CHAPTER I

INTRODUCTION

1.1 Statement and significance of the problem

The two major arthritic diseases are rheumatoid arthritis (RA) and osteoarthritis (OA). RA is an inflammatory and autoimmune disorder, affecting about 1 percent of the world's population (1). OA, on the other hand, is the most common of all joint diseases, and one of the most frequent causes of physical disability. The prevalence of OA increases with age, affecting 80% of people over age 65, and about 135 million people worldwide (2). RA is a systemic disease, affecting multiple joints throughout the body, while OA is usually localized to one or two joints that have been subjected to chronic use or injury. The common thread linking RA and OA is irreversible destruction of the cartilage, tendon and bone in the affected joints. The progressive degradation of this connective tissue seen in both disease leads to pain, loss of joint function, and substantial decrease in quality of life.

At first, the loss of cartilage was considered a particularly early stage of the disease and a problem related mainly to mechanical factor (in OA) or an innocent bystander due to synovitis at the inflammatory stage of the disease (in RA). In the last two decades, however, scientific advances in the field of arthritis have allowed the discovery of several important mechanisms involved in the degradation and destruction of articular cartilage (3).

Articular cartilage has a simple avascular structure composed of a large amount of extracellular matrix (ECM) and a small number of chondrocytes and is the major target tissue for destruction in both RA and OA. In arthritis, excessive degradation of cartilage ECM components by proteinases is key to the destructive process. Cartilage ECM is composed mainly of proteoglycans including the major proteoglycan, aggrecan, and others minor proteoglycan (decorin, fibromodulin, lumican and biglycan etc) and collagens such as type II collagen and others minor collagens (type IX, XI and VI collagens). Depletion of proteoglycan from articular cartilage is a common initial change in these joint diseases with subsequent degradation of the collagen fibrils. After the initiation of collagen fibril fibrillation and laceration of the articular cartilage begins and is result of the destruction of the arcade structures of the collagen fibrils in the articular, which is composed of the superficial, transitional, radial, and calcified zones.

The primary cause of the degradation of the ECM exceeds its synthesis in cartilage is thought to be increased proteolytic enzyme activity. Matrix metalloproteinases (MMP) have been implicated in the excessive matrix degradation that characterizes the cartilage degradation of OA (4). The expressions of several MMPs are high in cartilage of OA patients (e.g. MMP-3, MMP-13 and MMP-14). The recently discovered groups of metalloproteinases, called “aggrecanases,” are now thought to play a major role in aggrecan breakdown. Three members of the ADAMTS family of proteinases (a disintegrin and a metalloproteinase domain with thrombospondin motifs), ADAMTS-1, ADAMTS-4 and ADAMTS-5, have been identified as aggrecanases (5). As the disease progresses, the local pH of the cartilage may fall and cathepsin B, cathepsin L, and cathepsin K from the chondrocytes may

participate in further cartilage destruction (6, 7). A deficiency in the tissue inhibitor of MMP (TIMPs) (8) clearly favors the excessive proteolysis observed in the disease articular cartilage.

Cytokines produced by the synovium and chondrocytes, especially interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α), play a significant role in the degradation of cartilage (9), and prostaglandin and leukotrienes may also be involved (10). The production of nitric oxide (NO), which is stimulated by proinflammatory cytokines, is involved in cartilage catabolism (11) and also may induce the apoptosis of chondrocytes (12). The NO and PGE2 produced by activated chondrocytes in diseased cartilage may modulate disease progression in OA and should therefore be considered as potential targets for therapeutic intervention (10).

There are many pharmacologic treatment of arthritis such as nonsteriodal anti-inflammatory drugs (NSAIDs). The ability of different NSAIDs is to inhibit COX, the enzyme that catalyzes the synthesis of cyclic endoperoxides from arachidonic acid to proinflammatory and other forms of prostaglandin (13). Moreover, the intra-articular administration of steroids such as glucocorticoids and administration of hyaluronan (HA) have recently become more widely accepted in the armamentarium for OA pain (14).

Nowadays there is a new group of drug for OA called disease-modifying osteoarthritis drugs (DMOADs), although none of these drugs has yet been reproducibly shown to alter the natural history of the disease (15). They include the following: inhibitors of MMP (16), growth factors and cytokine manipulation (17), genetic therapy (18), and sulfated and nonsulfated sugar (19) such as glucosamine sulfate for oral administration and intramuscular in human (20).

Thai medicinal plants are interested to use for study as anti-arthritic drug for further developing DMOADs. Two plants were investigated for this purpose, *Alpinia galanga* and *Sesamun indicum.*

There were many reports of *Alpinia galanga* Linn. effects including antimicrobial, carminative, stomachic, anti-flatulent, anti-itching and anti-rheumatic effects (21). Its antirheumatic action resulted in suppression of synthesis of cyclooxygenase-1 and -2 (22). In addition, the aqueous acetone extract from rhizomes of this plant has possible inhibitory effects on lipopolysaccharide (LPS)-induced nitric-oxide production (23). Moreover, in 2001 Altman and Marcussen reported that high pure extract of this plant was shown to significantly reduce symptoms of OA of the knee (24). Nevertheless, the active compound, which result this anti-arthritic effects, was still unknown.

For *S. indicum,* there were many reports of the anti-inflammatory activity of sesamin, which is lignan in *Sesamun indicum* seeds (25-28). Sesamin inhibit delta-5 desaturase activity, resulting in an accumulation of dihomo-(-linolenic acid) (DGLA) that can displace arachidonic acid and decrease the formation of pro-inflammatory mediator, such as prostaglandin E_2 and leukotriene B_4 (25, 26). Moreover, sesamin and the other active compound, sesamolin, suppressed LPS-induced NO production in microglia and macrophage through inhibition of signal transduction pathway or nuclear transcription factor (27, 28).

Therefore, it is interesting to study other anti-inflammatory effects of the active compounds extracted from two plants. For *A. galanga*, firstly the main active compound was isolated and its anti-inflammatory effect was further studied. For *S.*

indicum, the anti-inflammatory effect was studied using well-known active compound, sesamin.

The anti-inflammatory effect was studied using several models. Firstly screening model, porcine cartilage explant was used and induced inflammation by interleukin-1 beta (IL-1β). The molecular mechanism of each pure compound was investigated in human articular chondrocyte (HAC) induced by IL-1β and only one compound, which had the best effect, was used for further study. The inhibition of IL-1β signaling pathway in HAC was performed and moreover the papain-induced OA rat model was used for investigation of the anti-arthritic effect of that compound in the *in vivo* model.

1.2 Literature reviews

1.2.1 Articular cartilage

Cartilage is a type of dense connective tissue. It is composed of specialized cells called chondrocytes that produce a large amount of ECM composed of collagen fibers, abundant ground substance rich in proteoglycan, and elastin fibers. Cartilage is classified in three types, elastic cartilage, hyaline cartilage and fibrocarilage, which differ in the relative amounts of these three main components (29).

Cartilage is found in many areas in the body, including the articular surface of the bones, the rib cartilage, the ear, the nose, the bronchial tubes and the intervertebral discs. Its mechanical properties are intermediate between bone and dense connective tissue like tendon (29).

Unlike other connective tissues, cartilage does not contain blood vessels. The chodrocytes are fed by diffusion, helped by the pumping action generated by

compression of the articular cartilage or flexion of the elastic cartilage. Thus, compared to other connective tissues, cartilage grows and repairs more slowly (29).

Articular cartilage is the hyaline cartilage, which is smooth, glistening white tissue that covers the surface of all the diarthrodial joints in the human body. The figure of articular cartilage covers the joint surfaces was shown in Figure 1.1. As its name implies, articular cartilage is critical in the movement of one bone against another. Articular cartilage has an incredibly low coefficient of friction, which coupled with its ability to beat very large compressive loads, makes it ideally suited for placement in joints, such as the knee and hip (29).

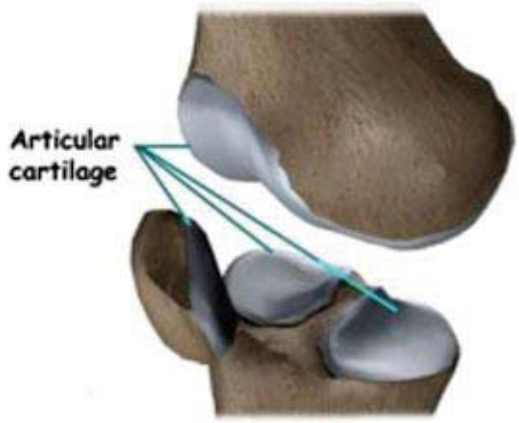


Figure 1.1 Articular cartilage or hyaline cartilage covers the joint surfaces. (http://www.eorthopod.com/public/patient_education/6613/meniscal_surgery.html)

Articular cartilage is not a homogeneous tissue. Instead, it has vary complex composition and architecture that permits it to achieve and maintain proper biomechanical function over the majority of human lifespan. Articular cartilage is composed mainly of water (70 – 80% by wet weight). The solid phase of articular cartilage consists primarily of type II collagen and proteoglycan. Collagen forms network of fibrils, which resist the swelling pressure generated by proteoglycans. Because of the tendency of peoteoglycan to noncovalently interact with hyaluronic acid (HA), form huge aggregates that become trapped in the collagen network. Because of their numerous negatively charged sulfate groups, these proteoglycan aggregates attract cations, which in turn bring in water to minimize differences in osmotic pressure. Thus, type II collagen and proteoglycan create a swollen, hydrated tissue that resists compression (29).

