine whether the amino acid sequence is in keeping with aggrecan.
4.2 Materials and Methods

4.2.1 Radiolabelling of Cultures

Culture medium was replaced prior to labelling (20% FBS in Ham's F12 + 100 µg/ml ascorbic acid). To analyze proteoglycan core protein structure the cultures were incubated with $[^{14}C]$-serine (1 µCi/per well). Alternatively, the cultures were labelled with $[^3H]$-glucosamine (30 µCi/per well) or $[^{35}S]$-sulphate (4 µCi/per well) in order to analyze glycosaminoglycan chains or proteoglycan monomer size. The radioisotope was added to the inner well of the filter insert and incubated for 1, 3 or 24 hours prior to harvesting. $[^{35}S]$-sulphate and $[^3H]$-glucosamine both become incorporated into the newly synthesized proteoglycans and thus serve to control for variations in sulphation or carbohydrate content.

4.2.2 Proteoglycan Extraction

Matrix proteoglycans were extracted with 4 M guanidine hydrochloride in 50 mM sodium acetate, pH 5.8 containing 0.1 M 6-amino-hexanoic acid, 50 mM benzamidine HCl, 10 mM EDTA and 5 mM N-ethylmaleimide for 24 hours at 4°C. The proteoglycans from the matrix and supernatant were precipitated by the addition of three volumes of ice cold ethanol. After 24 hours at 4°C the precipitate was collected by centrifugation at 14000 rpm for 30 minutes and washed three times with 70% ethanol. The pellets were resuspended in 4 M guanidium HCl with protease inhibitors for determination of $[^{14}C]$-serine or $[^{35}S]$-sulphate incorporation in a scintillation counter and for column chromatography. After chromatography on Sepharose CL-2B columns, the proteoglycans
from the peaks of PG-R and PG-NR were collected and used for further analysis. 

$[^{14}\text{C}]$-serine labeled proteoglycans were resuspended in immunoprecipitation buffer (see below). Bovine cartilage ex vivo or monolayer cultures labelled for 24 hours with $[^{14}\text{C}]$-serine or $[^{35}\text{S}]$-sulphate served as controls. Proteoglycans from these cultures were extracted identically to the filter cultures.

### 4.2.3 Gel Filtration Chromatography of Proteoglycan Aggregates

#### 4.2.3.1 Binding of Total Endogenous Proteoglycans to Hyaluronic Acid

An ELISA method was used to determine whether proteoglycans can bind to hyaluronic acid. Microtiter well plates were coated with hyaluronic acid (2 µg) for 18 hours at 4°C. Varying amounts of proteoglycans (0-2 µg range) extracted from both the matrix and the medium at either day 10, 21, or 49 were allowed to bind to the HA coated plates for 4 hours. Plates coated with bovine serum albumin (BSA, 2 µg) were used as a means to assess non-specific binding. An anti-keratan sulphate IgG antibody (5D4, 1:3000, ICN Biochemical) was used to detect the proteoglycans bound to the plates after washing. An alkaline phosphatase-conjugated goat anti-mouse antibody (Biorad) was used as the secondary antibody. After addition of the alkaline phosphatase substrate the plates were read at a wavelength of 405 nm on a spectrophotometer (Titertek Multiskan MCC/340).
4.2.3.2 Aggregation of Newly Synthesized Proteoglycans with Hyaluronic Acid

Aliquots of the proteoglycans extracted, from the matrix of 10 and day 49 cultures, were applied to analytical Sepharose CL-2B columns (100 x 1 cm). Proteoglycan concentration was not determined in absolute terms but as chondroitin sulphate equivalents by reaction with dimethylmethylene blue dye following papain digestion as described in Chapter 2 (Farndale et al, 1986; Goldberg and Kolibas, 1990). The columns were eluted (6 ml/hr) with 4 M GuHCl in 0.05 M sodium acetate, pH 5.8 containing protease inhibitors. The peaks were dialyzed against distilled water and lyophilized. To determine the percent aggregation, aliquots of partially purified proteoglycans (isolated from the peaks of Sepharose CL-2B columns) were mixed with 2% hyaluronic acid (w/w)[sodium salt, rooster comb, Sigma] and incubated for 18 h at 4°C. The incubation mixture was then applied to an analytical Sepharose CL-2B column (100 x 1 cm), and eluted under associative conditions (0.15 M sodium acetate, pH 6.8 with protease inhibitors) at a flow rate of 6ml/hr. Fractions (2.0 ml) were analyzed for the amount of $[^{35}S]$-sulphate. Aggregation was defined as proteoglycan eluting at a $K_a V \leq 0.15$ (Rizkalla et al, 1990).

4.2.3.3 Affinity of Proteoglycans for Hyaluronic Acid

To determine whether the ability of proteoglycans to aggregate could be enhanced by alkaline pretreatment, purified newly synthesized proteoglycans (PG-NR) from day 10 cultures were suspended in 0.1 M Tris/ 0.05 M sodium acetate, pH 8.6 for 48 hr at 22°C before being mixed with hyaluronic acid. This alkaliine pretreatment has previously been
shown to improve the hyaluronate-binding properties of newly synthesized aggregan monomers extracted from chondrocyte culture (Plaas and Sandy, 1986). Hyaluronic acid was added at 2% (w/w) and the samples incubated for 18 hours at 4°C in 0.15 M sodium acetate, pH 6.8 with PI, before chromatography as described above (part 4.2.4.2). Proteoglycan concentration was determined as chondroitin sulphate equivalents as described above (Farndale et al, 1986; Goldberg and Kolibas, 1990).

