CHAPTER I
INTRODUCTION

1.1 Statement of the problems

The major clinical manifestations of rheumatoid arthritis (RA) and osteoarthritis (OA) are degraded cartilage, synovial, and bone tissue, resulting in severe mobility impairment. The severity of the disease is generally scored according to pain and mobility indices, and even though a number of standardized rating systems have been introduced, it remains difficult to quantify these parameters. In RA, inflammation parameters such as the erythrocyte sedimentation rate (ESR) and, more interesting, a highly sensitive assay for C-reactive protein (CRP) provide useful information about the general inflammation process. However, these biological markers are not joint specific and are poorly correlated with cartilage damage at the individual level. Thus, radiography continues to be the best-established method of assessing joint damage in OA/RA.

Change of joint space width assessed by radiography remains the gold standard, but it allows neither early detection of joint tissue damage nor efficient monitoring of the efficacy of treatment aimed at preventing joint destruction, because of its poor sensitivity and relative large precision error. Clearly, for identifying patients at high risk for destructive OA/RA and for monitoring the efficacy of new structure-modifying therapies, there is a need for better technique than radiography. Alternatively, specific and sensitive biochemical markers reflecting abnormalities of cartilage, synovium, and bone tissues may be useful for the investigation and monitoring of OA/RA (Garnero *et al.*, 2000).

Several assays have been developed that recognize nonspecific proteoglycan fragments epitopes from the core protein generated by metalloproteinase (Shikhman *et al.*, 2000), hyaluronidase, or fragments containing either KS or CS. According to the specificity of each antibody, a competitive enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody recognize these epitope can reflect mostly synovium or cartilage metabolism and reflect either aggrecan (hyaluronan-binding protein) destruction or synthesis (Saxne *et al.*, 1985).

A homologous N-terminal proteolytic fragment of aggrecan, termed the G1 domain, is responsible for binding to both link protein and hyaluronan (HA). An equivalent region is also found in the aggrecan-related proteoglycans (versican) in cartilage. These HABPs would represent candidate biochemical markers likely that the arthritis-associated protein tumor necrosis factor stimulated gene-6 (TSG-6) produced locally in inflamed joint and serve as a useful marker in arthritis (Kohda *et al.*, 1996). Serum derived hyaluronan-associated protein-hyaluronan (SHAP-HA) complex is also as joint marker that directly correlated to the degree of joint inflammation in RA (Kida *et al.*, 1999).

The aims of this study were to develop competitive ELISA method for hyaluronan binding proteins (HABPs) determination by using mAb 1H8 and evaluate the concentration of HABPs in human serum from normal subjects and patients with OA/RA. HABPs concentrations are compared to the concentration of HA in the same sample.

1.2 Literature review

1.2.1 Articular cartilage

Articular cartilage is a highly specialized tissue that allows for unique functions within synovial joints (Hunter, 1743). It forms the articulating surfaces in diarthrodial joints (figure 1) and endows a joint with low-friction surfaces and in conjunction with ligaments, tendons and menisci, transmits and distributes forces to the underlying bone. The biomechanical properties of articular cartilage result from a complex interaction of genetic, environment and growth leading to a heterogeneous (Buschmann *et al.*, 1996). Disruption of the cartilage framework results in alternations of its properties and lead to the patient's perception of pain, loss of motion, strength, or instability.

Articular cartilage consists of chondrocytes and an extracellular matrix with water. The chondrocytes are highly differentiated cells that account for only about 5% of the total volume and are quite metabolically active, despite limited ability to replicate. These cells are responsible for synthesizing and maintaining the extracellular matrix through the formation of proteoglycan, collagen, non-collagenous proteins, and glycoproteins. They also form degradative enzymes that are responsible for the normal turnover of these macromolecules (Stockwell, 1979).

The structure and composition of the articular cartilage vary throughout its depth, from the articular surface to the subchondral bone. These differences include cell shape and volume, collagen fibril diameter and orientation, proteoglycan concentration, and water content. The articular cartilage can be divided into 4 zones: the superficial zone, the middle or transitional zone, the deep zone, and the zone of calcified cartilage. The superficial zone is the uppermost zone of the cartilage and forms the gliding surface. The thin collagen fibrils are arranged parallel to the surface, the chondrocytes are elongated with the long axis parallel to the surface, the proteoglycan content is at its lowest level, and the water content is at its highest level. The middle or transition zone contains collagen fibers with a larger diameter and less apparent organization, and the chondrocytes have a more rounded appearance. The deep zone contains the highest concentration of proteoglycans and the lowest water content. The collagen fibers have a large diameter and are organized perpendicular to the joint surface.

In adults human, articular cartilage is aneural, avascular, and alyphatic. The subchondral plate in health human is impervious to blood vessels. Articular cartilage derives its nutrition by diffusion system (Mankin *et al.*, 1994). Nutrients must first diffuse across the synovial membrane into the synovial fluid and then through the dense matrix of the cartilage to reach the cartilage cells (McKibben *et al.*, 1966). There are no nerves in articular cartilage, the weight-bearing surfaces of the joint depend on nerve endings in the capsule, muscles, and subchondral bone for appreciation of pain and proprioception (Hogfervorst *et al.*, 1998).

1.2.2 Structural organization of articular cartilage and Extracellular Matrix

The extracellular matrix (ECM) consists of collagen 15-20% of the wet mass, proteoglycan 10-15% of the wet mass, non-collagenous proteins, glycoproteins and water (figure 3).

Articular cartilage is composed 65% to 80 % of water. The maximal volume contents of water in the superficial zone and is progressively reduced with increasing depth in adult human articular cartilage. Type II collagen, and types IX and XI collagens, are organized in fibrils that endow cartilage with its tensile properties. These collagens account for about 15% to 25% of the wet weight and about half of dry weight, except in the superficial zone, where they account for most of the dry weight (Muir, 1979). Type II collagen content is usually progressively reduced with increasing depth from the articular surface (Kempson *et al.*, 1973). The proteoglycan content, mainly the very large molecule called aggrecan, accounts for the compressive stiffness of cartilage. It is responsible for up to 10% of the wet weight or about one fourth of dry weight. Because its glycosaminoglycan chains can bind up to 50 times its weight of water. Aggrecan content increases with depth (Webber *et al.*, 1987). The histologic section of normal adult articular cartilage is shown in figure 2.

The cartilage matrix consists of distinct regions that can be identified by structural difference reveals by their morphology. All chondrocytes are surrounded by a thin pericellular matrix of up to approximately 2 μm thick that contains few well-defined collagen. It is rich in type IV collagen, fibrilin-1, and decorin. The chondrocytes and its pericellular matrix have been called a chondron.