The cartilage matrix consists of macromolecules in which collagen and proteoglycan (aggrecan) are the main representatives. These components are highly ordered from the cartilage surface to the deepest layers. Cartilage is divided into four zones with different functions: the superficial, middle or transitional, deep or radial, and calcified cartilage zones. Interestingly, there is no sharp boundary between the first three zones. The layers of cartilage were shown in Figure 1.2.

The superficial zone, the thinnest of the four, consists of fine collagen fibrils with tangential orientation, low proteoglycan content, and elongated chondrocytes aligned parallel to the surface (29). This zone is contact with synovial fluid, and is responsible for most of the tensile properties of cartilage that enable cartilage to resist shear and the tensile and compressive forces imposed by the movement of the articulation.

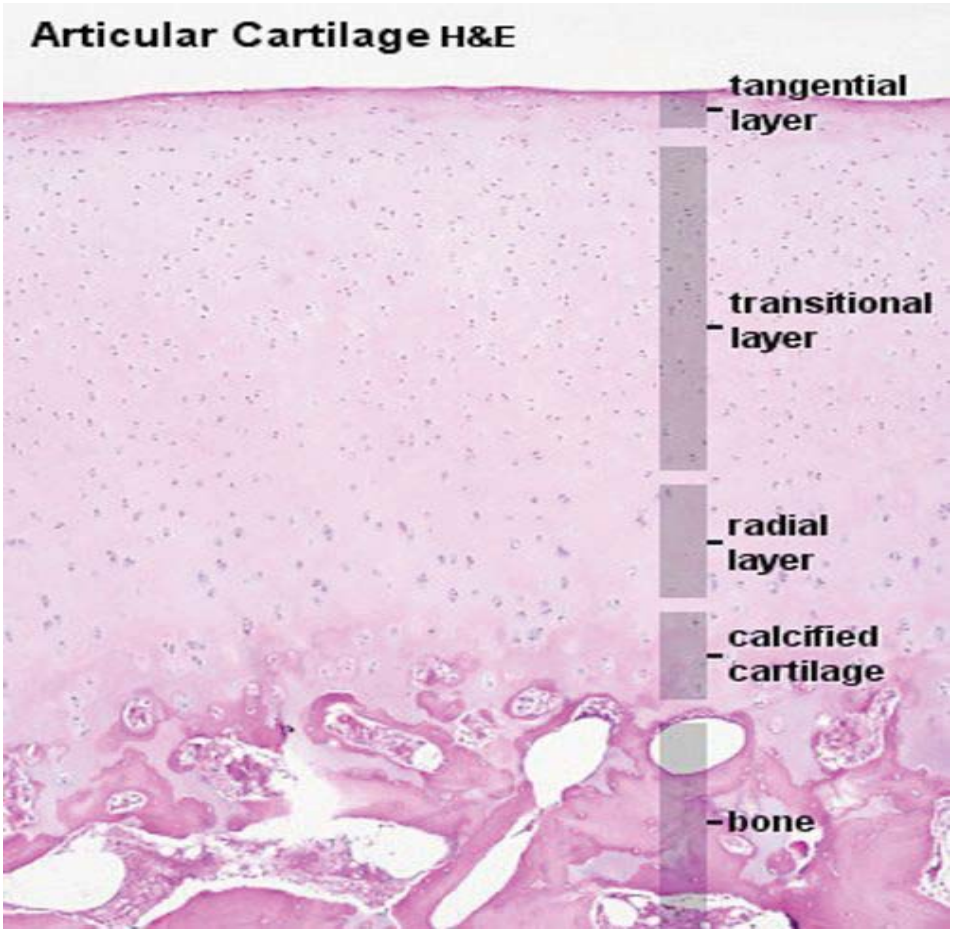


Figure 1.2 Section of bovine articular cartilage stained with Hematoxylin & Eosin (H&E). The layers of articular cartilage are easiest to identify in large joints. Note the changing orientations of the lacunae and isogenous groups at different depth in the cartilage. The changing orientations of chondrocytes and isogenous groups reflect the orientations of the collagen fibres in the matrix. The fibres are not visible in the slide. The darker hue of the cartilage close to the bone is caused by the calcification of the cartilage (adapted from http:// www.lab.anhb.uwa.edu.au/mb140/CorePages/Cartilage/Cartil.htm#CARTILAGE).

The middle zone represents 40 – 60 % of the total cartilage height. It is formed by proteoglycans and ticker collagen fibrils organized into radial bundles or layers. The chondrocytes in this zone are at low density and have a round shape.

In the deep zone, the chondrocytes have the same shape as those in the middle zone and are aligned perpendicular to the articular surface, but with low density. This zone contains the largest collagen fibrils in radial disposition (29), and aggrecan content is at its highest.

The calcified cartilage is divided from the other zones by the tidemark, and separates – physically and mechanically – the hyaline cartilage from the subchondral bone. Its main function seems to be anchored the cartilage to the bone as collagen fibrils from the radial zone penetrate into the calcified cartilage. In this zone, the cell population is very scarce and chondrocytes are hypertrophic.

The pericellular area surrounding the chondrocytes is made of a thin layer of non-fibrillar material, which most likely represents the synthetic products of the chondrocytes, such as proteoglycans and glycoproteins. Immediately adjacent to the pericellular area is the territorial cartilage matrix, which contains a dense meshwork of thin collagen fibers forming a capsule-like structure around the cells and providing mechanical protection to the chondrocytes (30).

Collagen, proteoglycans, and other proteins or glycoproteins represent only abount 20 % of the tissue wet weight; water and inorganic salts represent most of the remaining tissue. The water content is unevenly distributed. The highest concentration (80%) is found near the cartilage surface. Its concentration decreases gradually with increasing depth, to reach about 65% in the deep zone. Most of the water is extracellular. Inorganic salts, such as sodium, calcium, and potassium chloride, are

dissolved in the tissue water. The maintenance and flow of water in the tissue relies in its interaction with the macromolecules. The diffusion of water through the cartilage helps move the nutrients from the synovial fluid through the tissue, contributing to the nutrition of chondrocytes.

Collagen represents 50-60% of the cartilage dry weight. Collagen fibrils form a network that provides the shape and form of the tissue. Fibril diameters vary from 20 nm in the superficial zone to 70-120 nm in the deep zone. Type II collagen is specific to cartilage and is the primary collagen in this tissue (90-98% of the total tissue collagen). The most important mechanical properties of collagen fibrils are tensile stiffness and strength.

The proteoglycans constitute the second largest portion of the solid phase in articular cartilage, accounting for 5-10% of the tissue-wet weight. The proteoglycans in articular cartilage are complex supramolecular aggregate, and consist of a central HA filament to which multiple monomers are non-covalently attached. Proteoglycan monomers of the HA chain. A low-molecular-weight protein named like protein, stabilizes the bond between the monomer and the HA. The chemical structure of the monomer can be compared to a ‘test-tube brush’. Proteoglycan in the cartilage matrix are mainly of the large aggregating type (50-80%) and of the large non-aggregating type (10-40%). An aggregating proteoglycan is composed of a monomer formed by a protein backbone, the core protein. Numerous glycosminoglycan chains radiate at 90 from this core protein, extending stiffly. The glycosaminoglycan molecules are formed of a long chain of repeating polydimeric saccharides: chondroitin-4-sulfate, chondroitin-6-sulfate, and keratan sulfate, of which chodroitin sulfate is the most abundant (60-90%). The average chondrotin sulfate chain consists of 25-30%

repeating disaccharide units. Keratan sulfate chains are shorter and consist of only 5 or 6 repeating dimeric units.