4.2.4 Glycosaminoglycan Chains

4.2.4.1 Glycosaminoglycan Chain Length

Cultures were labelled at either day 10 or 49 for 24 hours with \([^{35}\text{S}]\)-sulphate (4 µCi per well) in order to label the newly synthesized glycosaminoglycan chains of proteoglycans retained (PG-R) and not retained (PG-NR) within the matrix. Proteoglycans were extracted as previously described (part 4.2.3). Sepharose CL-6B gel chromatography was used to investigate GAG chain length. Proteoglycan extracts were dialyzed overnight at 4°C against distilled water and incubated with sodium borohydride (1 M NaBH\(_4\)/0.2 M NaOH) for 24 hours at 37°C which results in β-elimination of the glycosaminoglycan chains. The samples were neutralized with 5% acetic acid. Alternatively, proteoglycans extracts were digested with papain [10 µg/ml in 20 mM ammonium acetate, 1 mM EDTA and 2 mM DTT] for at least 18 hours at 65°C. Samples (300 µl) were examined by Sepharose CL-6B chromatography (100 x 1cm) in 0.2 M sodium acetate buffer, pH 5.5, at a flow rate of 6 ml/h. Elution profiles for the glycosaminoglycan chains were generated by quantifying counts per minute (cpm) \([^{35}\text{S}]\)-
sulphate within each 2 ml fraction. The partition coefficient and dispersity were
determined for the glycosaminoglycan chains. Dispersity of the proteoglycans was
measured as the difference in Kav at half-maximal peak height

4.2.4.2 Carbohydrate Analysis of Proteoglycans Extracted from Filter Cultures

The ratio of galactosamine to glucosamine was determined using HPLC and Pico-
Tag system (Waters). The samples were hydrolyzed and derivatized. The derivatized
sample was dissolved in sample diluent and placed on the Waters reverse phase HPLC
system. The sugars (galactosamine and glucosamine) were detected at 254 nm. Amino
acid analysis on the same samples was performed in order to normalize hexosamine
content to protein. Hydrolysis of amino acids was performed using 6 M HCl at 105°C for
20 hours under vacuum. Amino acids were determined using a Pico-Tag amino acid
analyzer. Protein content was estimated by summation of the amino acid residues
(excluding cysteine/cystine). There was no correction for any destruction of the residues
that may occur on hydrolysis. These analyses were done at the Biotechnology Service
Centre at the Hospital for Sick Children.

4.2.5 Core Protein Isolation

4.2.5.1 Chromatographic Purification

Cultures were labelled with [14C]-serine for 24 hours and the proteoglycans from
the matrix and medium were extracted as described above (part 4.2.2) and applied to
Sepharose CL-2B columns (100 x 1 cm) in 4 M guanidinium hydrochloride buffer in
the presence of protease inhibitors, at a flow rate of 6 ml / hour. The peak fractions from
the elution profile were dialyzed against distilled water and lyophilized. The protein
content of the sample was determined by the bicinchoninic acid (BCA) protein assay
(Pierce Chemical) with BSA used as a standard. The proteoglycans were deglycosylated
using trifluoromethanesulphonic acid (TFMS) [Sigma] as described below (part 4.2.7).

4.2.5.2 Immunoprecipitation of Keratan Sulphate (KS) Containing Proteoglycans

Alternatively, \(^{14}\text{C}\)-serine labelled core proteins were isolated by
immunoprecipitation. By using antibodies to specific epitopes, different proteoglycan
populations can be isolated before TFMS deglycosylation. This method was used in an
effort to separate the different populations of large proteoglycans. Antibodies reactive
with keratan sulphate were used to isolate KS containing proteoglycans (eg. aggrecan)
while separating them from proteoglycans only containing chondroitin sulphate (CS) or
heparan sulphate (HS) chains (eg. versican, perlecan respectively).

Cultures were labelled with \(^{14}\text{C}\)-serine for 24 hr and the proteoglycans from the
matrix and medium were extracted as described above. The proteoglycans were
resuspended in immunoprecipitation buffer (50 mM sodium acetate buffer, pH 5.0; 500
mM sodium chloride, 0.1 % SDS, 1% NP-40, 0.02 % sodium azide). An antibody
reactive with keratan sulphate (5D4)[ICN Biochemical] was added to 30-40 µg of
proteoglycans and incubated 16 hours at 4°C while rotating. Protein G is added to the
samples (10 µl / µg antibody) and incubated and rotated for 2 hours at room temperature. The samples are centrifuged at 14000 rpm for 2 minutes to pellet the proteoglycan-antibody-protein G complexes. The pellets were washed a minimum of six times with immunoprecipitation buffer. The antigen-antibody complex was eluted from the immobilized protein G by incubation with the elution buffer (0.1M glycine-HCl buffer, pH 2.5). The proteoglycans reactive with the KS antibody are recovered in the supernatant after centrifugation and desalted using microconcentrators (Microcon 30). The immunoprecipitated proteoglycans were treated with TFMS as described below in order to examine their core proteins.

4.2.6 Chemical Deglycosylation of the Core Protein Using Trifluoromethanesulphonic Acid

4.2.6.1 TFMS treatment of Filter Culture Proteoglycans

Proteoglycan core proteins were deglycosylated using a modification of a method described by Edge et al (1981). Lyophilized proteoglycan was dissolved in cold trifluoromethanesulphonic acid (TFMS)/anisole (2:1 v/v) at 5 mg/ml at 0°C. After a two hour incubation, the reaction was stopped by adding two volumes of cold diethyl ether and three volumes of cold 50% aqueous pyridine. The samples were centrifuged and the ether phase discarded. The pellet was washed twice using 95% ethanol, dried in a speed vacuum, and resuspended in Laemmli's buffer.
4.2.6.2 Electrophoresis and Transfer of the Deglycosylated Core Protein

Samples deglycosylated using TFMS were dissolved in Laemmli buffer containing 10% (w/v) SDS, 0.1% (w/v) bromophenol blue, 20% glycerol and 5% (v/v) β-mercaptoethanol and heated at 100°C for 3 minutes. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 6% gels was performed using the Novex system (Helixx). Selected molecular weight standards (range 36-250 kD) were used to estimate core protein size. Electrophoretically separated samples were transferred onto polyvinylidene difluoride (PVDF) membranes in 12.5 mM TRIS/ 96 mM glycine buffer containing 20% methanol. The membranes were stained with 0.1% Ponceau S (50% methanol) and excised for amino acid composition determination.

4.2.7 Internal Protein Sequence Analysis and Peptide Mapping of Filter Culture Proteoglycans

4.2.7.1 Enzymatic Digestion of Proteoglycans with Trypsin

PG-NR was isolated as previously described (part 4.2.6). Briefly, the core proteins of both explant and filter culture supernatant proteoglycans were isolated after labelling with [¹⁴C]-serine in serum free medium (Ham's F12). After immunoprecipitation with a keratan sulphate antibody, the proteoglycans were resuspended in 100 mM sodium bicarbonate for enzymatic digestion in solution. Trypsin (Sigma, sequencing grade) was added to the samples [E:S, 1:20, w/w] and digestion performed at 37°C for 1, 3, 6, or 24 hours. After digestion, Laemmli’s buffer was added and the digestion products were separated on 4-12% SDS-PAGE gels followed by
transfer to PVDF. Membranes were stained with Coomassie blue to visualize the peptides.