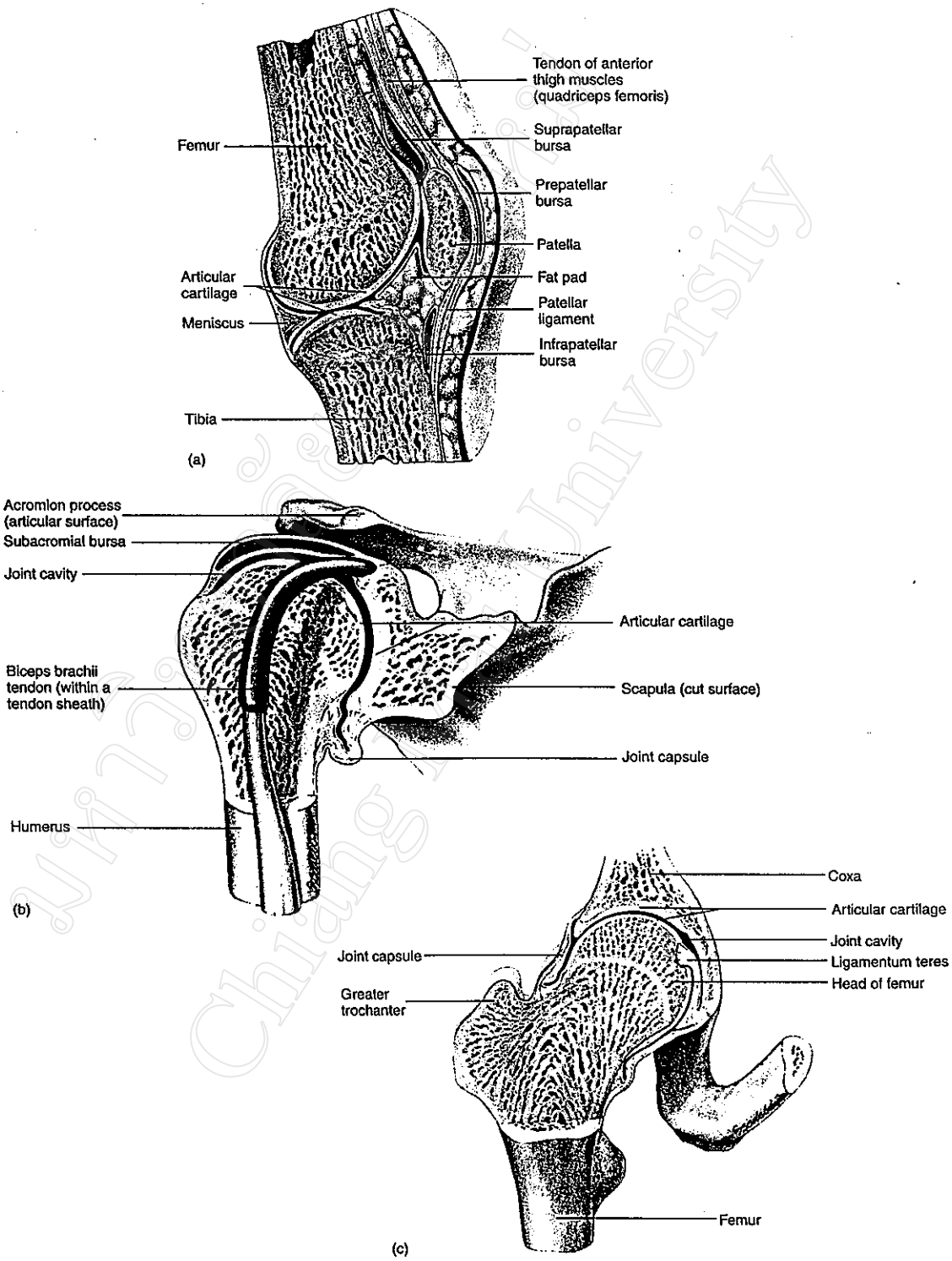

Figure 1 Three selected joints (a) Sagital section through right knee joint. (b) Frotal section through the right shoulder. (c) Frontal section through the right hip joint (Seeley *et al.*, 1999).

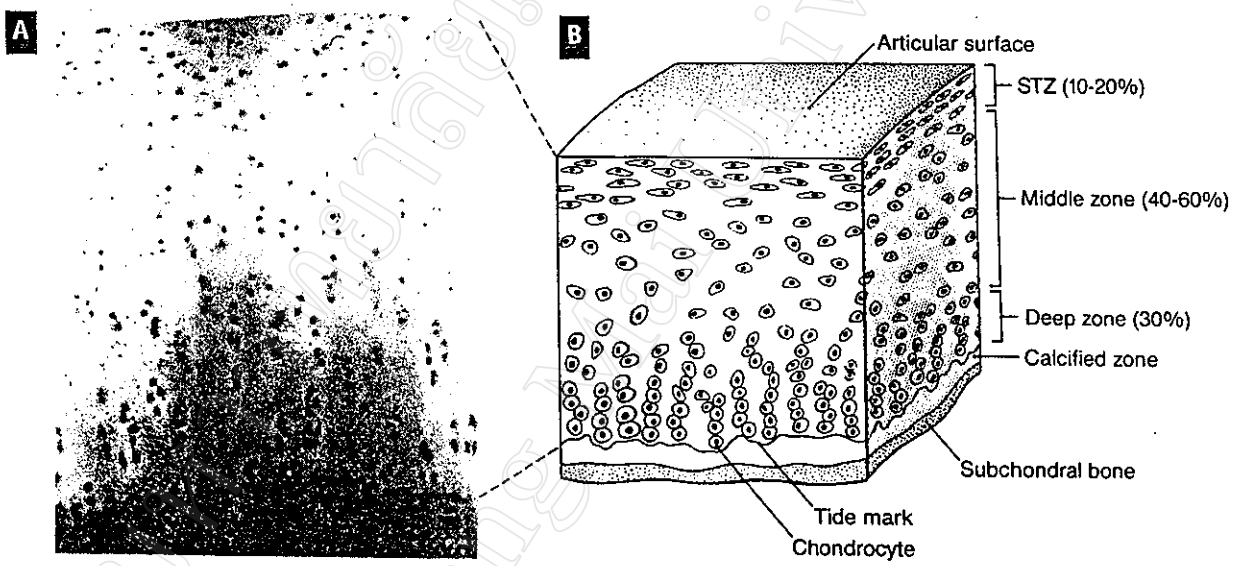

Figure 2 Hitologic section of normal articular cartilage showing. (B) Schematic diagram of chondrocyte organization in the 3 major zones of the uncalcified cartilage (Koopman, 2001)

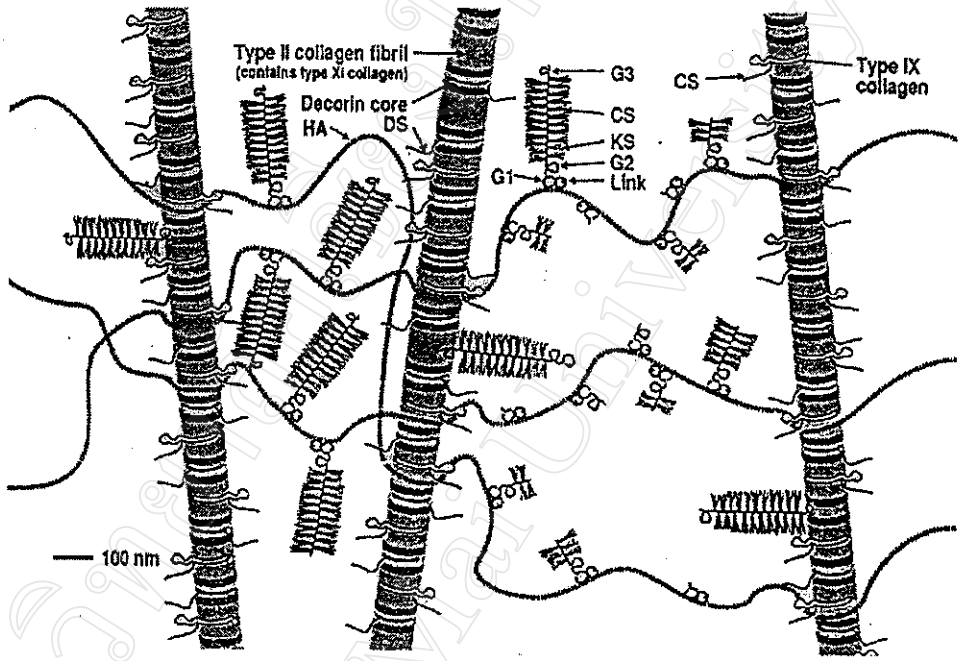

Figure 3 Organization of type II collagen fibrils, containing types IX and XI collagens, link protein and aggrecan, which binds to hyaluronan (HA) in the extracellular matrix of articular cartilage. HA interacts directly or indirectly with collagen fibrils in a periodic manner. This collagen is covalently bound to and has a periodic distribution on type II collagen. Decorin also binds to type II collagen through its core protein in a periodic manner (Poole *et al.*, 1982).

1.2.2.1 Glycosaminoglycan

Glycosaminoglycans (GAGs) consist of long-chain, unbranched, repeating disaccharide units. Chondoitin sulfate 4-and 6-isomers; keratan sulfate (KS); and dermatan sulfate (DS) are the most prevalent glycosaminoglycan in cartilage. Each chain is composed of 25 to 30 repeating disaccharide units, giving an average chain weight of 15 to 20 kD. The keratan sulfate constituent of articular cartilage, which resides primarily in the large, aggregating proteoglycan, is not as well defined as the chondoitin sulfates. The keratan sulfate composition and degree of sulfation vary in human articular cartilage. Keratan sulfate chains from human articular cartilage are shorter than chondoitin sulfate chains with an average molecular weight of 5 to 1 kD. Hyaluronan is also glycosaminoglycan. But it is not sulfated (figure 4).

All the glycosaminoglycan chains found in cartilage have repeating carboxyl and/or sulfate groups. In solution, these groups become ionized and require positive counterions such as Ca^{2+} and Na^{+} to maintain overall electroneutrality. Eighty percent to 90% of all proteoglycans in cartilage are of the large, aggregating type, called aggrecan. They consisted of a long, extended protein core with up to 100-chondoitin sulfate and 50-keratan sulfate GAGs chains covalently bound to the protein core. In young individuals the concentration of keratan sulfate is relatively low, and chondoitin 4 sulfate is the predominant form of chondoitin sulfate. With increasing age, the concentration of keratan sulfate increases and chondoitin 6 sulfate becomes the predominant form of chondroitin sulfate. Structural and functional diversity of glycosaminoglycans was shown in table 1.