1.2.2 Composition of articular cartilage matrix

1.2.2.1 Collagens

The key feature of all collagens is the triple helix, a coiled-coil structure in the form of a right-handed helix of 1.5 mm diameter, composed of three polypeptide chains (α-chains) (31-33). (Figure 1.3) A structure requirement for the assembly of polypeptide chains into collagen triple helix is the occupation of every third position by a glycine residue, resulting in the (Gly-X-Y)n repeat structure characterizing all collagens. The α-chain form a stretched, left-handed helix with a pith of 18 amino acid residues per turn (34) and asseble around a central axis in a manner allowing all glycine residues to be positioned in the center of the triple helix. The more bulky side chains of the amino acids in the X and Y position occupy the outer positions, where they are available for lateral interactions with adjacent collagen molecules to form fibrils. Another tropical feature of the collagen triple helix is the high content of proline and hydroxyproline. The hydroxyl groups of 4-hydroxyl-proline are essential for the formation of intramolecular hydrogen bonds and thus critically determine the thermal stability of each collagen triple helix (33, 35).

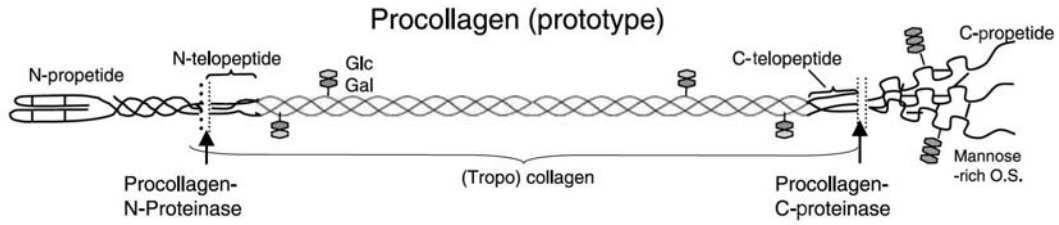


Figure 1.3 Type I procollagen as a prototype of fibril-forming collagens. In types I, II, and III procollagens N- and C-terminal propeptides are removed after secretion by specific proteases, in types XI and V the N-propeptides are larger and only partially cleaved. The C-propeptides contain AsN-linked mannose-rich oligosaccharides, while the triple helical part contains only hydroxylysine-linked glucosyl-galactosyl disaccharides or monosaccharides (36).

The backbone of all cartilaginous tissue of the vertebrate body is a heterofilbril containing type II collagen as the predominant collagen type, into which type XI collagen is incorporated. The cartilage collagen fibril is decorated with fibril-associated collagens with interrupted triple helices (FACIT colalgens), mostly type IX collagen, which is covalently linked to type II collagen (37, 38). The large n-terminal noncollagenous domain of FACIT collagens reach out of the fibril into the adjacent matrix space and may serve to anchor the collagen fibril in the proteoglycan matrix. Young growth cartilage contains about 85-90% type II collagen with 5-10% type XI and 5-10% collagen XI, while adult articular cartilage may have as little as 1% collagen IX and 3% collagen XI (39).

Recently two new fibril-forming collagens (type XXIV and XXVII) (40, 41), and two new FACIT-like collagens (type XX and XXII) (42, 43) were found to be expressed in cartilage, but their function is not yet known.

1.2.2.1.1 Cartilage collagen

The details of each collagen found in cartilage were described as follow;

Type II collagen

The major macromolecule of articular cartilage is type II collagen. It is composed of three identical polypeptide chains, αI(II)chains, that intertwine along most of their length to form a triple helix. The α-chains are synthesized as pro-α-chains, which possess large propeptides at both their N and C termini. The propeptides are separated from the central triple-helix-forming region by short telopeptides. The propeptides are required for normal trimer assembly during

synthesis within the chondrocyte, but are removed from the resulting procollagen by proteolysis following secretion into the extracellular space, leaving only the telopeptides attached to the triple helix. The N-propeptide is removed by a procollagen-N-protease (ADAMTS-3, a member of a subgroup of the adamalysin family, is most active on type II collagen (44) and the C-propeptide is removed by procollagen-C-protease (BMPI (45)). Two forms of type II procollagen can be generated. Type IIA procallagen, which processes a variable region, is mostly associated with newly formed cartilage during chondrogenesis, whereas type IIB procollagen is present in all cartilage.

The trimeric collagen molecule does not exist in isolation within the extracellular matrix, but in the form of collagen fibrils. These are formed by the lateral association of collagen molecules in a quarter staggered array. Each collagen molecule is joined to its neighbors via aldimine-derived crosslinks. These crosslink's join the telopeptide of one collagen molecule to the helix of another, stabilizing the fibril, preventing thermal or mechanical dissociation, and allowing the fibril to resist the tensile forces to which the cartilage is exposed *in vivo*. The initially formed aldemine crosslinks are subsequently modified to pyridinoline crosslink, which can serve as a marker for mature collagen (46). With increasing age, additional nonaldemine-derived crosslink (e.g. pantosidine) accumulate in the collagen fibrils (47), making them stronger and more resistant to proteolytic degradation. Indeed, mature collagen fibrils undergo little turnover under normal physiological conditions during the life of an individual.

The triple helical region of the type II collagen molecule is resistant to degradation by most proteases, but can be cleaved by the action of collagenases.

Memmalian collagenease act consecutively at a single site in each of the three α-chains to yield fragments that are about three-quarters and one-quarter the length of the intact molecule. Three mammalian collagenases exist and are named metalloprotease (MMP)-1, MMP-8, and MMP-13, with MMP-13 favoring the cleavage of type II collagen over the other fibrillar collagens (48). The collagen fragments can be denatured by further cleavage by gelatinases (MMP-2 and MMP-9). However, cleavage of the intact collagen fibril is a slow process, as it might contain hundreds of collagen molecules across its diameter.

The importance of type II collagen in cartilage formation and function is best illustrated by the consequence of type II collagen (*COL2A1*) gene mutation, which give both growth plate and articular cartilage abnormalities (49).

Type XI collagen

Type XI collagen is another fibrillar collagen that is cartilage specific. It is less abundant that type II collagen and its abundant decreases from about 10% of the total collagen in fetal cartilage to around 3% in adult articular cartilage (50). It is composed of three different α-chains that intertwine to form a heterotrimeric triple helix, which possesses the composition α1(XI), α2(XI), α3(XI). The α3(XI) chain is derived from the *COL2A1* gene, but differs from the α1(II) chain in post-translational modification (51). Type XI collagen is synthesized and secreted as a procollagen in a similar manner to type II collagen, but undergoes proteolytic removal of only its C-propeptide. The type XI collagen molecules do not form unique fibrils but occur in heterotypic fibrils in association with type II collagen molecules. The type XI collagen molecules reside at the center of the heterotypic fibril with their N-

propeptide protruding from its surface and limiting its lateral growth. Type XI collagen is most concentrated in the thin collagen fibrils that form the pericellular network. Growth in fibril width might involve proteolytic removal of the N-propeptide of the type XI collagen to permit the accretion of more type II collagen molecules or the fusion of adjacent fibrils.

Type IX collagen

Type IX collagen is a member of the FACIT (fibril-associated collagen with interrupted triple helix) family; it is also cartilage specific. It dose not form a collagen fibril itself but is instead present at the surface of the type II/type XI fibril. Its abundance decreases from about 10% of the total collagen in fetal cartilage to about 1% in adult articular cartilage (50). It is composed of three different α-chains that form a heterotrimeric molecule processing the composition α1(IX), α2(IX), α3(IX). The type IX collagen molecule consists of three triple helical domains (COL1-3) bordered by nonhelical domain (NC1-4). Interaction between the type IX collagen molecule and the collagen fibril takes place through the COL1 and COL2 domains, with the COL3 domain and the amino terminal NC4 domain projecting away from the fibril. The type IX collagen molecules are crosslinked to the type II collagen molecules of the fibril via aldemine-derived crosslink's (52). The cationic NC4 domain of the α1 chain has been suggested to act as a bridge between the collagenous framework and the interspersed extracellular matrix, particularly the anionic glycosaminoglycan chains of the proteoglycans, although this remains to be proven. In some tissue, the cationic NC4 domain is absent due to the use of an alternative promoter in the *COL9A1* gene (53). Type IX collagen might itself be considered a

proteoglycan, as it can be substituted by a chondroitin sulfate chain in the NC3 domain of its α2 chain. The functional significance of such substitution is unclear.

Other collagens

As with all connective tissue, articular cartilage also contains type VI collagen, which accounts for about 1% of its total collagen content (50). Its content is increases and tissue distribution altered in OA cartilage (54, 55). Type VI collagen is a nonfibrillar collagen composed of three distinct α-chains, with each type VI collagen molecule possessing a central triple helical domain flanked by large nonhelical terminal regions. The type VI collagen molecules assemble into a disulfide-bonded filamentous network, which is predominant in the pericellular matrix around the chondrocytes (54, 55). The proteoglycan biglycan, which interacts with type VI collagen, appears to be important for the assembly of the type VI collagen network. The type VI collagen does not appear to be covalently crosslinked to the other collagen types of the cartilage, but is associated with the fibrillar collagen framework of the tissue via the interaction of matrilin with decorin or biglycan, which might be bound to the collagen give rise to congenital muscular dystrophy; it is not clear how cartilage might be affected in these disorders. In the type VI collagen knockout mouse there is no gross abnormality in cartilage function.

