A NOVEL CARTILAGE BIOCHEMICAL MARKER (CM-1):
ISOLATION, PURIFICATION AND CHARACTERIZATION

by

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

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ABSTRACT

A Novel Cartilage Biochemical Marker (CM-1): Isolation, Purification and Characterization

Doctorate of Philosophy, 1999

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Fluorescent biomolecules within cartilage matrix can be used as specific markers of cartilage metabolism. This thesis originally aimed to investigate changes in articular cartilage (AC) collagen crosslinks associated with maturation, ageing and osteoarthritis (OA). Following hydrochloric acid (HCl) hydrolysis of AC, chromatographic analysis of collagen crosslinks revealed an apparently novel fluorescent compound (Cartilage Marker-1: CM-1), the characterization of which became the focus of the thesis. To isolate CM-1, freeze-dried bovine metacarpophalangeal AC was hydrolyzed in 6M HCl at 110°C for 24 hr. CM-1 was purified by reverse-phase HPLC and its elution was monitored using a fluorescence detector at excitation wavelength, \( \lambda = 306 \) nm and emission wavelength, \( \lambda = 395 \) nm. From bulk preparations, 12 \( \mu \)g (58 nmol) of CM-1 per gm dried AC was obtained. Purified CM-1 was characterized by mass spectrometry, nuclear magnetic resonance spectroscopy, and single crystal x-ray diffraction. CM-1 is a symmetrical aromatic compound, molecular weight 204, molecular formula \( \text{C}_{11}\text{H}_{5}\text{O}_{2} \), and a molar extinction coefficient 4700 \( \text{M}^{-1}\text{cm}^{-1} \) at maximal UV absorption (\( \lambda = 306 \) nm).

CM-1 has the IUPAC nomenclature of 2,6-dimethyl-difuoro-8-pyrone. CM-1 is a product of HCl hydrolysis of the carbohydrate moiety of cartilage but not cartilage collagen. A protocol was developed to quantify CM-1 in biological compounds and tissues. From preliminary results, CM-1 was detected in all hyaline cartilage and synovial fluids from human and various animal species. As well, CM-1 was detected in reconstituted AC matrix produced \textit{in vitro}. CM-1 concentration is higher in human neonatal AC (3 day old, 525 pmol/mg dried AC) versus old AC (\( >53 \) yr. old –range 182 to 238 pmol/mg). Similar differences were noted in young versus old rabbit cartilage. In human and bovine AC, CM-1 levels were higher in the middle-deep lamina versus superficial-middle lamina. CM-1 in OA cartilage was present in lower amounts (range: 0 to 96 pmol/mg dried AC) compared to intact AC (range: 63 to 236 pmol/mg). CM-1 has potential application as a unique biochemical marker in joint diseases involving articular cartilage degradation.
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This work would not have been completed without the contribution of the chemists at the Chemistry Department, University of Toronto, who deserve considerable credit for their enthusiasm to face the challenge of identifying the novel compound “Cartilage Marker -1” - Dr. Patricia Aroca, Dr. Tim Burrow, Dr. Allan Howart, Dr. Alex Young and in particular, Dr. Allan Lough who identified the molecular structure of CM-1 using single crystal x-ray diffraction.

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Dedicated

To My Beloved Mother
Dhian Kaur Gahunia

And

In Fond Memory Of My Dear Father
Harbhajan Singh Gahunia

Dearest Daddy, thank you very much for instilling in me to aspire “High” on the “Ladder of Success”, to be strong at all times, to be persevering, to believe in myself and to pursue my goals. Thank you for guiding me and showing me the path through “the good times and the rough times”.
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<table>
<thead>
<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
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<td>GuHCl</td>
<td>Guanidine hydrochloride</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HABR</td>
<td>Hyaluronic acid binding region</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HFBA</td>
<td>Heptafluorobutyric acid</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple Bond Correlation</td>
</tr>
<tr>
<td>HMQC</td>
<td>Heteronuclear Multiple Quantum Correlation</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>IL-1, IL-6</td>
<td>Interleukin-1,-6</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
</tr>
<tr>
<td>Kda</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KS</td>
<td>Keratan sulfate</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MGP</td>
<td>Matrix Gla protein</td>
</tr>
<tr>
<td>MHz</td>
<td>Mega Hertz</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
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## LIST OF ABBREVIATIONS (cont.)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N</td>
<td>Number of samples</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NP</td>
<td>Not present</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino Terminal</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OH-PRO</td>
<td>Hydroxyproline</td>
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<td>ON</td>
<td>Osteonectin</td>
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<tr>
<td>P A₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>pCOL II-C</td>
<td>Carboxyl- type II procollagen peptide</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycans</td>
</tr>
<tr>
<td>PITC</td>
<td>Phenylisothiocyanate</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomoles</td>
</tr>
<tr>
<td>PPi</td>
<td>Inorganic pyrophosphate</td>
</tr>
<tr>
<td>PRO</td>
<td>Proline</td>
</tr>
<tr>
<td>Pyd</td>
<td>Pyridinoline</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RT</td>
<td>Retention time</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SF</td>
<td>Synovial Fluid</td>
</tr>
<tr>
<td>YKL-40 or HC gp-39</td>
<td>Human cartilage glycoprotein (Also called as Chondrex)</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitors of matrix metalloproteinases</td>
</tr>
<tr>
<td>TMS</td>
<td>Trimethylsulfonyl</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UD</td>
<td>Under detection</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
CHAPTER 1 LITERATURE REVIEW

1.1 INTRODUCTION

During normal metabolic processes and in disease states tissues release various compounds into the blood and tissue fluids. Some of these compounds are specific to particular tissues and can, therefore, be used as diagnostic markers to monitor the status of the tissue. Cartilage is a specialised connective tissue that functions in joints and at other skeletal sites to resist compressive forces associated with locomotor and related motion. The ability of cartilage to perform this function can be compromised by changes in tissue properties that occur with age and as a consequence of diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA). These changes reflect metabolism that can be monitored by analysis of appropriate biological markers (Lohmander et al, 1992). Treatments designed to slow down or reverse the effects of abnormal metabolism can benefit enormously from early detection of the diseased state. Consequently, the identification of biological markers can be extremely valuable for clinical diagnosis of cartilage diseases.

Type II collagen is the major structural protein in cartilage and is also essentially unique to cartilagenous tissues. The tensile strength of the collagen fibres is dependent upon the formation of intra-molecular crosslinks that involve chemical modification of lysine side chains. Interaction of the lysines and modified lysines can generate complex heterocyclic compounds, some of which have fluorescent properties. Moreover, the type of crosslink formed can vary in different collagens and in different connective tissues.
These differences have been utilized to provide biochemical markers in biological fluids (Heinegard and Saxne, 1991; Richardson and Emery, 1996; Ratcliffe and Seibel, 1990; Lohmander and Felson, 1998). Fluorescent compounds can also be generated through the modification of carbohydrate structures. For example, protein glycation results in the formation of Amadori products or fructoselysine (Ahmed et al, 1986).

While fluorescence emission has been used extensively to study protein and carbohydrate structure and dynamics, few studies have utilized fluorescence emission for studies of articular cartilage. The purpose of this Chapter is to review the structure and function of articular cartilage and its constituent molecules in normal tissues and the changes that are generated through the effects of osteoarthritis. Various types of luminescence that are relevant to studies of biological tissues are also reviewed. This is followed by a comprehensive analysis of known cartilage biomarkers in biological fluids, with an assessment of their utility and limitations.

### 1.2 ARTICULAR CARTILAGE

Articular cartilage is a highly specialized connective tissue with biophysical properties consistent with its ability to withstand high compressive forces. It has a smooth surface lubricated by synovial fluid, which allows bones to glide over one another with minimal friction. The properties and organization of the macromolecular constituents of the cartilage matrix and their interaction with water molecules is the key to the shock absorbing properties of the tissue. Adult cartilage is typically avascular, alymphatic and aneural (Ghadiyally, 1978). Nourishment is provided through long-range
diffusion of the joint fluid. Diffusion of substances from blood flowing through vessels in the subchondral bone can also contribute to the nourishment of cartilagenous tissues in the immature animal. The unique biological and mechanical properties of articular cartilage depend on the architecture of the articular cartilage and on the interactions between the extracellular matrix and the chondrocytes that maintain the cartilage function (Buckwalter and Mankin, 1998).

1.2.1 Cartilage Heterogeneity and Compartmentalization

Articular cartilage comprises several morphologically distinct components that are involved in its attachment to the bone, in the formation of an articulating surface as well as in the formation of the compression-resistant core of the tissue. Thus, uncalcified articular cartilage is attached to the subchondral bone via a narrow layer of calcified cartilage. The interface between uncalcified and calcified cartilage is demarcated by a densely basophilic calcified line called the “tidemark” (Havelka et al, 1984). The heterogeneous uncalcified cartilage can be distinguished microscopically as three compartments, or laminae, which are parallel to, and extend from, the articular surface to the tidemark. The differences between the three laminae are based on chondrocyte morphology and distribution, molecular composition and concentration of collagen and proteoglycan, architecture of collagen, water content, and biomechanical properties of cartilage.

The lamina closest to the cartilage surface is called tangential lamina or lamina splendens or superficial lamina. Superficial lamina is characterized by small ellipsoid chondrocytes with their long axis parallel to the articular surface. The collagen fibres are
densely packed and orientated parallel to the articular surface. The middle lamina is
called transitional lamina. The transitional chondrocytes are large, round, and more
randomly distributed than those in superficial lamina. Collagen fibres form an oblique
transitional network in the middle lamina. The lamina superficial to the calcified
cartilage is called radial or deep lamina. The deep chondrocytes are largest and arranged
in longitudinal columns that are oriented perpendicular to the articular surface. Collagen
fibres are oriented radially in the deep lamina. The deep chondrocytes synthesize
alkaline phosphatase that is likely involved in the calcification of the subjacent calcified
lamina (Xu et al, 1994). The calcified lamina is characterized by small chondrocytes
embedded in a heavily calcified matrix (Hunziker, 1992; Mitrovic et al, 1983).

In adult human hip cartilage the water content decreases from 74% in the
superficial lamina to 67% in the deep lamina (Maroudas, 1977). In immature cartilage,
proteoglycan content is least in the middle lamina compared to the articular and
epiphyseal lamina. However, in mature cartilage proteoglycan content is lowest in the
superficial lamina (Stockwell, 1979; Maroudas et al, 1969). The collagen content is
highest in the superficial lamina and lowest in the middle lamina (Muir et al, 1970).
Cellularity is considerably reduced during development especially in the deep lamina.

Matrix compartmentalization studies have revealed a clear subdivision of the
middle and deep lamina into pericellular, territorial, and interterritorial matrices (Poole,
1998 and 1992). Each chondrocyte cell membrane is immediately surrounded by
pericellular (or lacunar) matrix which is characterized by the absence of fibrillar collagen
and abundance of proteoglycan. The pericellular matrix is composed of a mixture of
hyaluronan (Mason, 1981), sulphated proteoglycans, biglycan (Miosge et al, 1994).
glycoproteins, fibronectin (Glant et al., 1985), laminin (Durr et al., 1996), and collagen type VI and IX (Poole et al., 1997). Encapsulating the pericellular matrix is the territorial (or capsular) matrix, characterized by a fine network of fibrillar collagen (Poole et al., 1984 and 1985). The chondrocytes establish contact with these collagen fibrils by extending fine cytoplasmic processes. Adjacent to this is the outermost matrix compartment, the interterritorial region, which constitutes the largest domain of the matrix and which lies in the space between various territorial matrices. The interterritorial matrix is characterized by collagen fibres, which run in parallel interspersed with varying concentrations of proteoglycans, depending upon the lamina in which the chondrocytes lie.

1.2.2 Chondrons

Chondrons are the microanatomical, micromechanical and metabolically active functional units of articular cartilage. Anatomically a chondron comprises of the chondrocyte and its pericellular microenvironment (Muir, 1995). Morphologically, a transparent pericellular glyocalyx is present on the chondrocyte surface and is enclosed by a fibrillar pericellular capsule. The chondron plays an important role in maintaining the homeostasis of the articular cartilage.

Throughout life, articular cartilage undergoes continual internal remodeling. Chondrocytes can synthesize and secrete the major macromolecular components of the extracellular matrix and they can also degrade the matrix by releasing degradative enzymes such as collagenase and other metalloproteinases including stromelysin. During growth and development synthesis outweighs the degradation. In adults, matrix synthesis
is finely balanced by controlled matrix degradation (Roth and Mow, 1980; Hwang et al., 1992). Hence, chondrocytes continually replace the matrix macromolecules lost during normal degradation. However, disruption to the normal balance of synthesis and degradation can lead to variation in the intrinsic characteristics of cartilage laminae. This can lead to a gradual degeneration of the extracellular matrix that is responsible for the development of clinically recognizable disease(s) (Poole et al., 1993; Venn and Maroudas, 1977).

1.2.3 Extracellular Matrix

The extracellular matrix (ECM) of cartilage (95% cartilage volume) is a resilient gel comprising 60-80% (cartilage wet weight) tissue fluid with a complex macromolecular organization. The tissue fluid plays an important role in joint lubrication and wear resistance (Kuettner et al., 1991). The physicochemical properties of articular cartilage depend on the structure and organization of the ECM macromolecules. These consist mainly of type II collagen which in humans represents 55% of the dry weight (bovine - 64% to 67%), and proteoglycans, representing 35% of the dry weight. Evidence exists that there are interactions between proteoglycan aggregates and collagen fibers (Smith et al., 1967; Matthews, 1965; Hammerman et al., 1970). The intrinsic physical properties of collagen fibres (high tensile strength) and proteoglycan (high fixed charge density and compressive rigidity) primarily determine the biomechanical properties of cartilage. The compressive properties of articular cartilage are a function of the balance between the osmotic swelling generated by water bound to the sulphate and carboxylate groups of the proteoglycan and the tension developed in the collagen network.
surrounding the proteoglycan. The non-collagenous proteins in cartilage ECM include fibronectin, anchorin CII, tenascin, cartilage oligomeric matrix protein (COMP) and cartilage matrix glycoprotein (CMP) (Muller et al. 1998; Hedboom et al. 1992; Clark. 1990; Kuhne et al. 1998; Durr et al. 1996).

1.2.3.1 Proteoglycans

The proteoglycans (PGs), a diverse family of molecules, are strongly hydrophilic. These properties are of key importance in the lubrication of the joint bearing surfaces. Proteoglycans are composed of a protein core onto which one or more glycosaminoglycan (GAG) chains are covalently bonded. The GAG molecules are unbranched chains of repeating disaccharides. Hyaluronic acid (HA) is the only GAG that is not bound to a core protein and is non-sulphated. The heterogeneity of PG structure is a reflection not only of the variation in protein core, but also variation in the type and size of the GAG chains. Variation in the position of sulphation can also increase diversity in the chemical and physical properties of the GAG chains.

In articular cartilage, proteoglycans are present as PG monomers or as PG aggregates. PGs give cartilage its resistance to compression and its resilience. The GAG groups present in cartilage PGs are primarily chondroitin sulphate (87%) which exists both as chondroitin-4-sulphate (C4S) and chondroitin-6-sulphate (C6S) with keratan sulphate (6%) (KS) and HA (table 1.1). The CS chains are covalently attached to the protein core via a xylose residue linkage to specific serine residues, whereas KS chains are attached to protein via N- and O-linked glycosidic linkages to asparagine or serine/threonine, respectively. HA is a large polyanionic molecule that can have a
molecular weight up to 6 million.

In articular cartilage, large aggregating proteoglycans (aggrecan and versican) form 50-58% of the total proteoglycans whereas large non-aggregating proteoglycans form 40% of the total proteoglycan (Ratcliff and Mow, 1996). Aggrecan is a large proteoglycan molecule consisting of over 100 CS chains, 20-40 KS and 40 O- and N-linked oligosaccharides. Aggrecans have the ability to bind with hyaluronic acid via their HA-binding region and link protein stabilizes the interaction between aggrecan and HA.

The relative concentration of GAGs varies markedly with age. In immature cartilage, there is a preponderance of C4S and little KS. With advancing age, there is an appreciable increase in KS content and a corresponding fall in C4S (Inerot et al, 1978).
Table 1.1 Proteoglycans in Articular Cartilage.

<table>
<thead>
<tr>
<th>Proteoglycan Type</th>
<th>Disaccharide Repeating Units</th>
<th>Molecular Weight (KiloDaltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteoglycan Aggregate</td>
<td>-</td>
<td>3 x 10^3 to 3 x 10^6</td>
</tr>
<tr>
<td>Hyaluronic Acid (Hyaluronan)</td>
<td>N-acetylgalactosamine glucuronate linkage 1-3</td>
<td>4 to 8000</td>
</tr>
<tr>
<td>Proteoglycan Monomer (PGM) (Aggrecan)</td>
<td>-</td>
<td>1 x 10^3 to 3 x 10^3</td>
</tr>
<tr>
<td>Protein Core</td>
<td>-</td>
<td>200 to 250</td>
</tr>
<tr>
<td>Keratan Sulphate (KS)</td>
<td>N-acetylgalactosamine galactose linkage 1-4</td>
<td>4 to 19</td>
</tr>
<tr>
<td>Chondroitin-4-Sulphate (C4S) (Chondroitin Sulphate A)</td>
<td>N-acetylgalactosamine glucuronate linkage 1-3</td>
<td>5 to 50</td>
</tr>
<tr>
<td>Chondroitin-6-Sulphate (C6S) (Chondroitin Sulphate C)</td>
<td>N-acetylgalactosamine glucuronate linkage 1-3</td>
<td>5 to 50</td>
</tr>
</tbody>
</table>

1.2.3.2 Collagens

Collagen fibers maintain cartilage integrity by providing resiliency (Mayne, 1989; Akizuki et al, 1986). Five genetically distinct collagen types are known to exist in articular cartilage (table 1.2). Type II collagen, the principal fibrillar macromolecule, represents 90 to 95% of the total collagen in articular cartilage (Eyre et al, 1992 and 1987). Through its high tensile strength it provides the structural integrity to articular cartilage. Type XI collagen, a fibril forming collagen, contributes to about 2-3% of the total collagen and is incorporated in the type II collagen fiber in a ratio of about 1:30 in mature tissues (Eyre et al, 1987). Type XI collagen is thought to mediate physical interactions between collagen fibrils and PGs in cartilage and to regulate the size of the type II collagen fibres (Mendler et al, 1989; Cremer et al, 1998; Wu and Eyre, 1992).
Type IX collagen represents 1-2% of collagen in adult cartilage and at least 10% in foetal cartilage. Type IX collagen is located on the outside of the fibril and is covalently crosslinked to type II collagen. Type IX collagen is also distributed in ECM without association with Type II collagen (Wu et al, 1992; Bruckner et al, 1985; Wu and Eyre, 1984 and 1989). Type IX collagen is also considered to be a PG because its α2(IX) chain contains CS or dermatan sulphate (DS) GAG chains. The GAG chains in type IX collagen are thought to stabilize type II collagen fibril structure (Olsen, 1997). Type VI collagen, a short-helix molecule concentrated pericellularly that represents 1-2% of the total collagen (Hambach et al, 1998), helps mediate the attachment of chondrocytes to the macromolecular framework of the matrix. Type X collagen (1%), a short non-fibrillar collagen, is present in the calcified lamina (Nerlich et al, 1992). It is synthesized and deposited largely by chondrocytes of hypertrophic/calcified cartilage (Gannon et al, 1991), and is thought to play important role in the development of the growth plate and cartilage calcification.

Table 1.2  Collagen Types in Articular Cartilage.

<table>
<thead>
<tr>
<th>Collagen Type</th>
<th>Molecular Organization</th>
<th>Molecular Weight (KiloDaltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibril Constituents of Collagen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen Type II</td>
<td>α1(II)₃</td>
<td>290</td>
</tr>
<tr>
<td>Collagen Type IX</td>
<td>α1(IX) α2(IX) α3(IX)</td>
<td>250</td>
</tr>
<tr>
<td>Collagen Type XI</td>
<td>α1(XI) α2(XI) α3(XI)</td>
<td>300</td>
</tr>
<tr>
<td>Non-Fibril Constituents of Collagen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen Type VI</td>
<td>α1(VI) α2(VI) α3(VI)</td>
<td>500-550</td>
</tr>
<tr>
<td>Collagen Type X</td>
<td>α1(X)₃</td>
<td>170</td>
</tr>
</tbody>
</table>
1.2.3.3 Non-Collagenous Proteins

Small non-collagenous proteins and glycoproteins are present in the cartilage ECM and are thought to be crucial for modulating several fibril properties (Hagg et al, 1998). Although glycoproteins form a small fraction (2-5%) of the cartilage ECM, they play an important role in matrix assembly and/or regulation of matrix metabolism (table 1.3). These matrix glycoproteins contain distinct and functionally active peptide domains that allow interactions with chondrocyte surface receptors as well as other matrix molecules.

Cartilage oligomeric matrix protein (COMP), a 524 kDa glycoprotein, is found in cartilage during chondrogenesis. It is preferentially localized in the territorial matrix surrounding the chondrocytes (Newton et al, 1994; Hedbom et al, 1992). COMP is markedly anionic due to its high content of aspartic and glutamic acid residues (acidic amino acids), as well as to its substitution with negatively charged sugars. Recently a new molecule called human cartilage glycoprotein (HC gp-39) (Hakala et al, 1993) or Chondrex, was discovered independently by Harvey et al (1998) and Johansen et al (1992, 1993 and 1996). Also termed YKL-40 (De Ceuninck et al,1998), Chondrex is a 40 kDa glycoprotein originally identified in the whey secretions of nonlactating cows (Rejman and Hurley, 1988). It is a major secretory glycoprotein of human chondrocytes and synovial-fibroblasts and has been reported to be produced by an osteosarcoma cell line (Johansen et al, 1992).

Biglycan, decorin and fibromodulin are members of a family of structurally related proteoglycan called the small CS/DS proteoglycans. They bind to the matrix macromolecules and thereby help to stabilize the matrix. Biglycan, a 100 kDa molecule
with a core protein of 38 kDa, is the predominant small PG of cartilage and contains two chains of CS/DS. Biglycan is localized to the pericellular matrix (Bianco et al. 1990). Decorin, a 74 kDa PG with a core protein of 36 kDa (Neame et al. 1989), is present throughout the interterritorial ECM, with increased amounts in the superficial laminae of articular cartilage (Poole et al. 1996). It is thought to mediate interactions between aggregating proteoglycan and collagen (Scott, 1993). Fibromodulin (59 kDa) represents 0.1-0.3% of the cartilage wet weight. It has a characteristic amino acid composition, with 14% of its residues being made up of leucine (Öldberg et al. 1989). Recently, Noyori et al. (1998) investigated the presence of non-collagenous proteins on the cartilage surface in normal bovine and human samples. Their study indicated that the surface cartilage has abundant fibromodulin (a collagen binding small proteoglycan) and a small amount of fibronectin, decorin, and biglycan. Fibronectin (FN) is a glycoprotein of the extracellular matrix which is composed of two similar disulphide linked polypeptide chains of approximately 250 kDa each. FN effects cell adhesion, morphology, migration, and differentiation as well as matrix assembly (Couchman et al. 1990; Clark, 1990; Burton-Wurster and Lust, 1986). FN plays an important role in the adhesion of chondrocytes to ECM and is implicated in tissue repair (Piperno et al., 1998). Tenascin is an oligomeric glycoprotein and functions in processes such as wound repair and formation of bone and cartilage. Tenascin is thought to influence interactions between the chondrocytes and the matrix and is involved in the assembly of the chondrocyte matrix. It is expressed in 220 and 320 kDa forms in articular cartilage (Savarese et al., 1996).

Cartilage matrix glycoprotein (CMP), a 54 kDa protein, is a major component of the cartilage ECM (Paulsson and Heinegard, 1984; Agraves et al., 1987). It is also
detected in the intervertebral disks, the connective tissue of the eye (Fife and Brant, 1984; Fife, 1986) and forms about 5% of the wet weight of aged tracheal cartilage. Although its function is not known, it has been suggested that CMP can bind to and bridge type II collagen fibrils (Tondravi et al, 1993). Matrix Gla protein (MGP), a vitamin K-dependent 10 kDa protein that inhibits calcification, was initially isolated from bone and is now known to be present in cartilage as well (Loeser and Wallin, 1991). Anchorin CII (31 kDa non-collagenous protein) appears to mediate in anchoring chondrocytes with type II (Mollenhauer et al, 1999 and 1984). Chondronectin is a cartilage matrix protein that specifically mediates the attachment of chondrocytes to type II collagen (Carsons and Horn, 1988). Link protein (MW = 45 kDa) binds to both cartilage aggrecan and HA in ECM thereby stabilizing their aggregation (Kahn et al, 1994).
Table 1.3  Non-Collagenous Proteins in Articular Cartilage.

<table>
<thead>
<tr>
<th>Glycoprotein / Protein</th>
<th>Molecular Weight (Kda)</th>
<th>Anatomic Location</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage Oligomeric Matrix Protein (COMP)</td>
<td>524</td>
<td>Territorial Matrix</td>
<td>Newton et al, 1994; Hedboom et al, 1992</td>
</tr>
<tr>
<td>Human Cartilage Glycoprotein (HC gp-39), Also called YKL-40 and Chondrex</td>
<td>40</td>
<td>ECM*</td>
<td>Hakala et al, 1993; Harvey et al, 1998; Johansen et al, 1992</td>
</tr>
<tr>
<td>Biglycan</td>
<td>100</td>
<td>Pericellular Matrix</td>
<td>Bianco et al, 1990</td>
</tr>
<tr>
<td>Decorin</td>
<td>74</td>
<td>Interterritorial Matrix, Superficial Lamina, ECM*</td>
<td>Neame et al, 1989; Poole et al, 1996</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>59</td>
<td>Superficial Lamina</td>
<td>Noyori et al, 1998</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>440</td>
<td>ECM*</td>
<td>Piperno et al, 1998</td>
</tr>
<tr>
<td>Tenascin (Two size variants)</td>
<td>220 &amp; 320</td>
<td>Territorial Matrix</td>
<td>Savarese et al, 1996</td>
</tr>
<tr>
<td>Cartilage Matrix Glycoprotein (CMP)</td>
<td>54</td>
<td>ECM*</td>
<td>Paulsson &amp; Heinegard, 1984; Agraves et al, 1987</td>
</tr>
<tr>
<td>Matrix Gla Protein (MGP)</td>
<td>10</td>
<td>ECM*</td>
<td>Loeser &amp; Wallin, 1991</td>
</tr>
<tr>
<td>Anchorin CII (Cartilage Annexin V)</td>
<td>31</td>
<td>Chondrocyte Membrane</td>
<td>Mollenhauer et al, 1984; Mollenhauer et al, 1999</td>
</tr>
<tr>
<td>Chondronectin</td>
<td>180</td>
<td>ECM*</td>
<td>Carsons &amp; Horn, 1988</td>
</tr>
<tr>
<td>Link Protein</td>
<td>45</td>
<td>ECM*</td>
<td>Kahn et al, 1994</td>
</tr>
</tbody>
</table>

* Extracellular Matrix, compartment not specified
1.3 BIOLOGICAL FLUORESCENCE

1.3.1 Fluorescence and Autofluorescence

Some molecules can absorb photons with a short wavelength (i.e. high energy) and emit light at a longer wavelength (i.e. lower energy). This emission is called fluorescence. Fluorescent compounds are characterized by one or more stable ring structures. The fluorescence emission of proteins is often used to study their structure and interactions in solution (Jameson and Reinhart, 1989).

Tissue autofluorescence is due to the fluorescence of endogenous molecules. The fluorescence characteristics of tissues depend upon their biochemical composition and histomorphological architecture, both of which undergo a change during maturation, ageing (Wolman et al, 1985; Van der Korst et al, 1977; Bellmunt et al, 1995; Vlassara et al, 1994) and in the progression or manifestation of disease(s) (Bissonnette et al, 1998; Banerje et al, 1998; Abiko et al, 1999; Van Schaik et al, 1999; Nagaraj et al, 1992). These changes are detectable as an alteration in the fluorescence spectral profile of the tissues. Autofluorescence of viable mammalian cells has been attributed to the autofluorescence from mitochondria and lysosomes (Andersson et al, 1998).

The application of fluorescence to the study of protein structure and dynamics has been extensively exploited to facilitate the understanding of complex biological problems. Fluorophores in biological tissues are formed by non-enzymatic, spontaneous
chemical modifications of proteins (Pongor et al. 1984; Njoroge et al. 1988) and glycation of proteins or oxidation of lipids resulting in fluorescent final product(s) (Nakamura et al., 1997). It is well established that glucose reacts non-enzymatically with free amino groups on proteins to form a stable amino 1-deoxyketosyl adduct (Cerami et al., 1987), also called the Amadori product or fructoselysine (Ahmed et al. 1986). This relatively stable Amadori product can then undergo various oxidation, dehydration, condensation and/or rearrangement reactions. Malonaldehyde and other lipid peroxidation products can interact with amino acids or amines to produce fluorescence products (Chio and Tappel, 1969).

1.3.2 Fluorescent Compounds in Articular Cartilage

Fluorescent molecules in articular cartilage are produced either by enzymatic (e.g. collagen crosslinks) or non-enzymatic reactions (e.g. products of glycation or oxidation). A summary of the known fluorescent compounds in articular cartilage is shown in table 1.4.