4.2.7.2 Internal Sequence Analysis

Selected peptides generated by trypsin digestion were excised from PVDF membranes and submitted for microsequencing by the Peptide Sequencing Analysis Facility at the Biotechnology Service Centre. The samples were analyzed on a Porton gas-phase microsequencer (model 2090) with on-line PTH analysis. Five to ten N-terminal sequencing cycles were run for the peptides submitted.

4.2.7.3 Computer Analysis

The SwissProt annotated protein sequences database was used to compare amino acid composition and sequence analysis data to known proteoglycan sequences (expasy.hcuge.ch/sprot/). The AA Comp Ident program (Expasy Proteomic Tools: expasy.hcuge.ch/www/tools.html) was used to identify proteins by their amino acid composition. The Fasta sequence analysis program was used to determine sequence alignments with internal sequencing data (www.ebi.ac.uk/fasta3/).
4.3 Results

4.3.1 Radioactive Labelling Controls

As discussed in chapter 3, the newly synthesized proteoglycans retained in the matrix (PG-R) during the accumulation phase were heterogeneous with an average Kav of 0.44±0.02. The newly synthesized proteoglycans in the supernatant (non-retained [PG-NR]) at this time were larger in size with an average Kav = 0.23±0.01. In order to determine if the length of the radioactive labeling period affected the size of the proteoglycans as determined by dissociative chromatography, three and one hour labeling periods were compared to the 24 hour labelling period (Figure 4.1). After a three hour labelling period, the size difference between the proteoglycans retained and not retained was observed as demonstrated at 24 hours. After one hour of radiolabelling, there were insufficient proteoglycans in the supernatant to assess the proteoglycans not retained. However, the proteoglycans retained within the matrix had a similar Kav to the proteoglycans which had been labelled for 24 hours.

Table 3.1: Effect of Labeling Period on Partition Coefficient

<table>
<thead>
<tr>
<th>Labelling Period</th>
<th>PG-R Kav</th>
<th>PG-NR Kav</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>0.44±0.02</td>
<td>0.23±0.01</td>
</tr>
<tr>
<td>3 hours</td>
<td>0.45±0.01</td>
<td>0.24±0.02</td>
</tr>
<tr>
<td>1 hour</td>
<td>0.45±0.03</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND not determined
Figure 4.1: Proteoglycan hydrodynamic size and labelling period

Gel chromatography of proteoglycans extracted from the matrix (●) or supernatant (○) of 21 day old cultures. Cultures were labelled with $[^{35}\text{S}]$-sulphate for (A) 1 hour or (B) 3 hours. Proteoglycan hydrodynamic size was determined on Sepharose CL-2B columns under dissociative conditions.
4.3.1.1 [³H]Glucosamine Labelling

[³⁵S]-sulphate is incorporated into the newly synthesized glycosaminoglycan chains of the proteoglycan. This method is generally used to analyze sulphated proteoglycans because 99% of the isotope is incorporated by chondrocytes into these molecules (Thonar et al, 1986). However, the amount of sulphation of proteoglycans can vary and so the results were confirmed using an alternative label. Glucosamine is a hexosamine incorporated into glycosaminoglycan chains as it is present in keratan sulphate, heparan sulphate, and hyaluronic acid chains. The profiles obtained using this label represent proteoglycans bearing keratan sulphate, heparan sulphate chains or chondroitin sulphate (glucosamine to galactosamine conversion). The elution profiles using [³H]-glucosamine were similar to those obtained for PG-NR and PG-R labelled with [³⁵S]-sulphate (Figure 4.2) in that the partition coefficients were similar. These results indicated that the proteoglycan size difference did not represent proteoglycan populations with differences in sulphation.
Figure 4.2: Hydrodynamic size of $[^3\text{H}]-\text{glucosamine labelled proteoglycans}$
Gel chromatography (Sepharose CL-2B) of proteoglycans extracted from 21 day old cultures which were either retained (•) or not retained (○) in the matrix. Cultures were labelled with $[^3\text{H}]-\text{glucosamine}$ for 24 hours.
4.3.2 Endogenous Proteoglycan Aggregation with Hyaluronic Acid

The ability of the proteoglycans to aggregate with hyaluronic acid is a critical factor in proteoglycan retention. Proteoglycans may not be retained in the matrix during the accumulation phase because they lack a functional hyaluronic acid binding region. Total endogenous matrix and medium proteoglycans were assessed for their ability to bind to hyaluronic acid using an ELISA method. Early cultures (day 10) showed that proteoglycans extracted from the matrix had a higher ability to bind to hyaluronic acid compared to proteoglycans not retained in the matrix (Figure 4.3A). Proteoglycans from both compartments at day 21 showed similar curves thus demonstrating that they have comparable ability to bind to hyaluronic acid (Figure 4.3B). Proteoglycans from the supernatant of the maintenance phase bound to the HA plates but less efficiently until higher amounts of proteoglycans were used (Figure 4.3C).
Figure 4.3: Aggregation of endogenous proteoglycans with hyaluronic acid
An ELISA assay was used to assess proteoglycan binding to hyaluronic acid during the accumulation phase ([A] day 10 [B] day 21) and maintenance phase ([C] day 49) of the filter cultures. Proteoglycans were added to microtiter well plates coated with hyaluronic acid. Proteoglycans bound to hyaluronic acid were detected using an anti-keratan sulphate antibody and measured spectrophotometrically (supernatant proteoglycans - open symbols, matrix proteoglycans - closed symbols)
4.3.3 Proteoglycan Aggregation with Hyaluronic Acid

During the accumulation phase there are two different populations of proteoglycans synthesized that demonstrate differential retention within the matrix. Aggregation with hyaluronate is the principle mechanism of proteoglycan retention. Therefore these two populations were examined to determine if a differential ability to aggregate with HA was influencing their distribution.