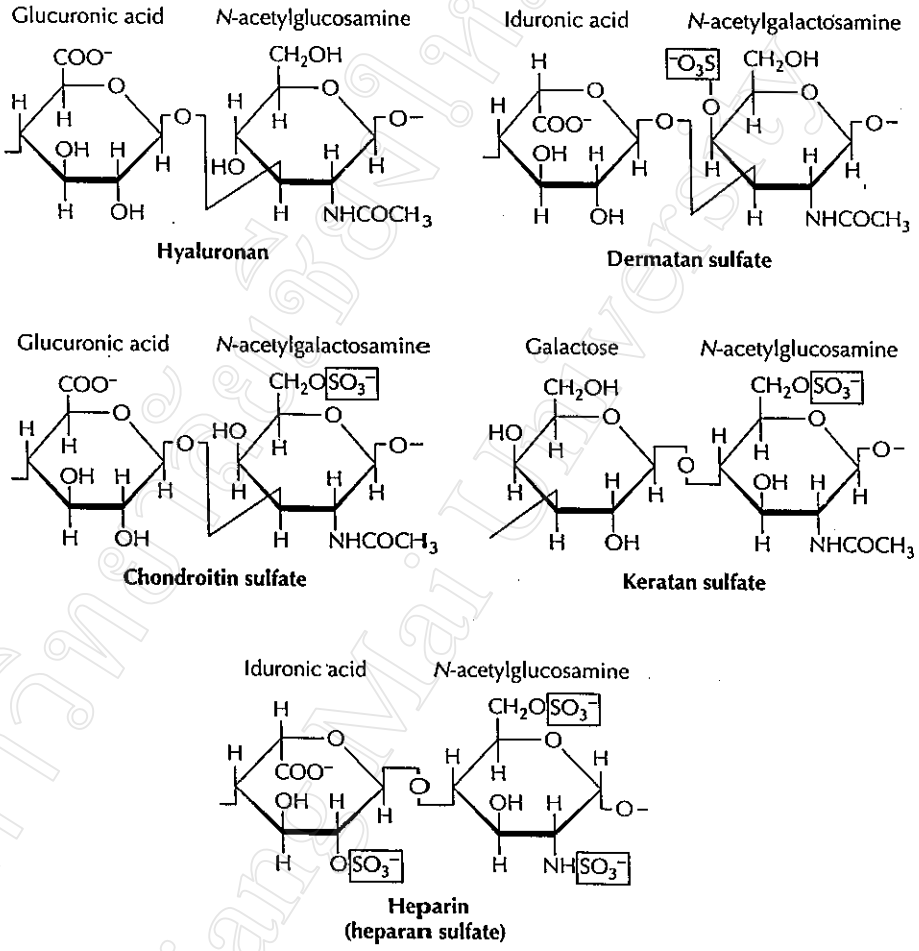

Figure 4 Major types of glycosaminoglycans. Glycosaminoglycans consist of repeating disaccharide units. With the exception of hyaluronan, the sugars frequently contain sulfate. Heparan sulfate is similar to heparin except that it contains fewer sulfate groups (Cooper, 2000).

Table 1 Structural and functional diversity of glycosaminoglycans (Yanagishita, 1997).

Glycosaminoglycans	Function
Chondroitin Sulfate	
GlcAβ1-3GalNAc(4S)β1-4	Receptor for malaria-infected erythrocytes, Receptor for CD44
GlcAβ1-3GalNAc(6S)β1-4	Binding of pleiotrophin to 6B4 proteoglycan
GlcAβ1-3GalNAc(4S,6S)β1-4	Binding of granules in mast cell, Macrophage receptor for lipoprotein lipase, Anticoacglulant activity
GlcA(2S)β1-3GalNAc(6S)β1-4	Neutrient growth promoting activity
Dermatan Sulfate	
IdoAα1-3GalNAc(4S)β1-4	Binding to HGF
IdoA(2S)α1-3GalNAc(4S)β1-4	Binding domain for heparin cofactor II
Keratan Sulfate	
GlcNAc(6S)β1-3Galβ1-4	Transparency of cornea, Mark of immunogenicity of G1 domain of aggrecan
GlcNAc(6S)β1-3Gal(6S)β1-4	
Heparin Sulfate, Heparin	
(IdoA(2S)α1-4GlcNSα1-4)3	Binding domain for FGF2
(IdoA(2S)α1-4GlcNS(6S)α1-4)3	Binding domain for FGF1
(IdoA(2S)α1-4GlcNSα1-4IdoA(2S)α1-4GlcNS(6S) α1-4IdoA(2S)α 1-4GlcNSα1-4IdoA(2S)α1-4GlcNS(6S)	Binding domain for HGF
GlcNAcα1-4GlcAβ1-4GlcNS(3S)α1-4IdoA(2S)α1-4GlcNS(6S)	Binding domain for antithrombin III

1.2.2.2 Proteoglycans

The proteoglycans are a family of glycoconjugates with a central core protein to which glycosaminoglycans (GAGs) side chains are covalently linked post-translationally. These GAG chains are acidic molecules, and they participate in a wide variety of binding interactions with other matrix macromolecules, cations and water (Kiani *et al.*, 2001).

Aggrecan belongs to a family of aggregating proteoglycan cause of their ability to interact with hyaluronan in a non-covalent fashion. The family is also called hyalectans because of a second domain that can bind carbohydrate. It has a core protein about 220 kD. At the N-terminus is a globular domain, G1, consisting of an immunoglobulin (Ig) repeat A second globular domain, G2, is present in aggrecan, but not in other family members, and consists of two further tandem repeats (PTRs). An important interglobular domain (IGD) separates the two globular structures. In human aggrecan, there follows keratan sulfate (KS)-substituted region, follow by a large chondoitin sulfate (CS)-substituted domain. Attachment of these GAGs chains occurs on the serine of serine-glycine dipeptide sequence present in this region, and one molecule of aggrecan contain up to 100 CS chains, 30 KS chains and many O- and N-linked oligosaccharides (Wight *et al.*, 1991). At the C-terminus of core protein, there is a third globular domain, consisting of three subdomains. In the human, there are two epidermal growth factor (EGF) repeats. The central subdomain has a lectin-like structure, whereas the last portion resembles a complement regulatory protein module. These domains play a role in the secretion of recombinant products (Zheng *et al.*, 1998) and translocation of the core protein (Domowicz *et al.*, 2000).

A third molecule that stabilizes interaction between hyaluronan and aggrecan is link protein, which comprises approximately 0.05% of the wet weight of cartilage. It is synthesized as a single gene product and glycosylated to generate two mature forms of 48 kD and 44 kD. Its molecular structure is closely resembles that of aggrecan G1 domain, consisting of an Immunoglobulin (Ig) repeat and two link modules. Link protein interacts with hyaluronan and also interacts with aggrecan core protein G1 domain to form a ternary complex that provide the tissue with its capacity to load bearing and resist deformation (Neame *et al.*, 1993). Only

nanomolar concentration of this peptide is shown subsequently to stimulate synthesis of proteoglycans and collagen (McKenna *et al.*, 1998).

All three components of this ternary complex are synthesized and exported by chondrocytes, and the assembly process is in extracellular matrix. The association of aggrecan with hyaluronan in stable complexes is a key feature of the unique matrix that characterizes cartilage. These interactions are critical for the maintenance of cartilage matrix architecture. The various stages involved in the synthesis and secretion of these molecules were shown in figure 4.

The other small proteoglycans termed biglycan and decorin, both of which have a protein core approximately 30 kD and play roles in maintaining the structure of the extracellular matrix by interact with type I and type II collagen fibrils (Xu *et al.*, 1998). Fibromodulin is another small proteoglycan present in cartilage and contains keratan sulfate. This molecule also plays roles in providing tissue biomechanical properties. These small proteoglycans are present in a wide variety of tissues but may be absent from growth plate cartilages which fibrils have a very short life span (Poole *et al.*, 1986). These structural molecules of adult articular cartilage matrix were shown in table 2.