Fluorescence of connective tissue components can result from non-enzymatic reaction products of sugars or oxidized lipids with proteins (Hormel and Eyre, 1991). Age related protein changes in cartilage, in particular collagen, have been investigated using ultraviolet light-induced fluorescence (Hoerman et al, 1969). The fluorescence that appears in ageing human intervertebral disks has been attributed to covalent adducts (possibly derived from non-enzymatic reactions with carbohydrates or lipids) linked to the collagen and probably to other long-lived matrix proteins. Such modifications to structural proteins may contribute to the degeneration of ageing disks.
1.3.2.1 Amino Acids

The absorption maximum of most proteins is near 280 nm due to absorption by the aromatic side chains of the tyrosine and tryptophan residues. At wavelengths from 295 to 305 nm, the tryptophan residues of proteins are selectively absorbed (Kierdaszuk et al. 1995). Tryptophan, tyrosine, and phenylalanine, which also has an absorption near 260 nm, have intrinsic fluorescence. Intrinsic fluorophores are fluorescent molecules found within the cell or tissue structures. However, some molecules are weakly fluorescent. Hence an extrinsic fluorophore such as acridine orange (for nucleic acids), fluorescein or quinacrine, is added which then binds to the particular cell component or molecule of interest thereby enhancing the fluorescence. The molecular structures of tryptophan, tyrosine, and phenylalanine are shown in figure 1.1.
(A) Fluorescent amino acids

![Chemical structures of tryptophan, tyrosine, and phenylalanine]

Tryptophan  Tyrosine  Phenylalanine

(B) Fluorescent biochemical markers

![Chemical structures of pyridinoline, deoxypyridinoline, and pentosidine]

Pyridinoline  Deoxypyridinoline

Ribose  Lysine  Arginine

Pentosidine

Figure 1.1  The Molecular Structure of Fluorescent Compounds in Articular Cartilage. (A) Fluorescent amino acids are tryptophan, tyrosine and phenylalanine. (B) Fluorescent biochemical markers are pyridinoline, deoxypyridinoline and pentosidine. Note that fluorescent molecules have a stable ring structure.
Table 1.4  Fluorescent Compounds in Articular Cartilage.

<table>
<thead>
<tr>
<th>Compound/ Amino Acid</th>
<th>Molecular Weight (Daltons)</th>
<th>UV Absorption Maxima (A- nm)</th>
<th>Emission (A- nm)</th>
<th>Molar Absorption Coefficient (M⁻¹.cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>204</td>
<td>280</td>
<td>-</td>
<td>5600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>219</td>
<td>-</td>
<td>4700</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>181</td>
<td>274</td>
<td>-</td>
<td>1400</td>
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<td></td>
<td></td>
<td>222</td>
<td>-</td>
<td>8000</td>
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<tr>
<td></td>
<td></td>
<td>193</td>
<td>-</td>
<td>48000</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>165</td>
<td>257</td>
<td>-</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>206</td>
<td>-</td>
<td>9300</td>
</tr>
<tr>
<td></td>
<td></td>
<td>188</td>
<td>-</td>
<td>60000</td>
</tr>
<tr>
<td>Pyridinoline</td>
<td>429</td>
<td>295</td>
<td>395</td>
<td>5700</td>
</tr>
<tr>
<td>Deoxypyridinoline</td>
<td>413</td>
<td>295</td>
<td>395</td>
<td>5000</td>
</tr>
<tr>
<td>Pentosidine</td>
<td>335</td>
<td>395</td>
<td>395</td>
<td>4500</td>
</tr>
</tbody>
</table>
1.3.2.2 Pyridinoline and Deoxypyridinoline Collagen Crosslinks

Collagen fibrils are stabilized by covalent crosslinks formed between adjacent collagen chains (intramolecular crosslink) and adjacent collagen molecules (intermolecular crosslink). Crosslinking of collagen fibrils occurs extracellularly via an enzyme mediated reaction (Pokharna et al, 1995). Lysyl oxidase, a 30 kDa copper requiring enzyme, initiates crosslink formation. This enzyme catalyzes the oxidative deamination of certain -NH₂ groups in collagen. It acts on specific lysine or hydroxylysine residues in the telopeptide region at each end of the collagen molecule. Intramolecular crosslinks in collagen are derived from lysine side chains at the nonhelical region near the N-terminal. The resulting aldehydes combine either with neighbouring aldehydes to form aldol condensation products (ACP), or with amino groups from lysyl or hydroxylysyl residues to form "bifunctional crosslinks" through a Schiff-based reaction. For the formation of ACP, the enolate ion derived from one aldehyde adds to the carbonyl group of the other aldehyde. These carbonyl-derived crosslinks found in neo-synthesized collagen are easily reduced by exogenous reagents such as lithium bromide and sodium borohydride which are used to form stable derivatives to identify crosslinks. In vivo, the reducible immature crosslinks initially formed are then converted to mature non-reducible compounds (mature crosslinks) (Knott and Bailey, 1998; Frazer, 1998). Ultimately the hydroxypyridinium crosslinks, pyridinoline (Pyd) and deoxypyridinoline (Dpyd) are formed. These intermolecular crosslinks, which are naturally fluorescent compounds, are formed by condensation of two hydroxylysine residues and one lysine residue i.e., between residues near the N-terminal of one collagen
molecule and the C-terminal of another. Four residues in each collagen molecule can participate in these crosslinks: a lysine near the N-terminal, a lysine near the C-terminal, and hydroxylysines in helical regions near the ends of the molecule (residues 87 and 930).

Pyridinoline (Pyd) was first isolated from rat tail tendon and characterized in the mid 1970's by Fujimoto et al (Fujimoto, 1977; Fujimoto et al, 1978). Pyd is present in collagen-containing tissues such as cartilage, synovial membrane, meniscus, bone and ligament. It is far more abundant in cartilage than it is in bone (Takahashi et al, 1996) and its concentration remains relatively constant in cartilage with age (Takahashi et al, 1994 and 1997; Chen et al, 1998; Eyre et al, 1988). Besides Pyd, deoxypyridinoline (Dpyd) is also abundant in bone and dentine.

1.3.2.3 Pentosidine

Fluorophores are formed by the non-enzymatic glucosylation and fructosylation of certain proteins such as native collagen (Fujimori, 1989). Glycation (non-enzymatic glycosylation), crosslinking, and fluorophore formation of collagen occur both in vivo and in vitro. In vivo, native collagen becomes more glycated which significantly increases collagen fluorescence with ageing (Odetti et al, 1994) and as a consequence of diabetes (Monnier et al, 1986). Different patterns of collagen autofluorescence could be associated with the diseased state. A fluorescent dimer (β-components) of two collagen α-chains, crosslinked as a result of glycation, was found in pepsin digests of tail tendons from older rats (Tanaka et al, 1988).
Pentosidine, a condensation product of arginine, lysine, and ribose, is an end product of advanced glycation (Grandhee and Monnier, 1991). It is formed by sequential glycosylation and oxidation reactions. The formation of pentosidine requires aerobic conditions, and antioxidative conditions inhibit its formation (Dyer et al., 1991; Fu et al., 1992). Pentosidine was isolated from human dura mater and characterized by Sell and Monnier in 1989. They reported that the pentosidine concentration in tracheal cartilage is 182 pmoles/mg of collagen (Sell and Monnier, 1989). Using fluorometry and high pressure liquid chromatography (HPLC) of hydrolysates, quantitation of pentosidine in various collagen-rich tissues such as dura mater, skin, ocular lens, and cartilage has shown that the amount of pentosidine increases exponentially with age (Sell and Monnier, 1990; Bank et al., 1998; Graham, 1996; Monnier, 1990). Akimbo et al. (1991) also reported that the amount of pentosidine per collagen molecule in human articular cartilage increases linearly with age. However, the amount of Pyd per collagen was found to remain constant and did not correlate with age. Pyd and pentosidine crosslinks have fluorescent maxima at different wavelengths, a property that provides the means of their identification on HPLC (Uchiyama et al., 1991).
1.4 SPONTANEOUS DEGENERATIVE ARTHRITIS

1.4.1 Etiology, Histology and Biochemistry

Osteoarthritis (OA) is an extremely common and often debilitating form of degenerative arthritis (Setton et al. 1993). Despite its clinical prevalence, OA remains difficult to define, characterize, or assess (Pritzker, 1994). OA is a slow progressive disease and is associated with defective integrity of articular cartilage, in addition to related changes in the underlying trabecular and cortical bone and at the joint margin, in particular the synovium (Altman et al. 1986; Oegema et al. 1997). Gross macroscopic observations show that in OA the normally whitish-blue translucent cartilage takes on an opaque yellowish appearance. Surface irregularities due to fissuring and pitting are followed by an extensively ulcerated area leading to partial or full cartilage thickness erosion. These erosions, which are initially focal, become confluent and progress to large denuded areas, particularly in the load-bearing zone (Dieppe, 1995).

Cartilage extracellular matrix composition in early OA is characterized by elevated water content with freshly-administered water binding more avidly to OA than to normal cartilage (Mankin and Thrasher, 1975; Venn and Maroudas, 1977). Elevated metabolic activity in human OA cartilage is an early event. OA cartilage is characterized by a significant increase of mRNA and corresponding matrix proteins throughout the entire depth of the tissue. Although PG synthesis is markedly increased in OA compared to normal cartilage, the rate of PG turnover is also increased resulting in a decrease in
total PG and/or GAG content, which is directly proportional to the OA severity (Maroudas et al, 1973). PG synthesized by OA cartilage chondrocytes is different in structure from PG molecules produced by normal cartilage. The depletion of GAG does not uniformly affect all of GAGs species. For example, KS is more severely affected than CS (Mankin et al, 1981). Further, GAGs are shorter, there is an increase in the number of PG fragments, a decrease in size of its subunits with diminished and/or defective aggregation, an increase in C4S compared to C6S, and an increased CS/KS ratio. Increased levels of aggrecan, decorin, biglycan, fibromodulin, and link protein have been reported in human OA cartilage (compared to age matched controls) (Cs-Szabo et al, 1997). An increase in the cytoplasmic anchorin CII (annexin V epitopes) and their release into the pericellular and interterritorial matrix has also been documented (Mollenhauer et al. 1999). Human cartilage explant studies have shown that tenascin levels increase in OA cartilage and in the superficial lamina of damaged cartilage in particular (Chevalier et al, 1996; Salter, 1993).

Although the total collagen content of OA cartilage varies little (Lane and Weiss, 1975), type I, III, VI, and X collagens are often increased (Pullig et al. 1999; Girkontaite et al. 1996; Aigner et al, 1993). Also, OA cartilage collagen on average is less thermally stable and more soluble than normal (Herbage et al, 1972). Poole and colleagues (1993) have detected collagen degradation in OA cartilage by using an antibody to unfolded type II collagen molecules. Collagen fibre diameter and orientation may also show considerable variation from normal (Weiss, 1973). A switch to type I collagen synthesis with a decrease in the synthesis of type II collagen is observed in OA cartilage. Type I collagen appears around chondrocyte lacunae, but type II collagen is retained in the
interterritorial cartilage. Under physiological conditions type II collagen fibrils contain more water than type I fibrils (Grynpas et al. 1980; Studer et al. 1996). Therefore, an increased type I collagen and decreased type II collagen could account for decreased water content in severely OA tissue. Recently, Pfander et al (1999) reported that fibrillated surface of OA articular cartilage has enhanced deposition of type I collagen, fibronectin, and tenascin. Other studies have also confirmed the increased fibronectin level in human osteoarthritic articular cartilage (Homandberg et al. 1998; Jones et al. 1987; Chevalier, 1993). Van der Mark et al (1992, 1995) demonstrated the synthesis of type X collagen by OA chondrocytes. Type X collagens were irregularly distributed around chondrocyte clusters in fibrillated OA cartilage. Average pentosidine levels in OA cartilage are low.

1.4.2 Animal Models of Osteoarthritis

Osteoarthritis (OA) can be induced or the disease may occur spontaneously in animals (Bendele and Hulman, 1998; Tulamo et al, 1996). Thus, OA may result from mechanical disturbances due to surgical procedures (e.g. meniscectomy or transection of the medial collateral or cruciate ligaments) or from the administration of single major or multiple impact of the joints or to joint immobilization. Intraarticular chymopapain injection into the joint cavity and response to vitamin A or hormones such as corticosteroids or testosterone, can also induce OA. The advantages in studying the induced OA models include the enhanced evolution of the disease and more importantly, the ability to study the early stage of OA. The main disadvantage of induced animal models is that they often mimic only certain parts of the disease process. For example,
postoperative inflammation following surgical intervention may be a significant contributing factor in the enhancement of OA. Further, the depletion of cartilage matrix macromolecules is highly acute and has little in common with the chronic, slowly progressive alterations seen in clinical or experimental OA.

Animal models in which cruciate ligament rupture and meniscal tears have been introduced in the knee joint have shown substantial increases in the level of degradative enzymes, and matrix components such as aggrecans and COMP immediately after the injury (Ratcliffe et al, 1993 and 1994; Fife and Brandt, 1989; Panula et al. 1998; Williams et al. 1988). In a rabbit model of antigen-induced arthritis, the PGs were lost from the cartilage matrix most rapidly during the acute phase of the disease, whereas only minor changes in cartilage PG content were observed from day 14 onwards. A similar release of cartilage PGs into the synovial fluid could also be evoked by a single intra-articular injection of human recombinant interleukin-1 (IL-1). In a chick model of OA, decorin was depleted from the surface lamina (Masse et al. 1997).

1.4.3 Osteoarthritis vs Ageing

Osteoarthritis was once believed to be "a disease of the elderly". However, OA is now regarded as distinct from and superimposed on ageing processes. Ageing itself may not be a cause of OA but may cause changes in chondrocytic function required for the OA process to be perpetuated. Ageing leads to alterations in the composition of the matrix and the activity of the chondrocytes, this in turn could accelerate the degradation of the cartilage. Both bone density and the incidence of OA are known to vary with age in humans (Sokoloff, 1969). Mitrovic et al (1983) documented age-related decrease in
cell density in all laminae of the human femoral condyle articular cartilage, though more
markedly in superficial lamina. Vascularity of the calcified lamina of cartilage (a sign of
remodeling) is well developed after 55-65 years of age. Lane et al (1977) observed age-
related decline in calcified cartilage thickness in human femoral condyles with attenuated
number of tidemarks after the 6th decade. These findings suggest that remodeling of the
bone appears to cease with increasing age. Reduction in the water content from 70% -
80% (normal wet weight) to 50% - 65% (wet weight) accompanies ageing process
especially in the deeper lamina (Venn. 1978).

1.5 Joint Arthropathy

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder that mainly
affects the joint tissues. Although RA is of unknown etiology, autoimmunity plays a
pivotal role in its chronicity and progression (Harris, 1990; Mitchell, 1985). The initial
pathologic event in RA appears to be activation and/or injury of synovial microvascular
endothelial cells and as the disease progresses to more chronic stages, the synovium
becomes massively hypertrophic and edematous. With the progression of RA and in
response to cytokines such as Il-1, chondrocytes synthesize excessive amounts of
collagenase and stromelysin which degrade the type II collagen and proteoglycan of
articular cartilage matrix. In addition, a decreased synthesis of collagen and
proteoglycans have been reported (Mitchell, 1985). The loss of cartilage could be
accelerated by the precipitation of rheumatoid factor containing immune complexes in
the superficial lamina of the cartilage. The net result is progressive thinning and loss of
cartilage over the entire surface of the joint.
Articular crystal deposits are associated with a variety of acute and chronic joint disorders. Endogenous crystals such as monosodium urate, calcium pyrophosphate dihydrate, and basic calcium phosphate (hydroxyapatite) have been shown to be pathogenic. These endogenous crystals produce disease by triggering the cascade that results in cytokine-mediated cartilage destruction. The two common crystal arthropathies are: gout, caused by urates, and pseudogout, associated with calcium pyrophosphate. Gout is a disease involving urate crystal deposition in the synovial fluid leading to synovial hyperplasia, fibrosis and pannus formation which in turn destroys the underlying cartilage. The urates may also deposit on the articular cartilage surface hence accelerating the cartilage degradation. CPPD crystal deposition can occur in tendons, ligaments, synovium, and in articular cartilage (Ryan and McCarty, 1993). The conditions leading to crystal formation are not entirely known but include altered activity of the enzyme, inorganic pyrophosphatase, which degrades pyrophosphate, resulting in its accumulation and eventual crystallization with calcium. Patients with CPPD crystal deposition showed unaltered inorganic pyrophosphate (Pi) levels in plasma and urinary excretion. However, the synovial fluid Pi level is elevated.
1.6 BIOCHEMICAL MARKERS OF ARTICULAR CARTILAGE

1.6.1 Introduction

Biochemical markers (Ratcliffe et al. 1996; Chevalier. 1997; Kleesiek et al. 1987; Cameron et al, 1994; Schneiderman et al, 1995) in body fluids are valuable tools in clinical medicine. Biochemical markers can be used to study growth and development, detect latent disease, or to monitor pre-existing disease and its treatment. The most direct measure of synovial tissue metabolism is a biochemical assessment of the catabolic or anabolic products of the extracellular matrix of cartilage, synovium, and/or bone found in the body fluids namely, synovial fluid, serum, plasma or urine. Biochemical markers from anabolic and catabolic processes are released from the synovial tissues to the synovial fluid during both the destructive and repair phases of a pathological process. These metabolic products are then carried to the blood stream via the lymphatic ducts. In the blood circulation they are taken up by the kidney either directly or after modification by the liver and excreted in the urine (Lomander et al. 1990 and 1991).

Since changes can occur in the rate of turnover of the ECM macromolecules in joint diseases, elevated levels of biochemical markers in the serum can reflect increased synthesis, an increased release due to accelerated tissue catabolism or to a decreased clearance rate from serum.
An ideal biochemical marker has the following characteristics:

1) it can be used to detect and monitor the early stage of disease, 2) it is tissue specific, 3) it is specific for anabolic or catabolic processes, 4) it is sensitive to changes in disease activity, 5) it can reflect a relevant aspect of the disease, 6) it is easy to follow because of readily available samples, 7) it is inexpensive to assay, and 8) it can be assayed reliably, reproducibly, and quantitatively (Thonar et al, 1993).

1.6.2 Biochemical Markers in Human Studies

Aggrecan has been considered as an excellent marker for cartilage damage (Lohmander et al, 1999; Ishiguro et al, 1999; Thonar et al, 1991). The concentration of PG and/or its components in the synovial fluid is greatly affected by both disease activity and the stage of disease progression. However, serum concentrations of PG are of limited value due to the rapid clearance of the PG from the circulation and lymph. Using immunochemical and biochemical assays, high concentrations of immunoreactive PGs, KS epitope, sulphated GAGs (Murakami et al, 1998; Shinmei et al, 1995) or hyaluronidase activity (Nagaya et al, 1999) have been reported in the synovial fluid of patients with joint diseases such as rheumatoid arthritis (RA), OA, gout, and reactive arthritis (Saxen et al, 1987; Vaatainen et al, 1998). Potential cartilage biochemical markers produced as a consequence of aggrecan metabolism are summarized in table 1.5. The increased levels of PG in synovial fluid of patients with acute reactive disease can be attributed to the accelerated rate of cartilage PG degradation in response to the acute injury. Thus, PG levels in synovial fluid from the same group of patients were found to decrease during the steroid-induced duration of the disease. In RA patients, the loss of
cartilage, as evaluated by joint space narrowing, correlated with a decline in synovial fluid PG concentrations. This could relate to the late stage of RA. Lohmander et al (1989) studied patients with various post-traumatic knee joint lesions (knee trauma, cruciate ligament tear with or without meniscus tear, meniscus tear only, and chondromalacia patellae) of different duration. Compared to the normal (control) population, patients with post-traumatic cruciate ligament injuries showed elevated synovial fluid PG levels. A slight to moderately elevated level of synovial PGs persisted for as long as 5 to 7 years after the initial trauma. Elevated levels of synovial PG or its components in the patients with no apparent degenerative cartilage changes could represent increased metabolic events of repair after trauma. It appears that high levels of cartilage PG components in synovial fluids particularly indicate the active phases of cartilage metabolism or of increased matrix depletion. Aggrecan fragments from the hyaluronic acid binding region (HABR), such as HABR-FVDIPEN (Phe-Val-Asp-Ile-Pro-Glu-Asn) and HABR-FMDIPEN, are released into both articular cartilage and synovial fluid by matrix metallopeinase induced degradation of aggrecan (Olszewski et al, 1996). A recent study reported an increase in the cartilage metabolic markers namely pCOL-II-C, MMP-3, Δdi-HA and the Δdi-6S/Δdi-4S ratio in synovial fluid of OA and osteonecrotic patients (Iwase et al, 1998). Lohmander et al (1999) have also investigated the synovial fluid concentrations of aggrecan fragments from a total of 385 patients with knee joint injury, OA and acute pyrophosphate arthritis and tested their reactivity with chondroitin sulphate 846 epitope. Although an increased reactivity of the 846 epitope was observed in all the patients, it was highest in OA patients. Further, upon comparison with other markers of matrix turnover, epitope 846 reactivity correlated
positively with COMP (r-s = 0.421) and CPII (r-s = 0.307).

Pyridinium crosslinks, pyridinoline (Pyd) and deoxypyridinoline (Dpyd), have been used extensively as reliable markers for the destruction of collagens. Urinary levels of Pyd and Dpyd provide information about the intensity of catabolic changes in the connective tissues in various physiological and pathological states (Black et al. 1988; James et al. 1990). Both clinical and animal studies have shown urinary Pyd to be a marker of bone and cartilage breakdown in joints and Dpyd as a specific marker of bone loss (Tordjman et al, 1994). Using an immunoassay, Robins et al (1986) reported significantly elevated levels of Pyd in the urine of OA and RA patients (table 1.6) while Black et al (1989) found that an elevated level of Pyd excreted in the urine correlated with disease activity.

Recently pentosidine, a crosslink of ageing collagen, was detected in articular cartilage and body fluids of patients with bone and joint disorders (Pokharna and Pottenger, 1997; Brama et al, 1999; Miyata et al, 1998). An increased pentosidine level has been reported in age-matched OA cartilage. Extremely high levels of pentosidine have been detected in the skin and ocular lens as well as in the plasma and urine of patients with diabetes and uremia (Miyata et al, 1999). Takahashi et al (1997) reported that the mean concentration of pentosidine in articular cartilage from RA patients was 214 μmol/mol of hydroxyproline that corresponds to 228 pmol/mg of collagen (table 1.6).
Table 1.5 Cartilage Biochemical Markers: Aggrecan Metabolism Detected in Body Fluid(s).

<table>
<thead>
<tr>
<th>CARTILAGE MARKER (Aggrecan Derived)</th>
<th>MARKER REFLECTS</th>
<th>BODY FLUID</th>
<th>MARKER LEVEL WITH GROWTH OR JOINT DISEASE(S)</th>
<th>REFERENCE(S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratan Sulphate</td>
<td>Proteoglycan Anabolism; Proteoglycan Catabolism</td>
<td>Synovial Fluid; Serum</td>
<td>↑ with growth; ↑ with OA; ↑ with RA; ↑ with chondrolysis; ↑ with repetitive joint usage</td>
<td>Lohmander et al., 1999; Thonar et al., 1993; Williams et al., 1991; Lohmander et al., 1989</td>
</tr>
<tr>
<td>Hyaluronate</td>
<td>Synovium &amp; Cartilage Metabolism</td>
<td>Synovial Fluid; Serum</td>
<td>↑ with OA; ↑ with RA; ↓ exposed by enzymatic cleavage and/or found in newly synthesized PGs; ↑ with mild OA; ↓ with severe OA</td>
<td>Leipold et al., 1989; Chevalier X., 1997</td>
</tr>
<tr>
<td>Neoepitopes 3B3(−); 3B3(+) 7D4; 846; BC3; BC4</td>
<td>Cartilage Repair</td>
<td>Synovial Fluid; Serum</td>
<td>↓ with OA; ↓ with RA; ↓ exposed by enzymatic cleavage and/or found in newly synthesized PGs; ↑ with mild OA; ↓ with severe OA</td>
<td>Frisbie et al., 1999; Ishiguro et al., 1999; Lohmander et al., 1999; Ralcliffe et al., 1993</td>
</tr>
<tr>
<td>Large Fragments - Glycosaminoglycan-Rich Core Protein</td>
<td>Early Chondrolysis</td>
<td>Synovial Fluid</td>
<td>↑ with mild OA</td>
<td>Saxen et al., 1987</td>
</tr>
<tr>
<td>Proteoglycan Containing Hyaluronic Acid Binding Region Site</td>
<td>Late Chondrolysis</td>
<td>Synovial Fluid</td>
<td>↑ with severe OA</td>
<td>Olszewski et al., 1996</td>
</tr>
<tr>
<td>Aggrecan Link Protein</td>
<td>Proteoglycan Metabolism</td>
<td>Synovial Fluid</td>
<td>↑ with OA</td>
<td>Ratcliffe et al., 1996; Ratcliffe et al., 1994</td>
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<tr>
<td>Proteoglycan Core Protein Beta-D-Xylosyltransferase</td>
<td>Proteoglycan Metabolism</td>
<td>Synovial Fluid; Serum</td>
<td>↑ with OA; ↑ with RA</td>
<td>Kleesiek et al., 1987</td>
</tr>
<tr>
<td>Sulphated Glycosaminoglycan Chondroitin-4-Sulphate; Chondroitin-6-Sulphate.</td>
<td>Proteoglycan Metabolism</td>
<td>Synovial Fluid</td>
<td>↑ with growth; ↓ with severe OA; ↑ with osteonecrosis</td>
<td>Ishiguro et al., 1999; Iwase et al., 1998; Murakami et al., 1998</td>
</tr>
</tbody>
</table>
## Table 1.6  Cartilage Biochemical Markers: Collagen and Non-Collagenous Proteins Detected in Body Fluid(s).

<table>
<thead>
<tr>
<th>CARTILAGE MARKER</th>
<th>MARKER REFLECTS</th>
<th>BODY FLUID</th>
<th>MARKER LEVEL WITH GROWTH OR JOINT DISEASE(S)</th>
<th>REFERENCE(S)</th>
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<tr>
<td><strong>COLLAGEN DERIVED PROTEINS</strong></td>
<td></td>
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<tr>
<td>Carboxy-terminus Type II Procollagen (CP II)</td>
<td>Collagen Synthesis</td>
<td>Synovial Fluid; Serum; Urine</td>
<td>↓ with RA; ↑ with joint injury; ↑ with early OA; ↓ with severe OA</td>
<td>Frisbie et al., 1999 Ishiguro et al., 1999 Lohmander et al 1999 Shinmei et al., 1995</td>
</tr>
<tr>
<td>Collagen Crosslinks</td>
<td>Collagen Degradation</td>
<td>Synovial Fluid; Serum; Urine</td>
<td>↑ with growth and maturation; ↑ with OA; ↑ with Osteoporosis; ↓ with skeletal injury; ↑ with repetitive joint usage; ↓ with RA</td>
<td>Tordjman et al., 1994 James et al., 1990 Black et al., 1988 Robins et al., 1986</td>
</tr>
<tr>
<td>Pentosidine</td>
<td>Cartilage Ageing</td>
<td>Synovial Fluid; Serum; Urine; Plasma</td>
<td>↑ with ageing; ↑ with OA; ↑ with RA; ↑ with diabetes; ↓ with uremia</td>
<td>Chen et al., 1998 Miyata et al., 1998 Takahashi et al., 1997 Odetti et al., 1992</td>
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<tr>
<td>Hydroxyproline</td>
<td>Collagen II Breakdown</td>
<td>Urine</td>
<td>↓ with OA; ↓ with Osteoporosis; ↑ with skeletal injury;</td>
<td>Thomas et al., 1994</td>
</tr>
<tr>
<td><strong>NON-COLLAGENOUS PROTEINS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cartilage Oligomeric Matrix Protein (COMP)</td>
<td>Early destruction of superficial cartilage lamina &amp; / or anabolic cartilage response</td>
<td>Serum</td>
<td>Involved in cartilage repair; Present during chondrogenesis; ↓ with OA; ↑ with early RA; ↑ with traumatic knee injury</td>
<td>Kuhne et al., 1998 Lohmander et al., 1994</td>
</tr>
<tr>
<td>Cartilage Matrix Glycoprotein (CMG)</td>
<td>Cartilage Degradation</td>
<td>Serum</td>
<td>↑ with OA</td>
<td>Fife et al., 1989 Fife et al., 1988</td>
</tr>
<tr>
<td>Human Cartilage Glycoprotein-39 (HC gp-39, YLK-40, Chondrex)</td>
<td>Cartilage Degradation Joint Inflammation</td>
<td>Synovial Fluid; Serum</td>
<td>↓ with OA; ↓ with RA</td>
<td>Harvey et al., 1998 Johanson et al., 1996 Johansen et al., 1993</td>
</tr>
<tr>
<td>Osteonectin</td>
<td>Chondro-Differentiation</td>
<td>Synovial Fluid</td>
<td>↑ During growth; ↑ with RA</td>
<td>Nakamura et al., 1996</td>
</tr>
<tr>
<td>Fibronectin Intact Fibronectin Fragments</td>
<td>Wound Repair; Joint Inflammation</td>
<td>Synovial Fluid</td>
<td>↓ with OA; ↓ with RA</td>
<td>Homandberg et al., 1998</td>
</tr>
</tbody>
</table>
Elevated levels of cartilage matrix glycoprotein (CMG) (Fife et al., 1994; Fife, 1988) and chondronectin (Carsons and Horn, 1988) in the synovial fluid of OA patients have been reported (table 1.6). However, serum levels of CMG were inconsistently elevated in the patients with trauma-related arthropathies and did not correlate with the severity of arthroscopic or radiological lesions (Fife et al., 1991). Cartilage oligomeric matrix protein (COMP) has been documented as a marker of cartilage turnover and degeneration and levels were found to be increased in the serum and synovial fluid of OA patients (Kuhne et al., 1998; Lohmander et al., 1994). However, in a study of 37 patients with hip OA, the COMP levels did not show any correlation with the radiographic findings. Levels of collagenase and stromelysin showed higher values in synovial fluid than in serum (Manicourt et al., 1994; Ishiguro et al., 1996; Martel-Pelletier et al., 1994; Maeda et al., 1995).

Harvey et al. (1998) reported that Chondrex level (assay using a commercially available kit) increased in serum and synovial fluid of patients with both degenerative and inflammatory joint disease. These findings suggest that Chondrex may reflect aspects of joint destruction in addition to inflammation. Chondrex concentrations were about 2.5-fold greater in the serum of patients with inflammatory or degenerative joint disease compared to healthy adults. In synovial fluids Chondrex concentrations were 10 to 15-fold higher than in serum suggesting that in the patients with joint disease, most of the Chondrex found in the serum may be produced in the joint. A decreased Chondrex value from baseline levels in patients treated with disease-modifying anti-rheumatic drug therapy was found to reflect the clinical improvement observed in responders, whereas the value was maintained or increased in nonresponders. Although the Chondrex
concentration was similar in women and men, a decreased Chondrex value was observed with ageing in women but not in men.