Aggregation of the newly synthesized proteoglycans ([\(^{35}\)S]-sulphate labelled) extracted from the matrix (PG-R) and the supernatant (PG-NR) of the filter cultures after 10 days in culture was examined. Approximately 75 ± 7 % of the matrix proteoglycans (PG-R) have the ability to aggregate with hyaluronate. However, only 35 ± 5 % of the proteoglycans not retained (PG-NR) have the ability to aggregate (Figure 4.4). This difference in ability to aggregate is significant (Student’s t-test, p<0.01). Based on these results it appears that approximately 65% of the newly synthesized proteoglycans (NS-PG) in the culture medium were unable to aggregate with hyaluronate under the conditions used. This suggests that the release of NS-PG into the culture medium is due in part to their inability to aggregate with hyaluronic acid.
Figure 4.4: Aggregation of newly synthesized proteoglycans with hyaluronic acid

[A] PG-R and [B] PG-NR (day 10) labelled with $[^{35}\text{S}]$-sulphate were incubated with hyaluronic acid (2%w/w) and applied to analytical Sepharose CL-2B columns, and eluted under associative conditions. Proteoglycans eluting at a $K_{av} \leq 0.15$ were considered aggregated.
4.3.4 Aggregability of Proteoglycans after Alkaline Pretreatment

PG-NR's inability to aggregate with hyaluronate under these conditions may be due to a lower affinity or a complete inability to bind to hyaluronate. To differentiate between these two possibilities, the percent aggregation by PG-NR monomers using associative Sepharose CL-2B was determined after alkaline pretreatment (Figure 4.5). Alkaline pretreatment was used to enhance the conversion of the proteoglycans to a higher affinity form. Such alkaline pretreatment has been previously shown to improve the hyaluronate-binding properties of newly synthesized monomers by chondrocytes in culture (Plaas and Sandy, 1986). The percent aggregation of PG-NR was enhanced by 12 ± 3.8 % (n = 4) by pretreating the samples for 48 hours at pH 8.6 before the addition of excess hyaluronate (Figure 4.6). The difference between alkaline and no pretreatment was significant (Student's t-test, p<0.05). The data suggests that within PG-NR, there may be a population of proteoglycans that are unable to aggregate with hyaluronate under the conditions tested.
Figure 4.5: Aggregability of PG-NR with hyaluronic acid after alkaline pretreatment
Proteoglycans from the supernatant (PG-NR) of day 10 filter cultures were treated with an alkaline solution prior to the addition of hyaluronic acid and then chromatographed on analytical Sepharose CL-2B columns under associative conditions. [A] alkaline pretreatment [B] no alkaline pretreatment. Aggregation with hyaluronic acid was considered as a Kav ≤ 0.15.
Figure 4.6: Percentage aggregability of PG-NR with hyaluronic acid after alkaline pretreatment
Proteoglycans from the supernatant (PG-NR) of filter cultures were treated with an alkaline solution prior to the addition of hyaluronic acid and then chromatographed on analytical Sepharose CL-2B columns under associative conditions. Percentage aggregation of PG-NR ± alkaline pretreatment is compared to PG-R.
4.3.5 Glycosaminoglycans Analyses

4.3.5.1 Glycosaminoglycan Chain Length

Several structural properties of proteoglycans can contribute to their hydrodynamic size. Core protein length and the size and number of glycosaminoglycan (GAG) chains can influence the size of the proteoglycan monomer. These properties were analyzed to determine if they differ in PG-NR as compared to PG-R. The size of GAG chains produced by NaBH₄ or papain treatment of proteoglycans was determined by gel filtration chromatography on Sepharose CL-6B columns. Comparison of the mean Kav demonstrated that PG-NR and PG-R have GAG chain lengths that are not statistically different (Student’s t-test, p>0.05) [Table 4.1]. Dispersity of the proteoglycans was measured as the width of the peak at half-maximal peak height. PG-NR and PG-R demonstrated similar dispersity. Figure 4.7 shows typical elution profiles of the GAG chains.

Table 4.1: Glycosaminoglycan Chain Length

<table>
<thead>
<tr>
<th></th>
<th>$K_{av}$</th>
<th>dispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG-R</td>
<td>0.35±0.02</td>
<td>0.27±0.02</td>
</tr>
<tr>
<td>PG-NR</td>
<td>0.32±0.03</td>
<td>0.24±0.02</td>
</tr>
</tbody>
</table>

* values from NaBH₄ treatment
Figure 4.7: Glycosaminoglycan chain length
Elution profiles of glycosaminoglycan chains after gel filtration chromatography on Sepharose CL-6B columns. (A) sodium borohydride or (B) papain treated proteoglycans from PG-R (●) and PG-NR (○) during the accumulation phase.
4.3.5.2 Carbohydrate Composition

The previous experiment demonstrated that the average size of the GAG chains did not vary between the two populations of PG. However, the number of GAG chains substituted on the core protein may also affect its size. The ratios of CS / μg protein and KS / μg protein will give an estimation of the number of GAG chains substituted on a core protein. In order to characterize the GAG chains of PG-R and PG-NR further, carbohydrate composition was determined. In Table 4.2, the hexosamine ratios (molar ratio) and hexosamine to protein are shown for PG-NR and PG-R at day 10 (mean ± SEM, n = 4). Since the populations analyzed can consist of more than one proteoglycan species (newly synthesized and pre-existing PG), only day 10 analyses were done to minimize the contribution of pre-existing proteoglycans. The ratio of galactosamine to protein is significantly different between the matrix and supernatant (p<0.05, Student's t-test)

Table 4.2: Galactosamine /Glucosamine Ratios

<table>
<thead>
<tr>
<th></th>
<th>galactosamine/glucosamine</th>
<th>galactosamine / protein</th>
<th>glucosamine / protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matrix</td>
<td>6.2±2.5</td>
<td>0.55±0.13</td>
<td>0.13±0.06</td>
</tr>
<tr>
<td>Supernatant</td>
<td>7.1±1.1</td>
<td>1.14±0.18 *</td>
<td>0.17±0.04</td>
</tr>
</tbody>
</table>

galactosamine (CS) / glucosamine (KS and HS) molar ratios
* Student's t-test, p<0.05
4.3.6 Core Protein Analysis

4.3.6.1 Core Protein Size

SDS-PAGE was used to determine the molecular weights of the newly synthesized core proteins from explant cultures following deglycosylation by TFMS. Proteoglycans extracted from cartilage as well as the in vitro cartilagenous tissue (day 10 and 49) migrates as a diffuse high molecular weight band that remains at the top of a 6% polyacrylamide gel. The TMFS method of removing GAG chains was developed in order to examine the core protein size by minimizing the contribution of sugars and GAG and/or residual GAG stubs to the protein migration. Cartilage explant proteoglycans were used for method development (Figure 4.8). These served as controls to ensure that aggrecan could be identified using this method. Proteoglycans extracted from the matrix showed core protein sizes of 225 and 187 kD. The 225 kD band is consistent with the size expected for aggrecan suggesting that TMFS treatment could be used to estimate core protein size. The 187 kD band likely represents the 225 kD core protein which has been proteolytically modified.