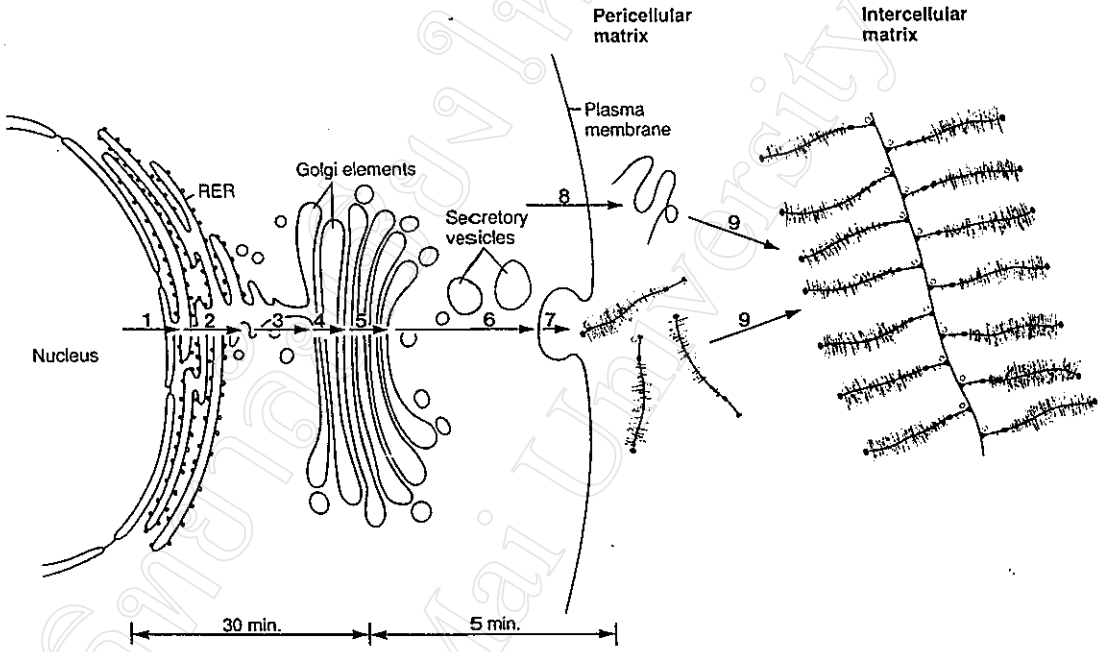

Figure 5 Diagram depicting the various stages involved in the synthesis and secretion of aggrecan and link protein by a chondrocytes. (1) The transcription of the aggrecan and link protein genes to mRNA. (2) The translation of the mRNA in the endoplasmic reticulum (rER) to form the protein core of the aggrecan. (3) The newly formed protein is transported from the rER to (4) the cis and (5) medial-trans Golgi compartments where the glycosaminoglycan chains are added to the protein core. (7) They are released into the extracellular matrix. (8) Hyaluronan is synthesized separately at the plasma membrane. (9) Aggrecan, link protein, hyaluronan come together to form proteoglycan aggregates in ECM. (Hardingham, 1992)

Table 2 Structural macromolecules of adult articular cartilage matrix (Koopman, 2001).

Molecule	Size Structure/ Organization	Distribution/ Comment	Function
Collagens			
Type II	Forms banded fibrils; 80-90% of total collagen	Throughout matrix	Tensile strength
Type VI	Forms microfibrils in pericellular sites	Pericellular, mid, and deep zones	Unknown
Type IX	Cross-linked to type II. Has single CS or DS-chain; hence a proteoglycan	Throughout matrix	Tensile properties of type II fibril?
Type X	Associated with type II fibrils and in pericellular network	Pericellular, territoral hypertrophic cartilage	Unknown
Type XI	Present in type II fibrils	Throughout cartilage	Tensile properties of type II fibril?
Type XII and XIV	Both are homotrimeric; resemble type IX in structure and sequence; both are proteoglycans if chondoitin sulfate chains are present	Each is present in distinct sites in association with collagen fibrils	May be part of collagen fibrils
Proteoglycans			
Aggrecan	$3x10^6$ kD; largest proteoglycan. Binds to hyaluronan through G1 globular domain	Throughout matrix but deficient at articular surface	Provides compressive stiffness through hydration of high fixed charged density
Versican	263 kD core protein containing G1 and G3 domains	Binds to Hyaluronan	Unknown
Biglycan	M_r, 76.3 kD; M_r core 38 kD; one CS or DS chain	Most concentration under articular surface; codistributed with decorin	Unknown

(Continue)

Decorin	M_r, 76.3 kD; M_r core 38 kD; one CS or DS chain	Codistributed with biglycan binds to collagen fibril	Regulates collagen fibril formation
Fibromodulin	M_r, 58 kD Has up to 4 N-link, KS chains	Absent from prehypertrophic and hypertrophic cartilage	Regulates collagen fibril formation
Lumican	M_r, 58 kD. Has up to 4 N-linked KS chains	Unknown	Regulates collagen II fibril formation
Link protein	M_r, 38.6 kD	Throughout matrix, binds to aggrecan and hyaluronan	Stabilizes attachment of aggrecan/versican to HA via G1 domain
Other molecules			
Annexin V	M_r, 34 kD (also called anchorin CII)	Cell surface/matrix vesicles	Receptor for type II collagen
Fibronectin	M_r, 440 kD	Mainly in fetal and diseased cartilages	Promotes attachment of cells interacts with collagen and GAGs
Cartilage matrix protein	3 subunits each of M_r, 54 kD linked via disulfide bridges	Absent from healthy articular cartilage and intervertebral disc	Unknown
Cartilage oligomeric protein	5 subunits of 100-115 kD, disulfide bonded	Cartilage is primary location	Unknown, but defect produces pseudoachondroplasia
Elastin	Found only in elastic cartilages such as ear and epiglottis	Throughout elastic cartilages	Elasticity of cartilage
Hyaluronan	M_r, 1,000-3,000 kD	Interacts directly or indirectly with collagen fibrils; aggrecan and versican bind to it via G1 domain	Retention of aggrecan and versican in matrix
Matrix γ-carboxyglutamic acid (g1a)	84 residuce protein	Pericellular (adult), diffuse (fetal)	Inhibits calcification
Tenascin	200 kD subunit forming a Six-armed glycoprotein	Might be absent from mature cartilage	Involved in chondrogenesis

1.2.2.3 hyaluronan-binding proteins in cartilage

The functions of HA are mediated through specific interactions with hyaluronan-binding molecules. Hyaluronan binding proteins (HABPs) are proteoglycan. Most of them have a defined HA-binding site (table 3). This region is a tandem repeat domain of 100 amino acids sequence called proteoglycan tandem repeat (PTR). This region is a very well conserved during evolution and come from a same ancestor protein (Barta *et al.*, 1993). HABP with PTR can be designed to the large group of link module like protein. The best-characterized proteins of this family are CD44, aggrecan, link protein, versican, tumor necrosis factor stimulated gene-6 (TSG-6), neurocan and hyaluronectin. Other domains are also common to many of these HABPs: epidermal growth factor like, lectin like and Ig like domain. HABPs molecules have variants and are the result of an alternative splicing (figure 6).

Some HABPs are cell receptors such as CD44 and receptor for HA-mediated motility (RHAMM) and allow cells to interact with the matrix. CD44 is expressed in a wide range of different cell types (Bajorath *et al.*, 1998), and implicated in a number of important cellular functions including metastasis, T cell signaling and activation (Knutson *et al.*, 1996). RHAMM is not found in most normal tissues and its expression is up-regulated in Ras transformed fibroblast (Turley *et al.*, 1991). Neurocan and hyaluronectin are very abundant in brain and play an important role in brain development (Watanabe *et al.*, 1995). TSG-6 is found in synovial fluid of patients with rheumatoid arthritis (Wisniewski *et al.*, 1993). It is produced locally in inflamed joints and serve as a useful marker in arthritis (Wisniewski *et al.*, 1996).

Aggrecan is the only member of this family containing an additional globular domain, G2, which has a structure similar to G1 and is separated from it by the interglobular domain, IGD, at the N-terminus (figure 7). Keratan sulfate and chondroitin sulfate glycosaminoglycan chains are attached to the core protein between G2 and a third globular domain, G3. Aggrecan forms multi-molecular hydrophilic aggregates by interaction of the G1 domain with hyaluronan and link protein, thereby conferring on tissue the ability to deform reversibly.

Table 3 Some of the most abundant HA-binding proteins and their different characteristics (Bost *et al.*, 1998).