Another cartilage marker of considerable interest is the carboxyl-terminal type II procollagen peptide (pCOL II-C). Its level in synovial fluid is elevated in the patients with OA and traumatic arthritis (Shinmei et al, 1993). Since type II collagen is essentially unique to cartilage, pCOL II-C levels in synovial fluid could reflect the synthetic activity of type II collagen of chondrocytes in the diseased joint. Osteonectin (ON), an abundant extracellular protein particularly in the ECM of hypertrophic chondrocyte lamina, is classified as a marker protein in chondrodifferentiation (Haas and Holock, 1996; Nakamura et al, 1996).

Phospholipase A2 (P A2) plays a key role in membrane phospholipid degradation by initiating a cascade of events leading to the production of proinflammatory prostaglandins (Bomalaski et al, 1991). It is produced in large amounts by both cartilage and synovial membrane, and increased serum P A2 levels have been reported both in OA and RA (Pruzanski et al, 1991). Pro-inflammatory cytokines, Il-1, Il-6 and TNFα are produced in the synovium and/or cartilage and are released into the circulation (table 1.7). Levels of these cytokines in the synovial fluid or serum reflect disease activity and/or process (Webb et al, 1998; Kutukculer et al, 1998; Kubota et al, 1998). Serum levels of metalloproteinases, e.g. collagenase and stromelysin, and tissue inhibitors of matrix metalloproteinases type 1 (TIMP) involved in ECM degradation are reported to increase with disease activity.
Table 1.7  Cartilage Biochemical Markers: Cytokines, Matrix Metalloproteinase, Matrix Metalloproteinase Inhibitors, Enzymes, and Growth Factors Detected in Body Fluid(s).

<table>
<thead>
<tr>
<th>CARTILAGE MARKER</th>
<th>MARKER REFLECTS</th>
<th>BODY FLUID</th>
<th>MARKER LEVEL WITH JOINT DISEASE(S)</th>
<th>REFERENCE(S)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MATRIX METALLOPROTEINASE AND ITS INHIBITORS CYTOKINES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromelysin (MMP-3)</td>
<td>Cartilage Destruction</td>
<td>Synovial Fluid; Serum</td>
<td>↑ with mild OA; ↑ with RA</td>
<td>Panula et al., 1998; Ishiguru et al., 1996</td>
</tr>
<tr>
<td>Collagenase (MMP-1)</td>
<td>Cartilage Destruction (Collagen)</td>
<td>Synovial Fluid; Serum</td>
<td>↑ with OA; ↑ with chondromalacia patella</td>
<td>Vaatainen et al, 1998; Maeda et al., 1995</td>
</tr>
<tr>
<td>Tissue Inhibitor of Metalloproteinases Type 1 (TIMP)</td>
<td>Cartilage Repair</td>
<td>Synovial Fluid; Serum</td>
<td>↑ with mild OA; ↑ with chondromalacia patella</td>
<td>Panula et al., 1998</td>
</tr>
<tr>
<td><strong>CYTOKINES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interleukin 1</td>
<td>Cartilage Destruction, Joint Inflammation</td>
<td>Synovial Fluid; Serum; Plasma</td>
<td>↑ with mild OA; ↑ with RA</td>
<td>Webb et al., 1998; Kutukcüler et al., 1998; Kubota et al., 1998</td>
</tr>
<tr>
<td>Interleukin 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ENZYMES AND GROWTH FACTORS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipase A2</td>
<td>Membrane Phospholipid Degradation</td>
<td>Serum</td>
<td>↑ with OA; ↑ with RA; ↑ with chondromalacia patella</td>
<td>Vaatainen et al, 1998; Pruzanski et al., 1991</td>
</tr>
<tr>
<td>Transforming Growth Factor-Beta (TGF-β)</td>
<td>Joint Inflammation</td>
<td>Synovial Fluid; Serum</td>
<td>↑ with OA; ↑ with RA</td>
<td>Cameron et al., 1994</td>
</tr>
<tr>
<td>Insulin Like Growth Factor-1 (IGF-β1)</td>
<td>Cartilage Repair</td>
<td>Synovial Fluid; Serum</td>
<td>↑ with OA</td>
<td>Schneiderman et al., 1995</td>
</tr>
<tr>
<td>Tumor Necrosis Factor Alpha (TNF-α)</td>
<td>Joint Inflammation</td>
<td>Synovial Fluid; Serum</td>
<td>↑ with RA</td>
<td>Webb et al., 1998; Kutukcüler et al., 1998</td>
</tr>
</tbody>
</table>
1.6.3 Biochemical Markers in Animal Studies

In a rat model of antigen-induced arthritis, elevated levels of PG, GAG, and KS have been found to correspond with the initial state of acute inflammatory response. Towards the late stage of the disease the levels of PG and its components decline to levels comparable to the normal levels in the synovial fluids. This decline could be related to the decreased disease activity. Mehraban and Moskowitz (1989) used a rabbit OA model that was generated by partial menisectomy and reported that no significant changes were detectable in the serum KS epitope level. On the other hand, Leipold et al (1989) used a canine model of OA to study serum KS and HA. The intra-articular injection of chymopapain, a cartilage degrading enzyme, dramatically increased the serum KS epitope levels within 24 hours after injection. Further, when the levels of KS epitope and HA were investigated in a strain of Labrador retrievers known to develop spontaneous OA, no significant differences either in serum KS epitope or HA concentrations were noted. In a canine model of OA generated by transection of the anterior cruciate ligament, Brandt and Thonar (1989) reported that throughout the experimental period, which lasted until 14 weeks after surgery, the serum KS epitope level remained in the normal range. The levels varied considerably between dogs, indicating intra-species difference. However, examination of serial samples from the same dog (6 of 9 dogs) studied showed an increase of at least 10% in serum KS epitope concentrations. Recently, Frisbie et al (1999) investigated the serum and synovial fluid concentrations of chondroitin sulphate epitope 846, carboxyl terminal type II procollagen peptides (pCOL II-C) and keratan sulphate as biochemical markers for the diagnosis of
osteochondral fragmentation in horses. They reported an increase in the serum epitope 846 and pCOL II-C concentrations (27 of 34 i.e., 79%) and associated them with increased synthesis of cartilage aggrecan. An increase in the synovial fluid level of epitope 846 but not of pCOL II-C or keratan sulphate was also noted.

Differences in the relative amounts of Pyd and Dpyd are believed to be indicative of different forms or stages of arthritic disease involving various degrees of bone and cartilage degradation. Animal studies have also shown a positive correlation between the increase in the urinary collagen crosslink level and the extent of cartilage and subchondral bone lesions suggesting crosslink measurements as a means to assess OA activity (Tordjman et al. 1994; Scott et al, 1993; 't Hart et al, 1998; Seibel et al, 1989).

1.6.4 Limitations of Cartilage Biochemical Markers

Identification of appropriate biological markers for disease activity in OA or other types of arthritis is a challenging and complex endeavour. To date several markers have been used in clinical and animal studies either by itself or in combination. However, only a few studies specify the stage (severity level) of the disease. Lack of the specification of the stage of disease could result in the misinterpretation of data because the level of joint markers could increase in one stage and decrease in another stage of the disease.

Several PG cleavage products and enzyme activities (e.g. collagenase, stromelysin) were shown to reflect formation as well as degradation of cartilage. Clinical studies have demonstrated that the concentration of KS epitope and HA in serum are on average higher in OA patients than in normal group (Thonar et al, 1987; Sweet et al,
The increased level of KS was associated with cartilage destruction or response to acute injury. However, the overlap between healthy and diseased individuals was almost complete (Ratcliffe and Seibel, 1990). The large range of normal values and small, if any, changes with disease combine to make a single observation of little diagnostic use. For example, the values in the upper range of normal may reflect high metabolic turnover or could actually reflect degenerative joint disease. Substantial inter-individual variability was observed in the increase of KS level, which was consistently higher in the OA patients. KS is also present in aorta and cornea; therefore it is not a cartilage specific molecule. Further, increased levels of serum KS did not reflect the cartilage histological changes. Some animal studies also indicate that serum KS is not a reliable marker for the activity of OA (Spector et al, 1992). Although HA has been documented amongst the best candidates as markers for cartilage metabolism, HA is more a marker of synovial membrane hyperplasia and hyperactivity rather than that of cartilage per se. Thus, determining the concentrations of serum KS epitope and HA as reliable markers for the diagnostic test for OA cartilage damage seems to be presently of limited value.

COMP detected in the superficial cartilage lamina is released only in the process of chondrolysis. In OA patients, COMP is modified only in the presence of substantial and sustained local overproduction. Also, neither plasma nor serum levels of CMG have been found to reflect the extent of cartilage degradation. Although an increased Chondrex (YLK-40) level in the synovial fluid and serum of patients with OA has been reported, suggesting that Chondrex may be a useful new marker for assessing cartilage degradation in joint diseases, it is not cartilage specific. Chondrex has been identified in
human synoviofibroblasts and Chondrex mRNA is expressed strongly in chondrocytes and liver and it is weakly expressed in brain, kidney and placenta, and in small amounts in heart, lungs, skeletal muscle, pancreas, mononuclear cells, and skin fibroblasts.

Sinigaglia et al (1991) have assessed the urinary and synovial concentrations of Pyd crosslinks in RA and OA patients. Urinary concentrations of both Pyd and Dpyd were significantly greater in RA than in OA patients. The synovial fluid from both groups showed only relatively small amounts of Pyd. This is indicative of either a flaw in the experimental design or in the tissue processing or alternatively, it could support the hypothesis of an extraskeletal origin of Pyd in chronic joint diseases. Although pyridinium crosslinks have been extensively used as markers of bone resorption, inconsistency in the published results questions their utility as bone specific resorption markers. Crosslink levels in knee joint articular cartilage of partially meniscectomized rabbits were compared with those occurring during ageing. Both pyridinium crosslinks and pentosidine were assayed on reverse phase HPLC. The total Pyd content did not change with age or OA; a result which does not corroborate the previous findings. The total pentosidine concentration, as expected, increased significantly with age but remained constant with OA (Odetti et al, 1992). Although the Pyd/Dpyd ratio is used as a marker to distinguish between destruction of cartilage and bone collagen, a recent report by Hein et al (1997) questioned the usefulness of Pyd/Dpyd ratio in urine. Using reverse phase HPLC they examined the levels of both Pyd and Dpyd in the urine and serum levels of 38 RA patients. A correlation between serum and urine concentrations was demonstrable for Pyd, but not for Dpyd. Since bone metabolism is at a higher rate than the cartilage metabolism, crosslink levels from urine or serum samples reflect bone
metabolism rather than cartilage. Further, a great variability with strong, weak or no correlation between the urinary crosslinks and the clinical activity (MacDonald et al. 1994) have been reported. Pyd and Deoxy are products of collagen turnover from bone, cartilage, tendon and ligament. It is difficult to distinguish between Pyd and Deoxy of type I collagen, which is mostly bone derived and type II collagen which is cartilage specific. Pyd levels but not the Dpyd were significantly elevated in the patients with active inflammatory disease and strongly correlated with the inflammatory activity of the disease. An accurate quantitative marker of bone and cartilage breakdown should be used as a tool for monitoring disease activity in OA, RA and possibly in other joint diseases.

A biochemical marker, which primarily reflects cartilage metabolism, would be useful for assessing the stage of OA and in evaluating new therapeutic regimens. An ideal cartilage biochemical marker should be sensitive to change in cartilage structure and/or biochemistry and would reflect disease progression over time. To obtain accurate and reliable results, levels of the marker should be correlated with the severity of the joint disease. Tissue sample or tissue fluid sample processing plays a crucial role in the result outcome. For example, exhaustive analytical preparation of urine samples could lead to substantial loss of the marker hence inconsistency in the results.

An ideal set of biologic markers would distinguish and measure arthritis activity and progression. To date, an ideal marker for cartilage metabolism and destruction is still not available. Currently available biochemical markers for the detection of degradation processes of cartilage are largely unspecific. Further studies should be directed towards defining the biological and pathological profiles that are capable of distinguishing
cartilage lesions from bone and synovium. Also these studies should be able to separate cartilage catabolic from anabolic activity as well as determine and/or monitor the extent and stage of the cartilagenous lesions.
CHAPTER 2  HYPOTHESES, RATIONALE, AND AIM OF CARTILAGE MARKER-1 PROJECT

2.1 INTRODUCTION

The original focus of the thesis was to investigate the changes in cartilage collagen architecture, and biochemical composition with respect to cartilage maturation, ageing, and degradation. In particular, I was interested in investigating changes in the concentration of the cartilage collagen crosslink, pyridinoline, associated with maturation and osteoarthritis. Osteoarthritis (OA) is a degenerative joint disease that involves slow progressive loss of articular cartilage. The functional characteristics of the chondrocytes of OA cartilage are altered. Biochemical, pathological and histochemical analysis has shown that OA is associated with altered biosynthetic activity of chondrocytes, with changes in cartilage composition, and molecular organization (Hunziker, 1992). This eventually results in cartilage erosion and inadequate tissue repair. The OA cartilage collagen fibre diameter and orientation show considerable variation from normal. Also, an increase in type I and decrease in type II collagen as well as higher concentration of type VI (Pullig et al, 1999) and type X collagen have been reported (von der Mark et al, 1995).

Crosslinks that stabilize the collagen fibrils in connective tissues have been used as markers for tissue destruction. Pyridinoline (Pyd) and deoxypyridinoline (Dpyd) are examples of collagen crosslinks that are formed enzymatically, whereas pentosidine is a non-enzymatically formed crosslink. Elevated levels of pyridinoline in synovial fluid and
urine have been reported in OA patients (Robins et al., 1986). It is important to detect the early stage of a disease so that therapeutic modulations can be implemented either to halt or slow the progression of disease. Therefore, I initially attempted to investigate the changes in collagen crosslink concentration both in ex vivo and in vitro experimental systems. While establishing the chromatographic procedure to quantitate pyridinoline crosslinks in hydrochloric acid hydrolyzed tissue samples (cartilage, ligament, tendon, meniscus and bone), I observed a novel fluorescent peak that was present in articular cartilage samples but not in other dense connective tissues. This peak eluted approximately fifteen minutes after the known pyridinolinium peaks. An extensive literature search did not reveal any documentation of this peak. As the compound, "Cartilage Marker-1 (CM-1)", appeared to have considerable potential as a marker of cartilage metabolism, its isolation and characterization became the focus of the thesis.

2.2 HYPOTHESIS

The fluorescent compound, Cartilage Marker-1, is found exclusively in articular cartilage and can be used as a measure of cartilage metabolism in normal and diseased states.

2.3 RATIONALE

While the fluorophores pyridinoline and pentosidine (Robins et al., 1986; Black et al., 1989; Takahashi et al., 1994 and 1997) are accepted as biomarkers of degradation in
bone and connective tissue, respectively, they are not unique to articular cartilage. Therefore, their measurement is not helpful in assessing cartilage degeneration in OA or RA. From preliminary chromatographic analysis of crosslinks in cartilage and other dense connective tissues (such as bone, ligament, meniscus, etc.) an unknown fluorescent peak was found in addition to the pyridinium peaks in articular cartilage hydrolysates. This unknown peak was consistently either absent or below the detection limit in samples from tissues. The uniqueness of CM-1 in cartilage samples prompted us to investigate the likelihood that CM-1 could be of scientific and clinical significance in arthritis. Therefore, the identification of CM-1 and its characterization in normal and OA cartilage became the focus of my thesis.

2.4 GENERAL OBJECTIVES

The general objective of this thesis was to isolate and characterize CM-1 and assess its suitability as a biochemical marker of cartilage metabolism.

2.4.1 Analytical Objective

The analytical aim of the thesis was to determine the molecular structure of CM-1.

2.4.2 Biological Objective

The biological aim of this project was to elucidate the potential of CM-1 as a cartilage specific biochemical marker.
2.5 SPECIFIC AIMS

The specific aims of this project are classified as follows:

2.5.1 Analytical Aims

1. To purify CM-1 so that its full characterization could be done in terms of its physical and chemical properties.
2. To determine the molecular structure of CM-1.
3. To develop the method for the preparation of a CM-1 calibrator which will enable the analytical quantification of CM-1.

2.5.2 Biological Aims

1. To examine the validity of CM-1 as a cartilage specific molecule by investigating the presence of, and quantifying CM-1 in various tissues from different species.
2. To identify the cartilage extracellular matrix macromolecule (collagen or proteoglycan) associated with elution of CM-1. This could subsequently enable us to identify the precursor molecule of CM-1 and to understand the mechanism by which CM-1 is formed.
3. To develop and optimize the assay for quantifying CM-1 in tissues.
4. To investigate the presence of, and quantify CM-1 in the cartilagenous matrix generated by chondrocytes in culture.
CHAPTER 3  PURIFICATION OF CARTILAGE MARKER-1

3.1  INTRODUCTION

The original focus of this thesis was to quantitate and investigate the changes in collagen crosslink levels with respect to maturation, ageing, and the progression of OA. While establishing the protocol (Eyre et al. 1988) in our laboratory, the analysis of collagen crosslinks (pyridinoline and deoxypyridinoline) also revealed a novel fluorescent peak in addition to the crosslinks. This peak (Cartilage Marker-1, CM-1) appeared to be unique to cartilage-containing tissues. Therefore, it was thought that CM-1 could be a useful indication of cartilage metabolism associated with normal growth, ageing and in diseases involving cartilage matrix degradation such as osteoarthritis. The isolation and characterization of this novel compound, CM-1, then became the focus of my thesis.

High pressure liquid chromatography (HPLC) is one of the bulk preparatory and analytical methods by which unknown compound(s) are isolated and purified. Prior to identifying the molecular structure of CM-1 by using various spectroscopic tools, it was necessary to develop a method to purify CM-1 from articular cartilage. The purpose of the work outlined in this chapter was to develop a method to isolate and purify CM-1 from cartilage and to prepare CM-1 calibrators so that CM-1 can be analytically quantified in various tissues.
3.2 MATERIALS AND METHODS

3.2.1 Experimental Animals

Fully encapsulated bovine metaphalangeal joints (n=350, age < two yr.) were obtained from the abattoir (Ryding Regency, Toronto), and dissected within 24 hours of death. Most of the excised articular cartilage was used for cartilage culture experiments and the remaining cartilage was used for the purification of CM-1, or for the preparation of a CM-1 calibrator. The excised articular cartilage was freeze-dried (at least 24 hours) and stored at -70°C until the day of experiment.

3.2.2 Cartilage Hydrolysis and Preparation

Eight to ten grams of lyophilized cartilage flakes were placed in a 500 ml Pyrex bottle and hydrolysed in a sand bath with 320-400 ml (1 mg: 40 μl) of 6M HCl for 24 hours at an average temperature of 110°C. The hydrolyzed cartilage was cooled and evaporated in a round Pyrex flask (Fischer Scientific) using a rotoevaporator. The dried hydrolysate was resuspended in a total volume of 25 ml of 50% HPLC grade methanol (Caledon Laboratories) and 50% of HPLC grade water (Omnisolve from Fischer Scientific). After rigorous vortexing, the black slurry was filtered through a sterilized 0.22 μm Gelman filter and aliquoted into 2 ml Hewlett Packard HPLC injection vials. A total of 116 gm of dried cartilage was used to isolate and purify CM-1 for chemical analysis.
3.2.3 High Pressure Liquid Chromatography Purification of CM-1

The CM-1 separation method involved a total of three cycles of sample injection-peak collection-lyophilization-resuspension stages. The HPLC system consisted of Hewlett Packard HPLC pumps (model 1050), a Hewlett Packard automatic injector (series 1100), a Hewlett Packard Diode Array absorbance detector (model 1050) and a Perkin Elmer fluorescent detector (model LC 240). The following solvents were used: Hydrochloric acid (Sigma), HPLC grade water (Omnisolve from Fischer Scientific), HPLC grade methanol (Caledon Laboratories), and HPLC grade acetonitrile (Caledon Laboratories). CM-1 was HPLC purified using three steps with either a different mobile phase or different concentrations of the mobile phase. For each step, CM-1 was collected manually and care was taken to discard the tails of the CM-1 peak to minimize any possible co-eluting and contaminating compound.

3.2.3.1 Purification of CM-1 - STEP 1 (30% Acetonitrile)

One ml of the filtered cartilage hydrolysate was injected onto the HPLC semi-preparatory Phenomenex Sphereclone C18 column (dimension of 250 X 10 mm, pore size of 5 µm, ODS of 2). The mobile phase comprised of 30% acetonitrile in 70% distilled water, flow rate was 4 ml/min and back pressure was about 170-190 bars. The fluorescence was set at excitation \( \lambda = 306 \text{ nm} \) and emission \( \lambda = 395 \text{ nm} \). The eluant fractions corresponding to CM-1 peak were manually collected from all the sample injections and the fractions were pooled, evaporated (Fraction A), and resuspended in 50% methanol/50% water solution.
3.2.3.2  Purification of CM-1 - STEP 2 (20% Acetonitrile)

The lyophilized fraction A of CM-1 was resuspended in 5 ml of 50% methanol in distilled water and an aliquot of 1 ml CM-1 solution was injected into the HPLC system using a new sphereclone semipreparatory C18 column (dimension of 250 X 10 mm, pore size of 5 μm, ODS of 2). 20% acetonitrile was used as a mobile phase to separate any compound co-eluting with CM-1. The flow rate was 4 ml/min and the pressure was 190 bar. The CM-1 peak eluant was manually collected and the tails of the peak were discarded. The CM-1 fractions collected from all the injections were pooled and evaporated (Fraction B).

3.2.3.3  Final Purification of CM-1 - STEP 3 (50% Methanol)

The lyophilized fraction B (from purification step 2) was resuspended in 4 ml of 50% methanol in distilled water and 2 ml of CM-1 in solution was injected into the HPLC system (C18 column dimension of 250 X 4.6 mm, pore size of 5 μm, ODS of 2). A mobile phase comprising of 50% methanol/50% distilled water was used. The flow rate was 1 ml/min and pressure was 190 bar. The CM-1 peak eluant was manually collected. The tails of the peak were discarded. The CM-1 fractions collected from all the injections were pooled and lyophilized (Fraction C). The flask containing the purified CM-1 was washed six times with 1 ml of 100% deuterated methanol. The wash containing CM-1 was transferred into a pre-weighed test tube and dried with nitrogen.

The control sample (700 ml of 6M HCl) was subjected to similar experimental protocol and conditions as the CM-1. The control sample was hydrolyzed at a temperature of 110°C for 24 hours in the sand bath, lyophilized and resuspended in 15 ml
of 50% methanol in HPLC grade water. The control was filtered and injected onto the HPLC system with a new semi-preparatory sphereclone C18 column. The eluant was manually collected from retention time of 19 min for a period of 3 minutes, lyophilized, and resuspended in 50% methanol and 50% water and the control sample was then processed for the purification steps 2 and 3 (sections 3.2.3.2 and 3.2.3.3).

### 3.2.4 Preparation of Purified Cartilage Marker-1 for NMR Studies

Purified CM-1 (1.4 mg) was resuspended in 80 μl of 100% deuterated methanol, briefly vortexed and placed in a 3 mm inner diameter tube (Nerelac Glass Ware) which was especially used to acquire data by nuclear magnetic resonance (NMR) spectroscopy. The above procedure was repeated twice making a total volume of 240 μl. The transfer of deuterated methanol was done quickly and carefully to prevent any proton exchange with the environment which could interfere with the NMR spectroscopy data acquisition. The mass spectrometry and NMR spectroscopy (described in Chapter 4) was done on CM-1 in deuterated methanol solution and also the control in similar solution. The NMR tube containing CM-1 in solution (5.7 μg/μl) was then placed in the freezer at -90°C.

### 3.2.5 Preparation of a Calibrator for Quantifying Cartilage Marker-1

A calibrator for CM-1 quantitation was prepared from 16.62 gm dried calf metaphalangeal articular cartilage. The CM-1 was isolated and purified using the protocol described for CM-1 purification (section 3.2.3). The purified CM-1 powder was then dissolved in 10 ml of 50% methanol/50% water. The UV absorbance was measured at maximum 306 nm wavelength. For analytical purposes on HPLC, the pyridinoline, deoxypyridinoline, and CM-1 standards were combined to give one external calibrator.
3.3 RESULTS

3.3.1 Purification of CM-1

A total of 1.4 mg of CM-1 was purified from 116 gm of dried calf metacarpophalangeal articular cartilage (12 µg or 58 nmols of CM-1/gm of dried articular cartilage.

During the first purification step, CM-1 eluted at retention time (RT) of 19 min (figure 3.1 and figure 3.2). The fluorescence was monitored using an excitation wavelength of 306 nm which is the maximal UV absorbance for CM-1.

![HPLC Chromatograph of CM-1 Using a Fluorescence Detector During Purification Step 1.](image)

Figure 3.1 HPLC Chromatograph of CM-1 Using a Fluorescence Detector During Purification Step 1. Typical elution time of CM-1 was 19 minutes.
Typical elution time of CM-1 was 19 minutes. The control sample did not show any peak at RT 19 min (figure 3.3). This indicated that CM-1 is not a product of hydrochloric acid alone. The control sample corresponding to the RT of 19 min was manually collected for a period of 3 minutes.

The control sample consisted of 6M HCl (without cartilage) which was subjected to similar experimental protocol as the CM-1 during purification step 1. Note the absence of a peak at RT of 19 minutes.
During the second purification step using 20% acetonitrile, the retention time of the CM-1 peak was 41 min (figure 3.4 and figure 3.5). Control fractions in purification step 2 were collected from RT 40 minute for seven minutes duration (figure 3.6).

**Figure 3.4** HPLC Chromatograph of CM-1 Using a Fluorescence Detector During Purification Step 2. The elution time of CM-1 is 41 minutes.

**Figure 3.5** HPLC Chromatograph of CM-1 Using a UV Absorbance Detector During Purification Step 2. Typical elution time of CM-1 was 41 minutes.
The control sample consisted of 6M HCl (without cartilage) which was subjected to similar experimental protocol as the CM-1 during purification step 2. Note the absence of a peak at retention time of 41 minutes.

During the third purification step using 50% methanol, CM-1 peak eluted at 16 minutes (figure 3.7 and figure 3.8). The control fractions from purification step 3 were collected for ten minutes duration from RT of 16 minutes (figure 3.9).
Figure 3.7  HPLC Chromatograph of CM-1 Using a Fluorescence Detector During Purification Step 3. Typical elution time of CM-1 was 15.5 minutes.

Figure 3.8  HPLC Chromatograph of CM-1 Using a UV Absorbance Detector During Purification Step 3. Typical elution time of CM-1 was 15.5 minutes.
Figure 3.9  HPLC Chromatograph of Control Sample Using a UV Absorbance Detector During Purification Step 3. Note the absence of a peak at RT of 15.5 minutes.
3.3.2 Crystallization of Purified Cartilage Marker-1

Pale yellow crystals of CM-1 formed within 8-10 days when the CM-1 solution (5.7 mg/ml) was kept at temperature of -9°C. The crystals reached their final size after several additional days at -20°C (figure 3.10). The supernatant was removed in order to analyze the crystal structure by single crystal x-ray diffraction (See chapter 4). As expected, no crystals were observed from the control sample kept at -9°C for a month.

A.

B.

C.

Figure 3.10 Photomicrographs of a CM-1 Crystal Observed Under Polarized Light Microscopy. A small piece was used for single crystal x-ray diffraction.
3.3.3 Preparation of a Calibrator for Quantifying Cartilage Marker-1

A total of 0.3 mg of CM-1 was purified giving a yield of 18 μg of CM-1/gm of dry weight of calf metaphalangeal articular cartilage. The measured UV absorbance at maximum 306 nm wavelength was 0.69 AU. The molar extinction coefficient of CM-1 in 50% methanol was 4700 M⁻¹.cm⁻¹, compared to 5700 M⁻¹.cm⁻¹ for pyridinoline and 5000 M⁻¹.cm⁻¹ for deoxypyridinoline. Since CM-1 is novel and there is no available reference material, one "unit" of CM-1 was arbitrarily defined as the quantity present within one ml of neutral pH water exhibiting a UV-maximum absorbance of 1 absorbance unit at 306 nm. Subsequent to this work the molecular weight of CM-1 was determined to be 204 (please see chapter 4). Therefore, one unit = 213 nmol. For analytical purposes on HPLC, the pyridinoline, deoxypyridinoline and CM-1 standards were combined to give one external calibrator (figure 3.11).

![Figure 3.11 A Typical Calibrator Used for Quantitative Analysis of Crosslinks and CM-1 in Various Tissues. The calibrator consisted of pyridinoline (Pyd), deoxypyridinoline (Dpyd) and Cartilage Marker-1 (CM-1).]
3.4 CONCLUSIONS

A methodology has been developed to isolate and purify CM-1. During bulk preparation of CM-1 a total of 1.4 mg of CM-1 was purified from 116 gm of dried calf metacarpophalangeal articular cartilage (12 μg or 58 nmol of CM-1/gm of dried articular cartilage). The CM-1 calibrator preparation yielded 18 μg of CM-1/gm of dried calf metaphalangeal articular cartilage. The difference in the CM-1 yield of the bulk preparation and calibrator preparation can be attributed to the discard of CM-1 in the anterior and posterior tail of the peak and slight loss of the CM-1 due to more exhaustive procedure used in the bulk preparation of CM-1. Also, when CM-1 was prepared in bulk, the conditions for optimized production of CM-1 was in progress. Therefore, the ratio of the amount of dried cartilage to the volume of HCl used for hydrolysis in the bulk preparation of CM-1 was not in its optimal state for quantification.

The CM-1 was crystallized in 100% methanol to yield 100% pure CM-1 (slightly yellowish crystal). The crystals of CM-1 were grown from a CM-1 solution of 5.7 mg/ml (pH=7) at -9°C for 8 to 10 days and then 3 to 5 days at -20°C. The chemical analysis to determine the molecular structure of CM-1 is presented in chapter 4. A control sample (6M HCl without the cartilage) was also prepared in the similar method as the CM-1. No crystals were formed when seeded for crystallization for up to a period of one month.
CHAPTER 4 IDENTIFICATION OF THE MOLECULAR STRUCTURE OF CARTILAGE MARKER-1

4.1 INTRODUCTION

A protocol was developed to isolate and purify CM-1 in solution (5.7 mg/ml) or crystal state (see Chapter 3). Having purified CM-1, the next logical step was to determine its molecular characteristics. In organic chemistry, spectroscopic techniques measure the different types of molecular motion, identifying specific functional groups and how they are connected. Hence, to determine the molecular structure, i.e., the chemical identity and atomic arrangement of CM-1, the following analytical techniques were used: mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and single crystal x-ray diffraction (Crystallography). These techniques were performed in various facilities of University of Toronto in collaboration with specific operators and the consultants as mentioned below in the respective sections.