Immunoprecipitation was used to extract keratan sulphate containing proteoglycans from PG-NR. Other large proteoglycans, for example versican and perlecan, are present in small amounts in cartilage. By immunoprecipitating with a keratan sulphate antibody, aggrecan and any KS rich proteoglycans will be separated from the other large proteoglycans. After immunoprecipitation, the newly synthesized
PG-R showed a major band at 187 kD and minor bands at 500, 300, 225 kD (Figure 4.9). In contrast the PG-NR demonstrated a major protein at a molecular weight of 500 kD.
Figure 4.8: Newly synthesized explant proteoglycan core proteins after TFMS deglycosylation

Proteoglycans were extracted from explant cultures and deglycosylated with TFMS. Core proteins were resolved on 4-12% SDS-PAGE gels. Autoradiogram of [\(^{14}\)C]-serine labelled core proteins from the matrix and supernatant of explant cultures.
Figure 4.9: Newly synthesized filter culture proteoglycan core proteins after TFMS deglycosylation

Proteoglycans were extracted from the matrix and supernatant of filter cultures and deglycosylated with TFMS. Core proteins were resolved on 4-12% SDS-PAGE gels. Autoradiogram of [14C]-serine labelled core proteins of PG-R and PG-NR.
N-glycosydic linkages to N-acetyl glucosamine which are present in N-linked oligosaccharides and keratan sulphate substituted asparagine residues can be retained after TFMS treatment of proteoglycans (Edge et al, 1981). In order to determine if there are residual carbohydrates contributing to the different core protein sizes observed after TFMS treatment, staining with toluidine blue (sulphated proteoglycan) and Western blotting with an antibody reactive with keratan sulphate (JD5) were performed. Toluidine blue did not stain the gel after TFMS treatment and no reactivity for keratan sulphate was seen on the blot (data not shown).

4.3.6.2 Core Protein Amino Acid Composition

As there appeared to be multiple core proteins the amino acid compositions of the different molecular weight bands (500, 300, 225, and 187 kD) were determined. The amino acid compositions of the four protein bands obtained after TFMS treatment were in keeping (high rank) with aggrecan as determined by the Expasy AACompIdent program (Table 4.3). The amino acid compositions of bovine aggrecan, human aggrecan and human versican taken from the literature were included in the table for comparison. The sequence of bovine versican is not currently available. This data suggested that the 500 kD protein is likely a dimer of aggrecan which would explain its slower migration. Alternatively, it might have aggrecan-like components, but this is considered less likely.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>500kD matrix</th>
<th>300kD matrix</th>
<th>225kD matrix</th>
<th>187 kD matrix</th>
<th>Bovine aggrecan (1)</th>
<th>Human aggrecan (2)</th>
<th>Human versican (3)</th>
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</tbody>
</table>

Amino acid composition is expressed as residues of amino acids/1000 amino acid residues
4.3.7 Peptide Mapping of PG-NR

PG-NR was partially purified by column chromatography and then underwent trypsin digestion followed by SDS-PAGE on 4-12 % gels. The gels showed many peptides. Their apparent molecular weights were in the range of ~4-96 kD (Figure 4.10A). There were two prominent peptides at 64 and 50 kD. In some experiments a small band corresponding to a larger fragment (150 kD) was found probably representing incomplete cleavage. The $[^{14}C]$-serine core protein preparation from filter culture and ex vivo culture showed similar peptide bands. The gel patterns of trypsin digest fragments of proteoglycans from filter cultures and their controls varied more in the amounts of the various bands rather than the pattern.

4.3.8.1 Internal Sequencing of PG-NR

N-terminal sequences of two fragments of PG-NR were determined after enzymatic digestion of the core proteins with trypsin and their separation by SDS-PAGE. The two fragments (64 and 50 kD) were used because they were labelled with $[^{14}C]$-serine and were therefore peptides from newly synthesized proteoglycans. These two peptides had sequences similar to internal sequences of aggrecan suggesting that PG-NR is aggrecan (Figure 4.10B).
Figure 4.10: SDS-PAGE of trypsin digested proteoglycan core proteins isolated from cartilage explant and filter cultures

[A] Proteoglycans from the supernatant of explant and filter cultures following trypsin digestion. Peptides derived from proteoglycan core proteins were resolved on 4-12% SDS-PAGE gels, transferred to PVDF, and stained with coomassie blue. [B] Partial amino acid sequence of aggrecan shown with sequence alignments of the N-terminally sequenced peptides from PG-NR after trypsin digestion.
4.4 Discussion

During the period of matrix accumulation, two populations of proteoglycans were observed in filter cultures that demonstrated differential retention within the matrix. The proteoglycan populations retained in the matrix (PG-R) had a smaller hydrodynamic size than those not retained (PG-NR). The proteoglycan not retained in the matrix decreased in amount with time in culture. In the maintenance phase, no large proteoglycan with the size of PG-NR was detected. As determined by gel filtration chromatography, the average size of the GAG chains did not vary between the two populations of PG, however, the ratio of galactosamine to protein was significantly different in proteoglycans found in the matrix when compared to the supernatant. This suggests that PG-NR had a greater degree of substitution with CS chains than PG-R either as a result of biosynthetic or proteolytic processes. In order to differentiate between these two possibilities PG-NR was further characterized.