Type of interaction with HA	HABPs	Location	Molecular mass of core protein (kD)	Function
Ionic (PTR)	Aggrecan	Cartilage	~220	Cartilage elasticity and compressibility
	Link protein	Cartilage	~ 44-49	Cartilage elasticity and compressibility
	Versican	Fibroblast	265	Cell adhesion, migration and proliferation
	CD44	Ubiquitous	80-200	Tumor cell invasion, cell adhesion and migration
	TSG-6	Synovial fluid	35	Anti-inflammatory effect (rheumatoid arthritis)
	Neurocan	Brain	136	Cell adhesion and motility during brain development
	Hyaluronectin	Brain	68	Development and tumor progression
Ionic	RHAMM	Fibroblast	~56	Cell migration and cell adhesion in Ras transformed cells
Covalent	SHAP	Pathology	85	ECM stabilization
Ionic	CESF	Cumulaus oophorus	126	Oocyte maturation

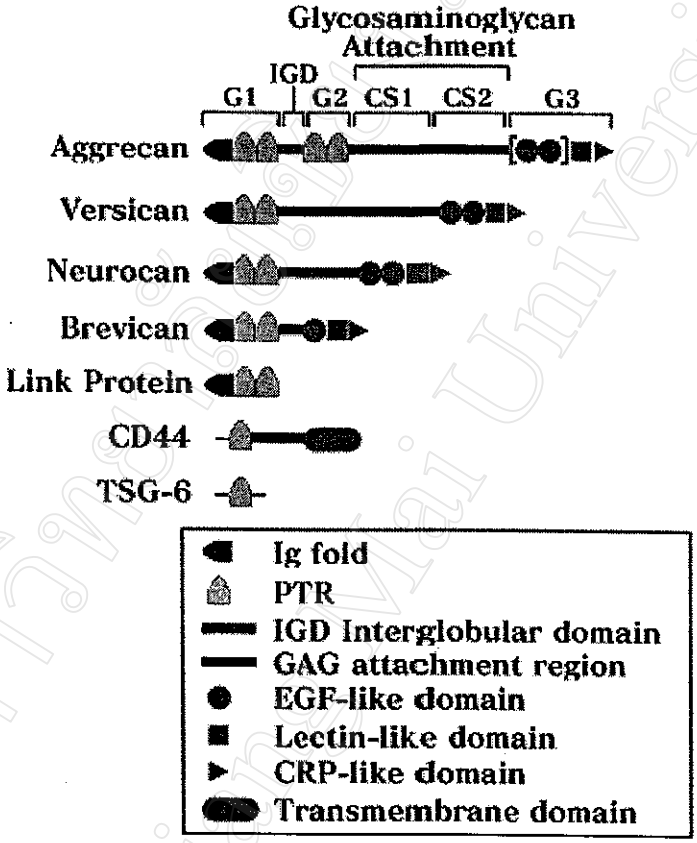

Figure 6 The hyaluronan-binding family of proteins related to aggrecan. Schematic models of the domain structures of the proteoglycans aggrecan, versican, neurocan and brevican; the cell surface HA-receptor CD-44 and the matrix molecules TSG-6 and link protein. The brackets in aggrecan mark the alternatively spliced EGF-like domains (Hardingham *et al.*, 1998).

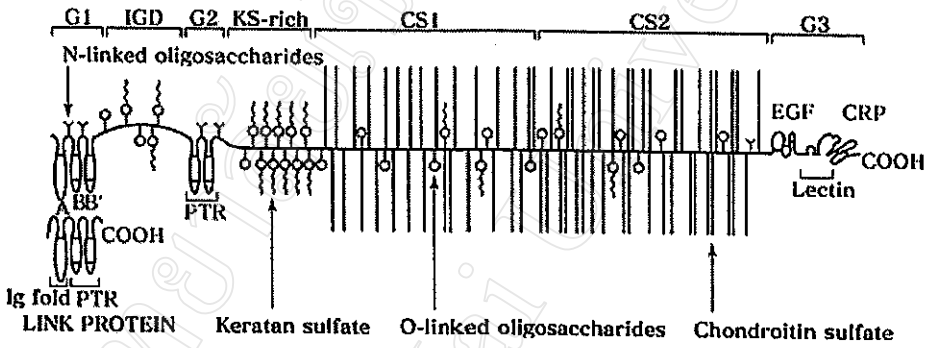

Figure 7 Cartilage Proteoglycan (aggrecan) structure. Aggrecan contains 3 globular domains (G1, G2, and G3) and 2 extended region, which form the interglobular region between G1 and G2, and the main glycosaminoglycan attachment region is composed of a variable keratan sulfate region and 2 chondoitin sulfate regions are distinguished by their sequence patterns. The domain structure of link protein also shown, which is similar to the aggrecan G1 domain. In aggregates the G1 domian of aggrecan binds to hyaluronan and this binding is stabilized by link protein, PTR (proteoglycan tendem repeat) (Hardinham, 1998)

1.2.3 Articular cartilage metabolism

As human articular cartilage ages, the proliferative response of chondrocytes to serum and growth factors decreases progressively (Guerne *et al.*, 1995). There is a decrease in overall proteoglycan synthesis, which is correlated with an increase in an age-related accumulation of pentosidine, a cross-link formed by lysine, a sugar, and arginine (De Groot *et al.*, 1999). This non-enzyme glycation results from a spontaneous reaction of reducing sugars, such as glucose, with free amino groups of proteins. The content of pentosidine can increase 50-fold in human cartilage from age 20 to 80 years (Bank *et al.*, 1998).

Progressive extracellular degradation also occurs, resulting in a reduction in the molecular size of aggrecan. Intact molecules containing the G3 globular domain are less commonly seen in aging (Dudhia *et al.*, 1996), whereas smaller keratan sulfate-rich molecules appear in increasing numbers (Franzen *et al.*, 1981). The G1 globular domain increases considerably in content. Most of this exists as the G1 domain bound to hyaluronan in the absence of the remainder of the molecule (Roughley *et al.*, 1985). This complex reflects proteolytic cleavage between the G1 and G2 globular domains of aggrecan. These degradation products accumulate in the deep zone (Bayliss *et al.*, 1978), remote from the chondrocytes in the interterritorial matrix. The half-life of the G1 domain has been estimated about 25 years, whereas that of whole molecule is only 3.4 years (Maroudas *et al.*, 1998). Link protein is also progressively reduced in molecular size during development and aging, as a result of cleavage close to the N-terminus. The accumulation of these breakdown products reflects progressive damage to the extracellular matrix overtime and lack of turnover and replacement of these damaged molecules (figure 8).

Degradation of cartilage matrix involves to the extracellular cleavage of matrix molecules, primary by protease, but also by free radicals released from chondrocytes. When degradation is induced in healthy cartilage, the aggrecan is lost rapidly. Subsequently type II collagen is degraded. The collagen fibril-associated proteoglycans (decorin, biglycan, and lumican) are more resistant to degradation. Degradation products can remain in the extracellular matrix, either because they can form part of larger macromolecular structure such as collagen fibril or because they represent a molecule that remains bound, such as part of the aggrecan monomer which remains bound by G1

domain to hyaluronan. Aggrecan fragments lacking the G1 domain, which are released from the macromolecular organization, are free to diffuse through matrix, where they can be locally endocytosed by chondrocytes or enter synovial fluid and the lymphatics. Degradation products of type II collagen and the proteoglycan aggrecan, including the G1 domain, can be detected in synovial fluid

1.2.4 Degenerative joint disease

The destruction of joint cartilage is of central importance in human arthritic disease. Degradation of cartilage matrix involves in the extracellular cleavage of matrix molecules, primarily by protease, but also by free radicals released from chondrocytes. When degradation is induced in healthy cartilage, the proteoglycan aggrecan is lost rapidly. Subsequently type II collagen is degraded. The degradation products can remain in the extracellular matrix, either because they are form part of a larger macromolecular structure such as collagen fibril, the fragments of aggrecan that remain bound to hyaluronan (figure 9). Aggrecan fragments lacking the G1 domain, which are released from the macromolecular organization, are free to diffuse through matrix, where they can be locally endocytosed by chondrocytes or enter synovial fluid and the lymphatic (Witter *et al.*, 1987).

Cartilage matrix degraded fragments can stimulate the release of proteolytic enzymes from phagocytes, leading to further antigen release and support the chronic self-perpetuating cycle of cartilage destruction (Guerassimov *et al.*, 1999).