Mass spectrometry is a microanalytical technique requiring only a few picomoles of sample to obtain characteristic information regarding the molecular weight and sometimes the structure of the analyte. In all cases, some form of energy is transferred to the analyte molecules to effect ionization resulting in the detection of the parent molecule which can further be subjected to fragmentation to detect the daughter fragments. Nuclear magnetic resonance spectroscopy is an analytical tool that requires at least 1 mg of sample in order to determine the molecular structure. NMR is most commonly used to study $^1$H nuclei (protons) and $^{13}$C nuclei. Although any atom possessing a nuclear spin
can be studied, NMR can be done only when certain nuclei in the sample have magnetic moment and is capable of magnetic spin when electrical energy is provided. Single-crystal x-ray crystallography is the most common means of obtaining a detailed picture of a molecule, allowing the resolution of individual atoms by interpreting the diffraction of x-rays from many identical molecules in an ordered array such as a crystal. Usually to resolve the identity of a given molecule, several spectroscopic tools are used to determine the molecular structure in solution (mass spectrometry and NMR spectroscopy) and in the solid crystal state (crystallography). Although the methods mentioned above have their strengths and weaknesses, they definitely coexist as complementary methods to resolve the molecular structure.

4.2 MATERIALS AND METHODS

4.2.1 Mass Spectrometry

Mass spectrometry of CM-1 was performed in two Mass Spectrometry laboratories: Mass Spectrometry Laboratory, Carbohydrate Research Centre (University of Toronto, Operator: Dr. Mary Cheung) and the Mass Spectrometry Laboratory, Chemistry Department (University of Toronto, Operator: Dr. Alex Young). Mass spectrometry experiments were done on 5 different preparations of CM-1. The control sample consisted of material obtained from the identical processing of an equal volume of 6M HCl (without cartilage) as was used in the preparation of CM-1. The conditions for preparing the control sample were the same as those used for the preparation of CM-1.
CM-1 was analyzed by Electrospray Ionization Mass Spectrometry (ESIMS) and Electron Impact Mass Spectrometry (EIMS).

4.2.1.1 Electrospray Ionization Mass Spectrometry (ESIMS)

The ESIMS experiments were carried out on the Perkin Elmer/Sciex (Concord, Ontario) API-III triple quadrupole mass spectrometer. The mobile phase consisted of a solution of 50% acetonitrile and 50% water, 1 mmol ammonium acetate, and 0.1% acetic acid. The mobile phase was pumped at a flow rate of 0.020 ml/min using an LKB Bromma (Sweden) HPLC pump. The voltage applied to the tip of the ion spray needle was 5.00 kvolts and the voltage applied to the orifice was 80 volts. One to 20 μl of the sample solution was injected into the mass spectrometer. The ESIMS method detects the parent ion in solution.

Another experiment, the Tandem Mass Spectrometry (MS/MS), gave information on the structure of the selected parent ion and its fragmented daughter ions. An aliquot of CM-1 in solution was injected and ionized on a needle probe i.e. where ions were formed. MS/MS scans were obtained by mass selecting a parent ion that was observed in the normal mass spectrometry scan into the second quadrupole. The pressure of the collision gas (argon) in the second quadrupole determined the degree of fragmentation, and was set such that the collision gas target (CGT) had a value of 200 to 250 bars.

4.2.1.2 Electron Impact Mass Spectrometry (EIMS)

For EIMS, a Micromass 70-250S (double focusing) Sector Mass Spectrometer operating at an accelerating voltage of 8 keV was employed. Approximately 0.5μl of
CM-1 in solution (5.71 mg/ml) was applied to the platinum coil of a Direct Chemical Ionization (DCI) probe tip, inserted into the source of the mass spectrometry and rapidly heated at 20 mA/s to desorb the sample as the mass spectrometer was scanned at a rate of ca 1 scan per second. The source temperature was 250°C and source pressure was 10⁻⁶ mbar.

To further confirm the structural analysis, high-resolution experiments were conducted on two separate preparations of CM-1 and at two different times. The instrument was operated at 10,000 resolution (10% valley) $2\Phi = 5.7$ PPM based on 27 measurements of the molecular ion of cholesterol.

### 4.2.2 Nuclear Magnetic Resonance Spectroscopy

To elucidate the chemical structure and atomic connectivity of CM-1 in solution the following NMR spectroscopic experiments were acquired: one-dimensional (1-D) proton ($^1$H) and carbon ($^{13}$C), and two-dimensional (2-D) Heteronuclear Multiple Quantum Correlation (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC) NMR spectroscopy. The NMR spectra of CM-1 were analyzed at the NMR laboratory in the Chemistry Department (University of Toronto, Operators: Dr. Patricia Aroca and Dr. Tim Burrow) using a 500 MHz Varian UNITY NMR spectrometer. Data were acquired prior to and post crystallization of the purified CM-1. A 3 mm closed NMR tube containing 1.4 mg of CM-1 in 240 µl of deuterated methanol (5.7 mg/ml) was placed in the 3 mm Nalorac direct microprobe for analysis. The experiments were run at a temperature of 40°C unless otherwise specified. Previous attempts to run CM-1 in a 5 mm direct detection probe at 500 MHz were unsuccessful due to the extremely low concentrations involved.
4.2.2.1 One-Dimensional NMR Spectroscopy

The proton ($^1$H) spectrum was recorded at room temperature using a single pulse experiment. The 1-D $^1$H spectrum was of sufficiently high digital resolution and signal to noise ratio that the spin-spin coupling was observable in the NMR spectrum. The spectrum consisted of 16 scans acquired using a 60° pulse and a 0.976s recycle delay. Another $^1$H NMR spectrum was repeated with a 20s repetition time in order to determine the extent of $T_1$ discrimination on the integration of the signals. The $^{13}$C NMR spectrum was recorded at room temperature using a single pulse experiment. It was obtained with 4711 scans, using a 90-degree pulse and a 50s recycle delay.

4.2.2.2 Two-Dimensional NMR Spectroscopy

Two-dimensional heteronuclear magnetic quantum correlation (HMQC) experiment detects the attached protons selectively (i.e., it correlates protons directly attached to each carbon nucleus). The two-dimensional heteronuclear multiple bond correlation (HMBC) experiment correlates heteronuclei e.g. carbon nuclei with protons not only through one-bond, but also through long range couplings. The HMBC enabled us to follow a CH$_n$-CH$_n$ network, even including quaternary carbon. The method of HMBC however does not provide the information to allow the assignment of carbons with no direct proton bonds. There is a sensitivity advantage in HMBC experiment in that it uses the equilibrium magnetization derived from the protons. Since this magnetization is proportional to the population difference of the proton energy levels (rather than the lower $^{13}$C population difference) a stronger NMR signal results per scan. Secondly, for a given magnetization the strength of the NMR signal increases with the
frequency of observation so that a larger signal is expected for protons compared to $^{13}$C nuclei because of the higher proton observation frequency.

The HMQC and HMBC experiments were recorded using the Nalorac 3 mm indirect microprobe. A 2-D decoupled HMQC spectrum was run in the phase sensitive mode, with a $^{13}$C spectral window of 20120 Hz and a $^1$H spectral window of 4620 Hz. The experiments were done at room temperature (20°C). A 1024 data point was acquired pre scan with a 1s recycle delay. The 2D NMR spectrum consisted of 70 time increments with 1024 scans per time increment (1D spectrum). During the acquisition GARP $^{13}$C decoupling was applied and a BIRD nulling long relaxation delay of 0.3s was used. The heteronuclear experiments were recorded essentially as described by Dayi et al (1996).

### 4.2.3 Single Crystal X-Ray Diffraction (Crystallography)

The crystal structure of CM-1 was analyzed in the Crystallography Laboratory, Chemistry Department, University of Toronto (Operator: Dr. Allan Lough). The molecular structure and atomic connectivity of CM-1 in a crystal phase was investigated using Single-Crystal X-ray Diffraction Analysis.

A pale yellow crystal was selected, mounted on a glass fibre and stabilized by embedding in epoxy. Single x-ray diffraction was observed from a crystal of volume 13.72 x $10^{-3}$ mm$^3$. Data were collected at room temperature on a Nonius KappaCCD diffractometer using graphite monochromated MoKα radiation (β = 0.71073Å). Three hundred and sixty frames of $1^\circ$ rotation in phi were exposed for 60 seconds each. The first 15 frames of data were used to index the crystal lattice. Indexing, integration, and
scaling were carried out using the DENZO package (Otwinowski and Minor, 1997). Lorentz and polarization corrections were carried out but no absorption correction was applied.

The structures were solved and refined using the SHELXTL\APC (Sheldrick, 1994) package. Refinement was by full-matrix least squares on F^2 using all data (negative intensities included). The weighting scheme was \( w = 1/[\Phi^2(\text{Fo}^2) + (0.0883\text{P})^2] \) where \( \text{P} = (\text{Fo}^2 + 2\text{Fe}^2)/3 \). Hydrogen atoms were included in calculated positions and treated as riding atoms.

4.3 RESULTS

4.3.1 Mass Spectrometry

4.3.1.1 Electrospray Ionization Mass Spectrometry

Data obtained from ESIMS experiment clearly showed a striking difference between the two collected fractions i.e. CM-1 versus control (figure 4.1 and figure 4.2). The \([(\text{H}_2\text{O})_4 \text{MeOH}]^+ \) fragment originates from the methanol:water solvent (50:50) in which the sample (CM-1 or control) was dissolved. The species of MW 204 appeared over a relatively narrow time range as the probe was heated and could be "separated" from the bulk of the impurity in the spectrum.
Figure 4.1 Mass Spectrum of CM-1 Obtained from the Ion Spray Mass Spectrometry. The peak at 205 m/z depicts the protonated CM-1.

Figure 4.2 Mass Spectrum of Control Sample Using Ion Spray Mass Spectrometry. Note the absence of peak corresponding to 205 m/z.
4.3.1.2 Electron Impact Mass Spectrometry (EIMS)

The 70 eV Electron Impact (EI) spectrum of CM-1 had the molecular ion of m/z 204.04 Daltons as the base peak and few abundant fragment ions, which suggested a low ionization potential, stable structure for the molecular ion, consistent with an aromatic structure. The molecular ion (m/z 204) decomposed through loss of m/z 29 (CHO) to afford m/z 175 (C_{10}H_{13}O_3); a loss of m/z 79 (C_5H_2O) to yield m/z 125 (C_6H_5O_3) and a fragment of m/z 80 (C_5H_4O). All elemental compositions were verified by high-resolution measurement (figure 4.3). The species of MW 204 appeared over a relatively narrow time range as the DCI probe was heated and thus could be "separated" from the bulk of the impurity spectra. High-resolution measurements on both the CM-1 preparations at two different times yielded values of 204.04 amu and 204.04 amu for the molecular ion. When these values were submitted for possible "fits" for C, H, O (0-4), N(0-2) and P(0-2), the possibility that emerged was C_{11}H_{8}O_{4}. This identification was also supported by examination of the isotopic distribution of the molecular ion. The fragment at 175.04 amu was identified using the above criteria as C_{10}H_{13}O_3 arising via CHO loss from the appropriate 204. The fragment at 125.02 amu was also identified as C_6H_5O_3 arising from the loss of C_5H_2O from the respective 204. The fragment at m/z 80.03 amu was identified as C_5H_4O. From an examination of the isotopic distribution of the molecular ion, the correct formula for CM-1 appeared to be C_{11}H_{8}O_{4}. From the data, the molecule was considered as consisting of two portions - 125 amu and 79 amu (which appeared as a protonated fragment at m/z 80). The 79 portion had an aldehyde function which was readily cleaved and had the formula C_5H_2O (C_5H_4O when protonated). The 125 portion had the formula C_6H_5O_3 and was thought to have a trihydroxy benzene structure.
Figure 4.3  Mass Spectrum of CM-1 Using Tandem Mass Spectrometry. The peak at 204 m/z depicts CM-1. The molecular ion (m/z 204) decomposed through loss of m/z 29 (CHO) to afford m/z 175 (C_{10}H_{17}O_{3}); a loss of m/z 79 (C_{6}H_{3}O) to yield m/z 125 (C_{6}H_{5}O_{3}) and a fragment of m/z 80 (C_{6}H_{4}O).
4.3.2 Nuclear Magnetic Resonance Spectroscopy

4.3.2.1 One-Dimensional NMR Spectroscopy

The $^1$H NMR spectrum showed two peaks of interest, one at 2.5 ppm (methyl singlet) and another at 6.6 ppm (olefinic singlet) (figure 4.4). All $^1$H chemical shifts were recorded with respect to trimethylsulfonyl (TMS). The singlet peaks indicate that these functional groups neighbour no other protons. The methanol and water proton peaks were the prominent peaks in the spectrum. The other weak resonances observed in the figure 4.4 originated from impurities.

![NMR Spectroscopy Diagram](image.png)

Figure 4.4 One-Dimensional Proton Nuclear Magnetic Resonance Spectra of CM-1 in Deuterated Methanol. Two proton environments were noted at 2.5 ppm and 6.6 ppm corresponding to the CM-1 Methyl and Methine protons respectively.
A typical $^{13}$C NMR spectrum of CM-1 showed six singlets. Taking into account the restraining condition that the molecular weight of this molecule is 204 Dalton (as determined by the MS data) (figure 4.5). It was concluded that there is a high degree of symmetry in the CM-1 molecule. This symmetry simplified the $^{13}$C NMR spectrum to six signals. The presence of only singlets indicated that all carbons attached to protons were isolated from each other. Peak assignment was performed in standard $^{13}$C chemical shifts (Wehrli, 1976). There were three resonances apparent in the aromatic region of the spectrum, 156 ppm, 158 ppm and 161 ppm with a relative ratio of 2:1:2. The other singlets were observed at 14.4 ppm (methyl carbon), 100.5 ppm (olefinic carbon) and 139 ppm. The assigned carbons are shown in figure 4.5.

![Diagram showing carbon chemical shifts in ppm](image)

**Figure 4.5 One-Dimensional Carbon Nuclear Magnetic Resonance Spectra of CM-1 in Deuterated Methanol.** Six carbon environments were noted at 139 ppm, 156 ppm, 158 ppm, and 161 ppm corresponding to the CM-1 molecule aromatic region of the spectrum. The carbon peaks at 14.4 ppm, and 100.5 ppm corresponds to the methyl and olefinic carbon respectively.
4.3.2.2 Two-Dimensional NMR Spectroscopy

The 2-D HMQC showed two carbon-proton attached peaks (figure 4.6). The proton singlet at 2.5 ppm (methyl singlet) correlated to the carbon at 14.4 ppm whereas the proton singlet at 6.6 ppm correlated to the carbon at 100.5 ppm. The HMBC experiment confirmed the presence of six $^{13}$C atoms as seen in 1-D $^{13}$C spectrum shown in figure 4.7.

![Figure 4.6 Two-Dimensional Heteronuclear Multiple Quantum Cohesion Nuclear Magnetic Resonance Spectra of CM-1 in Deuterated Methanol. This spectrum shows the presence of the two proton groups at 2.5 ppm and 6.6 ppm which are directly attached to the corresponding carbon atoms at 14.4 ppm and 100.5 ppm, respectively.](image-url)
Figure 4.7  Two-Dimensional Heteronuclear Multiple Bond Correlation Nuclear Magnetic Resonance Spectra of CM-1 in Deuterated Methanol. This spectrum shows the presence of the two proton groups at 2.5 ppm and 6.6 ppm and the carbon environments to which they are not directly attached i.e. 139 ppm, 156 ppm, 158 ppm, 161 ppm, 14.4 ppm, and 100.5 ppm.
4.3.3 Single Crystal X-Ray Diffraction (Crystallography)

4.3.3.1 Structure of Cartilage Marker-1 Molecules in 3-D Crystalline Phase

The crystals diffracted x-rays isotropically to at least 0.8 Å in resolution. The lattice parameters of the colourless crystals belong to the orthorhombic system. A summary of selected crystallographic data is given in table 4.1. From the systematic absences, space group Cmcm or Cmc2_1 was assigned to the pale yellow crystal of CM-1. The structure solution and refinement proved the space group to be the latter, with cell dimensions a = 12.87 (1) Å, b = 10.81 (1) Å, c = 13 (1) Å, alpha = 90 Å, beta = 90 Å and gamma = 90 Å (figure 4.8).

The crystal structure has two independent molecules that lie on mirror planes. The mirror plane bisects the molecules through atoms O1A, C1A and O2A for molecule A, and O1B, C1B and O2B for molecule B. The planar molecules stack in the c-direction and align almost parallel to the 0 1 0 plane of the crystal lattice. There is a slight rotation of approximately 6° for molecule A and 4° for molecule B, with respect to the 0 1 0 planes. This rotation (or tilt) was about the long axis of the molecule (figure 4.9). The molecules were rotated by 180° every one half translation of the unit cell, in the c-direction, as a virtue of the 2_1 screw axes. An intermolecular distance of 2.4Å between the C=O group and a methyl hydrogen may be a weak non-bonded interaction interaction but there were no other untoward intermolecular interactions. Weak Van der Waals forces hold the adjacent molecules together in the crystal.
Table 4.1  Summary of Crystal Data, Details of Intensity Collection and Least-Squares Refinement Parameters of C_{11}H_{18}O_{4}.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empirical Formula</td>
<td>C_{11}H_{18}O_{4}</td>
</tr>
<tr>
<td>Molecular Weight, M,</td>
<td>204.17</td>
</tr>
<tr>
<td>Crystal Size, mm</td>
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</tr>
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<td>Crystal Class</td>
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</tr>
<tr>
<td>Space Group</td>
<td>Cmc2_1</td>
</tr>
<tr>
<td>Temperature, K</td>
<td>123(1) K</td>
</tr>
<tr>
<td>Unit Cell Dimension at a, Å</td>
<td>12.87(1) Å</td>
</tr>
<tr>
<td>Unit Cell Dimension at b, Å</td>
<td>10.81(1) Å</td>
</tr>
<tr>
<td>Unit Cell Dimension at c, Å</td>
<td>12.97(1) Å</td>
</tr>
<tr>
<td>Volume, V as Å³</td>
<td>1804.6(3) Å³</td>
</tr>
<tr>
<td>Number of Molecules per Unit Cell, Z</td>
<td>8</td>
</tr>
<tr>
<td>Density, D\text{calc} g cm\text{⁻³}</td>
<td>1.50 g/cm\text{³}</td>
</tr>
<tr>
<td>Absorption Coefficient, (\mu(\text{MoKα})), cm\text{⁻¹}</td>
<td>1.16</td>
</tr>
<tr>
<td>Number of Electrons per Unit cell, F(000)</td>
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</tr>
<tr>
<td>(\theta) Range For Data Collected, °</td>
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</tr>
<tr>
<td>Independent Reflections</td>
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<td>Number of Observed Data [(</td>
<td>l</td>
</tr>
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<td>Final R Indices, (R_1, [</td>
<td>l</td>
</tr>
<tr>
<td>(wR_2) (all data)</td>
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</tr>
<tr>
<td>Goodness of Fit on (F^2)</td>
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<tr>
<td>Maximum Peak in Final (\Delta F) map, eÅ⁻³</td>
<td>0.26</td>
</tr>
</tbody>
</table>
Figure 4.8  Three-Dimensional Orientation of CM-1 Determined from Single Crystal X-Ray Diffraction. CM-1 belongs to Space Class of Orthorhombic and Space Group of Cmc21.

Figure 4.9  Three-Dimensional Orientation of CM-1 Determined from Single Crystal X-Ray Diffraction. CM-1 belongs to Space Class of Orthorhombic and Space Group of Cmc21.
4.3.3.2 Molecular Structure of Cartilage Marker-1

The most striking structural features of CM-1 were the symmetrical, rigid nature of the CM-1 and the stacking of the CM-1 molecules. CM-1 is a very stable aromatic molecule consisting of 11 carbon atoms, 8 hydrogen atoms and 4 oxygen atoms. Each molecule has an axis of symmetry (mirror plane) (figure 4.10). Each monomeric unit consists of a methylated furan molecule. The two-methylated furan molecules are arranged symmetrically on the opposite ends of the central pyrone ring. The chemically reactive site of CM-1 is located at the carbonyl-keto region of the central pyrone ring. The methyl group of furan could enable small side-chain movements of the CM-1 molecule. Each molecule is stacked back to back with the carbonyl group alternating between the two opposite sides of the molecule and showed uniformity between various stacks. The arrays of the CM-1 molecules appeared in an orderly fashion.

Figure 4.10 Molecular Structure of CM-1 (204 Dalton) Determined from Single Crystal X-Ray Diffraction. A CM-1 molecule has a total of 11 carbons, 8 hydrogens and 4 oxygens.
4.5 CONCLUSIONS

Both low resolution and high-resolution mass spectrometry showed that the parent peak of purified CM-1 molecule has a molecular weight of 204 and possible molecular formula of $C_{11}H_8O_4$. The $^{13}$C NMR spectrum clearly confirmed that both the methyl carbons (14.4 ppm) and methine carbon (100.5 ppm) are isolated from other protons. The $^{13}$C singlet at 100.5 ppm is an olefinic carbon with an attached proton and the $^{13}$C singlet at 161 ppm is assigned to the carbon in the aromatic ring directly connected to the methyl group. The $^{13}$C singlets at 156 ppm and 161 ppm correspond to the fully conjugated aromatic/olefinic carbons. The 158 ppm peak belongs to a carbonyl carbon in the aromatic ring with no attached protons. Assuming that the MS data is correct then the $^{13}$C NMR spectrum clearly showed that the molecule in question has a very high degree of symmetry. In addition, both $^1$H and $^{13}$C NMR spectroscopy confirm the fact that there are only two $^1$H environments present, explaining the simplicity of the uncoupled $^{13}$C NMR spectrum.

The results from the various analytical techniques used to identify CM-1 corroborate well, confirming the molecular structure of CM-1 and its atomic connectivity. The absence of CM-1 in the control sample clearly showed that CM-1 was not merely an artifact from 6M HCl and its container. Mass spectrometry data proposed a molecular weight of 204 for CM-1 and a possible molecular formula of $C_{11}H_8O_4$. NMR spectroscopy experiments clearly showed six unique carbon environments and two unique proton environments in CM-1. In conjunction with the MS data, the NMR
spectroscopy results indicated a high degree of symmetry in CM-1 and proposed the structure of CM-1 in solution. Since each half of CM-1 molecule is a mirror image of the other, therefore only six carbon atoms and 4 hydrogen atoms were assigned from the \(^1\)H and \(^{13}\)C NMR spectra. The single crystal x-ray diffraction of CM-1 conclusively provided the structure of CM-1 in the solid state, which agreed with the NMR data in the solution state. Thus, the solid state structure of CM-1 is also that of CM-1 in the solution state. The molecular structure of CM-1 that was provided by NMR spectroscopy in solution and x-ray crystallography in crystal state conclusively showed that there was no structural change upon crystallization of CM-1. The molecular structure of CM-1 outlined in this chapter can thus define this cartilage specific product as 2,6-dimethyl-difuro-8-pyrone (figure 4.11).

Figure 4.11  Molecular Structure of CM-1 (204 Dalton) with IUPAC Nomenclature of 2,6-dimethyl-difuro-8-pyrone.  Blue = carbon atoms (11); White = hydrogen atoms (8); red = oxygen atoms (4).
CHAPTER 5 OPTIMIZATION OF CARTILAGE MARKER-1 YIELD

5.1 INTRODUCTION

A calibrator for the analytical measurement of CM-1 was prepared (see Chapter 3) in order to determine the amount of CM-1 in various tissues. However, it was imperative to optimize the experimental conditions for quantifying CM-1 in tissue samples to obtain maximum yield of CM-1. Therefore, the purpose of the various experiments outlined in this chapter was to investigate and optimize the experimental conditions for quantifying CM-1 in tissue samples. The experiments outlined in this chapter address the questions pertaining to the optimal time of hydrolysis of articular cartilage, the stability of CM-1 with prolonged acid hydrolysis, the choice of hydrolyzing agent (acid versus base) to generate CM-1, the requirement of ion-pairing agent for the fluorescent detection of CM-1, the optimal setting for ultraviolet light wave absorption, and the stability of CM-1 at varying storage temperature.

A prerequisite of a successful compositional analysis of proteins and peptides is a complete hydrolysis with a quantitative recovery of the amino acid residues in the hydrolysate. However, no procedure completely hydrolyses proteins to constituent amino acids without partial loss of certain amino acid residues (Davidson, 1997). The peptide bonds linking the amino acids are first broken by hydrolysis. Since peptide bonds are stable at neutral pH, catalysis by acid or base is employed. Since HCl is a very strong proton donor, the conventional method of hydrolysis utilizes 6M HCl at 110°C in a
sealed evacuated tube (Weiss, 1998). Unfortunately, under these conditions, all of the tryptophan and cysteine, most of cystine, and some of the threonine and serine are destroyed. If metals are present, methionine and tyrosine are partially lost. Glutamine and asparagine are quantitatively deamidated to glutamate and aspartate. Mild acid hydrolysis cleaves the rare Asp-Pro bond, while strong acid hydrolysis cleaves all the peptide bonds. Base hydrolysis is commonly used to analyse tryptophan, tyrosine and phenylalanine. However, base catalysed hydrolysis destroys serine, threonine, arginine, and cysteine.

Hydrolysing a protein in acid for a single hydrolysis interval, normally 24 hours leads to inaccurate estimates of the amino acid composition of that protein due to an effect of the time of hydrolysis on peptide bond cleavage and amino acid degradation. The simultaneous yield and decay of amino acids during the hydrolysis of a protein was described by a compartmental model with parameters for the hydrolysis and loss rates specific to each amino acid in a protein. Darragh et al (1996) documented that most of the amino acids underwent some degree of loss during hydrolysis.

The terminology of pyranose and furanose ring structures is based on the fact that the stable ring structures of monosaccharides are similar to the ring structures of either pyran or furan. Ketoses may also show ring formation (e.g. D-fructofuranose or D-fructopyranose). In the case of glucose in solution, more than 99% is in the pyranose form and less than 1% is in the furanose form (figure 5.1 and figure 5.2). Deoxy sugars are those in which the hydroxyl groups attached to the ring structure has been replaced by a hydrogen atom. They are obtained on hydrolysis of certain substances that are important in biologic processes. An example is the deoxyribose occurring in nucleic
acids (DNA) and as a carbohydrate of glycoproteins, the L-fucose (figure 5.3).

Disaccharides are sugars composed of 2 monosaccharide residues united by a glycosidic linkage. The physiologically important disaccharides are maltose, sucrose, and lactose. Hydrolysis of sucrose yield a crude mixture called "invert sugar" because the strongly levorotatory fructose thus produced inverts the previous dextrorotatory action of the sucrose. Polysaccharides such as starch (form of α-glucosidic chain) yields only glucose on hydrolysis and Inulin (starch found in tubers and roots of dahlias, artichokes, and dandelions) yields fructose on hydrolysis. High temperature HCl has been documented for amino-sugar hydrolysis during the analysis of monosaccharide composition (Guttman, 1997). In strong acids the glycosidic linkages are hydrolysed and under oxidizing conditions, limited hydrolysis of the polysaccharide chains may occur (Porath, 1971).

Figure 5.1  Structure of Pyranose Form of Glycose
Figure 5.2  Structure of Furanose Form of Glucose.

Figure 5.3  Structure of L-Fucose.
5.2 MATERIALS AND METHODS

5.2.1 Hydrolysis of Articular Cartilage for Varying Time Points

Fully encapsulated bovine metacarpophalangeal joints (age < two yr.) were obtained from the abattoir. The joints were dissected within 24 hours of death. The articular cartilage was excised and lyophilized. Approximately 50 mg of dry weight cartilage per test tube were hydrolysed in an oven with 1000 µl of 6M HCl (Sigma Scientific) at 110°C for varying time points. Each time point had 3 to 5 replicates. The hydrolysate was lyophilized and dissolved in 1000 µl of mobile phase. The mobile phase comprised of 24% HPLC grade acetonitrile (Caledon), 1% HFBA (Sigma Scientific) and 75% distilled water. The hydrolysate was briefly vortexed and then filtered through a 0.45 µm pore Gelman filter. An aliquot of 15 µl hydrolysate was applied to the HPLC column (Phenomenex Sphereclone analytical C18 column with dimension of 250 X 4.6 mm, pore size of 5 µm, and ODS of 2). The flow rate was 1 ml/min and back pressure was about 170-190 bars. Elution of pyridinoline, deoxypyridinoline, and CM-1 were monitored using a fluorescence detector, which was set at an excitation of 295 nm and emission of 395 nm. The peaks of interest were quantitated by generating a calibration curve using the pooled Pyd/Dpyd HPLC calibrator (Metra Biosystems, Inc., Pyd = 7.5 ng/µl and Dpyd = 3.2 ng/µl) and the CM-1 calibrator (1 Unit = 213 nmol/ml) prepared in house.
5.2.2 Hydrolysis of Different Amounts of Articular Cartilage

Articular cartilage weighing 25, 50, 75 and 100 mg were hydrolyzed with 1000 μl of 6M HCl for 24 hrs at 110°C. Each concentration combination was repeated three times. An aliquot of 30 μl hydrolysate was applied to the HPLC analytical C18 column.

5.2.3 Hydrolysis of Purified Cartilage Marker-1 for 24 Hours

A 10 μl aliquot of purified CM-1 (5.7 μg/μl) was lyophilized. The lyophilized CM-1 was resuspended in 7 ml of 50% HPLC grade methanol. The UV absorption of CM-1 was measured at 306 nm with Shimatzu Spectrophotometer. Methanol was evaporated and CM-1 was hydrolysed for 24 hours in 500 μl of 6M HCl at 110°C. CM-1 was then lyophilized and resuspended in 7 ml of 50% HPLC grade methanol, and the UV absorption of CM-1 was measured. Methanol was evaporated and the samples were resuspended in 500 μl of the mobile phase and injected into the HPLC analytical column.