The large proteoglycan (Kav 0.23) has keratan sulphate GAG chains and so immunoprecipitation was used to enrich for these proteoglycans for analysis. Although it limits which proteoglycans are analyzed it does eliminate the confusion that might have occurred as a result of the other large proteoglycans which are known to be present in cartilage. By immunoprecipitating with a keratan sulphate antibody, keratan sulphate-containing proteoglycans were separated from the other large proteoglycans such as perlecan, which is substituted with heparan sulphate and versican, which is substituted with chondroitin sulphate.
In sequencing studies, Upholt et al (1994) reported that the aggrecan core protein is in the size range of 218-248 kD, depending on which exons were spliced into the G3 domain of the mRNA. Since the sizes of the core proteins published can vary greatly depending on the method of preparation and analysis (different from this study), it is difficult to directly compare the reported sizes in the different studies. The larger size core proteins in other studies could be attributed to the retained N- and O-linked oligosaccharides and residual GAG stubs as the core protein size was most commonly determined following enzymatic deglycosylation. In the current study, although TFMS treatment results in the removal of the majority of carbohydrate, N-glycosidic linkages to N-acetyl glucosamine found in KS substituted asparagine residues and N-linked oligosaccharides are retained and may be contributing to the core protein size observed. Thus the biochemical methods available to determine core protein size have limitations.

The gel pattern of core proteins of different sizes as shown in Figure 4.10 do not necessarily imply the existence of different core proteins. Postsynthesis proteolytic cleavage of a single core protein could give rise to the smaller proteoglycan core protein (187 kD). The larger core protein sizes (300 and 500 kD) may reflect incomplete deglycosylation. Although we examined for residual carbohydrate we only examined for the presence of residual keratan sulphate. The other method of toluidine blue staining is relatively crude. Protein composition analysis suggested that the 500, 300, 225 and 187 kD proteins of PG-R are all in keeping with aggrecan. Some of the differences observed between the amino acid composition of the core proteins and the aggrecan core protein can be partially explained. Serine, glutamate, proline, and glycine are the predominant
amino acids in aggrecan, however, the core proteins from filter cultures seem to have less serine and proline than one would expect for aggrecan. Serine and threonine are destroyed (up to 85% of the residues) in the reaction to prepare the amino acids for analysis which may explain this difference. Proline peaks may not have separated from the ammonia on the HPLC column well enough to obtain an accurate estimation of its content. Proteolytic processing of the core protein, particularly for the 187 kD protein, can cause variability in the amino acid composition. As well there are several age related changes in amino acid abundance that can occur such as a decrease in serine, glycine and an increase in arginine and tyrosine (Roughley and White, 1980). Smaller increases in lysine, phenylalanine, methionine, valine, alanine, and proline and decreases in glutamate may also occur with age (fetal-58 years).

Peptides maps of PG-NR after trypsin digestion and internal sequence analysis of two of these peptides were used to determine the identity of PG-NR. $[^{14}C]$-serine labelled peptides were used to ensure that the bands sequenced were those of the newly synthesized proteoglycan core protein of PG-NR. Approximately 78 fragments should be obtained by cleavage with trypsin, although, fewer are resolved using SDS-PAGE. Sequence alignment analysis indicated that the internal sequences obtained were similar to the known sequence of bovine aggrecan. Of note the sequences obtained did not have arginine or lysine residues prior to the N-terminal amino acid as expected and it is possible that the trypsin preparation although sequencing grade may have had contaminating enzymes. The sequences did not align with versican, a large proteoglycan with homology to aggrecan.
The results presented in this study suggest that PG-NR is aggrecan and the size differences are likely due to proteolytic modification. This conclusion comes from the observations that 1) the tryptic peptide maps are very similar to the tryptic maps of articular cartilage aggrecan, 2) the amino acid composition of the core protein was similar to bovine aggrecan core protein, 3) two internal sequences aligned with aggrecan, 4) PG-NR contains keratan sulphate, and 5) the GAG chains of PG-NR and PG-R are the same length. It is not evident why the core protein of PG-NR is larger in size (~500 kD) than that of aggrecan (225 kD). It is possible that the 500 kD band represents a dimer of the 225 kD core protein. Why proteoglycan core proteins of PG-NR would dimerize and those from ex vivo cultures not dimerize remains unclear especially as the results from ex vivo PG analysis suggested that TFMS treatment is suitable for the analysis of the core protein sizes. As well others have used this approach to determine core protein size (Goodstone et al, 1996). There may be subtle differences in these proteoglycans that were not detected by the methods used that predispose them to dimerization.

The concept that newly synthesized aggrecan molecules can undergo rapid extracellular processing has been supported by the work of Thonar et al (1988) who found "CS-poor" monomers extracellularly in bovine chondrocyte cultures within 15 minutes of its synthesis. The proteoglycan population(s) present in the matrix of the tissue generated in this culture system may represent the cleavage of the G3 domain and possibly the CS-2 region, which would result in changes in hydrodynamic size. Conceivably, this proteolytic cleavage could occur rapidly and affect the size of PG-R while it resides in the matrix. If a significant amount of chondroitin sulphate chains were
lost, the glucosamine/galactosamine ratio would be increased. Likewise, the ratio would change if there were an increase in substitution of the core protein with glycosaminoglycan chains. The glycosaminoglycan chain length is similar in both populations of proteoglycans and thus substitution of the core protein with GAG chains or proteolytic cleavage within the GAG attachment region would account for the size difference observed in the proteoglycans during the accumulation phase.

Vilm and Fosang (1994) characterized the proteoglycans and proteoglycan fragments in human articular cartilage and also demonstrated proteolytic modification of aggrecan. The aggrecan populations comprised four different core proteins of molecular weights 380, 320, 240, 180 kD after enzymatic deglycosylation. Young and old human articular cartilage contained the same sizes of proteoglycan core proteins however their relative abundance varied. In addition, older tissue contained a larger proportion of C-terminally truncated aggrecan, many of which have lost their entire chondroitin sulphate-bearing domains. Malemud et al (1995) also showed proteoglycans of various sizes. These authors examined proteoglycans synthesized by chondrocytes in human osteoarthritic femoral head cartilage and nonarthritic articular cartilage while in explant cultures. Explants of nonarthritic articular cartilage synthesized proteoglycan core proteins with apparent molecular weights of 520, 480, and 390 kD on SDS-PAGE gels after enzymatic deglycosylation. The medium compartment contained principally the ~520 kD proteoglycan core protein. Chondrocytes in osteoarthritic cartilage synthesized only the ~ 520 kD core protein. Thus the largest proteoglycan reported in that study was synthesized by both osteoarthritic and nonarthritic chondrocytes and distributed in both
the matrix and supernatant compartments whereas the other large proteoglycans were not present in the medium.