Type X collagen is a cartilage-specific collagen that, under normal circumstances, is confined to the hypertrophic zone of the growth plate, where it participates in endochondral ossification. It is a homotrimeric molecule, with one central triple helical domain and nonhelical termini. It is thought to form a hexagonal array mediated via its C-terminal NC1 domain. Although type X collagen is not a

component of normal articular cartilage, it is present in OA cartilage (56), particular in the deep zones where clusters of hypertrophic chondrocytes are observed. Type X collagen can be cleaved within its triple helical region by MMP-1.

1.2.2.1.2 Collagen biosynthesis

Steps of collagen biosynthesis

There are seven steps for synthesis of collagen as described below;

First step of collagen synthesis are transcription and alternative spicing. Most collagen genes are transcribed into different mRNA species owing to alternative spicing, to a different extent of polyadenylation at the 3' end as in the case of type I, II, and III collagen, or to several transcription start sites in some collagen genes (57, 58).

The second step is translation. Collagens are synthesized a procollagen molecules in lumen of the rough endoplasmic reticulum (RER), transported to the Golgi and secreted (with the exception of membrane-spanning collagens) into the extracellular space via secretory vesicles. The nascent pre-proα-chains protrude after translation from the ribosome-bound collagen mRNA into the lumen of the RER with the help of signal-recognition particles and receptors. Immediately after intrusion, the signal peptide is cleaved off, and the nascent procollagen chains are modifies by hydroxylation and glycosylation before they align into triple helical procollagen molecules, which are secreted and processed before assembling into complex extracellular aggregates (59, 60).

The third step is hydroxylation and glycosylation. In the triple helical region of all procollagens about half of the proline residues in the Y-position of the Gly-X-

Y-triplets are converted to 4-hydroxyproline by the enzyme prolyl-4-hydroxylase, an event which occurs only on nascent collagen α-chains before the triple helix is formed (61-63). A few proline residues are converted to 3-hydroxyproline by the enzyme prolyl-3-hydroxylase (64, 65). The four subunits of the prolylhydroxylase encircle and slide along nascent pro α-chains, but are unable to hydroxylate prolyl-residues after the triple helix has formed. Therefore, the time when the α-chains are kept in a single-chain conformation is critical for the extent of hydroxylation of prolyl residues; the same intrue for hydroxylation of lysine residues. Prolyl-3- and -4-hydroxylase, as well as lysylhydroxylase, require Fe^{2+}, oxygen and 2-oxoglutarate as cofactors and ascorbic acid is required nonstochiometrically for uncoupled decarboxylation of 2- oxoglutarate. Scince non- or under-hydroxylated collagens are degraded to a large extent intracellular (66), the inhibition of prolylhydroxylase activity is considered a potent tool to control excess of collagen synthesis in fibrotic diseases (67).

The fourth step is alignment of the triple helix. Assembly of the procollagen chains to triple helical molecules occurs after hydroxylation of the nascent chains and completion of the C-propeptides. The correct conformation of the C-propeptides and of some N-propeptide is stabilized by the formation of intrachain disulfide bonds (68). This is catalyzed by the enzyme protein disulfide isomerase (PDI) (68, 69), a reducing enzyme with a catalytic site similar to thioredoxin and present in many plants and animal cells. Interestingly, PDI is identical to the β-subunit of prolyl-4-hydroxylase (61, 63), and thus has a dual role: as a β2 dimer it catalyzes and controls the formation of intrachain and interchain disulfide bonds in the C- and N-propeptides: in

combination with two α-subunits of the prolyl hroxylase it catalyzes the hydroxylation of proline.

Procollagen chain assembly involves a two-stage recognition event in the fibril-forming collagens (70). (1) The first association and chain recognition in type I procollagen is driven by the C-propeptides (68); essential for assembly are hydrophobic sequence patches with four hydrophobic amino acids that are conserved in their position in all fibril-forming collagens. (2) Rate limiting for the folding of the triple helix is a *cis-trans*-isomerization of prolyl peptide bonds in the α-chain, which is catalyzed by the enzyme peptide-prolyl *cis-trans*-isomerase (PPI) (69, 71). Critical support for a key role of PPI in collagen chainpfolding was the observation that cyclosporine, an inhibitor of PPI and other cyclins, blocks folding of procollagen I and III in cell culture (71). After alignment of the C-propeptides, in type I procollagen triple helix formation starts from the C-terminus and proceeds towards the N-terminus; it is driven by a patch of 3-4 Gly-Pro-Hypo triplets located at the C-terminal of the triple helix by providing a core stabilized by hydrogen bonds (70).

Although the role of C-propeptide in the chain assembly of fibril-forming collagens is firmly established, their role in chain selectively in cells that produce several collagen types is not clear yet. Interestingly, only procollagen chains containing eight cysteines in the C-propeptide are able to for hemotrimers (e.g. proα(I), α1(II), α1(III)), while procollagen chains lacking two or three of the eight cysteine residues such as pro α2(I) can only form heterotrimer (70).

The fifth step is chaperones involved in folding and processing of procollagen. Several chaperones such as BiP and HSP47 are involved in the post-translational modification of the procollagen molecule (72). It binds cotranslationally to the

nascent pro-α-chain in the RER and prevents unspecific chain folding and aggregation (73, 74).

The sixth step is procollagen processing. Following secretion of procollagen in secretory vesicles, the C-propeptides of the fibril-forming collagen, and the N-propeptides of type I and II, partially also of type III collagen are cleaved by specific proteases. Both procollagen-N-protienases and procollagen-C-protianases belong to a family of Zn^{2+}-dependent metalloproteinase's M12 (45, 75). The N-propeptidase exists in two splice variants in a longer form of 140 kDa (pNP1) and a shorter form of 70 kDa (pNP2) (76, 77). Both cleave the N-propeptide of type I and II procollagen (78), but only in the native state, leaving the N-telopeptide in the hairpin-loop configuration. The procollagen-C-proteinase cleaves the C-propeptides of type I, II, and III collagen, as well as laminin 5 and lysyl oxidase precursor (45).

The last step of collagen synthesis is cross-linking. The formation of intermolecular cross-links within newly formed collagen fibrils conveys the structural stability necessary for tissue function. The main processes involved in the formation initially of intermediate, chemically reducible cross-links, followed by maturation to more stable, nonreducible bonds, have been established over several years. A number of questions still remain. A key enzyme required for cross-linking of collagen α-chain and molecules is the enzyme lysyloxidase (79, 80), which catalyses the oxidation of the ε-amino group of certain lysine and hydroxylysine residues to aldehyde groups. Lysine- or hydroxylysine-derived aldehyde groups initially form covalent Schiff-base-type bonds with ε-amino group of other lysine and hydroxylysine residues in adjacent α-chains within the same or neighboring molecules.

1.2.2.2 Proteoglycan

Cartilage is particularly rich in extracellular matrix, with matrix making up 90% of the dry weight of the tissue (81). Proteoglycans were originally considered as mere structure components of extracellular matrix.

1.2.2.2.1 Structure of proteoglycan

Proteoglycans consist of a core protein and one or more covalently attached glycosaminoglycan chain (Figure 1.4). Glycosaminoglycans (GAGs) are linear polysaccharides, whose disaccharide building blocks consist of an amino sugar (*N*-acetylglucosamine, glucosamine that is variously *N*-substituted, or *N*-acetylgalactosamine) and a uronic acid (glucuronic acid or iduronic acid) or galactose. Figure 1.5 depicts characteristic features of the major types of glycosaminoglycans found in vertebrates. Hyaluronan or hyaluronic acid is shown for comparison: it does not occur covalently linked to proteoglycans, but instead interacts noncovalently with proteoglycans via hyaluronan-binding motifs. Generally, invertebrates produce the same types of glycosaminoglycans as vertebrates, except that hyaluronan is not present and the chondroitin chains tend to be nonsulfated. Most proteoglycans also contain *O*- and *N*-glycans typically found on glycoproteins. The glycosaminoglycan chains are much larger than these other types of glycans (e.g., a 20-kD glycosaminoglycan chain contains ~80 sugar residues, whereas a typical biantennary *N*-glycan contains 10–12 residues). Therefore, the properties of the glycosaminoglycans tend to dominate the chemical properties of proteoglycans (although *N*- or *O*-glycans on proteoglycans may have distinct biological properties as described for glycoprotein).

Proteoglycans were initially grouped together because of the high negative charges of their GAG chains, which make separation from other molecules by ion-exchange chromatography easy. The core protein size ranges from 10kDa to > 500 kDa, and the number of GAG chains attached varies from one to > 100. In addition, several proteoglycans carry GAG chains of more than one type and/or have additional N-linked or O-linked sugar modifications.