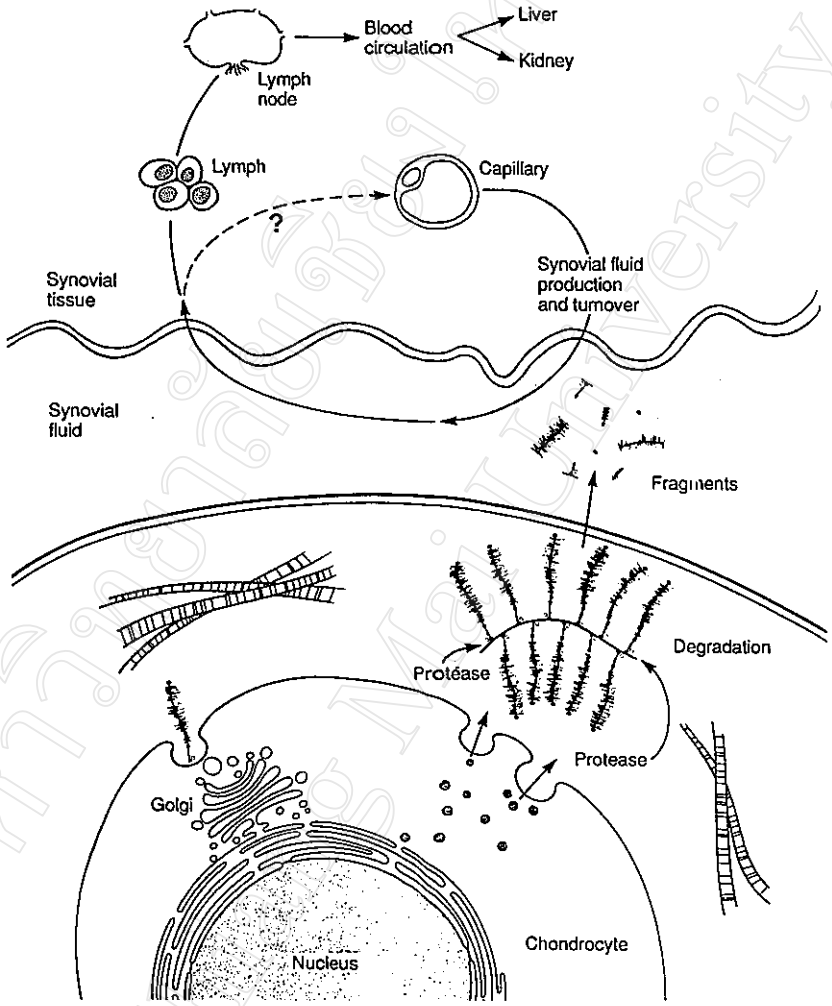

Figure 8 A schematic representation of the metabolic events controlling the proteoglycans in cartilage. (Hardingham *et al.*, 1992)

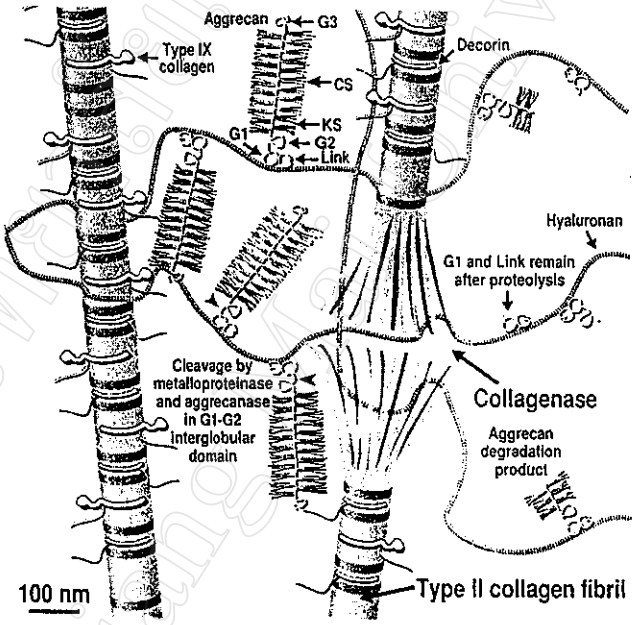

Figure 9 Diagrammatic representation of the organization of the proteoglycan aggrecan and type II collagen fibrils in cartilage matrix. Sites of attack on collagen and aggrecan by collagenases and other proteinase, respectively, are shown. (Koopman, 2001)

1.2.4.1 Osteoarthritis

Osteoarthritis (OA) is a common degenerative joint disease characterized by primary abnormalities in the articular cartilage and the most frequent cause of musculoskeletal disability (Moos *et al.*, 2000). Although the etiology of the disorder is still not clearly understood, osteoarthritis has been shown to be a family of disorders with cartilage as a target organ in which biomechanical factors play a central role and with risk factors such as age, weight, and occupation also of major importance.

The earliest histological changes reveal loss of extracellular cartilage matrix, loss of chondrocytes in the surface layers of articular cartilage (figure 9), reactive changes in the deeper chondrocytes manifested by cellular division and cloning in an apparent attempt at repair. Later, progressive loss of chondrocytes is seen at all levels, with marked thinning of the cartilage matrix and, in some instances, the development of fibrocartilage in place of lost hyaline cartilage. The surrounding synovium is largely unaffected (figure 10), although in later disease cartilage fragments may incite focal inflammatory lesions without the progressive and destructive pannus seen in typical inflammatory arthropathies (Goldman *et al.*, 2000).

A large number of cytokines (pro- and anti-inflammatory), antagonists, and growth factors are involved in OA pathophysiology. Tumor necrosis factor-α (TNF-α) and Interleukin-1β (IL-1β) are prominent and of major important to cartilage destruction. (Abbas *et al.*, 2000). Both cytokines can stimulate production and induce chondrocytes and synovial cells to produce other cytokines, such as IL-6, IL-8 and leukocyte inhibitory factor (LIF), as well as stimulate protease and prostaglandin E_2 (PGE_2) production. PGE is known to be an inflammatory mediator because it mediates cytokine induced bone resorption (Moriuchi-Murakami, *et al.*, 2000).

The G1 domain of aggrecan is commonly present in the synovial fluid of OA patient. It is relatively free of KS and often denatured, since it is unstable to bind to HA. Exposure of epitopes of link protein and the G1 domain to the immune system in these patients elicit the local production of antibodies and the expression of cellular immunity to link protein and the G1 domain in the circulation (Zhang *et al.*, 1998).

1.2.4.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic system inflammatory disorder that may affect many tissues and organs including skin, blood vessels, heart, lungs, and muscles, but principally attacks the joints, producing a non-suppurative proliferative synovitis that often progresses to destruction of the articular cartilage (figure 10). Although the cause of RA remains unknown, the autoimmunity plays a pivotal role in its chronicity and progression.

Rheumatoid arthritis occurs worldwide in all ethnic groups. Prevalence rates are range from 0.3 to 1.5% in most populations and females are two to three times more likely to be affected than males. Rheumatoid arthritis is a continuing autoimmune reaction in which T cells play a central role with the local release of inflammatory mediators and lytic cytokines that ultimately destroy the joint. Therefore involved in the causation are genetic susceptibility, primary exogenous arthritogen, autoimmune reaction within synovial membranes, and mediators of the joint damage (figure 11 and table 4).

The pathology hallmark of RA is synovial membrane proliferation and outgrowth associated with erosion of articular and subchondral bone and often involve malignant tumor, proliferating inflammatory tissue (pannus) may subsequently lead to destruction of intra-articular and periarticular structures and result in the joint deformities and dysfunction seen clinically. The events initiating of the destructive process are unknown. The earliest findings include microvascular injury and proliferation of synovial cells, accompanied by interstitial edema and perivascular in filtration by mononuclear cells, predominantly T lymphocytes. Continuation of the process leads to further hyperplasia of lining cell and the normally acellular subsynovial stroma becomes engorged with mononuclear inflammatory cells. T lymphocyte appears to be activated, presumably by some unknown antigen are presented type A synoviocytes, macrophages, dendritic cells, and B lymphocytes. Important pro-inflammatory cytokines appear to be linked in a cascade, with TNF-α at the apex promoting the subsequent elaboration of IL-1, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-1 induces the production of metalloproteinases and prostaglandin E_2 by synoviocytes. This cytokine also promotes the degradation and inhibits the synthesis of proteoglycan by chondrocytes, as well as enhances resorption of calcium from bone (Goldman *et al.*, 2000).