The other characteristic of PG-NR was that it was found predominately in the supernatant and was not retained in the matrix. Proteoglycan retention in the extracellular matrix occurs through several mechanisms. The ability of the proteoglycans to aggregate with hyaluronate is a critical factor in proteoglycan retention and their subsequent accumulation and which our data suggests was the reason that more proteoglycans were not retained in the early stages of the cultures. Proteoglycans may not be retained in the matrix during the accumulation phase because they lack a functional hyaluronate binding region due to: 1) proteolytic processing and loss of the HABR 2) low affinity for HA or 3) proteoglycans that do not bind with HA (non-aggregating).

Western blotting of PG-NR revealed large molecular weight core proteins reacting with the G1 specific antibody (1C6) suggesting that there are proteoglycans (aggrecan) within the population of PG-NR that contained a HABR. This raised the possibility that these proteoglycans did not have sufficient affinity for hyaluronate. By treating PG-NR with a mild alkaline solution prior to associative chromatography, proteoglycan aggregation increased by almost a third, which is consistent with this hypothesis. It is possible that the addition of link protein may have further increased the percent of proteoglycans that can form stable aggregates. Nevertheless, these results suggest that some of the newly synthesized PG-NR had not undergone the maturation process necessary for its aggregation and retention within the matrix. Studies have shown
that the HABR of proteoglycans undergoes a maturation process that results in an increased affinity for hyaluronate. Several cysteine residues are located in the HABR region and these are capable of forming disulphide bonds. Disulphide bonding within the HABR has been suggested to be the key to the affinity maturation process (Plaas and Sandy, 1986; Sandy et al, 1990). Static compression or acidic medium slows the conversion to the high affinity form (Sah et al, 1990). Areas of high proteoglycan concentration are acidic when compressed due to the increase in their fixed charge density which would explain why static compression slows conversion. An acidic pH may occur due to the fact that thiol groups are ionized at pK values in the range of 8-10 (Wetlauffer, 1998). Sah et al (1990) found that the conversion of newly synthesized proteoglycans to a form that would bind HA with high affinity occurred with t\textsubscript{1/2} of approximately 5.7 hours at pH 7.45 whereas others have suggested it can be as long as 24 hours (Sandy et al, 1989). We found similar to Sandy et al (1989) that there is a proportion of the newly synthesized proteoglycan monomers that rapidly lose or never acquire the ability to bind hyaluronate.

In conclusion PG-NR and PG-R contain glycosaminoglycan chains that are similar in length. However, the hexosamine content of these proteoglycan populations suggests differences in substitution of the core protein with GAG. It is likely that the proteoglycan retained in the matrix undergoes proteolytic cleavage within the CS attachment region resulting in a decreased hydrodynamic size and a lower hexosamine ratio. PG-NR has a core protein similar to aggrecan. The presence of PG-NR in the supernatant appears to be due to its decreased ability to aggregate with hyaluronic acid.
Chapter 5: Conclusions

In the present study, we have developed an articular chondrocyte culture system in which isolated chondrocytes placed on teflon collagen-coated filter inserts accumulate extracellular matrix and formed cartilagenous tissue. This tissue had features similar to the *in vivo* articular cartilage. The chondrocytes synthesized large proteoglycans and type II collagen, the major components of cartilage. Furthermore, synthesis of these macromolecules indicated that the chondrocytes maintain their phenotype while in filter culture. Using [*14C*]-proline incorporation and autoradiography as well as western blot analysis, no synthesis of type I collagen was detected, which would have been indicative of cellular dedifferentiation. Although, it is possible that low levels of type I collagen were synthesized but were not detectable by the methods used. The cartilagenous tissue showed organization of cells and collagen into superficial and deeper layers similar to that described previously for articular cartilage (Weiss et al, 1968; Clarke, 1971). The superficial chondrocytes were flattened and the deeper cells were spherical. There was increased cellularity in the deep zone as compared to the superficial and mid zone of the tissue. The collagen fibrils in the superficial layer were oriented parallel to the surface whereas in the deeper zone they surrounded the chondrocytes. The tissue consists of approximately 80 % water which is the range described for articular cartilage (65-80 %)[Torzilli, 1985]. By 35 days, the extracellular matrix contains approximately three-fold more collagen than proteoglycan similar to *in vivo* cartilage. Cultures initiated with deep zone chondrocytes generated tissue with a mineralized zone comparable to the calcified zone in articular cartilage.
The proteoglycans synthesized by the chondrocytes grown in filter culture have a large hydrodynamic size and contained keratan sulphate. The endogenous (or unlabelled) proteoglycans had hydrodynamic sizes similar to or smaller than the newly synthesized proteoglycans, which suggests that the proteoglycans were undergoing proteolytic degradation over time in the matrix. This was supported by the pulse chase experiments, which demonstrated that the newly synthesized proteoglycans retained in the matrix became progressively smaller over time. Studies on the catabolism of [35S]-sulphate labelled newly synthesized aggrecan in explant bovine cartilage cultures have shown that aggrecan within the matrix decreases in size with time (Campbell et al, 1989; Illic et al, 1992, Illic et al, 1995). In calf cartilage explants, approximately 80% of proteoglycans can form aggregates (Sah et al, 1990). A similar proportion of proteoglycans synthesized by chondrocytes in filter culture can form aggregates. The proteoglycans in vivo have been shown to go through a maturation process in which their affinity for hyaluronic acid increases. Similarly the non-retained proteoglycans of filter cultures demonstrated increased aggregability after alkaline pretreatment, which has been shown to enhance the maturation process.

The newly synthesized proteoglycans retained (PG-R) and not retained (PG-NR) in the matrix demonstrated a difference in hydrodynamic size. The GAG chain length was not significantly different in these proteoglycan populations. However, hexosamine analysis suggested a difference in the substitution of the proteoglycans with chondroitin sulphate chains. The difference in GAG substitution could be a result of either biosynthetic or proteolytic processes and could account for the difference in
hydrodynamic size observed between PG-R and PG-NR.