Glycosaminoglycan (except for in keratan sulfate (KS) synthesis is initialed by sequential addition of four monosaccharides (Xylose (Xyl), galactose (Gal), galactose, and glucoronic acid (GlcA). From this linker tetrasaccharide, the sugar chains are extended by addition of two alternating monosaccharides, an animosugar and GlcA. In heparin and heparan sulfate (HS), the aminosugar is *N*-acetyl-glucosamine (GlcNAc) and in chondroitin sulfate (CS)/dermatan sulfate (DS), it is *N*-acetyl-galactosamine (GalNAc) (Figure 1.5). The extent of epimerization of GlcA to iduronic acid (IdoA) and the sulphation pattern of the disaccharide units distinguish heparin from HS, and DS from CS (Figure 1.5). In KS, the GAGs are initiated as *N*-linked or *O*-linked oligosaccharides and extended by addition of GlcNAc and Gal.

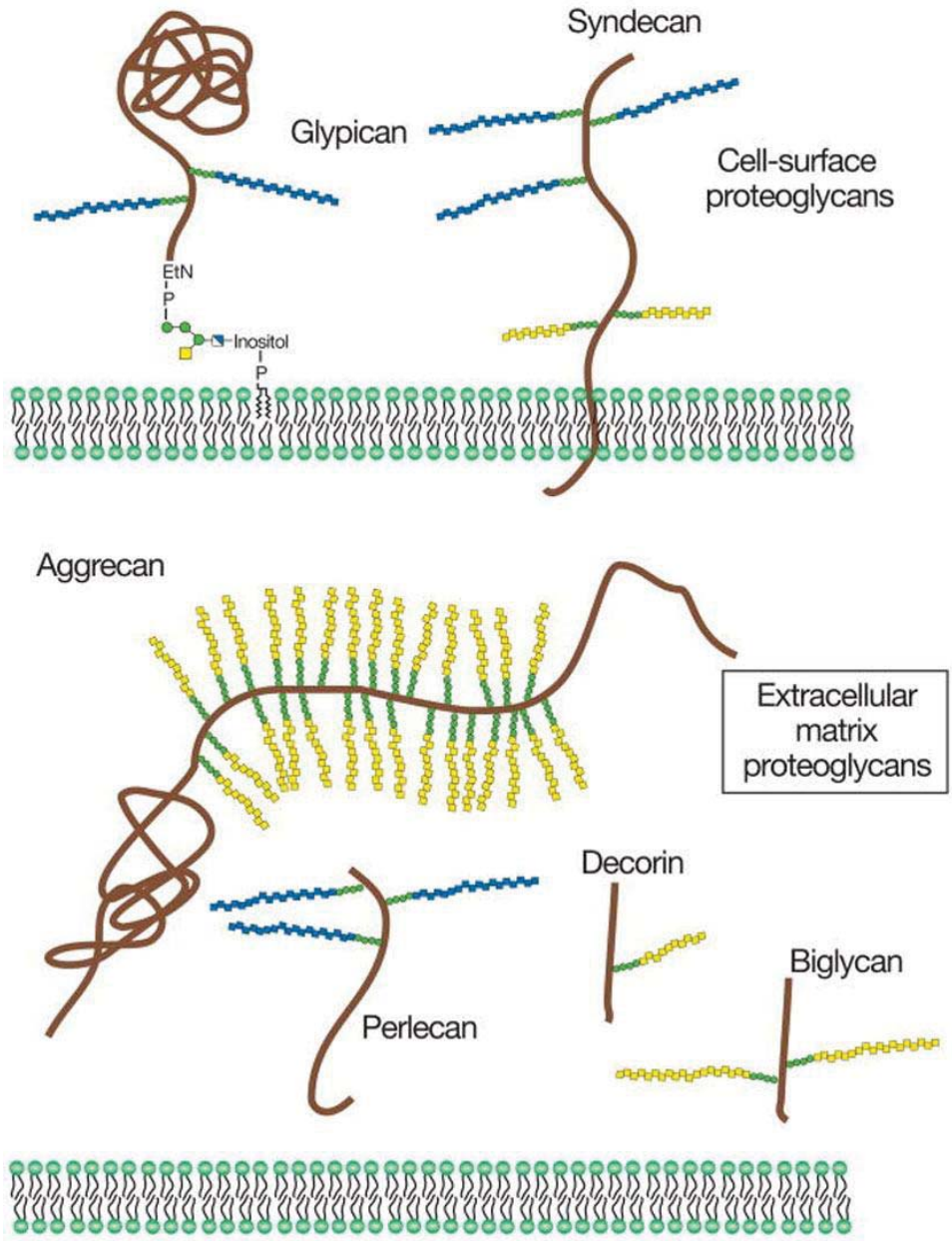


Figure 1.4 Proteoglycans consist of a protein core (*brown*) and one or more covalently attached glycosaminoglycan chains ([*blue*] HS; [*yellow*] CS/DS). Membrane proteoglycans either span the plasma membrane (type I membrane proteins) or are linked by a GPI anchor. ECM proteoglycans are usually secreted, but some proteoglycans can be proteolytically cleaved and shed from the cell surface (not shown) (82).

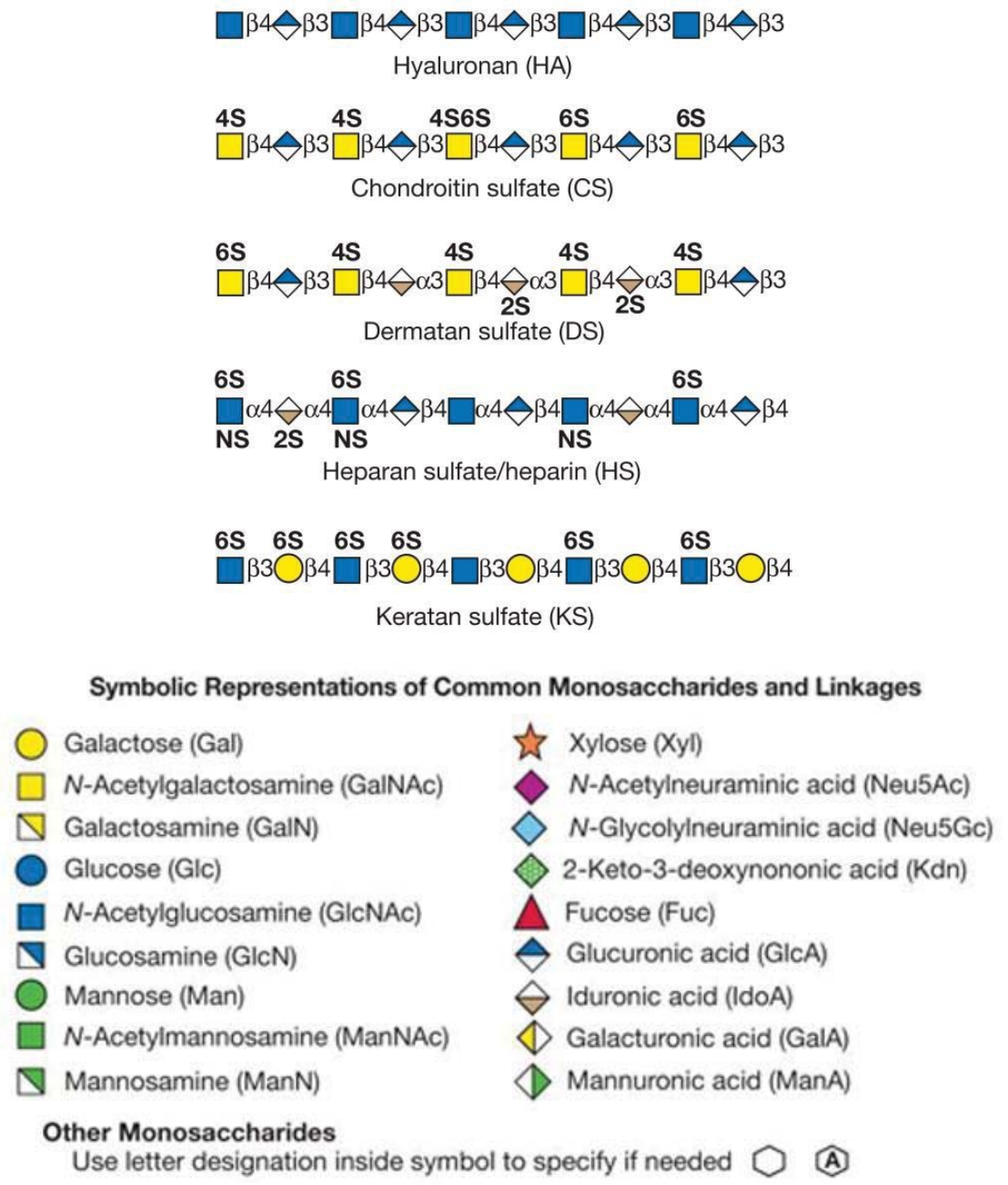


Figure 1.5 Glycosaminoglycans consist of repeating disaccharide units composed of an *N*-acetylated or *N*-sulfated hexosamine and either a uronic acid (glucuronic acid or iduronic acid) or galactose. Hyaluronan lacks sulfate groups, but the rest of the glycosaminoglycans contain sulfates at various positions. DS is distinguished from CS by the presence of iduronic acid. Keratan sulfates lack uronic acids and instead consist of sulfated galactose and *N*-acetylglucosamine residues (82).