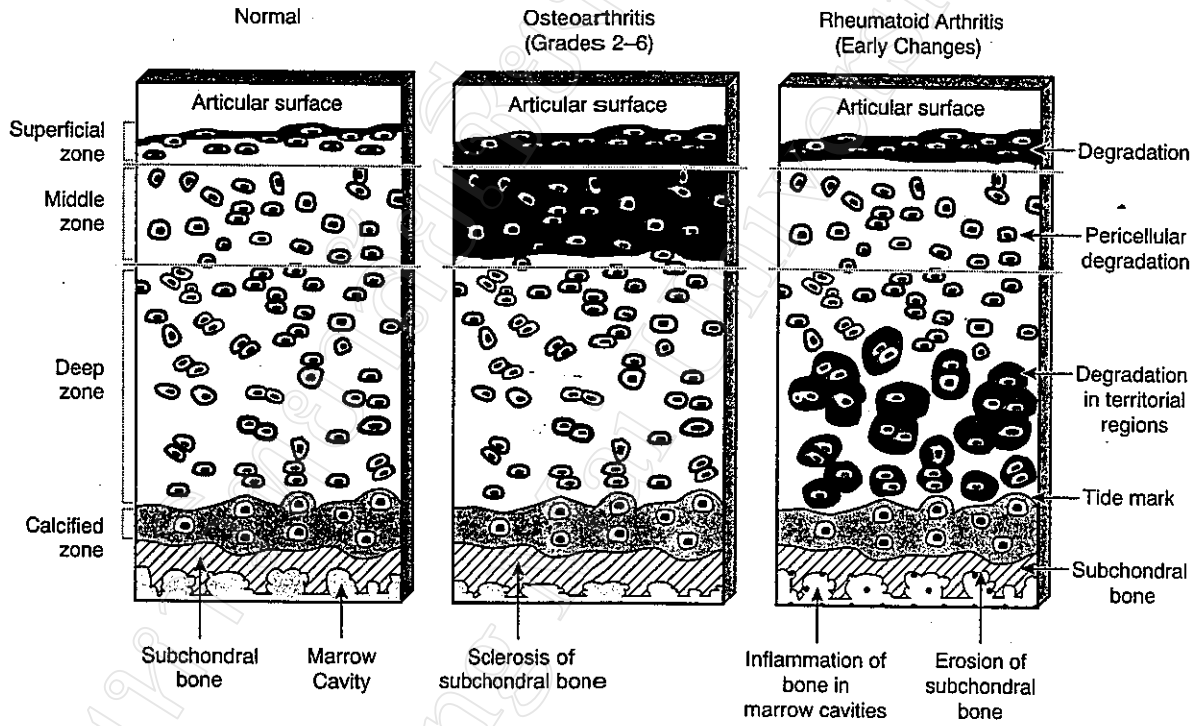

Figure 10 Diagrammatic representation of zones of normal cartilage (left) and sites of degradation of type II collagen in rheumatoid and osteoarthritic femoral cartilage showing sites of damage. Pericellular degradation in normally seen throughout healthy cartilage (Poole, 1993)

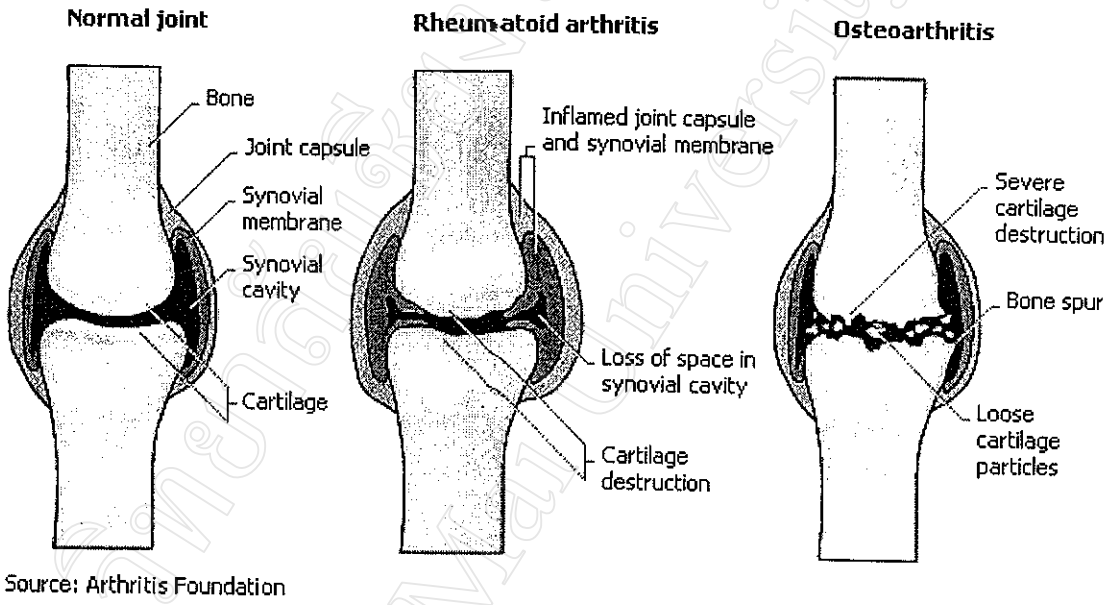

Figure 11 Diagrammatic representation of healthy, rheumatoarthritic, osteoarthritic joints. healthy joints are composed of cartilage and lubricating fluid, called *synovial fluid*, encased in a joint capsule, or *synovial membrane*. In osteoarthritis, joint cartilage is destroyed and, in some cases, bony outgrowths known as *bone spurs* develop. In rheumatoid arthritis, white blood cells in the synovial membrane divide, grow, and multiply, producing inflammation, pain, and stiffness in the joint, which may eventually lead to cartilage destruction (Microsoft corporation, 2001)

Table 4 Comparison of osteoarthritis and rheumatoid arthritis (Chandrasoma *et al.*, 1998).

	Osteoarthritis	**Rheumatoid arthritis**
Basic process	Degenerative	Immunologic, inflammatory
Site of initial lesion	Articular cartilage	Synovium
Age	50 plus	Any, but peaks at age 20-40 years
Sex	Male or Female	Female > male
Joints involved	Especially knees, hips, spine; asymmetric involvement	Hands, later large joints; multiple symmetric involvement
Fingers	Herberden's nodes	Ulnar deviation, spindle swelling
Nodules	No	Rheumatoid nodules
Systemic features	None	Uveitis, pericarditis, etc.
Constitution symptoms	None	Rheumatoid factor; high erythrocyte sedimentation rate; anemia, leukocytosis, hyperglobulinemia
Joint fluid	Clear, normally viscous; no inflammatory cells	Clear; low viscosity, high protein; neutrophils, some lymphocytes; immunoglobins, complement, rheumatoid factor

1.2.5 Biochemical markers of joint disease

A variety of different proteins have been proposed as the candidate of biochemical markers and these include matrix components, products of matrix degradation, cytokines, proteinases, and enzyme inhibitors (Garnero *et al.*, 2000). The characteristics of a biological prognostic marker that predicts future damage will be different from a marker that reflects continuing tissue destruction. A prognostic marker should correlate with modifiable, fundamental points in tissue destruction.

Early studies of biological markers by looking at the gross amounts of either proteoglycan or collagen that are released and established. Proteoglycan components are usually released before collagen fragments (Poole, 1993). It becomes clear that the synthesis of new matrix is also increased in disease as the tissue attempted to repair and so specific markers that could distinguish between repair and degradation are important (Eastell *et al.*, 1993).

Numerous proteoglycan monomers aggregate to bind to a chain of HA via a link protein to form larger molecular complexes. During cartilage degradation, most of these components are released into the synovial fluid and form there into the circulation. Thus, concentrations of aggrecan, chondroitin sulfate, or keratan sulfate related epitopes in body fluids are regarded as sensitive markers of cartilage breakdown and metabolism (table 4)(Dahlberg *et al.*, 1992).

Quantification of these tissue-derived macromolecules or fragments can reflect changes in the rate of synthesis or catabolism of the matrix of the different joint tissues. Immunoassays for such potential markers have indeed been developed and used in studies of both joint diseases include OA and inflammatory joint disease (Petersson *et al.*, 1998). Various epitopes have been identified in the extracellular matrix using both polyclonal antibodies.

Table 5 Molecular markers of cartilage metabolism (Dieppe, 1995).