5.2.4 Hydrolysis of Purified Cartilage Marker-1 for Different Time Points

100 μl of CM-1 calibrator was lyophilized and resuspended in 3.0 ml of 50% methanol and UV absorbance was measured. From the above mentioned CM-1 solution, five aliquots of 500 μl each were rehydrolyzed with 1000 μl 6M HCl at 110°C for 3, 6, 12, 24 and 48 hrs. The hydrolyzed CM-1 was lyophilized and resuspended in 500 μl of 50% methanol and UV absorbance was measured. Methanol was then evaporated and each sample was resuspended in 500 μl of the mobile phase and injected into the HPLC analytical column. The results of hydrolysis of CM-1 were compared to the unhydrolyzed CM-1 calibrator which was taken as the baseline recovery of 100%.
5.2.5 Hydrolysis of Articular Cartilage with Varying Concentrations of NaOH/HCl

Dried articular cartilage samples (50 mg per test tube) were hydrolysed at 110°C with 1000 µl of different concentrations of HCl and NaOH for 6 hrs (3 replicates). Each hydrolysate was lyophilized and processed as mentioned in section 5.3.1.

5.2.6 Hydrolysis of Articular Cartilage with Different Acids and Bases

Articular cartilage samples, fifty mg of dry weight per test tube, were hydrolysed in an oven (110°C) with 1000 µl of 50% each of acetic acid, nitric acid, sulphuric acid or ammonium hydroxide for 24 hrs (3 replicates). Each hydrolysate was lyophilized and injected onto HPLC analytical column and analyzed for the presence of CM-1.

5.2.7 Effect of Mobile Phase pH on the Retention Time of Cartilage Marker-1

The established method for Pyd, Dpyd and CM-1 was followed using mobile phase (25% acetonitrile and 75% buffer comprised of 20 mM potassium phosphate monobasic and dibasic). The pH of mobile phase was adjusted from 1.0 to 5.0 by adding 0.1M HCl or 0.1M NaOH. The retention time of Pyd, Dpyd and CM-1 was noted.

5.2.8 Requirement of Heptafluorobutyric Acid for Production of Cartilage Marker-1

Hydrolyzed cartilage samples were injected (30 µl) onto the analytical column using mobile phase with and without 1% HFBA. The retention time and peak area of Pyd, Dpyd and CM-1 was noted.
5.2.9 Fluorescent and Ultraviolet Light Absorption of Cartilage Marker-1

The ultraviolet absorbance maximum of CM-1 was investigated in neutral, aqueous solution, in HCl (0.1M and 6M) and NaOH (0.1M and 10M) using a Shimatzu Spectrophotometer. The fluorescence of emission of CM-1 at different excitation wavelengths was investigated.

5.2.10 Solubility of Cartilage Marker-1 in Various Solvents

The ultraviolet absorbance maxima of CM-1 was investigated in methanol, distilled water, chloroform, hexane, ether and ethanol.

5.2.11 Storage of Cartilage Marker-1 at Room Temperature for Eight Months

The UV absorbance of CM-1 calibrator (30 \( \mu \text{g/ml} \) of 50\% methanol) was measured. An aliquot of CM-1 calibrator was kept at room temperature (21°C) for a period of nine months and another aliquot was kept in a storage box in a -70°C freezer for an equal length of time. The UV absorbance of CM-1 calibrator aliquot was measured after eight months of respective storage procedure.

5.3 RESULTS

5.3.1 Hydrolysis of Articular Cartilage for Varying Time Points

The CM-1 yield was maximal after 3 hrs of hydrolysis (240 ± 9 pmol/mg of cartilage), with no further increase thereafter. However, a slight decrease in CM-1 yield
to 195 ± 2 pmol/mg dried cartilage was observed after 48 hrs of hydrolysis. This indicates that prolonged hydrolysis of articular cartilage could result in degradation of CM-1. Thus, optimal time of hydrolysis of articular cartilage to get the best yield of CM-1 is 3 hrs (figure 5.4). The maximum yield of Pyd was after 24 hrs (1683 ± 63 pmol/mg of dried cartilage) of digestion corroborating previous reports (Eyre et al. 1988). Even after 48 hrs of cartilage hydrolysis, Pyd content (1603 ± 27 pmol/mg) did not significantly change. On the other hand, Dpyd showed a maximum yield after 15 hrs (171 ± 16 pmol/mg dried cartilage) and at 48 hrs its value was decreased to 125 ± 4 pmol/mg dried cartilage.
Figure 5.4  Hydrolysis of Articular Cartilage for Varying Time Points. Fifty mg of dried articular cartilage (AC) per test tube (3 to 5 replicates) were HCl hydrolyzed at 110°C for varying time points as described under the methods. Each sample was resuspended in mobile phase and injected into the HPLC analytical C18 column to quantitate the amount of (A) pyridinoline (Pyd), (B) deoxypyridinoline (Dpyd) and (C) cartilage marker-1 (CM-1). The graph of CM-1 (D) shows the results obtained from 5 minutes to 12 hours of HCl hydrolysis. The results are expressed as the mean ± SEM of the replicates.
5.3.2 Hydrolysis of Different Amounts of Articular Cartilage

The yield of CM-1 per mg of dried cartilage (131 ± 9 pmol/mg) was significantly less (~ 50%) when 25 mg of cartilage was hydrolyzed with 1000 μl 6M HCl compared to the CM-1 yield (243 to 256 pmol/mg) when 50 to 100 mg of cartilage was hydrolyzed under similar condition (figure 5.5). These data shows that the ratio of tissue dry weight to the volume of hydrolysing agent (6M HCl) could play an important role in determining the yield of CM-1. The optimal ratio of tissue dry weight to volume of HCl is 1 (mg): 20 (μl). At this ratio the yield of CM-1 is maximal. It is possible that at this ratio there is equilibrium between CM-1 formation and CM-1 degradation. However, when the volume of HCl is in excess, the equilibrium between CM-1 formation and CM-1 degradation appears to shift such that the yield of CM-1 is decreased.

5.3.3 Hydrolysis of Purified Cartilage Marker-1 for 24 Hours

The typical UV absorption spectrum of CM-1 consisted of a larger peak at 306 nm with UV absorption value (AU) of 1.88 and a smaller peak at 278 nm of 1.00 (figure 5.6). However, after treatment of purified CM-1 with 500 μl 6M HCl, the typical UV absorption profile of CM-1 was altered into a profile with three peaks (figure 5.7). Upon hydrolysis of CM-1 with 6M HCl for 24 hours, the UV absorption at 306 nm decreased to 0.47 AU (decrease by 75%) and that at 276 nm decreased to 0.49 AU (decrease by 51%) as shown in table 5.1.
Figure 5.5 Hydrolysis of Different Amounts of Articular Cartilage. Varying amounts of dried articular cartilage (AC) (3 replicates) were HCl hydrolyzed at 110°C for 24 hours as described under the methods. Each sample was resuspended in mobile phase and injected into the HPLC analytical C18 column to quantitate the amount of cartilage marker-1 (CM-1). The above graphs show (A) the total yield of CM-1 and (B) yield of CM-1 per mg of dried articular cartilage. The results are expressed as the mean ± SEM of the replicates.
Figure 5.6  Ultraviolet Absorbance of CM-1 Prior to Hydrolysis with 6M HCl at 110°C for 24 hours.

Figure 5.7  Ultraviolet Absorbance of CM-1 after Hydrolysis with 6M HCl at 110°C for 24 hours.
Table 5.1 Pre- and Post Hydrolysis of Purified CM-1 with 6M HCl at 110°C for 24 Hours.

<table>
<thead>
<tr>
<th>HYDROLYSIS OF CM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis of CM-1 (6M HCl)</td>
</tr>
<tr>
<td>Pre-Hydrolysis</td>
</tr>
<tr>
<td>Post-Hydrolysis</td>
</tr>
</tbody>
</table>

5.3.4 Hydrolysis of Purified Cartilage Marker-1 for Different Time Points

For the interpretation of this data unhydrolyzed aliquot of CM-1 calibrator was assumed to yield 100% CM-1. A decrease in CM-1 yield to 64% was observed at 3 hrs of CM-1 hydrolysis and was maintained up to 12 hrs (figure 5.8). However the CM-1 yield decreased to 19% by 24 hrs and further to 4.5% by 48 hrs of CM-1 hydrolysis.

![Figure 5.8 Hydrolysis of CM-1 with 6M HCl for Varying Time Points](image)

The yield of unhydrolyzed CM-1 was considered 100%.
5.3.5 Hydrolysis of Articular Cartilage with Varying Concentrations of NaOH / HCl

Cartilage Marker-1 and the pyridinium crosslinks are released only by the hydrolysis of articular cartilage with 6M HCl (figure 5.9). Hydrolysis of articular cartilage with NaOH did not produce CM-1 or pyridinoline crosslinks.

![Graphs showing hydrolysis of articular cartilage with varying concentrations of NaOH and HCl](image)

Figure 5.9 Hydrolysis of Articular Cartilage with Varying Concentrations of HCl or NaOH. Fifty mg of dried articular cartilage (AC) (3 replicates) was hydrolyzed with varying concentrations of HCl or NaOH at 110°C for 6 hours as described under the methods. Each sample was resuspended in mobile phase and injected into the HPLC analytical C18 column to quantitate the amount of (A) pyridinoline (Pyd), (B) deoxypyridinoline (Dpyd), and (C) cartilage marker-1 (CM-1). The results are expressed as the mean ± SEM of the replicates.
5.3.6 Hydrolysis of Articular Cartilage with Acids and Bases

The CM-1 peak was not generated by acetic acid, nitric acid, sulphuric acid or ammonium hydroxide. The data corroborates other studies showing the HCl hydrolysis is essential for the formation of the CM-1 molecule.

5.3.7 Effect of Mobile Phase pH on the Retention Time of Cartilage Marker-1

When processed cartilage samples were injected on the column with mobile phase of pH ranging from 1.0 to 5.0, a significant shift (to the left) in the retention time of the pyridinium crosslinks was noted indicating its polar nature. However, there was no significant shift in the retention time of the CM-1 indicating that either it is an amphoteric or nonpolar compound.

5.3.8 Requirement of Heptafluorobutyric Acid for Production of Cartilage Marker-1

Heptafluorobutyric acid (HFBA) did not have an effect on the retention time or the peak area of CM-1. However, without HFBA the Pyd and Dpyd peak areas were decreased and the RT shifted.

5.3.9 Fluorescence and Ultraviolet Light Absorption of Cartilage Marker-1

The ultraviolet absorption spectrum at neutral pH showed absorption maxima at 306 nm (1 AU). However, at acidic or basic pH, the intensity of ultraviolet absorptivity of the CM-1 was less than at neutral pH. The UV absorbance at 0.1M HCl and 0.1M
NaOH were 0.7 AU and 0.6 AU respectively (figure 5.10). Although the absorbance decreased with increased concentration of acid and base compared to aqueous solution, the wavelength at maximum absorption remained the same at 306 nm. With the optimal excitation of CM-1 at 306 nm, the emission was at 395 nm (figure 5.11).

![Figure 5.10](image)

**Figure 5.10  Effect of pH on Ultraviolet Absorption Spectra of Purified CM-1.** The measurements were taken in (a) neutral, aqueous solution; (b,d) in 0.1 and 6M HCl; (c, e) in 0.1 and 10M NaOH. The UV absorption maximum was unchanged at 306 nm wavelength with each pH. The absorptivity was not affected by pH (baseline for “a” is offset).
Figure 5.11 Fluorescence Emission Spectra of Purified CM-1. The excitation wavelengths are indicated on the left axis. For CM-1, the emission is 395 nm, with optimal excitation at 306 nm. For each emission scan, deflection above its baseline reflects relative emission intensity at the wavelength shown at the bottom. Optimal excitation wavelength is 295 nm for pyridinoline and 335 nm for pentosidine.

5.3.10 Solubility of Cartilage Marker-1 in Various Solvents

CM-1 is highly soluble in water and methanol. CM-1 is insoluble in hexane and chloroform.

5.3.11 Effect of Storing Cartilage Marker-1 at Room Temperature for Eight Months

The UV absorbance of CM-1 (0.74 AU) at absorption wavelength of 306 nm when stored in the freezer was similar to that of the reference calibrator (0.69 AU). However, the calibrator aliquot which was kept in the room temperature decreased to 0.17 AU (A decrease of 75%) (table 5.2). It appears that either the room temperature
and/or possibly the room light itself caused degradation of CM-1 when kept at room temperature for a prolonged period of time. Pyd and Dpyd are known to be light sensitive. This experiment indicated that the storage method is an important parameter in maintaining the stability of CM-1.

Table 5.2 Determination of the Stability of CM-1 in Different Storage Conditions.

<table>
<thead>
<tr>
<th>STABILITY OF CM-1</th>
<th>Storage Condition</th>
<th>CM-1 Calibrator (10 µg/ml)</th>
<th>UV Absorption (306 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 Days after preparation</td>
<td>0.69 AU</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 Months later (Room)</td>
<td>0.17 AU</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 Months later (Freezer –70°C)</td>
<td>0.74 AU</td>
<td></td>
</tr>
</tbody>
</table>
5.4 DISCUSSION AND CONCLUSIONS

The goal of the experiments documented in this chapter was to establish a protocol that would maximize the yield of CM-1 from the hydrochloric acid hydrolysate of articular cartilage because CM-1 quantification is dependent upon the adequate generation, recovery and fluorescence of CM-1. Some guidelines have been established which, when combined with logic and suitable experimental design, lead to the optimal recovery of CM-1. With hydrochloric acid and high temperature conditions, articular cartilage yielded a chromophore, CM-1, with maximal absorbance at 306 nm. Amongst the known weak and strong acids such as acetic acid, sulphuric acid, nitric acid and hydrochloric acid, only 6M HCl generated CM-1. Similar results were observed in the fluorescence detection of pyridinoline and deoxypyridinoline. Gupta et al (1997) recently reported that sucrose concentrations higher than 10% caused interference in the ninhydrin assay of samples hydrolysed with 6M HCl. In contrast, hydrolysis with 13.5M NaOH did not show any interference by sucrose. They proposed that certain final products form with HCl hydrolysis that are not formed even with high concentrations of NaOH. This could explain why hydrolysis of articular cartilage with NaOH did not result in the formation of CM-1, Pyd or Dpyd. While investigating a sensitive determination of furosine in acid hydrolysates of foods Henle et al (1995) reported that the formation of furosine during hydrolysis of food samples increased linearly with acid concentration (4 to 8 mol/L). My results also showed that Pyd, Dpyd and CM-1 were generated at 6M HCl but not at low concentrations of HCl.
The results of the hydrolysis of purified CM-1 indicated that CM-1 is degraded when treated with 6M HCl at 110°C. The percent of degradation increased with the time of hydrolysis and after 24 hours of hydrolysis, 75 to 80% of CM-1 was degraded. Although the aromatic structure of CM-1 is maintained with prolonged hydrolysis of articular cartilage (shown in section 5.3.1), purified CM-1 does not withstand prolonged hydrolysis. The carbonyl (C=O) group of CM-1 molecules has a partial negative charge (δ-) on the oxygen atom and a partial positive charge (δ+) on the carbon atom. The protons of HCl (acting as an electrophile) are attracted to the oxygen. This could lead to an intermediate that can accentuate the positive charge on the carbon atom, making it more attractive to a nucleophile, in this case water. Therefore, one plausible explanation of this observation is the lack of steric hindrance (provided by other cartilage molecules) which directly exposes CM-1 to the harsh treatment of HCl. The covalent bonds of the aromatic structure are broken, perhaps at the reactive site of the molecule i.e. the keto group of central pyrone ring. This may lead to rearrangement of the molecule and/or further breaking of the weak bonds resulting in increased susceptibility of CM-1 to electrophilic attack. Further, this in turn may result in the hydrolysis of C=O bond followed by opening of ring structure and decreased fluorescence of CM-1 molecule by a process similar to the observations of Hashimoto et al (1995, 1996). They investigated the susceptibility of hydrolytic cleavage of pyroglutamyl-peptide bond (pGlu-peptides) when treated with dilute (1M) HCl and/or at 60°C and found that the N-terminal portion of pGlu-peptides was extremely labile to HCl hydrolysis resulting in a ring-opened product of the pyrrolidone moiety of the pGlu residue as well as a cleavage product of the pGlu-peptide linkages. However, when treated with 1M HCl at 60°C, the ring-opening
reaction predominated over the cleavage reaction in hydrolysis of the peptides. The rate of hydrolysis was affected by the reaction temperature that in turn had an effect on the predominant nature of the ring-opening reaction versus the cleavage reaction. This study could explain our observation of CM-1 degradation when treated with 6M HCl at varying time points.

Heptafluorobutyric acid (HFBA), a 214 Dalton molecule, is an ion pair reagent used in mobile phase for reverse phase HPLC separation of proteins and peptides. The use of ion-pairing reagents greatly enhances the fluorescence and resolving power of compounds separated by reverse phase HPLC. To quantify pyridinoline and deoxypyridinoline in hydrolyzed tissue samples, HFBA is an essential component of the mobile phase. However, the use of HFBA did not make any difference to the resolving power of CM-1. CM-1 differs from other known fluorescent biological molecules present in articular cartilage namely, Pyd and Dpyd which has an optimal excitation wavelength at 295 nm and pentosidine at 335 nm.

In conclusion, CM-1 is generated from hydrochloric acid hydrolysis of articular cartilage. The ratio of tissue dry weight to the volume of 6M HCl should not significantly deviate from 1 (mg):20 µl otherwise the results could be misinterpreted. It is also essential to store CM-1 calibrator in the freezer to prevent its degradation. Unlike the requirement of heptafluorobutyric acid for the generation of fluorescence of pyridinoline and deoxypyridinoline, HFBA is not required in the mobile phase for CM-1 fluorescence.
CHAPTER 6 CHARACTERIZATION OF TISSUE SPECIFICITY OF CARTILAGE MARKER-1

6.1 INTRODUCTION

The evaluation of disease activity and cartilage injury in the patients with osteoarthritis, rheumatoid arthritis, chondromalacia patella or osteochondritis requires a reliable biochemical marker. Although several markers are currently used to evaluate the presence of disease, only a few markers specify the stage (severity level) of the disease. The purpose of the experiments outlined in this chapter was to investigate the validity of CM-1 as a cartilage specific molecule by investigating the presence and quantifying CM-1 in various tissues and from different species. Additionally I investigated the validity of CM-1 as a marker of cartilage degradation.

6.2 MATERIALS AND METHODS

Tissue samples obtained from various sources (human autopsy, human knee joint transplant and various animals) were frozen and kept at -40°C until the day of experiment. Human samples were obtained from post-mortem. In addition, articular cartilage was also obtained from knee joint transplants. Synovial fluid samples were obtained as knee joint effusion samples from patients with knee joint arthropathy. Due to difficulty in obtaining immature human samples, tissues were obtained from only a single three-day-old infant and analyzed for the presence of CM-1.
For CM-1 quantification, the samples were lyophilized, weighed and then hydrolyzed with 1000 µl of 6M HCl for 24 hours at 110°C on a sand bath. To process the synovial fluid samples, 1000 µl of synovial fluid were hydrolyzed with 6M HCl for 24 hours at 110°C. The hydrolysed tissue or synovial fluid samples were lyophilized, dissolved in 1000 µl of mobile phase (24% acetonitrile, 1% HFBA and 75% deionized water), briefly vortexed and filtered with 0.45 µm-pore Gelman filter. An aliquot of 30 µl hydrolysate was applied to the HPLC analytical column attached to Hewlett Packard HPLC system. The C18 column (Phenomenex Spherisorb with dimension of 250 x 4.6. pore size of 5 µm and ODS of 2) was protected by a guard cartridge. The flow rate was 1.0 ml/minute. CM-1 fluorescence was monitored with a Perkin Elmer Fluorescence detector at 295 nm excitation and 395 nm emission. All sample peaks were quantitated using the CM-1 calibrator that was prepared as described in section 3.3.5. The detection limit of CM-1 in the various tissues was 1.7 ng or 8.5 pmol per µl of sample solution injected. The values of CM-1 are expressed per mg dried tissue sample.
6.3 RESULTS

The results presented in this chapter are preliminary because the number of samples investigated for the presence of CM-1 in various tissue samples were limited.

6.3.1 Human Specimens

6.3.1.1 Tissues

Most of the tissue samples examined from the 3-day-old human infant, as shown in table 6.1, detected the presence of CM-1. Articular cartilage (525 pmol/mg dry weight articular cartilage) and sternum (295 pmol/mg dry weight sternum) showed high amounts of CM-1. However, in adult human tissue samples only articular cartilage, vertebral disk, and trachea showed the presence of CM-1 (table 6.2 and figure 6.1). The amount of CM-1 present in adult articular cartilage (> 53 yr. old – range: 182 to 238 pmol/mg dried articular cartilage) was about 2.5 fold less than that present in the 3-day-old cartilage (table 6.3). CM-1 was also detected in the acid hydrolyzed articular cartilage containing tissues such as vertebral disk, sternum and trachea. CM-1 was not detected in the various tissue samples when it is not acid hydrolyzed. CM-1 was not detected from adult non-cartilagenous tissue samples (ligament, tendon, meniscus, bone, esophagus, stomach, intestine, brain, heart, aorta, lungs, muscle, adipose tissue, liver, and kidney).
Table 6.1  Amount of CM-1 Detected in Tissue Samples Obtained from a Newborn Baby (3-day-old) Diagnosed with Congenital Heart Disease.

<table>
<thead>
<tr>
<th>TISSUES EXAMINED</th>
<th>CM-1/TISSUE (ng/mg)</th>
<th>CM-1/TISSUE (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Articular Cartilage</td>
<td>107</td>
<td>525</td>
</tr>
<tr>
<td>Trachea</td>
<td>6</td>
<td>29</td>
</tr>
<tr>
<td>Sternum</td>
<td>60</td>
<td>295</td>
</tr>
<tr>
<td>Bone</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>Meniscus</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>Liver</td>
<td>7</td>
<td>35</td>
</tr>
<tr>
<td>Spleen</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>Adrenal Cortex</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>Intestine</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>Kidney</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Muscle (Cardiac)</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Brain – Occipital</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Lungs</td>
<td>5</td>
<td>26</td>
</tr>
</tbody>
</table>

NP refers to not present
Table 6.2: Amount of CM-1 Detected in Adult Human Tissue Samples.

<table>
<thead>
<tr>
<th>TISSUE EXAMINED</th>
<th>SAMPLE SIZE (n)</th>
<th>CM-1/TISSUE (ng/mg ± SEM)</th>
<th>CM-1/TISSUE (pmol/mg ± SEM)</th>
<th>RANGE (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Articular Cartilage</td>
<td>10</td>
<td>41 (± 3)</td>
<td>200 (± 16)</td>
<td>182 to 238</td>
</tr>
<tr>
<td>Vertebral disk</td>
<td>4</td>
<td>51 (± 18)</td>
<td>250 (± 87)</td>
<td>28 to 442</td>
</tr>
<tr>
<td>Trachea</td>
<td>5</td>
<td>15 (± 5)</td>
<td>75 (± 23)</td>
<td>13 to 131</td>
</tr>
<tr>
<td>Sternum</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Bone</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Tendon</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Meniscus</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Ligament</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Pancreas</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Stomach</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Spleen</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Gall Bladder</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Brain</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Intestine</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
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<td>Kidney</td>
<td>3</td>
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<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Rectum</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Aorta</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Muscle</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Fat</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Prostate Gland</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Pituitary</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Lungs</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Skin</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 6.1  Chromatographic Profiles Showing Fluorescence (295 nm Excitation, 395 nm Emission) of Acid Hydrolysates of the Human Tissues.  
"n" represents the number of samples examined for the presence of crosslinks and CM-1.  
A representative chromatograph of the tissue type is shown above.  Pyd eluted at 9.5 to 11 min; Dpyd eluted at 10.5 min; and CM-1 eluted at 23 to 24 min.  "AC" stands for articular cartilage.  Note: CM-1 was detected only in articular cartilage containing tissues.
The CM-1 was quantified in intact femoral condyle articular cartilage excised from the left and right femoral condyle (n=4). The average CM-1 is 208 pmol/mg of dry weight of old adult articular cartilage (table 6.3). CM-1 content of intact articular cartilage was lower in the superficial-middle lamina (70 ± 21 pmol/mg dried articular cartilage) compared to both the middle-deep lamina (143 ± 34 pmol/mg dried articular cartilage) and entire thickness (151 ± 41 pmol/mg dried articular cartilage) of old adult articular cartilage (table 6.4).

Table 6.3  Amount of CM-1 Quantified in Old and Young Adult Intact Articular Cartilage Obtained from Autopsy. [Male (M) and Female (F)].

<table>
<thead>
<tr>
<th>Autopsy No.</th>
<th>Sex / Age (Years)</th>
<th>Cartilage Marker-1 / Articular Cartilage Dry Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Left Femoral Condyle pmol/mg</td>
</tr>
<tr>
<td>1</td>
<td>M / 76</td>
<td>274</td>
</tr>
<tr>
<td>2</td>
<td>M / 53</td>
<td>232</td>
</tr>
<tr>
<td>3</td>
<td>F / 62</td>
<td>260</td>
</tr>
<tr>
<td>4</td>
<td>M / 80</td>
<td>178</td>
</tr>
<tr>
<td>5</td>
<td>M / 44</td>
<td>251</td>
</tr>
<tr>
<td>6</td>
<td>M / 30</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 6.4  The Concentration of CM-1 in Intact Articular Cartilage Laminae of
Human Hip Joint.

<table>
<thead>
<tr>
<th>Femoral Head Intact Articular Cartilage (Hip Joint)</th>
<th>Cartilage Marker-1/Articular Cartilage Dry Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/mg (± SEM)</td>
</tr>
<tr>
<td>Entire Thickness (n = 4)</td>
<td>31 (± 8)</td>
</tr>
<tr>
<td>Superficial-Middle Lamina (n = 4)</td>
<td>14 (± 4)</td>
</tr>
<tr>
<td>Middle-Deep Lamina (n = 4)</td>
<td>29 (± 7)</td>
</tr>
</tbody>
</table>

6.3.1.2  Body fluids

CM-1 was not detected in serum (n = 10), plasma (n = 10) or urine (n = 10) samples. A summary of CM-1 content in the synovial fluid samples of patients with knee joint arthropathy is shown in table 6.5. Some of the chromatographic profiles of CM-1 synovial fluid are represented in figure 6.2 to figure 6.5. CM-1 eluted at 22 minutes. Out of 15 serum samples (during the early phase of the project) obtained from knee joint arthropathy patients, CM-1 was detected in 10 serum samples and CM-1 was either absent or below the detection limit in the remaining 5 serum samples. Further, CM-1 was generated only when the synovial fluid samples were heat treated with 6M HCl. Preliminary data appears to indicate a higher level of CM-1 in synovial fluid of patients with knee joint disease (9 ± 2 pmol/μl of synovial fluid) compared to normal (< 3 pmol/μl of synovial fluid). Direct analysis of synovial fluid samples (i.e., direct injection of synovial fluid into the HPLC column without HCl hydrolysis) failed to generate CM-1 as shown in figure 6.6.
Figure 6.2  A Chromatograph of the Hydrolyzed Synovial Fluid Sample Obtained from the Knee Joint of a 52-year-old Male with RA. The CM-1 peak eluted at 22 min.

Figure 6.3  A Chromatograph of the Hydrolyzed Synovial Fluid Sample Obtained from Osteoarthritic Joint of a 55-year-old Female. The CM-1 eluted at 22.7 min, pyridinoline at 10 min and deoxypyridinoline at 11 min.
Figure 6.4 A Chromatograph of the Hydrolyzed Synovial Fluid Sample Obtained from a 49-year-old Patient with Cutaneous Vasculitis. The CM-1 eluted at 22.7 min.

Figure 6.5 A Chromatograph of the Hydrolyzed Synovial Fluid Sample Obtained from a Patient with Urate Crystal Arthropathy. The CM-1 eluted at 22.7 min.
**Figure 6.6  Chromatograph of a Human Synovial Fluid Sample.** The synovial fluid was taken from the same patient and was injected into the HPLC analytical column either neat or it was subjected through HCl treatment. The chromatographic profile in (A) is not hydrolyzed and in (B) an equal aliquot of synovial fluid was hydrolyzed with 6M HCl at 110°C for 24 hours. Note that CM-1 was generated only when the synovial fluid sample was hydrolyzed.
Table 6.5  CM-1 Content of Synovial Fluid Samples from Patients with Knee Joint Arthropathy.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Joint Disease</th>
<th>Sex/Age (Years)</th>
<th>Cell Count/L</th>
<th>Mucin Clot</th>
<th>CM-1/SF** (ng/µl)</th>
<th>CM-1/SF** (pmol/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rheumatoid Arthritis</td>
<td>M / 52</td>
<td></td>
<td></td>
<td>2.1</td>
<td>10.4</td>
</tr>
<tr>
<td>2</td>
<td>CPPD*</td>
<td>F / 81</td>
<td>$89 \times 10^6$</td>
<td></td>
<td>0.6</td>
<td>2.8</td>
</tr>
<tr>
<td>3</td>
<td>OA*** and CPPD*</td>
<td>F / 81</td>
<td></td>
<td></td>
<td>2.1</td>
<td>10.3</td>
</tr>
<tr>
<td>4</td>
<td>Urate Crystal</td>
<td>M / 87</td>
<td>$1244 \times 10^6$</td>
<td>Tight</td>
<td>6.2</td>
<td>30.2</td>
</tr>
<tr>
<td>5</td>
<td>Urate Crystal</td>
<td>M / 75</td>
<td></td>
<td></td>
<td>1.0</td>
<td>4.7</td>
</tr>
<tr>
<td>6</td>
<td>Urate Crystal</td>
<td>M / 79</td>
<td>$50000 \times 10^6$</td>
<td>Tight</td>
<td>3.5</td>
<td>16.9</td>
</tr>
<tr>
<td>7</td>
<td>Cutaneous Vasculitis</td>
<td>F / 49</td>
<td></td>
<td></td>
<td>1.5</td>
<td>7.1</td>
</tr>
<tr>
<td>8</td>
<td>Rheumatoid Arthritis</td>
<td>F / 82</td>
<td></td>
<td></td>
<td>1.14</td>
<td>5.61</td>
</tr>
<tr>
<td>9</td>
<td>CPPD*</td>
<td>F / 88</td>
<td>$9175 \times 10^6$</td>
<td>Intermediate</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Calcium pyrophosphate dihydrate crystals  
** Synovial Fluid  
*** Osteoarthritis
6.3.1.3 Osteoarthritic Cartilage Lesions

Investigation of articular cartilage with osteoarthritic lesions showed a decline in CM-1 levels compared to the intact adult articular cartilage (table 6.6 and figure 6.7).