It is likely that the proteoglycan retained in the matrix undergoes proteolytic cleavage within the CS attachment region resulting in a decreased hydrodynamic size and a lower hexosamine ratio. Enzymes may proteolytically cleave the core protein of the proteoglycan while it is entrapped within the extracellular matrix. Proteoglycans not retained within the matrix are more likely to "escape" this proteolytic processing. In addition, aggrecan is known to demonstrate considerable heterogeneity due to variable glycosaminoglycan substitution. PG-R and PG-NR may represent populations of proteoglycans that biosynthetically have different amounts of carbohydrate attached to their core proteins.

After immunoprecipitation to enrich for keratan sulphate containing proteoglycans, the newly synthesized PG-R showed bands at 187 and 225 kD in keeping with the predicted size of aggrecan. Interestingly, the core protein size of PG-NR was 500 kD yet the results presented in this study demonstrated that PG-NR had features consistent with aggrecan. This increased size likely reflects dimerization or incomplete deglycosylation of the core protein.

The differential distribution of PG-R and PG-NR can be explained in two ways. Firstly, some of the newly synthesized PG-NR had not undergone the maturation process necessary for its aggregation and retention within the matrix. Secondly, similar to the findings described by Sandy et al (1989), there is a proportion of the newly synthesized
proteoglycan monomers that rapidly lose or never acquire the ability to bind hyaluronate.

I can only hypothesize as to why the chondrocytes produce a larger proteoglycan that is not retained in the matrix. It may be that the larger proteoglycan is synthesized in order to replace the fixed charge density generated by a high concentration of proteoglycans that has been lost either in vitro by isolation of the cells from its matrix or in vivo during disease. A proteoglycan that had not undergone the maturation process necessary to bind to hyaluronic acid with high affinity would be more mobile within the matrix and could find a binding site further removed from the cell. In this study it appeared that the majority of these larger proteoglycans never found a binding site and were lost to the supernatant. The larger proteoglycans may increase the viscosity of the supernatant in vitro or the synovial fluid in vivo. It is possible that the increased viscosity of the synovial fluid may affect joint function favourably.

There maybe differences between the in vitro generated tissue and the in vivo tissue that have not yet been identified as I have not examined for the presence of all of the components known to occur in cartilage. Future studies will be necessary to fully characterize the matrix present in filter cultures. For example, the small proteoglycans, decorin, biglycan and fibromodulin, are present in articular cartilage and it will be necessary to determine whether they are present in the in vitro generated cartilagenous tissue, as well as their amounts and localization. In addition, the distribution of the different types of collagens needs to be studied in the in vitro generated cartilagenous tissue. Link
protein and hyaluronic acid contents of the extracellular matrix with time in filter culture can also be determined.

The accumulation of matrix observed in filter culture is consistent with the observation that the cell-associated matrix undergoes progressive organization with time in culture such that it resembles the matrix in vivo. Why chondrocytes in filter culture accumulate matrix and form a continuous layer of cartilagenous tissue in vitro has not been elucidated but likely is a result of a number of factors. The chondrocytes are plated at high density on the type II collagen coated filter. This high density promotes the rounded morphology of the cells, which is important in the expression of phenotypic properties of the chondrocytes. It is possible that chondrocyte metabolism was affected by the collagen coated on the filter insert. The inner and outer wells of the filter insert provide nutrients to both sides of the developing tissue a feature that differs from monolayer cultures. The concentration and timing of ascorbic acid addition is also critical to cartilagenous tissue formation. The cells do not survive if ascorbic acid is added too early to the cultures.

Several other systems have been developed to generate cartilagenous tissue in vitro but each of these has limitations. Chondrocytes have been grown in scaffolds, which results in a three-dimensional culture and formation of cartilagenous tissue (Stanton, 1995; Freed et al, 1993). This method differs from filter cultures in that the chondrocytes are cultured within the scaffold rather than on its surface and as a result cellular attachment is randomly distributed. There have been two studies in which reconstituted cartilagenous tissue was formed in vitro in the presence of scaffolds. In the study by Freed et al (1993) chondrocytes
were grown within the polymer fibrous polyglycolic acid (PGA) for up to 30 days. Under these conditions the cells form cartilagenous-like tissue but it had many features that differed from the *in vivo* articular cartilage. The tissue did not show a cellular organization similar to *in vivo* articular cartilage. It had a zone of tissue on its periphery that did not show chondroid features histologically, as there were little if any sulphated proteoglycans. Chondrocyte dedifferentiation had occurred in these cultures, in contrast to the filter cultures, as the cells synthesized type I collagen. Proteoglycan size was not determined in that study. Finally, the collagen to proteoglycan ratio in the tissue was 1.3:1 rather than the 3:1 that occurs in the cartilagenous tissue formed in filter culture and in articular cartilage *in vivo*. This analysis suggests that the tissue generated in PGA cannot be used as a model for cartilage. In a study by Stanton et al (1995), chondrocytes were grown in a composite of gelfoam and agarose for 25 days and were only minimally characterized. The cells accumulated extracellular matrix and formed cartilagenous-like tissue but no cellular organization was evident in the photomicrograph of the tissue shown. As the culture had been harvested at 18 days, this lack of organization may reflect the short culture period. Type II collagen was detected as expected for cartilagenous tissue but no analysis for type I collagen was done, so it was not possible to be sure that no cellular dedifferentiation had occurred. Proteoglycan size was also not determined. Further study is required to determine whether the tissue generated in the gelfoam culture system is a suitable model for cartilage. Currently to our knowledge, the cartilagenous tissue generated in filter culture is more similar to articular cartilage *in vivo* than other *in vitro* generated cartilagenous tissues. As the cartilagenous tissue produced by filter cultures is sufficiently similar to *in vivo* cartilage, it should be useful to study the mechanism(s) regulating cartilage matrix
accumulate and maintenance.

Chondrocyte filter cultures provide a number of avenues for future study. Filter cultures are a unique system for the study of chondrocyte metabolism, matrix organization, and macromolecule interactions. Chondrocyte transplantation has attracted interest as a means to resurface damaged joints (Brittberg et al, 1994; Chen et al, 1997). Cartilagenous tissue generated in filter culture is useful for applications such as transplantation. Using tissue as opposed to cells for transplantation will enable the chondrocytes to maintain their differentiated state and allow them to maintain a matrix that already exhibits organization. Our analyses confirmed the ability of isolated articular chondrocytes to produce a cartilage-like matrix in vitro. These morphologic and biochemical analyses on the tissue formed in filter culture provide the basis for future research on this unique articular chondrocyte culture system.
List of References