Marker	Specimen	Tissue of origin	Biochemical features	Function
Aggrecan				
- Aggrecan core protein - Keratan sulfate - Chondoitin sulfate epitopes	Serum Synovial fluid	Cartilage	Structural components of cartilage extracellular matrix	Structural integrity plus organization of cartilage; fluid level reflect aggrecan/small proteoglycans synthesis/degradation
Cartilage oligomeric matrix protein	Serum Synovial fluid	Cartilage Synovium	Structural components of cartilage extracellular matrix	Structural integrity plus organization of cartilage; fluid level reflect cartilage degradation and matrix repair
CarBoxyterminal propeptide of type II Procollagen	Serum Synovial fluid	Cartilage	Part of collagen type II precursor molecule	Collagen synthesis; fluid levels reflect chondroblastic activity
Metalloproteinases and inhibitors - Stromelysin - Interstitial collagenase - Tissue inhibitors of metalloproteinases	Serum Synovial fluid	Cartilage	Proteolytic enzymes and inhibitors derived from chondrocytes and synovial cells	Organization, turnover plus repair of cartilage extracellular matrix; fluid levels reflect matrix degradation and repair
Hyaluronan	Serum	Synovium Connective tissues	Structural component of cartilage extracellular matrix	Structural integrity plus organization of cartilage: fluid level reflect synovial and connective tissue metabolism
Aminoterminal propeptide of type II procollagen	Synovial fluid Bone	Synovium Bone	Part of collagen type II precursor molecule	Collagen synthesis; fluid levels reflect synovial cell/fibroblastic activity

1.2.6 Monoclonal antibodies directed against proteoglycan epitope

The immunological methods offer a number of advantages for the detection and quantitation of all categories of markers of joint disease. They are more sensitive, more specific, large number of samples can be easily processed and require small amount of samples or purification in order to avoid the effects of interference from other components.

The use of monoclonal antibodies has several advantages because the epitope specificity and biological characteristic of the antibody can be easily to define. There have been developed and identified monoclonal antibodies that recognize subtle biochemical differences present in the extracellular matrix of articular cartilage from arthritic joints, so-called markers of arthritis.

In-patients with RA, synovial fluid levels of core protein from aggrecan were highest early in the disease (Saxne *et al.*, 1985), probably reflecting the decrease of cartilage mass, and therefore aggrecan concentration and also slightly decreased in OA (Lohmander *et al.*, 1993). Assays have been developed that measure the HA-binding region or the G1 domain that consisted of aggrecan and link protein by using monoclonal antibody.

In 1992s, Calabro and colleaques have developed monoclonal antibodies that specifically detect epitopes on aggrecan degradation products. Biochemical characterization of the specificities of these monoclonal antibodies indicated that mAb 1C6 recognized an epitope shared by both the G1 and the G2 domains, mAb 5C4 recognized an epitope shared by both LP and the G1 domain, mAb 7D1 recognized an epitope shared by both the G1 and the CS attachment domains, mAb 14A1 and 15B2 recognized epitopes in the CS attachment domain, mAb 14B4 recognized an epitope in the G3 domain, and mAb 13D1 recognized a ubiquitous epitope shared by the G1, G2, G3, and CS attachment domains of aggrecan and also Link Protein. Collectively the specificities of these antibodies confirm the occurrence of multiple repeated epitopes (both carbohydrate and protein in nature) throughout the different domain structures of aggrecan. These antibodies have been proven to be useful for identifying aggrecan-like molecules in several connective tissues other than cartilage (Calabro *et al.*, 1992).

In 1993s, Radhakrishnamurthy reported that the monoclonal antibody C8F4 was developed to the core protein. The monoclonal antibody recognize the core proteins from bovine nasal cartilage proteoglycan and human aorta proteoglycan and suggested that the mAb C8F4 recognize hyaluronic acid binding peptide (Radhakrishnamurthy *et al.*, 1993). Previous studies are showed in table 6.

In addition, monoclonal antibody 1H8 has been developed in department of Biochemistry, faculty of medicine, Chiangmai University. It is produced against embryonic shark A1 (Tiengburanatam, 1998) and has shown the reactivity against HABPs (Chuangbunyat, 2001).

To produce a monoclonal antibody (mAb) 1H8, Balb/c are immunized with shark A1, the B cells are isolated from spleen of mice. These B cells are then fused with an appropriate immortalized cell line. Myeloma cell lines are the best fusion partners for B cells, because the like cells tend to fuse and give rise to stable hybrids more efficiently than the unlike cells. In current practice, the myeloma cell lines that are commonly used do not produce their own immunoglobulin, and cell fusion is achieved by using polyethylene glycol. Hybrids are selected for growth in hypoxanthine, aminopterin and thymidine (HAT) medium; under these conditions, unfused myeloma cells will die because they can not use the salvage pathway, and unfused B cells can not survive for more than weeks because they are not immortalized, so that only hybrids will grow. The fused cells are cultured at optimum concentration at which each culture well is expected to initially contain only one hybridomas cell are detected and then tested for the presence of antibody reactive with shark A1 which is use for immunization.

The screening method is done by using ELISA technique. Once positive wells, containing producing the desired antibody, are identified and cloned. These cloned hybridomas produce monoclonal antibodies of a desired specificity.

Table 6 Monoclonal antibodies directed against the polysaccharide attachment region of cartilage proteoglycan (Caterson *et al.*, 1985).

Clone	Immunogen	Specificity	Antibody Subclass
Chondroitin sulfate family			
CS-56	Ventral membranes of gizzard fibroblast	Chondroitin sulfate	m-IgM
MO-225	Proteoglycan from chick embryo limb bud	Chondroitin sulfate Type D	m-IgM
MC21C	Adult rat bone protein	Chondroitinase-6-sulfate	m-IgM
LY111	Chicken type IX collagen containing chondroitin-4-sulfate	Chondroitinase-4-sulfate	m-IgM
4/8/2-B-4	PG digested with chondroitinase ABC	Δ Di-4S	m-IgG1
4/8/9-A-2	Same as above	Δ Di-4S	m-IgG1
5/6/1-B-5	CSPG digested with chondroitinase ABC	Δ Di-OS	m-IgG1
2-B-6	Same as above	Δ Di-OS	m-IgG1
5/6/3-B-3	Same as above	Δ Di-OS	m-IgM
2H6	PG from 10-day-old rat brain	Chondroitin sulfate	m-IgM
473	Extracts of monkey brain	Chondroitin sulfate	m-IgA
Keratan sulfate			
1/20/5-D-4	CSPG monomer from human articular cartilage digested with chondroitinase ABC	Keratan sulfate	m-IgG1
4/8/1-B-4	BNC-PG-Core digested with chondroitinase ABC	Keratan sulfate	m-IgG1
Core protein of proteoglycan			
6-B-6	Human ovarian fibroma	Dematan sulfate proteoglycan	m-IgG1
2-B-1	Human yolk sac tumor	Large proteoglycan (Versican)	m-IgG1
HK-102	HSPG from mouse EHS tumor	HS proteoglycan (Neurocan)	m-IgG2b
1-G-2	CSPG from 10-day-old rat brain	CS proteoglycan (Perlecan)	m-IgG1
6-B-4	PG from 10-day-old rat brain	CS proteoglycan (Phosphacan)	m-IgM
1H8	Shark A1	G1 domain (aggrecan and link protein)	m-IgM

1.2.7 Clinical application of hyaluronan-binding proteins

The introduction of specific immunoassays for the molecules found mainly or exclusively inside cartilage has permitted a variety of *in vivo* studies of cartilage matrix metabolism by the analyses of synovial fluids, sera/plasma, and urine. The concentrations of the cartilage proteoglycan aggrecan are, as expected, higher in synovial fluid of arthritic joints than in serum (Poole *et al.*, 1994). These molecules generally represent degradation products of synthetic and/or degenerative process. The largest breakdown products include the keratan sulfate-rich domain and the G1 and G2 globular domains of aggrecan. Degenerative joint disease in dogs, causing joint instability, rapidly result in a persistent increase of aggrecan and other products in synovial fluids of affected joint (Heinegard *et al.*, 1985). These increases may show prognostic value because they are likely to be indicator of the establishment of progressive degenerative joint disease. In inflammatory joint, the high levels of aggrecan in joint fluids may be prognostic for the increased joint destruction in RA (Saxne *et al.*, 1985).

These are contrasts to more advanced disease in which there has been more proteoglycan degradation, and the hyaluronan-binding region (G1 globular domain) and associated aggrecan components predominate, probably as a result of preferential retention in cartilage matrix through hyaluronan binding.

Aggrecan is a potent immunogen. Proteoglycan core protein elicits antibodies that are specific for the globular (G1, G2, and G3) and interglobular domains. Antibodies to aggrecan have been reported only in-patients in a study of RA (Glant *et al.*, 1980). Link protein shares homology with the G1 domain. Immunity to link protein is also observed on RA (Guerassimov *et al.*, 1997)

1.3 Objective of this study

1. To develop ELISA method for quantitation of total HABPs in serum by using monoclonal antibody 1H8.
2. To evaluate the level of hyaluronan-binding proteins in arthritic patient serum and compare with healthy subjects.
3. To compare the concentration of HABPs and HA in the serum of arthritic patients.