Table 6.6 CM-1 Content of Intact and Lesioned Femoral Condyle Articular Cartilage. The samples were obtained from osteoarthritis or rheumatoid arthritis joints (from knee joint transplant or autopsy). [Male (M) and Female (F)].

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Sex /Age (Years)</th>
<th>Cartilage Marker-1/Tissue Dry Weight (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intact Cartilage</td>
</tr>
<tr>
<td><strong>OSTEOARTHRITIS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>F / 54</td>
<td>236</td>
</tr>
<tr>
<td>2</td>
<td>M / 71</td>
<td>157</td>
</tr>
<tr>
<td>3</td>
<td>F / 55</td>
<td>68</td>
</tr>
<tr>
<td>4</td>
<td>M / 76</td>
<td>No Data</td>
</tr>
<tr>
<td>5</td>
<td>M / 71</td>
<td>154</td>
</tr>
<tr>
<td>6</td>
<td>F / 45</td>
<td>231</td>
</tr>
<tr>
<td>7</td>
<td>F / 58</td>
<td>No Data</td>
</tr>
<tr>
<td>8</td>
<td>F / 55</td>
<td>132</td>
</tr>
<tr>
<td><strong>RHEUMATOID ARTHRITIS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>F / 88</td>
<td>63</td>
</tr>
</tbody>
</table>
Figure 6.7  Graph Showing the CM-1 Level in Human Femoral Condylar Articular Cartilage. The squares (■) show the correlation of CM-1 level of articular cartilage obtained from normal joint, the right versus left knee joint. The circles (○) show the correlation of CM-1 level of lesioned versus intact articular cartilage obtained from osteoarthritic joints. Clearly the level of CM-1 of cartilage obtained from normal knee joints have higher concentrations compared to that obtained from diseased knee joints. In osteoarthritic joints the level of CM-1 is higher in intact cartilage compared to lesioned cartilage.
6.3.2 Bovine Samples

6.3.2.1 Tissues

Bovine tissue samples were obtained from the abbatoir. CM-1 was detected in calf metacarpophalangeal joint cartilage (362 ± 48 pmol/mg of dry weight cartilage) and intervertebral disk (24 ± 4 pmol/mg dry weight tissue). CM-1 was not present in calf ligament, tendon, bone, ocular lens, cornea, and elastic cartilage (table 6.7). A significant difference was noted between the superficial-middle (129 ± 52) and middle-deep laminae (448 ± 63) (paired t-test, p = 0.008) as well as superficial-middle lamina and entire cartilage thickness (p = 0.02). There was no significant difference between middle-deep and entire cartilage thickness (p= 0.36).

6.3.2.2 Synovial Fluid and Vitreous Humor

Synovial fluid was collected from the calf metacarpophalangeal joint. CM-1 was detected in 11 (4.54 ± 0.7 pmol/µl of synovial fluid) of a total of 13 joints (table 6.8). No CM-1 could be detected in the vitreous humor.
Table 6.7  Amount of CM-1 Detected in Bovine Tissue Samples.

<table>
<thead>
<tr>
<th>TISSUES EXAMINED</th>
<th>CM-1/TISSUE ng/mg (± SEM)</th>
<th>CM-1/TISSUE pmol/mg (± SEM)</th>
<th>RANGE pmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage Mid-Deep Lamina (n = 4)</td>
<td>91 (± 13)</td>
<td>448 (± 63)</td>
<td>331 to 626</td>
</tr>
<tr>
<td>Cartilage Sup-Mid Lamina (n = 4)</td>
<td>26 (± 11)</td>
<td>129 (± 52)</td>
<td>0 to 247</td>
</tr>
<tr>
<td>Cartilage Entire thickness (n = 3)</td>
<td>74 (± 10)</td>
<td>362 (± 48)</td>
<td>300 to 456</td>
</tr>
<tr>
<td>Vertebral Disk (n = 4)</td>
<td>5 (± 1)</td>
<td>24 (± 4)</td>
<td>16 to 29</td>
</tr>
<tr>
<td>Cornea (n = 10)</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>Ocular Lens (n = 3)</td>
<td>Unclear</td>
<td>Unclear</td>
<td>Unclear</td>
</tr>
<tr>
<td>Ligament (n = 10)</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>Bone (n = 5)</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>Tendon (n = 10)</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>Nasal Cartilage - Bridge (n = 1)</td>
<td>211</td>
<td>1035</td>
<td></td>
</tr>
<tr>
<td>Nasal Cartilage Periphery (n = 3)</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>Elastic Cartilage (ear) (n = 3)</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
</tr>
</tbody>
</table>

Figure 6.8  A Typical Chromatograph of the Hydrolyzed Synovial Fluid Obtained from Calf Metacarpophalangeal Joint.
Table 6.8  Amount of CM-1 Quantitated from Calf Synovial Fluid and Vitreous Humor.

<table>
<thead>
<tr>
<th>TISSUE FLUIDS</th>
<th>CM-1 / FLUID ng/μl (± SEM)</th>
<th>CM-1 / FLUID pmol/μl (± SEM)</th>
<th>RANGE pmol/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synovial Fluid (Normal) (n = 11)</td>
<td>0.9 (± 0.2)</td>
<td>4.5 (± 0.3)</td>
<td>0.86 to 8.60</td>
</tr>
<tr>
<td>Synovial Fluid (Normal) (n = 2)</td>
<td>UD</td>
<td>UD</td>
<td>-</td>
</tr>
<tr>
<td>Vitreous Humor (n = 9)</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

6.3.3 Rabbit Samples

An approximately 18-fold difference in the CM-1 content of young (6 weeks) versus adult (48 weeks) rabbit knee joint articular cartilage was observed the respective chromatographs in figure 6.9 and figure 6.10. In comparison the Pyd and Dpyd content increased 4 fold between young and old cartilage. The amount of CM-1 decreased with increasing age in rabbit articular cartilage from an average of 185 ± 40 (<2 weeks) to 27 ± 3 (48 weeks) pmol/mg of dry weight cartilage. CM-1 was present in small amounts in ligament, tendon, meniscus or bone of rabbits aged ≤ 10 weeks. CM-1 was not present from the same tissues in 48 week old rabbits.
Figure 6.9 A Chromatograph of the Hydrolyzed Articular Cartilage (Femoral Condyle) of a Seven-day-old Rabbit Knee Joint. The CM-1 eluted at 21 min. The pyridinoline and deoxypyridinoline eluted at 7.3 and 8.7 min respectively.

Figure 6.10 A Chromatograph of the Hydrolyzed Articular Cartilage (Femoral Condyle) of a Two-year-old Rabbit Knee Joint. The CM-1 eluted at 21 min whereas pyridinoline and deoxypyridinoline eluted at 7.5 and 8.5 min respectively.
Table 6.9  Amount of CM-1 in Rabbit Knee Joint (Femoral Condyle) Articular Cartilage with Maturation.

<table>
<thead>
<tr>
<th>RABBIT WEIGHT (AGE)</th>
<th>CM-1/CARTILAGE ng/mg (± SEM)</th>
<th>CM-1/CARTILAGE pmol/mg (± SEM)</th>
<th>RANGE pmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 230 gm (~ 4 to 14 days) (n = 5)</td>
<td>38 (± 8)</td>
<td>185 (± 40)</td>
<td>122 to 340</td>
</tr>
<tr>
<td>1 Kg (~ 6 weeks) (n = 7)</td>
<td>11 (± 1)</td>
<td>52 (± 5)</td>
<td>39 to 79</td>
</tr>
<tr>
<td>2 Kg (~ 10 weeks) (n = 5)</td>
<td>8 (± 1)</td>
<td>40 (± 4)</td>
<td>26 to 56</td>
</tr>
<tr>
<td>4 Kg (~ 18 weeks) (n = 4)</td>
<td>5 (± 1)</td>
<td>27 (± 3)</td>
<td>20 to 34</td>
</tr>
</tbody>
</table>

Table 6.10 Amount of CM-1 Quantitated from Young (≤ 2 kg) and Mature Rabbit (~ 4 Kg) Tissues.

<table>
<thead>
<tr>
<th>RABBIT TISSUE (WEIGHT)</th>
<th>SAMPLE (n)</th>
<th>CM-1/TISSUE ng/mg (± SEM)</th>
<th>CM-1/TISSUE pmol/mg (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligament (~≤ 2 Kg)</td>
<td>2</td>
<td>1.4 (± 0.5)</td>
<td>6.9 (± 3)</td>
</tr>
<tr>
<td>Meniscus (~≤ 2 Kg)</td>
<td>4</td>
<td>3.2 (± 0.4)</td>
<td>15.9 (± 1.8)</td>
</tr>
<tr>
<td>Bone (~≤ 2 Kg)</td>
<td>1</td>
<td>0.74</td>
<td>3.65</td>
</tr>
<tr>
<td>Ligament (~≤ 4 Kg)</td>
<td>4</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>Meniscus (~≤ 4 Kg)</td>
<td>3</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>Bone (~≤ 4 Kg)</td>
<td>8</td>
<td>NP</td>
<td>NP</td>
</tr>
</tbody>
</table>

6.3.4 Lamprey Specimens

To determine whether CM-1 is present in more primitive cartilage tissues from the sea lamprey were examined. The sea lamprey is a vertebrate with an entirely cartilagenous skeleton consisting of noncollagenous elastin-like proteins. The cartilage morphology is similar to that of elastic cartilage of higher vertebrates (Robson et al. 1997). The major connective tissue component of lamprey annular cartilage is lamprin, an insoluble non-collagen and non-elastin protein (Robson et al, 1993). CM-1 was absent from the Lamprey articular cartilage (n=5). This indicates that CM-1 is not present in cartilage samples that lack proteoglycans.
6.4 DISCUSSION AND CONCLUSIONS

From tissues other than cartilage the fluorescent peak corresponding to CM-1 was consistently below the detection limit. It appears that investigators using similar procedure did not observe the CM-1 because the conventional elution time of CM-1 is twice that of pyridinoline. Thus, in previous studies, the elution time for samples injected into the HPLC was no longer than 15 minutes. The uniqueness of CM-1 in cartilage samples prompted me to investigate the likelihood that CM-1 could be of scientific and clinical significance to arthritis.

Stockwell (1979) reported that the percent dry weight of proteoglycan and collagen in cartilage-containing tissues varies. For example, bovine nasal cartilage has 43% PG and only 35% collagen, whereas bovine articular cartilage has 14% PG and 72% collagen. Human epiphyseal cartilage has 15% PG and 37% collagen whereas human articular cartilage has 18% PG and 66% collagen. Further, Stockwell (1979) also reported the decrease in cell density with distance from the articular surface was more prominent (two fold) in adult human than in rabbit femoral condylar articular cartilage. Both observations, ie, the variation in the macromolecular content of the tissue source and variation in cellularity could explain the difference in CM-1 levels noted in human, bovine and rabbit articular cartilage.

Based on these studies, CM-1 appears to be a compound that is essentially cartilage specific and is present in the articular cartilage and cartilagenous tissues such as trachea, sternum, and intervertebral disk in human (both knee replacement and autopsy samples) and various animal species. The absence of CM-1 in lamprey elastic cartilage
and lower concentrations in tracheal elastic cartilage compared to hyaline articular cartilage indicates that CM-1 is not associated with an elastic component of cartilage. In the intervertebral disk, the bovine nucleus pulposus has galactosaminoglycan-rich and KS-rich PG monomers. The bovine nasal cartilage has chondroitin-sulphate-rich PG monomers. Examination of the origin of CM-1 within the cartilage thickness in calf and human samples shows that it is most abundant in the deep lamina and least in the superficial lamina. The data show that the amount of CM-1 per mg of dry weight articular cartilage increases from the superficial to deep lamina. This result correlates with our finding that CM-1 is a product of acid hydrolysis of the carbohydrate moiety of articular cartilage; the proteoglycan also increasing from the superficial to the deep cartilage lamina.

Although a limited number of young human and animal cartilage samples were analyzed for CM-1 content, there is a good indication that CM-1 is more abundant in young compared to old cartilage. Further, the presence of CM-1 in practically all tissues examined from young human and animal samples also indicate its potential as a marker for growth, development and/or tissue differentiation.

CM-1 was detected in small amounts in the synovial fluid samples of bovine metacarpophalangeal joints and rabbit and human knee joints. However, its presence was not detected in urine samples. Furthermore, the synovial fluid samples were analyzed prior to and post hydrolysis with 6M HCl. Direct analysis of synovial fluid samples, without the acid hydrolysis, failed to reveal any measurable CM-1. The fluorescent peak of CM-1 could be identified only in the hydrolyzed synovial fluid sample.
CM-1 content declined in articular cartilage with osteoarthritic lesions, but its level appears to increase in knee joint synovial fluid of patients with knee joint arthropathy. As CM-1 can be detected in synovial fluids this molecule is a candidate marker for cartilage breakdown in arthritis.
CHAPTER 7  INVESTIGATION OF THE PRESENCE OF CM-1 IN MOLECULAR COMPONENTS OF ARTICULAR CARTILAGE

7.1 INTRODUCTION

Articular cartilage is composed of primarily the macromolecules, collagen and proteoglycan. CM-1 is a fluorescent molecule that was originally discovered as a product of acid hydrolysis of articular cartilage. CM-1 could be an advanced glycosylation end product formed by cleavage reactions or condensation from its precursor molecule. In order to identify the precursor molecule of CM-1 and to study the mechanism by which it is formed, it is important to investigate its molecular origin in articular cartilage. The aim of the experiments presented in this chapter was to identify the cartilage component (collagen or proteoglycan) from which CM-1 is derived. This would facilitate the determination of the mechanism by which CM-1 is formed. Further, I also investigated the presence of CM-1 in various biological compounds and purified macromolecular components of cartilage. Based on its structure, one of the putative precursors of CM-1 could be ascorbic acid. Therefore, I examined the possibility that CM-1 is a condensation product of ascorbic acid following acid hydrolysis.
7.2 MATERIALS AND METHODS

7.2.1 Isolation and Analysis of Collagen

Pure type II collagen and type I collagen were obtained from Sigma chemicals. Type II collagen was also extracted from articular cartilage (sources: cartilage excised from bovine metacarpophalangeal joints and cartilage powder from Sigma Chemical). Type I collagen was purified in our laboratory from rat tail tendon. Cartilage samples were digested with pepsin twice for 48 hrs each at 4°C. An aliquot from the pepsin-digested fraction was analyzed for the presence of CM-1. Pure samples of type I and type II collagens obtained from various sources were hydrolyzed using 6M HCl at 24°C in an oven at 110°C). The hydrolysate was lyophilized and dissolved in 1000 µl of mobile phase (1% HFBA and 24% acetonitrile in distilled water). The hydrolysate was briefly vortexed and then filtered through 0.45 µm pore Gelman filter. An aliquot of 30 µl hydrolysate was applied to the HPLC column. The flow rate was 1.0 ml/minute. Pyridinium and CM-1 fluorescence was monitored at an excitation of 295 nm and emission of 395 nm.

7.2.2 Analysis of Proteoglycans

Cartilage powder was extracted with 4M guanidine HCl in 50 mM sodium acetate, pH 5.8, containing proteolytic enzyme inhibitors, 0.1M 6-amino-hexanoic acid, 10 mM EDTA, 50mM benzamidine HCl and 5 mM N-ethylmaleimide for 24 hours at
4°C. The proteoglycans were precipitated from the supernatant by addition of three volumes of cold ethanol. The pellets were collected by centrifugation at 14,000 rpm for 30 min, washed three times with 70% ethanol, and analysed for CM-1 by HPLC as described in section 7.3.1.

In another experiment, various components of proteoglycans were analyzed for the presence of CM-1. Proteoglycan monomer (source - porcine nasal cartilage), proteoglycan aggregate (source - porcine nasal cartilage) and glycosaminoglycan (source - porcine nasal cartilage) were generously provided by Dr. Graeme Hunter (London, Ontario). Chondroitin sulphate C (source - shark cartilage) chondroitin sulphate A (source - bovine trachea), keratan sulphate (source - bovine cornea), hyaluronic acid (source - human umbilical cord), and sodium hyaluronate were purchased from Sigma chemicals. Proteoglycans, both aggregates and monomer, as well as various types of glycosaminoglycans (GAG) such as chondroitin sulphate A and C, keratan sulphate, and hyaluronic acid were hydrolyzed and analyzed for the presence of CM-1. For comparison unhydrolyzed samples of the above mentioned molecules were also processed for HPLC analysis.

7.2.3 Investigation of CM-1 in Known Fluorescent Amino Acids

The fluorescent amino acids, tryptophan, tyrosine and phenylalanine (10 mg/ml each) were lyophilized, hydrolyzed (300 μl of 6M HCl for 24 hr at 110°C), resuspended in the mobile phase and injected on C18 column.
7.2.4 *Exogenous Ascorbic Acid as a Source of CM-1*

Articular cartilage (50 mg) was hydrolyzed (1000 µl of 6M HCl for 24 hr at 110°C) with 0.1 mg and 10 mg of ascorbic acid (Sigma Chemicals) following the protocol in section 7.3.1. The control samples comprised articular cartilage alone, i.e. without added ascorbic acid, and ascorbic acid alone. Each combination of the experiment was repeated three times.

7.3 RESULTS

7.3.1 *Isolation and Analysis of Collagen*

CM-1 was absent from all processed samples of collagen type II (n=6) and collagen type I (n=4), indicating that CM-1 is derived from another cartilage molecule.

7.3.2 *Analysis of Proteoglycans*

CM-1 was detected in the guanidine HCl extract of cartilage indicating that it could be a breakdown product of a soluble carbohydrate moiety of cartilage. However, when articular cartilage was directly hydrolyzed i.e., without the extensive extraction process, the CM-1 yield was much greater than guanidine HCl treated cartilage. This indicates that guanidine HCl could have a quenching effect on the fluorescence of CM-1.
7.3.3 Analysis of Proteoglycan Components

CM-1 was present in varying amounts in the purified proteoglycan components such as the proteoglycan monomer, proteoglycan aggregate, glycosaminoglycans, and chondroitin sulphate (figure 7.1). Due to variable sources and purification techniques used to purify the above-mentioned molecules, it cannot be confirmed which component of proteoglycan is CM-1 present in highest concentration. Furthermore, the unhydrolyzed samples of the same purified proteoglycan components did not show the presence of CM-1. These data clearly shows that CM-1 is associated with the carbohydrate moiety of articular cartilage. Also it confirms the finding that CM-1 is a product of HCl hydrolysis and heat. Therefore, one can further explore the precursor molecule of CM-1 within the carbohydrate fraction of articular cartilage.
Figure 7.1  CM-1 Content in Collagen and Various Components of Aggrecan.  
Note that the source of tissue from which the macromolecules were isolated varies.

Type II Collagen – bovine articular cartilage (Sigma Chemicals)
Proteoglycan Monomer - porcine nasal cartilage (Dr. Graeme Hunter.London, Ontario)
Proteoglycan Aggregate - porcine nasal cartilage (Dr. Graeme Hunter.London, Ontario)
Glycosaminoglycan - porcine nasal cartilage (Dr. Graeme Hunter.London, Ontario)
Chondroitin Sulphate C - shark cartilage (Sigma Chemicals)
Chondroitin Sulphate A - bovine trachea (Sigma Chemicals)
Keratan Sulphate - bovine cornea (Sigma Chemicals)
Hyaluronic Acid - human umbilical cord (Sigma Chemicals)
7.3.4 Investigation of CM-1 in Known Fluorescent Amino Acids

The CM-1 was not detected in hydrolyzed samples of tryptophan, tyrosine or phenylalanine. This data confirmed that CM-1 is not derived from a known fluorescent amino acid.

7.3.5 Effect of Exogenous Ascorbic Acid on the Production of CM-1

As shown in figure 7.2, hydrolysis of ascorbic acid alone did not produce CM-1. On the contrary when exogenous ascorbic acid was added to cartilage sample a quenching effect was noted reducing the fluorescence of CM-1 by 5% when 0.1 mg of ascorbic acid was added and to 95% when 10 mg of ascorbic acid was added.

![Figure 7.2 Effect of Exogenous Ascorbic Acid on CM-1 Yield in Articular Cartilage.](image)
7.4 CONCLUSIONS

CM-1 was absent from all the collagen type II (n=6) and collagen type I (n=4) samples that were processed. It appears that CM-1 is present in the carbohydrate moiety of articular cartilage namely proteoglycan monomer, proteoglycan aggregate, glycosaminoglycans and chondroitin sulphate.
CHAPTER 8  INVESTIGATION OF CARTILAGE MARKER-1 IN CARTILAGENOUS CULTURES

8.1 INTRODUCTION

Researchers have used chondrocytes culture and articular cartilage explants to study chondrocyte physiology and drug responses to diseases such as osteoarthritis. Various animal articular cartilage sources including osteogenic and embryonic cells have been used in *in vitro* experiments. Chondrocytes were shown to grow in monolayer, in suspension or on various substrates such as collagen gel or agarose gel (Buschmann et al. 1992). While chondrocytes cultured as monolayers displayed fibroblastic morphology and grew faster, those cultured as suspensions over agarose adopted a round morphology and formed clusters of cells reminiscent of chondrocyte differentiation in intact cartilage.

In a long-term primary monolayer culture (12 week), Hunter et al (1984) reported that ECM synthesized by the rabbit chondrocytes primarily contained both aggregating and dermatan sulphate proteoglycans (PGs) and type II collagen, with very little type I. Benya et al (1982) reported that the dedifferentiation exhibited by rabbit chondrocyte progeny during serial monolayer culture is reversible when cultures were suspended in agarose gel. Although monolayer cultures have shown the formation of ECM deposition, they lack the multilaminated morphology of articular cartilage observed *in vivo* (van Kampen and Veldhuijzen, 1982). Frequently, chondrocytes cultured as a monolayer lose their phenotypic (chondrocyte morphology) and synthetic expression (synthesis of ECM). The most common observation is the dedifferentiation of chondrocytes into fibroblast-
like cells associated with switch of the synthetic activity from type II collagen to type I collagen (Von der Mark et al, 1977). Long-term cultures of chondrocytes on collagen gel (for six weeks) were documented to maintain the chondrocyte morphology and synthesis of type II collagen and proteoglycans (Kimura et al, 1984; Shakibaei et al, 1993). Repair of articular cartilage defects was investigated in agarose embedded chondrocytes (Rahfoth et al, 1998). Recently chondrons (chondrocyte and its surrounding pericellular environment) were isolated from articular cartilage matrix and used to study chondrocyte metabolism in vitro (Lee et al, 1997).

Collagen, a very stable molecule, has a long half-life ranging from 300 days to years (Prokop et al, 1992). The half-life of PGs in vitro varies depending on the cartilage source and can range up to 30 days (Hascall et al, 1991). In human articular cartilage, Hardingham et al (1990) reported that the turnover time of aggregcan (aggregating PG) is 200-800 days. The PGs of cartilage explants obtained from mature bovine have a half life of approximately 11 days when kept in a medium supplemented with 20% foetal calf serum and 6 days when maintained in medium alone (Campbell et al, 1984). In comparison, cartilage explants obtained from immature bovine (calf) showed a PG half-life of 21 days in a medium supplemented with calf serum.

Ex vivo experiments and in vivo animal models have shown that cytokines such as TNFα, IL-1β, and IL-8 play important roles in inflammatory responses in osteoarthritis (OA), and rheumatoid arthritis (RA) in goat (Matsukawa et al, 1998; Henderson and Pettipher, 1989). The major impact of IL-1β in the pathophysiology of osteoarthritis (OA) and RA is the stimulation of synoviocytes and chondrocytes to produce cartilage
degrading enzymes such as collagenase and stromelysin (metalloproteinases) (Marinova-Mutafchieva et al, 1997; Caron et al, 1996; Baragi et al, 1995). IL-1β inhibits the synthesis of proteoglycans (Tyler et al, 1985) and type II collagen (Goldring et al, 1988) in vivo and in vitro (Frazer et al, 1994).

The in vitro articular cartilage macromolecular synthesis and content vary depending upon a number of factors such as the age of the chondrocyte source, culturing media condition, presence or absence of ascorbic acid, variation in the amount of ascorbic acid, cell density when plated and the nature of chondrocyte culture such as monolayer versus suspension and culturing substrate. Therefore, interpretation and comparison of data should be done carefully.

The cartilage culture system developed by Boyle et al (1995) was used to investigate the development of CM-1, pyridinium crosslinks and macromolecular content in reconstituted articular cartilage. The advantage of using this culture system is that the chondrocytes maintain their phenotypic expression when cultured on a Teflon filter. Further, the cartilagenous matrix showed that the chondrocyte morphology and macromolecules synthesis was similar to that observed in vivo. The goal of the study presented in this chapter was to investigate the presence and quantity of CM-1 in the cartilage culture system using the protocol documented by Boyle et al (1995) and to determine any difference in the amount of CM-1 in the cartilagenous matrix generated by chondrocytes isolated from rabbit knee joints at two weeks versus ten weeks of age. Also I wanted to investigate whether the collagen crosslinks, pyridinoline (Pyd) and deoxypyridinoline (Dpyd), are formed in vitro. This study on rabbit chondrocyte cultures is an extension of Dr. Boyles' experiments on bovine chondrocyte cultures.
8.2 MATERIALS AND METHODS

8.2.1 In Vitro Cartilage Culture

Articular cartilage was excised from rabbit knee joints (femoral condyle, tibial plateau and patella) from very young white New Zealand rabbits (aged 1-2 weeks, weight 145-160 gm) and mature white New Zealand male rabbits (aged 10 weeks, weight 2 Kg.). Chondrocytes were isolated by sequential enzymatic digestion using protease (0.5% of type IX bacterial from Streptomyces griseus, Sigma, USA) followed by collagenase (0.1% of bacterial collagenase from Clostridium histolyticum, Worthington Biochemicals Corporation, USA). Teflon filter inserts (Millicell CM®, Millipore Corp., Bedford, MA. USA) were placed into the wells of the 24 well culture dish, coated with type II collagen (0.5 mg/ml in 0.1N CH₃COOH), dried overnight and UV sterilized for 30 min. The cells were maintained in Ham's F12 medium with 5% rabbit serum and plated on the Teflon inserts at a density of 1.5 x 10⁶/cm². From day 7 onwards, the cells were supplemented with the medium containing 20% rabbit serum and 50 μg/ml ascorbic acid (added fresh at all times) on every alternate day. The cells were grown at 37°C, 100% humidity and 5% CO₂. The cartilage cultures were harvested at fixed time points i.e. day 6, 14, 21, 35, 56, 77 and 98 and stored at -20°C till the analysis day.
8.2.2 Histochemistry and Morphology

The cartilage cultures with filter inserts were immediately fixed with 10% formalin; paraffin embedded and 5 μm thick sections were stained with Haematoxylin and Eosin (to study cellularity), Toluidine blue (to stain the sulphated proteoglycans) and Picrosirius Red (to stain collagen). The cartilage thickness measurements were obtained from a Leitz dialux 22 microscope with a MTI-65 video camera, Hipad digitizing tablet and mouse, a camera lucida and the Bioquant Bone Morphometry program (R & M Biometrics). A low power objective lens (x 6.3) was used and the system magnification factor of x 74.4 was determined by calibrating with a 2 mm length stage micrometer. Cartilage thickness was measured extending from the articular surface to the Teflon insert. Ten measurements were taken from each slide. Two slides represented each time point per culture group and a total of three culture groups.

8.2.3 Electron Microscopy

The cartilage cultures were prefixed in 0.2M phosphate buffer glutaraldehyde (2%) and postfixed in 0.2M phosphate buffer glutaraldehyde with 1% osmium tetroxide. The cartilage blocks were washed in buffer, dehydrated in graded strengths of ethanol, infiltrated with polyethyleneoxide and embedded in Spurr. Thin sections were stained with uranyl acetate and lead citrate.

8.2.4 Water Content

The weight of cartilage cultures was measured before and after lyophilization
overnight. The difference between the wet weight and dry weight of the cultures was used to determine the percent water content of the cartilage cultures. The dry weight of the Teflon filter alone was subtracted from the dry weight of the cartilagenous matrix in order to obtain the exact dry weight.

8.2.5 Collagen Content

The cultures were digested with 20 µg papain/ml buffer (20 mM ammonium acetate, 1 mM EDTA and 2 mM DTT) for 36 hrs. A 20 µl or 30 µl aliquot of the sample was lyophilized and then hydrolyzed by a vapour phase/liquid phase reaction using 6M HCl containing 1% phenol for 22 hours at 110°C. After hydrolysis, the excess HCl was removed from the reaction vial under vacuum and the hydrolysates were redried (methanol : water : triethylamine in ratio of 2:2:1). The sample was then derivitized (methanol : water : triethylamine : phenylisothiocyanate in ratio of 7:1:1:1) for 30 min at room temperature. The derivatizing solution was removed under vacuum and sample was redried to remove any traces of PITC. The sample was resuspended in sample diluent (200 to 500 µl) and injected on a Waters reverse phase high pressure liquid chromatography (HPLC) system at 38°C column temperature. The total amino acid and hydroxyproline composition of the sample was determined using the Waters Pico-Tag amino acid method. The amino acid concentration was determined by comparison to amino acid standards obtained from Beckman (Beckman System 7300/6300) to which was added an equal volume of hydroxyproline standard. The total collagen content was estimated by multiplying the values for hydroxyproline by 10 (Berg, 1982). The Hydroxyproline/Proline ratio was calculated.
8.2.6 Proteoglycan Content

The amount of proteoglycan (from the papain digest aliquot) was estimated by measuring sulphated glycosaminoglycan content using the dimethylmethylen blue (DMMB) dye binding assay (Farndale et al. 1986; Goldberg and Kolibas 1990). Chondroitin sulphate (Sigma Chemicals, St. Louise, MO) was used to generate a standard curve. Colourimetric assays at UV absorbance 525 nm were performed in Titertek 96 well plates and read by Titertek Multiscan MCC/340 (Farndale et al, 1982) and proteoglycan content was calculated.