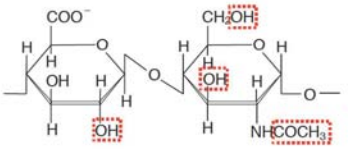
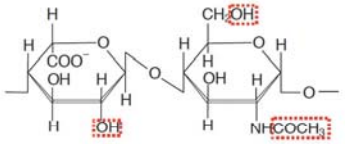
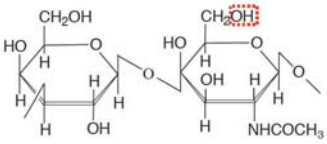
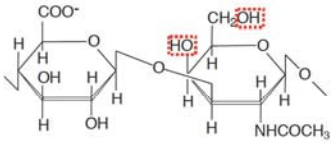
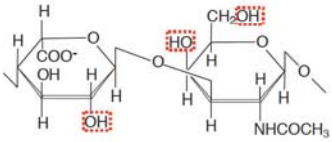
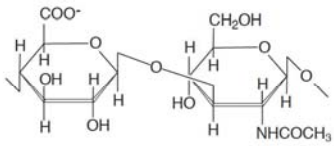
GAG	Hexuronic or Iduronic acid	Galactose	Hexosamine	Disaccharide composition
Heparan sulphate/ Heparin	D-glucuronic acid **(GlcA)** L-iduronic acid **(IdoA)**	-	D-glucosamine **(GlcNAc)**	 **GlcA β(1→4) GlcNAc α((1→4)**  **IdoA α(1→4) GlcNAc α(1→4)**
Keratan sulphate	-	Galactose **(Gal)**	D-glucosamine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

Figure 1.6 Structure of the different glycosaminoglycan chains. The structure of the repeating disaccharides in the different types of glycosaminoglycan chains is drawn without sulphation. The different sulphation positions in each GAG, are marked by encircling with a dashed red line (83).

Virtually all mammalian cells produce proteoglycans and secrete them into the ECM, insert them into the plasma membrane, or store them in secretory granules. The ECM determines the physical characteristics of tissues and many of the biological properties of the cells embedded in it. The major components of the ECM are fibrous proteins that provide tensile strength and elasticity (e.g., various collagens and elastins), adhesive glycoproteins (e.g., fibronectin, laminin, and tenascin), and proteoglycans that interact with other ECM components to provide a hydrated gel that resists compressive forces. Together, the various ECM components provide an extracellular environment that regulates cell proliferation and differentiation. Cells synthesize a diverse group of membrane proteoglycans. These typically have a type I orientation with a single membrane-spanning domain or a glycosylphospha-tidylinositol (GPI) anchor (Figure 1.4). Additionally, some cells concentrate proteoglycans along with other secretory products in secretory granules. Secretory granule proteoglycans are thought to help sequester and regulate the availability of positively charged components, such as proteases and bioactive amines, through interaction with the negatively charged glycosaminoglycan chains (83).

The tremendous structural variation of proteoglycans is due to a number of factors. First, a large number of core proteins have been identified, and these can be substituted with one or two types of glycosaminoglycan chains. Some proteoglycans contain only one glycosaminoglycan chain (e.g., decorin), whereas others have more than 100 chains (e.g., aggrecan). Another source of variability lies in the stoichiometry of glycosaminoglycan chain substitution. For example, syndecan-1 has five attachment sites for glycosaminoglycans, but not all of the sites are used equally. Other proteoglycans can be “part time,” that is, they may exist with or without a glycosaminoglycan chain or with only a truncated oligosaccharide. A given proteoglycan present in different cell types often exhibits differences in the number of

glycosaminoglycan chains, their lengths, and the arrangement of sulfated residues along the chains. Thus, a preparation of syndecan-1 represents a diverse population of molecules, each potentially representing a unique structural entity. These characteristics, typical of all proteoglycans, create diversity that may facilitate the formation of binding sites of variable density and affinity for different ligands (83).

1.2.2.2.2 Cartilage proteoglycan

Aggrecan

The core protein of aggrecan has a molecular weight of ~230 kDa and consists of three globular domains, G1, G2, and G3, and three interglobular domains, the keratan sulfate and chondroitin sulfate glycosaminoglycan attachment domains located between G2 and G3 and a short interglobular domain between G1 and G2 (84, 85). The N-terminus comprises the G1 globular domain, which interacts with hyaluronan and link protein. Mild trypsin treatment generates a protein fragment containing the G1 and G2 domains, which retains hyaluronan binding capacity, and thus is termed the hyaluronan-binding region (HABR). The G1 domain contains three looped subdomains, A, B and B'. Both the B and B' loops form disulfide bonding double loop structures called proteoglycan tandem repeat (PTR) units. The PTR loop cantains the functional site of the binding of aggrecan to hyaluronan. The secondary structure is significant and the HABR no longer binds hyaluronan under reducing conditions. The C-terminus comprises the G3 domain that includes as EGF module, a C-type lectin module and a complement regulatory protein (CRP) module. With aging, there is as increase in the population of aggrecan monomers that lack the G3 domain, most likely due to extracellular proteolytic degradation (85).

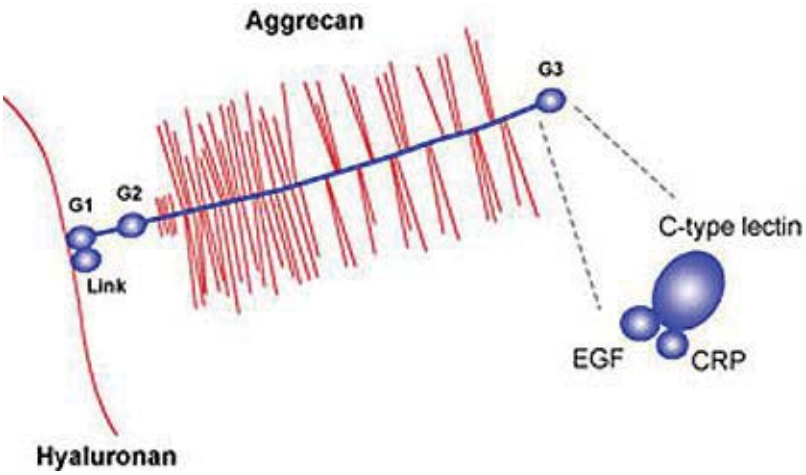


Figure 1.7 Aggrecan monomers contain two extended regions which carry the bulk of the glycosaminoglycan and three globular domains, G1 and G2 at the N-terminus and G3 at the C-terminus of the core protein. The C-terminal G3 domain comprises an alternatively spliced complement regulatory protein-like repeat at the extreme C-terminus, an adjacent repeat homologous with C-type animal lectins and an N-terminal epidermal growth factor (EGF)-like domain that is also subject to alternative splicing (adapted from http://www.cmb.lu.se/ctb/html/CartilageDes.htm).

Other hyaluronan-binding proteoglycans

Other hyaluronan-binding proteoglycans, namely, versican/PG-M, neurocan and brevican contain a G1 and a G3 domain similar to aggrecan (85). Thus these four large proteoglycans can be grouped into a family alternatively named for their common G1 or G3 functional domains, respectively, as the hyaluronan-binding proteoglycans or the lecticans. Until quite recently, the retention of these proteoglycans was thought to be based solely on their interaction with hyaluronan (85).

Small leucine-rich proteoglycan (SLRPs)

The family of small leucine-rich proteoglycans, termed the SLRPs (86) can be divided into three classes; members of each class can be indentified in cartilage.

Class I includes decorin and biglycan both of which bind TGF-β and are found in cartilage. Epiphyseal cartilage stains prominently for decorin and only weakly for biglycan, whereas in presumptive articular cartilage biglycan is found in the pericellular matrix whereas this developing zone is free of decorin (87). In adult articular cartilage decorin is present in the interterritorial matrix while biglycan is found in the pericullular matrix. Decorin is associated with collagen fibril as decorating proteoglycan and carries one chondroitin or dermatan sulfate side chain. The message level for decorin in cartilage is by far the most abundant of all the SLRP family members and shows increases with increasing age in human articular cartilage (88). The horseshoe shape of decorin, and the overall dimensions of this arch, support a model for its interaction with a single triple helix of collagen.