8.2.7 DNA Content

The DNA content of the culture samples was determined from an aliquot of papain digested cultures. The samples were reacted with Hoescht dye 33258 and the DNA quantitated fluorometrically with emission wavelength of 365 nm and excitation wavelength of 458 nm (Kim et al, 1988). Calf thymus DNA was used as the standard.

8.2.8 Pyridinoline, Deoxypyridinoline and Cartilage Marker-1 Content

Reconstituted cartilage was hydrolyzed with 500 µl of 6M HCl for 24 hours at 110°C in an oven. The hydrolysate was dissolved in 300 µl of mobile phase comprising of 24% acetonitrile, 1% HFBA and 75% distilled water. The hydrolysate was vortexed and filtered with 0.45 µm-pore Gelman filter. An aliquot of 30 µl hydrolysate sample was applied to the analytical HPLC column (A Phenomenex Spherisorb C18 column with dimension of 250 X 4.6 mm, pore size of 5 µm and ODS of 2 protected by a Brownlee C18 guard cartridge). The flow rate was 1.0 ml/minute. Pyd, Dpyd and CM-1
fluorescence was monitored with a Perkin Elmer Fluorescence detector at 295 nm excitation and 395 nm emission. An external calibrator comprising of a mixture of Pyd and Dpyd (purchased from Metra Biosystems) and in house CM-1 calibrator was used to generate a standard curve.

8.3 RESULTS

8.3.1 Histomorphology and Histochemistry

The chondrocytes plated as a monolayer had synthesized extracellular matrix that was evident from its multilayered structure by day 6. With increasing age of the cultures more matrix surrounded the chondrocytes (figure 8.1a and figure 8.1b). By day 21 changes in chondrocyte morphology could be visualized. The chondrocytes towards the superficial lamina were mostly elongated and flattened and those adjacent to the filters were bigger and round. Toluidine Blue staining intensity reflected lower proteoglycan concentration towards the superficial lamina compared to the deep lamina (figure 8.1a). Picro-sirius red stained sections was examined under polarized light microscope. Presence of collagen was evident by day 14. Cartilage collagen architecture, similar to in vivo articular cartilage, was seen by day 21 and more so by day 35. The collagen fibres were oriented parallel to the surface in the superficial lamina and perpendicular to the Teflon filter in the deep lamina. Collagen capsules were also seen surrounding the chondrocytes by day 35. The cartilage thickness varied slightly from one culture group to another. However, the thickness value increased with time (measured up to 56 days) in all the culture groups (table 8.1).
Figure 8.1a Photomicrograph of Regenerated Rabbit Cartilage Cultures (Day 21) Stained with Toluidine Blue Dye Showing the Presence of Proteoglycans. The chondrocytes towards the surface are flattened (similar to in vivo cartilage superficial lamina) and bigger and rounder chondrocytes adjacent (similar to in vivo cartilage deep lamina) to the Teflon filter (Objective 6.3)

Figure 8.1b Photomicrograph of Regenerated Rabbit Cartilage Cultures (Day 56) Showing the Presence of Proteoglycans. (Objective 6.3)
Table 8.1  The Regenerated Rabbit Cartilage Matrix Thickness. \((N = 5)\) at various time points of young rabbits (2 weeks old).

<table>
<thead>
<tr>
<th>Culture Day (sample size)</th>
<th>Cartilage Thickness ((\mu m)) Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6</td>
<td>31 ± 1</td>
</tr>
<tr>
<td>Day 14</td>
<td>66 ± 6</td>
</tr>
<tr>
<td>Day 21</td>
<td>113 ± 6</td>
</tr>
<tr>
<td>Day 35</td>
<td>187 ± 37</td>
</tr>
<tr>
<td>Day 56</td>
<td>254 ± 12</td>
</tr>
</tbody>
</table>

8.3.2  Electron Microscopy

Electron micrographs of the tissue cultures showed the increased synthesis of extracellular matrix from day 6 (figure 8.2a) to day 35 (figure 8.2b-d). The day 35 cartilage culture showed the unique architecture of collagen fibres which aligned parallel to the surface in the superficial lamina (figure 8.2b) and was perpendicular to the filter in the deep lamina (figure 8.2d). The collagen fibres in the interterritorial matrix was randomly oriented whereas those in the territorial matrix surrounded the chondrocytes (figure 8.2c).
Figure 8. 2A Electron Micrograph of Regenerated Rabbit Cartilage Cultures. (A) Day 6 culture showing the chondrocytes, (B) Day 35 showing flattened chondrocytes towards the superficial lamina and collagen fibres aligned parallel to the surface (similar to in vivo cartilage superficial lamina).
Figure 8.2B Electron Micrograph of Regenerated Rabbit Cartilage Cultures.

(C) The middle lamina of day 35 culture showing a part of the chondrocyte and the territorial and interterritorial matrix. (D) The deep lamina of day 35 culture showing the collagen fibres which are aligned perpendicular to the Teflon filter (similar to in vivo cartilage deep lamina).
8.3.3 Water Content

The dry weight of the reconstituted cartilage increased up to day 56 and plateaued thereafter (figure 8.3). The percent water content (figure 8.4) of the cartilagenous tissue from day 14 to day 98 (n = 55; range: 71 to 87) is 80% ± 1 (mean ± SEM). The reconstituted cartilage water content in vitro is comparable with its content in vivo (70% to 80%). The plausible reason for 60% ± 6 of water content in day 6 cultures (n = 6) could be attributed to the quick drying of the thin cartilage. As a consequence, the recorded wet weight was less than the actual value.
Figure 8.3 **Dry Weight of Reconstituted Cartilage Matrix at Various Time Points.**
The articular cartilage was reconstituted from chondrocytes isolated from ~2-week-old rabbit knee joint. The results are expressed as the mean ± SEM of the replicates given as n value.

Figure 8.4 **Percentage Water Content of Reconstituted Cartilage Matrix at Various Time Points.** The results are expressed as the mean ± SEM of the replicates given as n value in figure 8.3.
8.3.4 Collagen Content

The hydroxyproline content forms approximately 10% of total collagen by weight. Therefore, hydroxyproline content was multiplied by a factor of 10 to estimate the collagen content in the cartilage cultures. The result from young rabbit chondrocyte culture is presented in this section. The collagen content increased linearly from day 6 (15 µg ± 5) to day 35 (197 ± 10 µg). When the collagen content was standardized per milligram of cartilage, the collagen content for day 21 (104 µg ± 16 per mg dried cartilage) and day 35 (108 µg ± 10 per mg dried cartilage) was similar. Collagen hydroxylation using the OH-PRO/PRO ratio showed a trend of increased hydroxylation with culture age (figure 8.5). The total protein content of the cartilage cultures increased from 162 µg ± 16 at day 6 to 409 µg ± 8 at day 35.

8.3.5 Proteoglycan Content

The glycosaminoglycan content in the cartilage matrix of the cultures showed a linear increase from 8 µg ± 1 at day 6 to 152 µg ± 5 at day 35 (figure 8.6).
Figure 8.5  Collagen Content of Cartilage Matrix Reconstituted from 2-week-old Rabbit Chondrocytes. Dried cartilage from various incubation time points (5 replicates per time point) were pepsin digested as described under the methods. Each sample was injected into the HPLC column to quantitate the amount of (A) hydroxyproline, (B) collagen, (C) hydroxylation and (D) protein in the cultures. The results represent the cartilage cultures which were harvested at Day 6, 14, 21 and 35 and are expressed as the mean ± SEM of the replicates.
Figure 8.6  Glycosaminoglycan Content of Cartilage Matrix Reconstituted from 2-week-old Rabbit Chondrocytes. Dried cartilage from varying times (9 replicates per time point) were digested with papain and glycosaminoglycan (GAG) content was determined by dimethylmethylen blue dye binding assay and spectrophotometry as described under the methods. The results were shown as (A) GAG content per cartilage filter and (B) GAG content per mg of dried cartilage. The results represent the cartilage cultures which were harvested at Day 6, 14, 21 and 35 and are expressed as the mean ± SEM of the replicates.
8.3.6 DNA Content

Analysis of DNA content within each culture group remained the same when examined i.e., Day 6 up to Day 35. However, a slight variability in DNA content was noted between the culture groups. The results in table 8.2 were obtained from five culture groups and three filters from each group (N = 15). The DNA content of each cartilage culture group showed that either there was no cellular proliferative activity or an equilibrium existed between the rate of DNA synthesis and degradation (DNA turnover). This could be confirmed in a study involving thymidine incorporation in cartilage culture at various time points.

Table 8.2 The Amount of DNA in Cartilage Filter (N = 15) at Various Time Points of Young Rabbits (2-week-old).

<table>
<thead>
<tr>
<th>Culture Day</th>
<th>DNA (μg/filter) Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6</td>
<td>69 ± 1</td>
</tr>
<tr>
<td>Day 14</td>
<td>66 ± 8</td>
</tr>
<tr>
<td>Day 21</td>
<td>67 ± 8</td>
</tr>
<tr>
<td>Day 35</td>
<td>63 ± 5</td>
</tr>
</tbody>
</table>

8.3.7 Pyridinoline, Deoxypyridinoline and Cartilage Marker-1 Content

The number of cartilage filters used for the quantitative analysis of Pyd, Dpyd and CM-1 for the young and old rabbit cultures are shown in table 8.3 and table 8.4. The cartilage culture belonging to both young and old rabbit chondrocyte source increased in dry weight from day 6 to day 56 showing close weight values.
Table 8. 3  A Summary of the Number of Reconstituted Cartilage Culture, Crosslinks, and Cartilage Marker-1 Content when Chondrocytes were Excised from < 2-week-old Rabbit Knee Joints.

<table>
<thead>
<tr>
<th>Culture Day</th>
<th>Cartilage Culture</th>
<th>Pyd Content pmol/mg AC</th>
<th>Dpyd Content pmol/mg AC</th>
<th>CM-1 Content pmol/mg AC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6</td>
<td>n = 5</td>
<td>39 ± 20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Day 14</td>
<td>n = 11</td>
<td>83 ± 14</td>
<td>27 ± 5</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>Day 21</td>
<td>n = 11</td>
<td>100 ± 13</td>
<td>54 ± 12</td>
<td>0.72 ± 0.63</td>
</tr>
<tr>
<td>Day 35</td>
<td>n = 7</td>
<td>206 ± 44</td>
<td>63 ± 11</td>
<td>-</td>
</tr>
<tr>
<td>Day 56</td>
<td>n = 10</td>
<td>259 ± 40</td>
<td>61 ± 12</td>
<td>6.69 ± 2.64</td>
</tr>
</tbody>
</table>

Table 8. 4  A Summary of the Number of Reconstituted Cartilage Culture, Crosslinks, and Cartilage Marker-1 Content when Chondrocytes were Excised from < 10-week-old Rabbit Knee Joints.

<table>
<thead>
<tr>
<th>Culture Day</th>
<th>Cartilage Culture</th>
<th>Pyd Content pmol/mg AC</th>
<th>Dpyd Content pmol/mg AC</th>
<th>CM-1 Content pmol/mg AC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6</td>
<td>n = 10</td>
<td>16 ± 7</td>
<td>11 ± 4</td>
<td>0</td>
</tr>
<tr>
<td>Day 14</td>
<td>n = 7</td>
<td>7 ± 6</td>
<td>2 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>Day 21</td>
<td>n = 9</td>
<td>24 ± 16</td>
<td>24 ± 10</td>
<td>1.17 ± 0.6</td>
</tr>
<tr>
<td>Day 35</td>
<td>n = 12</td>
<td>69 ± 21</td>
<td>33 ± 10</td>
<td>2.23 ± 1.0</td>
</tr>
<tr>
<td>Day 56</td>
<td>n = 4</td>
<td>238 ± 3</td>
<td>52 ± 4</td>
<td>6.27 ± 6</td>
</tr>
</tbody>
</table>
Figure 8.7 Cartilage Culture Reconstituted from Chondrocytes Excised from <2-week-old Rabbit Knee Joints. The chromatographs show the pyridinoline (Pyd, RT=10 min), deoxypyridinoline (Dpyd, RT=11 min) and cartilage marker-1 (CM-1, RT=24 min) from cartilage cultures harvested at day 35 and day 98. Note that by day 98 the area of CM-1 peak is approximately five-fold more than day 35. RT stands for the retention time at which the compound is eluted from the column.

The CM-1 content was first noted at day 21 for both young (0.7 pmol/mg of cartilage) and old (1 pmol/mg of cartilage). By day 56 the CM-1 values increased to 6 pmol/mg of cartilage. Figure 9.8 shows the representative chromatograph for day 35 and day 98 in culture. These cultures were analyzed soon after harvesting. However, when the cultures (stored at -20°C) were analyzed two years later as shown in figure 8.8, 8.9, and 8.10, an underestimation of the exact CM-1 content in the cartilage cultures seemed to occur. There are two plausible explanations leading to the underestimation of CM-1 in the cultures. First, the time duration between harvesting the cultures and actual analysis could decrease the yield of CM-1. Secondly, the ratio of tissue dry weight to volume of HCl is 1 (mg):20 (μl) may have to be optimal. This ratio was determined at a later time.
point when the cartilage cultures were already processed for crosslink and CM-1 analysis. The cartilage cultures were hydrolyzed at a ratio of 1 (mg): 125 (µl) or more. At this hydrolyzing ratio, CM-1 yield is reduced to almost 99% due to degradation of CM-1 in excess of HCl. This could also explain the large SEM observed at day 56 in old cultures.

Both Pyd and Dpyd content increased from day 6 to day 56 in the young and old cartilage cultures. Although the Pyd and Dpyd content of young cultures were higher than old cultures at day 14, 21 and 35, their levels were comparable at day 6 (young = 39 pmol: old = 16 pmol/cartilage mg) and day 56 (young = 259 pmol and old = 238 pmol/cartilage mg).
Figure 8.8  Crosslink and Cartilage Marker-1 Analysis of Articular Cartilage Reconstituted from Chondrocytes Excised from < 2-week-old Rabbit Knee Joint. (A) Dried articular cartilage (AC) from various time points (number of samples [N] is shown in table 8.3) were hydrolyzed with 6M HCl at 110°C as described under the methods. Each sample was injected into the HPLC column to quantitate the amount of (B) cartilage marker-1 (CM-1), (C) pyridinoline (Pyd) and (D) deoxypyridinoline (Dpyd) content. The results (solid line) represent the cartilage cultures harvested at Day 6, 14, 21, 35 and 56 and are expressed as the mean ± SEM of the replicates.
Figure 8.9 Crosslink and Cartilage Marker-1 Analysis of Articular Cartilage Reconstituted from Chondrocytes Excised from 10-week-old Rabbit Knee Joints. (A) Dried articular cartilage (AC) from various time points (number of samples [N] is shown in table 8.4) were hydrolyzed with 6M HCl at 110°C as described under the methods. Each sample was injected into the HPLC column to quantitate the amount of (B) cartilage marker-1 (CM-1), (C) pyridinoline (Pyd) and (D) deoxypyridinoline (Dpyd) content. The results (solid line) represent the cartilage cultures harvested at Day 6, 14, 21, 35 and 56 and are expressed as the mean ± SEM of the replicates. Note that cartilage weight, and Pyd, Dpyd, and CM-1 content increased with culture time. The control sample (dash line) was Teflon filter alone without plating the chondrocytes.
Figure 8.10 Comparison of Crosslink and Cartilage Marker-1 Profile. (A) Dried articular cartilage (AC) was reconstituted from chondrocytes excised from < 2-week-old (solid line) and 10-week-old (dash line) rabbit knee joints. The graphs show the (A) cartilage weight, (B) cartilage marker-1 (CM-1), (C) pyridinoline (Pyd) and (D) deoxypyridinoline (Dpyd) content. The results represent the cartilage cultures harvested at Day 6, 14, 21, 35 and 56 and are expressed as the mean ± SEM of the replicates.
8.4 DISCUSSION AND CONCLUSIONS

The cartilage culture system developed by Boyle et al (1995) enabled the chondrocytes, which were plated on Teflon filter inserts, to reconstitute and maintain extracellular cartilagenous matrix. The chondrocytes synthesize extracellular matrix that was evident from its multilayered structure by day 6. With increasing age of the cultures more matrix surrounded the chondrocytes. By day 21 the chondrocyte morphology was similar to that seen in vivo. The chondrocytes towards the superficial zone were mostly elongated and flattened and those adjacent to the filters were bigger and round. Toluidine Blue staining intensity reflected lower proteoglycan concentration towards the superficial lamina compared to the deep lamina. Picro-sirius red stained sections was examined under polarized light microscope. Presence of collagen was evident by day 14. The day 35 culture extracellular matrix showed “Benninghoff’s gothic arch” organization of collagen fibres which were similar to that seen in vivo. Collagen fibres were oriented parallel to the surface in the superficial zone and perpendicular to the Teflon filter in the deep zone. Collagen fibres were observed around chondrocytes by day 35.

During the time period studied (up to 35 days), an increase in cartilage thickness, glycosaminoglycan and collagen content was observed. The glycosaminoglycan content of the cultures showed a linear increase from day 6 up to day 35. The amount of collagen in the cartilage culture at a given time point was determined from the hydroxyproline content. A linear increase in the collagen content was noted up to day 35. An increase in the degree of collagen proline hydroxylation was observed as indicated by
hydroxyproline to proline ratio.

The state of chemical maturation and mechanical stability of collagen may be related to the formation of intermolecular covalent crosslinks in connective tissues namely pyridinoline (Pyd) and deoxypyridinoline (Dpyd). During collagen degradation Pyd and Dpyd are released from the extracellular matrix into the joint. In the cartilage matrix, the concentration of these pyridinium crosslinks increases during early postnatal life. The experiments presented in chapter 8 were performed to investigate whether young and mature bovine articular chondrocytes cultured on Teflon filters synthesized collagens and pyridinium crosslinks. Further, the variation of de novo synthesized pyridinium crosslinks as a function of the age of the chondrocytes was investigated. Mature collagen specific pyridinium crosslinks were detected in the reconstituted cartilage at day 21. The increase in Pyd crosslink was most rapid between days 21 and 35 in young cultures and days 35 and 56 in mature cultures. Petit et al (1996) also reported the presence of collagen crosslinks in their culture system using alginate beads. However, they detected the crosslinks as early as day 7. It usually takes three weeks for the borohydride reducible bifunctional crosslinks to form mature crosslink. Therefore, theoretically three weeks appears to be the right time for the mature crosslinks to be detected in cartilage matrix. CM-1 increased with time in culture. This culture system will hopefully facilitate future studies to investigate the precursor molecule of CM-1.
CHAPTER 9: DISCUSSION AND CONCLUSIONS

Cartilage Marker-1 (CM-1) is a 204 Dalton carbohydrate derived compound that was isolated from calf metacarpophalangeal joint articular cartilage. It was detected from human and animal cartilage samples as well as articular cartilage containing tissues such as intervertebral disk, trachea and sternum. Human and bovine data have showed that CM-1 is much more abundant in articular cartilage obtained from young animals compared to old animals and its level decreases in cartilage with maturation and ageing. Anatomically, CM-1 level increases with the cartilage depth and in this respect it parallels the proteoglycan content. CM-1 was detected in the synovial fluids from patients with joint arthropathy and also from calf metacarpophalangeal joint (table 9.1). Young tissues (meniscus, ligament, and tendon) showed the presence of CM-1 both in human (3-year-old) and animals (< 2 years rabbit). Examination of the various non-cartilagenous tissues in mature individuals and animals did not detect CM-1 indicating that CM-1 is a cartilage specific molecule.
Table 9.1  **Biologic Distribution of Cartilage Marker-1.** The preliminary data outlined in this table shows that Cartilage Marker-1 is a cartilage specific molecule which is also detected in the synovial fluid of patients with joint arthropathy as well as in the normal synovial fluid of bovine metacarpophalangeal joints.

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>CM-1/Tissue (pmol/mg dry weight)</th>
<th>COMMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human Adult Hyaline Cartilage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a.) Articular Cartilage</td>
<td>158 to 274</td>
<td>↓ CM-1 with cartilage depth</td>
</tr>
<tr>
<td>b.) Superficial-Middle Lamina</td>
<td>20 to 109</td>
<td></td>
</tr>
<tr>
<td>c.) Middle-Deep Lamina</td>
<td>84 to 206</td>
<td></td>
</tr>
<tr>
<td>d.) Intervertebral Disk</td>
<td>28 to 442</td>
<td></td>
</tr>
<tr>
<td>e.) Trachea</td>
<td>13 to 131</td>
<td></td>
</tr>
<tr>
<td><strong>Adult Non-Cartilagenous Tissues</strong></td>
<td><strong>NIL</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Human Synovial Fluid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a.) Degenerative</td>
<td>1 to 10 pmol/μl</td>
<td>↓ CM-1 with OA progression</td>
</tr>
<tr>
<td>b.) Inflammatory</td>
<td>5 to 35 pmol/μl</td>
<td></td>
</tr>
<tr>
<td><strong>Human Osteoarthritic Cartilage</strong></td>
<td>0 to 122</td>
<td></td>
</tr>
<tr>
<td><strong>Human Neonate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a.) Articular Cartilage</td>
<td>525</td>
<td>↑ circulation of CM-1 or CM-1 present in myxoid matrix</td>
</tr>
<tr>
<td>b.) Sternum Cartilage</td>
<td>295</td>
<td></td>
</tr>
<tr>
<td>c.) Trachea</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>d.) Liver, Kidney, Intestine</td>
<td>&lt; 35</td>
<td></td>
</tr>
<tr>
<td><strong>Hyaluronan Source</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a.) Vitreous Humor (Bovine)</td>
<td><strong>NIL</strong></td>
<td></td>
</tr>
<tr>
<td>b.) Umbilical Cord (Human)</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>c.) Articular Cartilage (Porcine)</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td><strong>Bovine Articular Cartilage/Synovial Fluid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a.) Entire Cartilage Thickness</td>
<td>300 to 456</td>
<td>↑ CM-1 with cartilage depth</td>
</tr>
<tr>
<td>b.) Superficial-Middle Lamina</td>
<td>0 to 247</td>
<td></td>
</tr>
<tr>
<td>c.) Middle-Deep Lamina</td>
<td>331 to 626</td>
<td></td>
</tr>
<tr>
<td>d.) Synovial Fluid from Metacarpophalangeal Joint</td>
<td>1 to 9 pmol/μl</td>
<td></td>
</tr>
<tr>
<td><strong>Bovine Non-Cartilagenous Tissues</strong></td>
<td><strong>NIL</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Rabbit Articular Cartilage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a.) &lt; 14 days</td>
<td>122 to 340</td>
<td></td>
</tr>
<tr>
<td>b.) &gt; 6 weeks</td>
<td>20 to 79</td>
<td></td>
</tr>
<tr>
<td><strong>Rabbit Non-Cartilagenous Tissues</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a.) &lt; 10 weeks</td>
<td>4 to 16</td>
<td></td>
</tr>
<tr>
<td>b.) = 18 weeks</td>
<td><strong>NIL</strong></td>
<td></td>
</tr>
</tbody>
</table>
Successful isolation and purification of CM-1 has enabled the preparation of the calibrator for tissue quantitation of CM-1. The structure of CM-1 was determined and confirmed from its liquid and solid phase by using the various spectroscopy tools and single phase crystallography respectively. CM-1 is an orthorhombic, pale yellow crystal with aromatic ring structure, a molecular formula of \( C_{11}H_8O_4 \) and IUPAC nomenclature of 2,6-dimethyl-difuro-8-pyrone. It could be a product of condensation or cleavage as a consequence of acid hydrolysis. A control sample (6M HCl without the cartilage) was also prepared in the similar method as the CM-1. As expected, no crystals were formed when seeded for crystallization for up to a period of one month. The absence of CM-1 in the control sample showed that CM-1 is not merely an artefact from 6M HCl itself. The molecular structure of CM-1 that was provided by NMR spectroscopy in solution and x-ray crystallography in crystallized state conclusively showed that there was no structural change upon crystallization of CM-1. A summary of physical and chemical characterization of CM-1 is given in table 9.2.

CM-1 is a stable compound and its stability could be enhanced by enol-keto tautomerization where the \( \Pi \) bonds resonate to stabilize the ring structure. However, when pure CM-1 is treated with prolonged acid hydrolysis, a degradative effect was observed. This may relate to the nucleophilic elimination reaction on the carbonyl carbon atom which could further lead to either ring opening mechanism or rearrangement into a non-aromatic hence non-fluorescent compound.

Amongst the known weak and strong acids such as acetic acid, sulphuric acid, nitric acid and hydrochloric acid, only HCl the strongest acid, yielded CM-1 indicating that HCl is essential for CM-1 extraction. Heptfluorobutyric acid (HFBA), an ion
pairing reagent, is required for the optimal fluorescence of pyridinoline and deoxypyridinoline. Absence of HFBA in the mobile phase did not have any effect on the fluorescence of CM-1.

Table 9.2 Physical and Chemical Characterization of Cartilage Marker-1.

<table>
<thead>
<tr>
<th>METHODOLOGY</th>
<th>FEATURES</th>
<th>CHARACTERISTICS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Phase High Pressure Liquid Chromatography (HPLC)</td>
<td>Excitation</td>
<td>306 nm</td>
</tr>
<tr>
<td></td>
<td>Emission</td>
<td>395 nm</td>
</tr>
<tr>
<td></td>
<td>Mobile Phase</td>
<td>24% Acetonitrile</td>
</tr>
<tr>
<td></td>
<td>Retention Time</td>
<td>25 minutes</td>
</tr>
<tr>
<td>Fluorescence Spectroscopy</td>
<td>Excitation</td>
<td>306 nm</td>
</tr>
<tr>
<td></td>
<td>Emission</td>
<td>395 nm</td>
</tr>
<tr>
<td>Absorption Spectroscopy (UV Spectroscopy)</td>
<td>Solvent</td>
<td>50% Methanol</td>
</tr>
<tr>
<td></td>
<td>Maximum UV</td>
<td>306 nm</td>
</tr>
<tr>
<td></td>
<td>Absorbance</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Molar Extinction</td>
<td>4700 M$^{-1}.cm^{-1}$</td>
</tr>
<tr>
<td>Mass Spectrometry</td>
<td>Molecular Weight</td>
<td>204 Dalton</td>
</tr>
<tr>
<td></td>
<td>Molecular Formula</td>
<td>$C_{11}H_8O_4$</td>
</tr>
<tr>
<td></td>
<td>Empirical Formula</td>
<td>$C_{11}H_8O_4$</td>
</tr>
<tr>
<td>Nuclear Magnetic Resonance Spectroscopy</td>
<td>Proton Spectroscopy</td>
<td># of $^1H$ environments = 2</td>
</tr>
<tr>
<td></td>
<td>Carbon Spectroscopy</td>
<td># of $^{13}C$ environments = 6</td>
</tr>
<tr>
<td></td>
<td>HMQC</td>
<td>$2^1H$ groups attached to $^{13}C$</td>
</tr>
<tr>
<td></td>
<td>HMBS</td>
<td>$2^1H$ &amp; 6 $^{13}C$ environments</td>
</tr>
<tr>
<td>Single Crystal X-Ray Diffraction (Crystallography)</td>
<td>Empirical Formula</td>
<td>$C_{11}H_8O_4$</td>
</tr>
<tr>
<td></td>
<td>Molecular Weight</td>
<td>204 Dalton</td>
</tr>
<tr>
<td></td>
<td>Crystal Class</td>
<td>Orthorhombic</td>
</tr>
<tr>
<td></td>
<td>Percentage Composition</td>
<td>C (64.71%); H (3.92%); O (31.37%)</td>
</tr>
</tbody>
</table>
CM-1 was absent from all the collagen type II (n=6) and collagen type I (n=4) samples that were processed. Although, CM-1 was present in the proteoglycan components of articular cartilage namely, proteoglycan monomer, proteoglycan aggregate, glycosaminoglycans and chondroitin sulphate, due to different purification method and tissue source the derivative carbohydrate fraction of CM-1 could not be ascertained.

CM-1 is different from the fluorophores and chromophores reported by Hormel et al (1991). The collagen CNBr-peptide was measured at fluorescent profiles of 370 nm excitation and 440 nm emission. CM-1 is different from pyridinoline, based on its chromatographic behaviour and certainly different from glycated products that may exhibit fluorescent behaviour such as pyridoxamine, because of the very late elution time. For pentosidine, fluorescence excitation and emission wavelengths are optimal at 335 and 395 nm respectively (Bellmunt et al 1995, Uchiyama 1991, Odetti, 1994). The fact that CM-1 occurs principally in cartilage makes it unlikely that it reflects the presence of pentosidine or conventional amino acids, because these other compounds also exist in non-cartilagenous tissues. Although two fluorophores have been previously characterized in cartilage, pyridinoline and pentosidine, they are accepted as biomarkers for bone and connective-tissue degradation, their measurement is not helpful in OA or rheumatoid arthritis, because they are not specific to cartilage. Preliminary investigation showed that CM-1 levels declined with OA lesion cartilage compared to the intact cartilage tissue with progression of osteoarthritis. CM-1 may be useful in the evaluation of cartilage injury and repair in OA patients. Uchiyama et al (1991) studied fluorescent materials in articular cartilage, and despite characterizing two peaks of fluorescence
excitation. They concluded from their (short) chromatographic analysis, that there are only two types of molecules, pyridinoline and pentosidine, contributing to the fluorescence.

Although CM-1 is specific to hyaline articular cartilage, it was also present in other neonatal connective tissues. It is not known whether CM-1 is necessary to maintain cartilage integrity. Because CM-1 is small, chemically stable, and specific to cartilage, it has promise as a biochemical marker in arthritic conditions. CM-1 may be useful in the evaluation of cartilage injury and repair of patients with cartilage lesions.

**Putative Structure of CM-1 Precursor:**

CM-1 is an aromatic molecule consisting of two methylated furan rings and a central pyrone ring. Based upon the structure of CM-1, we had two speculations regarding the structure of the precursor molecule of CM-1. First CM-1 could be a carbohydrate derived from molecules such as ascorbic acid/sucrose/starch component of articular cartilage. Fennema (1985) presented an integrative flow chart of the major reactions that carbohydrates can undergo during the processing and handling of food. A portion of the flow chart relevant to the speculation of CM-1 precursor molecule is shown in figure 9.1. Since CM-1 has methylated furan and a pyran ring structure and was detected in the carbohydrate fraction of cartilage, it is very likely that one or more proteoglycan fractions could be the precursor molecule of CM-1. The results of the experiment in which exogenous supply of ascorbic acid was added to articular cartilage prior to HCl hydrolysis did not support that ascorbic acid as a precursor molecule of CM-1 (please see chapter 7, section 7.3.5).
Figure 9.1 Major Reactions that Carbohydrates can Undergo During the Processing and Handling of Foods. The abbreviations “nutr” and “Pot” stands for nutritive and potentially respectively. Note that articular cartilage has high concentrations of carbohydrates, namely proteoglycans (Fennema 1995).
Second, CM-1 could be derived from the lipid component of articular cartilage such as from the diketide/polyketide molecules. Diketides/polyketides are naturally-occurring and structurally diverse compounds, most often produced by microorganisms such as fungi and bacteria. The route by which these compounds are formed is one of the most common in nature. Polyketides are precursor molecules for a vast array of natural products with structures varying from simple aromatic compounds to large polycyclic compounds (Simpson, 1987). They are derived from highly functionalized carbon chains and are known to give modified or totally novel compounds mainly through condensation reactions (Staunton and Wilkinson, 1998). A hypothetical synthesis mechanism of CM-1 from polyketide is shown in figure 9.2.

Articular cartilage is one of very few body tissues which has substantial stores of lipid deposits (Kirkpatrick et al, 1982; Le Lous et al, 1981). Chondrocytes naturally accumulate lipid droplets and the individual fatty acids have shown both protective as well as destructive effect on cartilage degradation. Rabinowitz et al (1979) investigated the lipid composition of the tissues of human knee joints. They reported that the lipid profiles of articular cartilage, meniscus and ligaments were comparable where as the percent neutral lipid compositions varied among the different tissues of the knee. Using histochemical and chemical studies, Bonner et al (1975) showed a significant increase in the articular cartilage lipid level with advancing age. Lippiello et al, (1991) reported an association of lipid accumulation in articular cartilage and severity of osteoarthritis. They showed that the level of total fatty acids in articular cartilage elevated with increasing severity of osteoarthritic lesions. Our preliminary results showed that the level of CM-1 decreased with the presence of osteoarthritic lesion.
CM-1 does not appear to co-vary with conditions in which cartilage lipid levels are elevated, and CM-1 is not found in adipose tissue extracts. Therefore, if CM-1 is derived as a condensation product from a lipid precursor, thus is likely to be a lipid polyketide specific for cartilage.

Figure 9.2 Plausible Mechanism of CM-1 Formation from Polyketide Precursor.
CHAPTER 10 FUTURE EXPERIMENTS

10.1 INTRODUCTION

Biochemical markers of cartilage matrix metabolism in body fluids are used to diagnose and monitor cartilage degradation in certain diseases such as osteoarthritis and rheumatoid arthritis. Quantitative biochemical markers are used for monitoring early disease. Despite efforts toward developing a cartilage specific biochemical marker, to date, no biochemical marker has emerged that could be indicative of osteoarthritis progression. CM-1 is a novel compound which is specific to articular cartilage containing tissues in both human and animal samples. The preliminary data showed that CM-1 has the potential as a unique biochemical marker in joint diseases involving articular cartilage degradation. However, to better understand the biophysiological importance of CM-1 in the joint cartilage, it is crucial to investigate the precursor molecule. CM-1 could be a product of molecular condensation or a molecule cleaved from its precursor. CM-1 could be attached to a glycoprotein or carbohydrate itself and is cleaved by harsh HCl and heat treatment that may further undergo rearrangement and formation of a ring structure. The identification of the precursor molecule of CM-1 is likely of biologic importance. Therefore, future experiments should be directed in identifying the precursor molecule. The experiments outlined in chapter 7 indicated that CM-1 is eluted along with the carbohydrate fraction of articular cartilage. Therefore, the presence of CM-1 in glucose-derived fluorescent molecule and glycated proteins should be investigated.
One way of separating the carbohydrate fraction in cartilage is to digest the sample with various enzymes (such as papain, trypsin, collagenase, pepsin, proteinase) or a combination of enzymes. This could be followed by the separation of carbohydrate fraction by using lectin columns. Lectins form a remarkable group of proteins that possess the ability to react reversibly with specific sugar residues. This specificity enables them to bind polysaccharides and glycoproteins. Immobilised lectins have general application in a very wide variety of separation problems and are invaluable tools for isolating and separating glycoproteins, glycolipids, and polysaccharides. The binding reaction between a lectin and the specific sugar residue is analogous to the interaction between an antibody and an antigen. Con A Sepharose is generally used for the separation and purification of glycoproteins, polysaccharides and glycoproteins and in determining changes in composition of carbohydrate containing substances e.g. during development. Wheat Germ Agglutinin (WGA) is a 36,000 molecular weight protein with two identical subunits that binds preferentially to dimers and trimers of N-acetylglucosamine, and to oligosaccharide structures.

A consolidated characterization of CM-1 in human and animal samples was not possible in the present study due to limited number of available samples. Preliminary investigation in human and animal samples have shown that the CM-1 concentration is higher in young versus old cartilage. A thorough characterization of the change in CM-1 level with respect to normal growth, maturation and ageing should be pursued in an animal model in order to establish the baseline level of CM-1 in articular cartilage. It is imperative to identify the anatomic location from which cartilage samples are excised and the state of the knee joint. Hence, the photograph of the dissected joint should be taken
as well as the state of the knee joint reported grossly and histologically. Further, the amount of CM-1 in the articular cartilage and synovial fluid of the same knee joint of each animal should be correlated. Similar studies as those mentioned above should be extended in an experimental model of induced osteoarthritis. To characterize in humans, how levels of the CM-1 in articular cartilage and synovial fluid reflect cartilage matrix integrity, the level of CM-1 in the articular cartilage and synovial fluid should be correlated and the state of the joint studied both grossly as well as histologically. This could be done from autopsy cases with normal or osteoarthritic knee joint. CM-1 levels can be evaluated in synovial fluid samples collected from the knee joint of patients with various form of arthritis or joint arthropathy. CM-1 level from synovial fluid and articular cartilage of cadavers could be correlated. Similar studies from cadavers can be extended investigating the CM-1 levels in the synovial fluid of osteoarthritic knee joint and articular cartilage in particular the lesioned areas (fibrillated cartilage, osteophytic cartilage etc.).

Previous studies have shown that load bearing has an effect on the metabolism of cartilage macromolecules. The effect of loading on the production of CM-1 could be investigated by determining the anatomic variation in the distribution of CM-1 in the various quadrants of the joint, i.e. lateral and medial femoral condyle and tibial plateau and load bearing versus non-load bearing aspect of the joint.
10.2 PURIFICATION OF PRECURSOR MOLECULE OF CM-1

To determine more precisely the cartilage fraction containing CM-1, articular cartilage will be enzymatically degraded by using a combination of degradative enzymes (collagenase, trypsin, papain, and proteinase). Using a carbohydrate separation column such as wheat germ agglutinin and size-exclusion chromatography, fractions of the precursor compound will be separated. An aliquot of the collected fractions will be tested for CM-1. Three to four fractions containing the best yield of CM-1 will be used for further experimentation and also for immunising the animals for antibody production.

Articular cartilage will be excised from calf metacarpophalangeal joints using sterile technique and incubated for a month with excess and varying amounts of glucose under physiological conditions of pH and temperature. A control sample consisting of cartilage only (without glucose) will also be subjected to the above conditions. CM-1 will be quantified using HPLC as mentioned in chapter 5. Could CM-1 be formed from furosine, which is a by-product of acid hydrolyzed glycated protein? To verify the derivation of CM-1 from glycated compounds the presence of CM-1 will be investigated in acid hydrolysates of glycated neopentylamine, furosine, and browned poly-L-lysine. Also the presence of CM-1 in basic solutions should be investigated.

10.3 CHARACTERIZATION OF CM-1 IN SYNOVIAL FLUID AND ARTICULAR CARTILAGE

Preliminary reports (Chapter 6) in both human and rabbit samples have shown that CM-1 is present in higher concentration in young, immature articular cartilage
compared to old cartilage. It is speculated that the expression of CM-1 changes during growth and development and that the level of CM-1 in articular cartilage will decrease with maturation. CM-1 could be an important marker for growth and development. However, its level could be elevated in children suffering from cartilage related diseases such as Legg-Calve-Perthes disease.

Preliminary results (Chapter 6) have shown that CM-1 is a cartilage specific marker, which can be used as an indicator of disease stage. A well-defined study in a model of osteoarthritis such as that induced by cruciate ligament transection, meniscectomy or papain injection, is necessary to correlate the changes of CM-1 in cartilage and synovial fluid at various stages of OA.

10.4 MANIPULATION OF CARTILAGE CULTURE

The protocol for the tissue culture system outlined in chapter 8 (Boyle et al, 1995) will be followed for this experiment. Cartilage degradative enzymes such as collagenase, inflammatory cytokines and retinol will be used to induce degradative changes in articular cartilage. A very important change in the protocol used in chapter 8 would be to pool the cartilage filters per analysis. For example, six cartilage filters will be pooled together and hydrolyzed with 6M HCl such that the ratio of tissue to HCl is 1 mg/20 µl.

The cartilage culture system will be manipulated to inhibit synthesis of proteoglycans (GAG or core). Xylosides will be added to the culture media which will compete for GAG chain and result in core proteins with very little GAG.
10.5 DEVELOPMENT OF CM-1 DIAGNOSTIC KIT FOR CARTILAGE DEGRADATION

CM-1 will be purified as mentioned in chapter 4. There are two approaches to a diagnostic kit based on detection of CM-1: CM-1 fluorescence after HPLC extraction and antibody to CM-1. To be useful clinically, CM-1 must be detectable in blood, urine or synovial fluid. As we can detect CM-1 in synovial fluid, this should be the first target.

10.5.1 CM-1 fluorescence

The HPLC method to measure the CM-1 in synovial fluid, serum, and urine will be optimized so that clinical studies can be carried out. Various volumes of synovial fluid will be hydrolyzed with fixed volume of (0.5 ml) of 6M HCl and hydrolysed at 110°C for 24 hours. The hydrolysate will be dried, redissolved in the mobile phase, and 20 μL is injected onto a C18 column, with fluorescence measured at 295 nm excitation, 395 nm emission. The method will be characterized in terms of reproducibility, linearity, and accuracy (by spiking synovial fluid with known amounts of the CM-1 material). The optimal synovial fluid versus 6M HCl volume will be determined.

10.5.2 CM-1 Antibody

Since CM-1 is a very small molecule (204 Daltons) therefore it would require the attachment of a carrier molecule (ovalbumin, bovine serum albumin or KLH). In CM-1 there is only one site for carrier attachment i.e. at the carbonyl functional group of the pyrone ring. The CM-1-carrier complex will be used to immunise 4 rabbits and 4 goats.
To develop antibodies against the hypothesized precursor carbohydrate compound of CM-1, three fractions of enzymatically degraded articular cartilage containing the best yield of the CM-1 will be used as an immunogen to immunize 4 rabbits and 4 goats per fraction. For the carbohydrate fractions with the molecular weight less than 10, the preparation will be combined with Freund's complete adjuvant prior to injection into the animals.

Each animal will be immunized following a distinct immunization schedule over a period of up to two years. Long immunization schedules will be needed to get high affinity antibodies to small molecules. Pre-immune serum will be collected initially (~ 10 ml for rabbit and ~ 50 ml for goat). Serum samples will be collected from each animal 10 days to two weeks after each booster injection. Booster injections will be at least 3 weeks apart with the first booster injection given at least 1 month after the initial immunization. Animal bleeds will be collected on a monthly basis 2 weeks after each booster injection of the immunogen. The bleeds will be screened for the development of respective antibodies to the injected immunogen. If one or more animals do develop high affinity antibodies then larger volumes of blood would be collected (up to ~ 30 ml in rabbits and 250 ml in goats) at least three weeks apart. Blood samples will be collected by ear (rabbit) or jugular (goat) vein puncture. Antibodies will be purified using HPLC. Production of antibodies should greatly facilitate future research on the precursor of CM-1 in disease process and abnormality in growth process in children e.g. in the Legg-Calve-Perthes disease.

The antibodies developed against the precursor molecule of CM-1 will be purified
and utilized for immunoassays such as enzyme linked immuno-absorbent assay (ELISA), immunoradiometric assay (IRMA) or Radioimmunoassay (RIA). A reliable, reproducible, and easy to perform quantitative assay for measuring CM-1 could be used as a clinical tool for assessing cartilage degradation in tissue fluids. Preliminary results did not detect CM-1 in the serum or urine samples when measured using HPLC.Hopefully, the development of an easy to use diagnostic immunoassay kit will greatly improve the sensitivity and assay time involved to quantify CM-1 concentration in synovial fluid, serum and urine samples of patients with cartilage degradation. The diagnostic immunoassay kit has great potential to serve as a clinically relevant assay to determine hyaline cartilage matrix degradation.

10.6 CONCLUSIONS

CM-1 is a product of HCl hydrolysis whose biological function is unknown. CM-1 is extracted under harsh non-biologic conditions i.e., hydrolysis of articular cartilage with 6M HCl at 110°C for 24 hours. Such conditions do not occur biologically within the joint cartilage. However, its specificity to articular cartilage raises considerable interest to have its precursor molecule identified. To identify and synthesize the precursor molecule is a great challenge. It is important to isolate and investigate the possible biologic and physiologic function of the precursor compound of CM-1. Further work is required to clearly delineate the functional basis of CM-1 and its precursor and their contributions to the pathophysiology of a cartilage related diseases. As CM-1 is stable and can be measured in synovial fluid, it is a prime candidate for a specific biochemical marker for cartilage matrix degradation.
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APPENDIX A

CARTILAGE MATRIX STUDIES IN RECONSTITUTED BOVINE CARTILAGE

A1.1 INTRODUCTION

The putative role and mechanism of action of cytokines in the progression of arthritic diseases such as osteoarthritis (OA) has received particular attention because of the important interaction between articular cartilage and synovium in the pathophysiology of the diseased state. Maintaining matrix homeostasis in the normal adult cartilage phenotype requires normal turnover of matrix components, principally collagen and proteoglycan. Cytokines, such as interleukin 1 (IL-1) and tumor necrosis factor α (TNF-α), are known to favor tissue destruction by stimulating chondrocytes to produce metalloproteinases (collagenase, proteoglycanases and stromelysine) which are responsible for the damage of collagen and proteoglycans (PG) and also for suppression of collagen and proteoglycan synthesis. Hence, cytokines not only favor tissue destruction, but also inhibit tissue repair.

The purpose of the experiments outlined in this appendix was to investigate the effects of interleukin-1β (IL-1β) on cartilage reconstituted from chondrocytes isolated from the superficial and deep laminae of calf articular cartilage. In particular, the goal was to investigate the changes in pyridinoline (Pyd), deoxypyridinoline (Dpyd) and cartilage marker-1 (CM-1) level of IL-1β treated reconstituted cartilage matrix.
A1.2 MATERIALS AND METHODS

Articular cartilage was excised separately from the superficial-middle (SM) or middle-deep (MD) lamina of calf metacarpophalangeal joint using sterile technique as described by Boyle et al (1995). The cartilage slices were placed in a petri dish containing Ham's F12 and 1% antibiotics. Chondrocytes were isolated from the SM and MD extracellular matrix by sequential enzymatic digestion by using protease (1 hour, 37°C - 0.5% of type IX bacterial from Streptomyces griseus, Sigma, USA), washed three times with Ham’s F12 medium, and finally digested with collagenase A (overnight, 37°C - 0.1% of bacterial collagenase from Clostridium histolyticum, Worthington Biochemicals Corporation, USA). Teflon filter inserts (Millicell CM, Millipore Corp., Bedford, MA, USA) were placed into the wells of the 24 well culture dish, coated with type II collagen (0.5 mg/ml of 0.1N acetic acid), dried overnight, and UV sterilized for 30 min. The filter inserts were washed thoroughly with Ham’s F12 before plating the cells. The isolated SM and MD chondrocytes were washed extensively, resuspended in Ham’s F12 with 5% foetal bovine serum (FBS), and plated on the teflon inserts at a density of 3.0 x 10^6/cm^2. The cells were supplemented with the medium containing 20% FBS and 100 μg/ml ascorbic acid (added fresh at all times) from day 7 onwards every alternate working day. Some cartilage filter cultures were treated with 10 ng/ml interleukin-1β (IL-1β, human recombinant, Sigma) for 7 days (three changes of the Ham’s F12 with 20% FBS and IL-1β) prior to harvesting. The chondrocyte cultures were grown at 37°C (with 100% humidity and 5% CO₂), harvested at fixed time points (day 21, 35, and 56), and stored at -20°C till the analysis day.
The untreated (controls) and IL-1β-treated SM and MD cartilage cultures were analyzed for cartilage thickness and collagen, proteoglycan, pyridinoline, deoxypyridinoline and cartilage marker-1 content as described in chapter 8, section 8.2.

A1.3 RESULTS

A1.3.1 Histochemistry and Morphology

The thickness of the cartilage cultured from the SM and MD chondrocytes increased from Day 21 to 35 (figure A1). Beyond day 35, a slight increase in cartilage thickness was noted in both the groups (SM and MD). The cartilage reconstituted from the chondrocyte of the SM lamina was thinner than that reconstituted from the DM lamina for the same time point. Furthermore, the IL-1β treated cultures had thinner cartilage compared to the untreated controls for both SM and MD groups.

A1.3.2 Proteoglycan Content

The glycosaminoglycan (GAG) content of the SM and MD cartilage cultures increased from day 21 to day 56. However, the GAG content of the MD group at the time points studied was greater than those of the corresponding time points of the SM group (figure A2). The GAG content of both SM and DM groups decreased when treated with IL-1β for a period of 7 days (three media change) prior to harvesting at the given time point.
A1.3.3 Collagen Content

The collagen content of the SM and MD cartilage cultures increased from day 21 to day 56. The collagen content from SM and MD group was similar at the time points studied. The collagen content of both SM and DM groups decreased when cultures were treated with II-1β for a period of 7 days (three media change) in particular on day 56 (figure A3).
Figure A1  The Effect of Interleukin-1β (II-1β) 7-day Treatment on the Thickness of Cartilage. Cartilage was reconstituted from the chondrocytes which were isolated from the superficial-middle (SM) lamina versus middle-deep (MD) lamina of calf metacarpophalangeal articular cartilage.
Figure A2  The Effect of Interleukin-1β (IL-1β) 7-day Treatment on the Glycosaminoglycan Content of Cartilage. Cartilage was reconstituted from the chondrocytes which were isolated from the superficial-middle (SM) lamina versus middle-deep (MD) lamina of calf metacarpophalangeal articular cartilage.
Figure A3  The Effect of Interleukin-1β (IL-1β) 7-day Treatment on the Collagen Content of Cartilage. Cartilage was reconstituted from the chondrocytes which were isolated from the superficial-middle (SM) lamina versus middle-deep (MD) lamina of calf metacarpophalangeal articular cartilage.
A1.3.4 Pyridinoline, Deoxypyridinoline and Cartilage Marker-1 Content

The CM-1 in these cultures was not detected (figure A4 and figure A5) because the cultures were hydrolyzed at the ratio of 1 mg cartilage: 200 \( \mu l \) HCl. The optimal ratio of tissue dry weight to volume of HCl is 1 (mg): 20 (\( \mu l \)). Beyond this established optimal hydrolysis ratio CM-1 was shown to degrade. At the hydrolysis ratio used above 100\% of CM-1 is degraded. Furthermore, the addition of exogenous ascorbic (0.1 mg / ml) could have some quenching effect (~5\% decrease on 50 mg cartilage sample) on the production of CM-1. Alternatively, the two years duration between harvesting the cultures and analysing them could have played an important role in the degradation of CM-1.

The crosslinks Pyd and Dpyd increased in SM and MD cultures (figure A4 and figure A5). A decrease in the Pyd and Dpyd content of Il-1\( \beta \) treated cultures was noted at day 35 and more so at day 56. In general, the MD chondrocyte secretes a matrix which has more crosslinks than that secreted by the SM chondrocytes.
Figure A4  The Effect of Interleukin-1β (II-1β) 7-day Treatment on the Cartilage Weight, and Pyridinoline, Deoxypyridinoline and Cartilage Marker-1 Content of Cartilage. Cartilage was reconstituted from the chondrocytes which were isolated from the superficial-middle (SM) lamina of calf metacarpophalangeal articular cartilage.
Figure A5  The Effect of Interleukin-1β (II-1β) 7-day Treatment on the Cartilage Weight, and Pyridinoline, Deoxypyridinoline and Cartilage Marker-1 Content of Cartilage. Cartilage was reconstituted from the chondrocytes which were isolated from the middle-deep (MD) lamina of calf metacarpophalangeal articular cartilage.
A1.4 DISCUSSION

Several factors are known to influence the phenotype of chondrocytes in vitro. The behaviour of chondrocytes in culture may differ from in vivo characteristics. Boyle et al. (1995) have shown that under the culture conditions they described for reconstituted articular cartilage, chondrocytes maintain their normal phenotype as determined by the expression of type II collagen and aggregan.

Interleukin-1β (Il-1β) plays an important role in the destruction of cartilage matrix in arthritis. Different responses to Il-1β have been demonstrated by bovine chondrocytes derived from different laminae of cartilage in terms of PG and collagen synthesis. Il-1β has shown to inhibit the expression of mRNA for type II collagen (14f) and reduce the synthesis of type II collagen (Lefebvre et al., 1990).

Addition of Il-1β (10 ng/ml) to the cartilage cultures stimulated matrix degradation as demonstrated by the decrease in cartilage thickness, GAG, collagen, and crosslink content compared to the untreated cartilage cultures. Our results corroborate previous studies in which Il-1β has been shown to induce proteoglycan degradation in vitro (Saklatvala et al., 1984; Loulakis et al., 1992; Aydelotte et al., 1992). Unfortunately the effect of Il-1β on the CM-1 content could not be recorded because of underestimation of its content in the cultures analyzed.

Chondrocytes from the superficial lamina of articular cartilage developed matrix at a slower rate in vitro and appeared to show a greater vulnerability to the deleterious effect of Il-1β than cartilage derived from the chondrocyte from the deeper lamina.
A1.5 CONCLUSIONS

The bovine chondrocytes synthesized cartilaginous matrix in vitro showing the presence of collagen and glycosaminoglycan. The synthesized collagen formed mature collagen crosslinks namely pyridinoline and deoxypyridinoline. The GAG, collagen, and crosslink content were affected when the cultures were treated with interleukin-1β. Under the prevailing experimental condition, CM-1 was not detected in the bovine cultures.

A1.6 REFERENCES


APPENDIX B

The following three abstracts were presented as posters at the 10th Annual Meeting - OsteoArthritis Research Society International (OARSI) Congress at Vienna, Austria on September 16 to September 19, 1999. The following three abstracts were published in the Journal of Osteoarthritis and Cartilage (Volume 7 Supplement A).


A NOVEL ARTICULAR CARTILAGE BIOCHEMICAL MARKER: CHEMICAL CHARACTERIZATION

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AIM: While investigating collagen cross linking and autofluorescence in articular cartilage, a fluorescent peak of unknown characteristics in the HPLC eluate was detected. This peak is now termed "cartilage marker - 1 (CM-1)". We developed methods to successfully isolate, purify, and identify CM-1 from cartilage.

MATERIALS AND METHOD: Fully encapsulated bovine metacarpophalangeal joints (n=350, age < two yrs) were used as the source for articular cartilage. The articular cartilage was excised, lyophilized, and hydrolyzed using 6N HCl at 110°C for 24 hours. CM-1 was purified using reverse phase HPLC and monitored with a fluorescence detector (excitation 306 nm and emission 395 nm). Further purification (100%) was obtained by crystallizing CM-1 in 100% methanol. A control sample (6N HCl without the cartilage) was also prepared in the similar method as the CM-1.

RESULTS: A novel articular cartilage specific compound was detected, isolated and identified. This compound has an IUPAC nomenclature of 2,6-dimethyl-difuro-8-pyrone and molecular formula of C11H8O4. CM-1 was identified by mass spectroscopy, nuclear magnetic resonance spectroscopy, and single crystal x-ray diffraction. The results from the various analytical techniques used to identify CM-1 corroborate well confirming the molecular structure of CM-1 and its atomic connectivity as shown in figure 1. CM-1 is an aromatic compound with a high degree of symmetry, molecular weight of 204 daltons, maximum UV absorbance of 306 nm and a molar extinction coefficient of 4700 Mole\(^{-1}\) cm\(^{-1}\). CM-1 was absent in the control samples.

![Figure 1: CM-1 molecule has a total of 11 carbons, 8 protons and 4 oxygens.](image)

CONCLUSIONS: CM-1 appears to be a unique molecule present in articular cartilage in concentrations approximating collagen cross links. CM-1 can be detected in cartilage extracts by its characteristic fluorescence and eluate time relative to collagen cross links.
PRESENCE OF CARTILAGE MARKER-1 IN BOVINE AND RABBIT
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AIM: Cartilage Marker-1 (CM-1) is a novel cartilage specific compound recently isolated and characterized in our laboratory. We aimed to identify and quantify CM-1 in various calf and rabbit tissue samples; to determine the cartilage layer of origin of CM-1 (superficial-middle versus middle-deep layer); and to investigate the changes in CM-1 content with ageing in rabbit knee joint articular cartilage.

MATERIALS AND METHOD: Tissue samples were obtained from calf (16-20 weeks) and rabbit (<2, 6, 10 and 48 weeks) articular cartilage, synovial fluid, and intervertebral disc as well as several other noncartilagenous connective tissues. The samples were lyophilized, weighed, hydrolyzed with 6N HCl for 24 hours at 110° C and dissolved in mobile phase (24% acetonitrile, 1% heptafluorobutyric acid and 75% distilled water). An aliquot of 30 µl hydrolysate was applied to the HPLC analytical column attached to HPLC system. CM-1 fluorescence was monitored at 295 nm excitation and 395 nm emission. The sample peaks were quantified using the in house CM-1 calibrator.

RESULTS: CM-1 was detected from calf metacarpophalangeal joint cartilage (362 ± 48 pmol / mg of dry weight cartilage); synovial fluid (4.54 ± 0.7 pmol / ul of synovial fluid); and intervertebral disc (24.3 ± 4 pmol / mg dry weight) and in rabbit knee joint articular cartilage. CM-1 was not present in calf ligament, tendon, bone, ocular lens, cornea, elastic cartilage, or in rabbit (48 weeks) ligament, tendon, meniscus or bone. A significant difference was noted between the superficial-middle (129 ± 52) and middle-deep layers (448 ± 63) (paired T-test, p = 0.008) as well as superficial-middle layer and entire cartilage thickness (p = 0.2). There was no significant difference between middle-deep and entire cartilage thickness (p= 0.36). The amount of CM-1 decreased with increasing age in rabbit articular cartilage from an average of 185 ± 40 (<2 weeks), 52 ± 5 (6 weeks), 41 ± 4 (10 weeks) to 27 ± 3 (48 weeks) pmol / mg of dry weight cartilage.

CONCLUSIONS: CM-1 is a cartilage specific compound which is present in articular cartilage and intervertebral disc. CM-1 concentrate increases from the superficial to deep layer of articular cartilage. This result correlates with our finding that CM-1 is a product of acid hydrolysis of the carbohydrate moiety of articular cartilage.
EFFECT OF INTERLEUKIN-1β ON COLLAGEN, COLLAGEN CROSSLINKS AND PROTEOGLYCAN CONTENT OF RECONSTITUTED ARTICULAR CARTILAGE

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AIM: This study aimed to investigate the effects of Interleukin-1β (IL-1β) on cartilage reconstituted from the chondrocytes which were isolated from the superficial and deep layers of calf articular cartilage.

MATERIALS AND METHOD: Articular cartilage was excised separately from the superficial-middle (SM) or middle-deep (MD) layers of calf metacarpophalangeal joint using sterile technique. The isolated SM and MD chondrocytes were resuspended in Ham F12 with 5% fetal bovine serum and plated on teflon inserts at a density of \(3.0 \times 10^6/cm^2\). The cells were supplemented with the medium containing 20% FBS and 100 μg/ml ascorbic acid from day 7 (thrice a week). Some cartilage cultures were treated with 10 ng/ml IL-1β for 7 days (three changes of medium) prior to harvesting on day 21, 35, and 56. The control and IL-1β-treated cartilage cultures were analyzed for cartilage thickness, total protein, collagen, proteoglycan, pyridinoline, and deoxypyridinoline content.

RESULTS: Cartilage thickness of both treated and control cultures from SM and MD increased from Day 21 to 56. Cartilage reconstituted from chondrocytes of the SM layer was thinner than the DM layer. Furthermore, IL-1β treated cultures had significantly thinner cartilage compared to the controls. Treatment of the cartilage cultures with IL-1β for one week decreased the collagen content (by two fold) and the total protein content. The superficial layer was significantly lower than the deep layer in its collagen content \((p=0.003)\) and total protein content \((p<0.0001)\). The glycosaminoglycan (GAG) content of the SM and MD cartilage cultures increased from day 21 to day 56. However, the GAG content of the MD group at the time points studied was greater than those of the corresponding time points of the SM group. The GAG content of both SM and DM groups decreased when treated with IL-1β. The pyridinoline and deoxypyridinoline content of the cartilage cultures decreased with treatment on day 35 and 56.

CONCLUSIONS: Chondrocytes from SM layer developed matrix at a slower rate in vitro and appeared to be more susceptible to IL-1β induced damage to articular cartilage than the DM layers.
APPENDIX C

Cartilage Marker-1 (CM-1):

Novel Compound And Methods Of Diagnosis Using The Compound (2,6-Dimethyl-Difuro-8-Pyrone and related compounds)