The tissue localization and the potential interaction with other cartilage matrix

components have been less clearly defined for biglycan, which carries two chondroitin sulfate or dermatan sulfate side chains.

Class II SLRPs expressed in cartilage include fibromodulin, lumican and the protein known as PRELP (proline arginine-rich end leucine-rich repeat protein). Fibromodulin carries up to four keratan sulfate side chains (89). Fibromodulin has the capacity to decorate the surface of collagen fiber and therefore may regulate fibril diameter. The message levels for fibomodulin and lumican show increases with increasing age of human articular cartilage (88). Lumican is the major keratan sulfate proteoglycan in the cornea, but also shows widespread distribution in connective tissue, including cartilage. In young cartilage, lumican is found as a keratan sulfate proteoglycan while after IL-1 treatment, chondrocytes synthesize and secrete the lumican protein devoid of glycosaminoglycan substitution (90). PRELP exhibits protein sequence similarity to both lumican and fibromulin and has four potential *N*-linked glycosaminoglycan sites. Thus although it is a member of the SLR proteins, it apparently functions as a cartilage matrix protein with the capacity for matrix organization (91).

The class III SLRP expressed in cartilage is epiphycan (also referred to as PG-Lb). It is a dermatan sulfate proteoglycan that can be separated from decorin and biglycan from epiphyseal cartilage, from which its name derives (92). Two serine residues exhibit the typical glcosaminoglycan attachment consensus structure similar to aggrecan, decorin and biglycan. The expression of epiphycan during development of the growth plate lags behind that of aggrecan, and is excluded from both the layer of presumptive artilcular cartilage and the hypertrophic zone (93). Epiphycan was proposed that it could function to organize the matrix of the growth plate, especially

the zone of flattened chondrocytes wherein it is abundant.

1.2.2.2.3 Proteoglycan biosynthesis

There are four steps in biosynthesis of chondroitin sulfate proteoglycan including core protein synthesis, addition of the linkage oligosaccharides, formation of the GAG chains and sulfation of GAG chains. These processes occur in rough endoplasmic reticulum (ER) and Golgi apparatus, prior to secretion to extracellular matix or cell membrane.

The first step of proteoglycan synthesis is core protein formation. The nascent core protein biosynthesis is initiated by translation in the cytosol and the nascent polypeptide is transported into the lumen of the ER through the translocon, via a signal sequence-mediated process. After the nascent core protein is translocated to the ER lumen for addition of linker tetrasaccharide. The second and third steps are addition of the linkage oligosaccharide and formation of GAG chains (Figure 1.8).

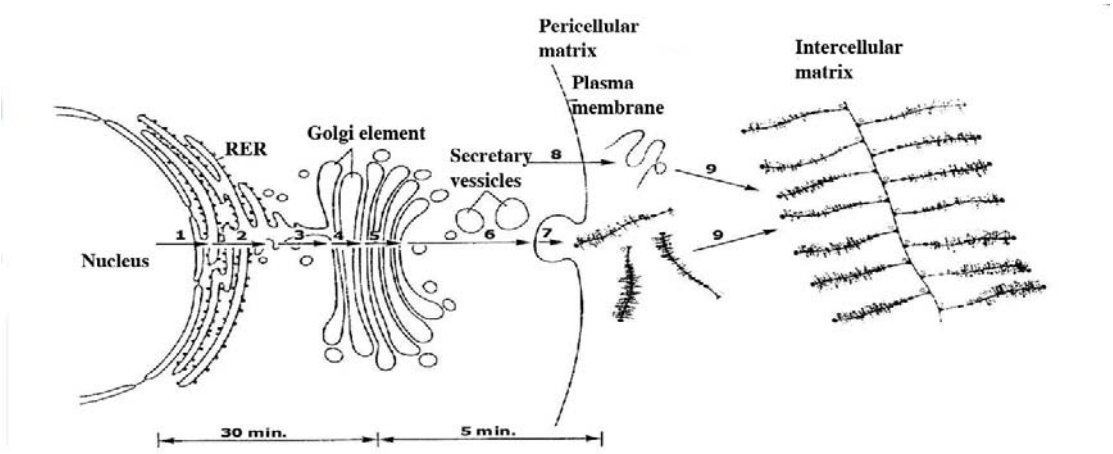


Figure 1.8 Schematic overview of synthesis and secretion of aggrecan, link protein and hyaluronan by a chondrocyte. The transcription of specific aggrecan and link protein genes to mRNA (1). The translation of mRNA in the rough endoplasmic reticulum (RER) to form the protein core (2). The newly-formed protein is transported from RER (3) through the cis (4) and medial-trans (5) Golgi compartments for glycosylation and sulfation. The molecules are transported via secertory vesicles (6) to the plasma membrane, where they are released (7). Hyaluronan is synthtesised at the plasma membrane (8). Finally, aggrecan, link protein and hyaluronan from the aggregates within the extracellular matrix (9) (94).

The cell takes up the building blocks for GAG synthesis, monosaccharides and sulphate, through specialized transporter complexes in the plasma membrane. Sugar and sulphate are then activated by nucleotide consumption in the cytosol to form UDP-sugars and 3'-phosphoadenosine 5'-phosphosulphate (PAPS), respectively. (Figure 1.9) Specific transporters then translocate UDP-sugars and PAPS (95) into the endoplasmic reticulum (ER) and Golgi lumens (96, 97). PAPS is the universal donor of sulphate to all sulfotransferases, both in the Golgi and the cytosol.

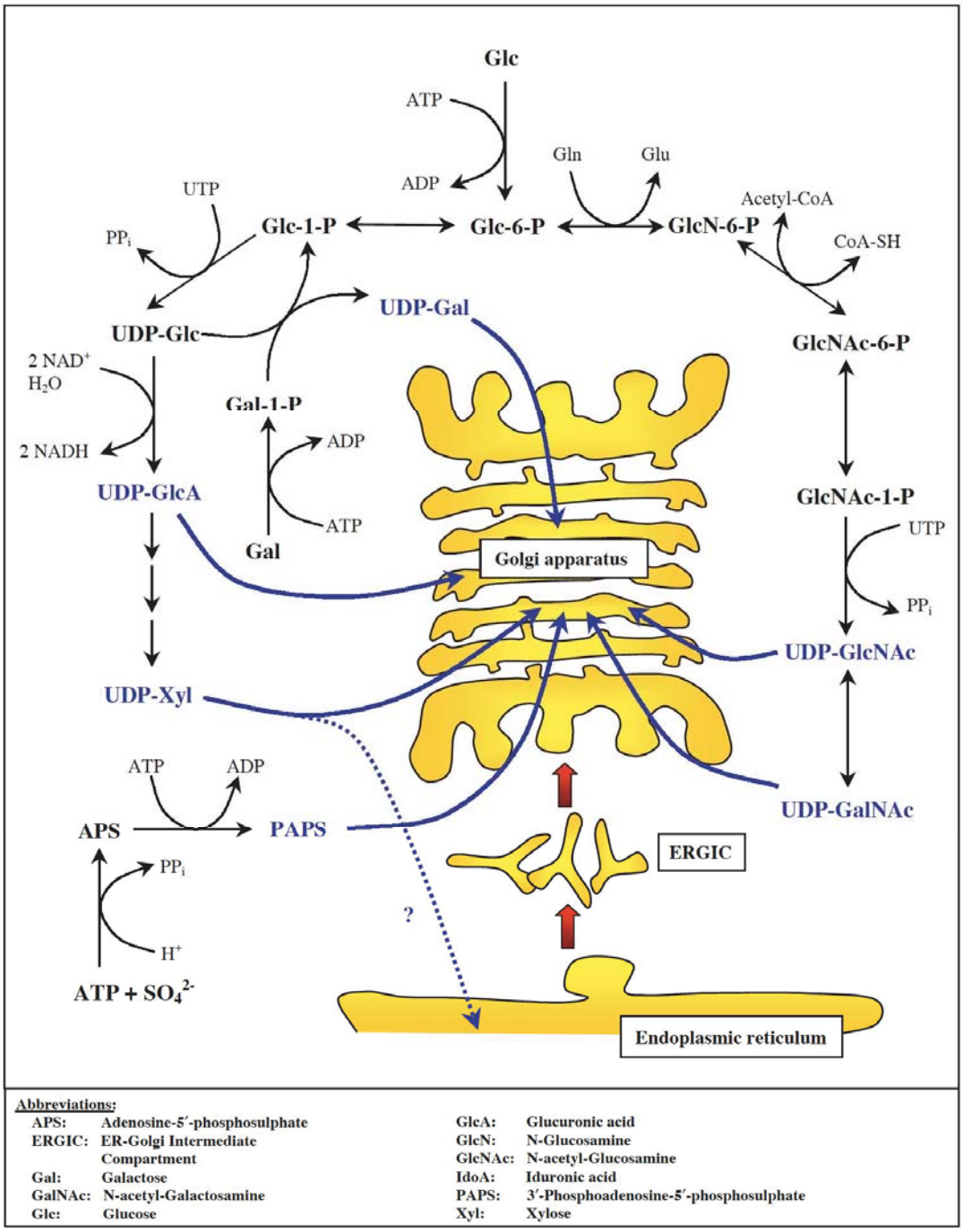


Figure 1.9 Synthesis pathway for the formation of UDP-sugars and PAPS. Synthesis pathway for the formation of UDP-sugar and PAPS needed for synthesis of PGs. Each UDP-sugar and PAPS is actively transported form the cytosol into the Golgi lumen, and into the lumen of ER by a corresponding transporter (83).

Although the lumen of the Golgi apparatus is the main site for GAG synthesis, the formation of the linker tatrasaccharide might start earlier in the secretory pathway. GAG synthesis is initiated by sequential addition of four monosaccharides (xylose (Xyl), Galactose (Gal), Galactose and glucuronic acid (GlcA) Figure. 1.10)

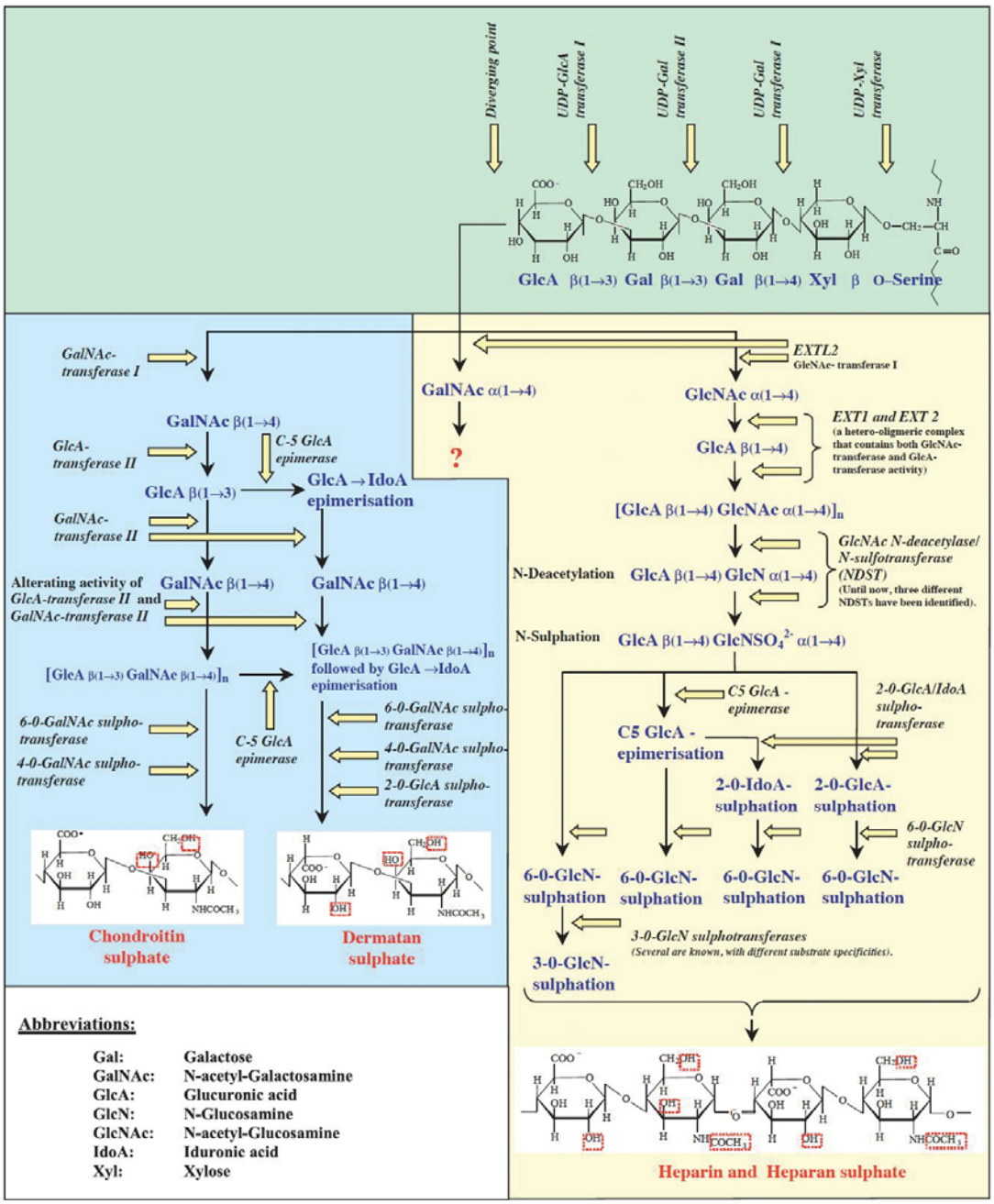


Figure 1.10 The different steps in the synthesis of CS, DS, HS and heparin glycosaminoglycan chains of the GlcA-Gal-Xyl-linker region (83).

The third step is formation of GAG chain. After completion of the linker tetrasccharide, the addition of the saccharide determines whether the GAG chain becomes CS/DS or HS/heparin. This sugar is GlcNAc in the case of HS/heparin and GalNAc in the case of CS/DS. GlcNAcT I (98) and GalNAcT I mediate addition of these sugars (99) and are postulated to be distinct from these enzymes used in elongation of the GAG chain of both CS (100) and HS (101). It has been shown that an α-GalNAcT is able to add GalNAc to the linker tetrasccharide to produce structure that have 'unproductive' pentasccharide because a β-linkage is required for further elongatio of CS chain. This enzyme could be important for regulation, capping some linker tetasaccharides with α-GalNAc and preventing their elongation. Several other factors might also regulate GAG-type synthesis including the amino acid sequence flanking the serine residue, access of UDP-sugars and the presence of GAG-synthesising enzymes in the Golgi, and modifications of the linker region.

For the synthesis of HS/heparin, the addition of the sixth sugar and of all the following sugars, to HS and heparin precursors in catalysed by a bifunctional 70 kDa enzyme (102). It catalyses addition of alternating GlcA and GlcNAc units. More recently, it has become evident that two different protein are involved in the catalysis of HA and heparin porimerization (103, 104). Both of these are products of tumor suppressor gene family (*EXT1* and *EXT2*) of the hereditary multiple exostoses gene family—as is GlcNAcT I (*EXTL2*). The *EXT1* and *EXT2* gene products are both needed for normal HS synthesis, but the exact role of each protein is not clear. The fourth step of HS/heparin synthesis, GAG chain modifications, are occurred at various positions. The modifications include the following: (1) deacetylation/N-sulphation of

GlcNAc unit in HS and heparin; (2) epimerization of GlcA to IdoA in HS and heparin; (3) *O*-sulphation in various positions of the disaccharides of HS and heparin.

For the synthesis of CS/DS, in the case of the CS chain in decorin, which has a single GAG chain, N-terminal deletions in the propeptide result in synthesisof a form of decorin that has a shorter GAG chain (105). The reason for this early termination is not know. Elongation of CS chains is mediated by two distinct transferases that operate by alternate transfer of GlcNAc and GlcA (106). The enzymatic activity that transfers GlcA to the growing GAG chain, glucuronosyl transferase II (107), is different from the activity that transfers GlcA to Gal to complete the linker region (99). The modification of CS/DS chain including: (1) epimerization of GlcA to IdoA in DS; (2) O-sulphation in various positions of the disaccharides of CS/DS.

Although linker tetrasaccharide synthesis seems to be a common pathway for both HS synthesis and CS synthesis, not only are elongation and modification of CS/DS and HS/heparin GAG chains catalysed by different set of enzymes, but these events also take place in different subdomains of the Golgi apparatus.

For sorting of PGs after reaching the plasma membrane, PGs can take part in binding and uptake of signaling molecules. Cell surface GAGs are internalized and have been detected in endocytic organelles and in the nucleus (108-110).

Cells secrete matrix proteoglycans directly into the extracellular environment (e.g., the basement membrane proteoglycans, SLRPs, serglycin, and members of the aggrecan family). However, others are shed from the cell surface through proteolytic cleavage of the core protein (e.g., the syndecans). Cells also internalize a large fraction of cell-surface proteoglycans by endocytosis (Figure 1.11). These internalized proteoglycans first encounter proteases that cleave the core protein and heparanase

that cleaves the HS chains at a limited number of sites, depending on sequence. These smaller fragments eventually appear in the lysosome and undergo complete degradation by way of a series of exoglycosidases and sulfatases. The main purpose of intracellular heparanase may be to increase the number of target sites for exolytic degradative enzymes. CS and DS proteoglycans follow a similar endocytic route, but endoglycosidases that degrade the chains before the lysosome have not been described.

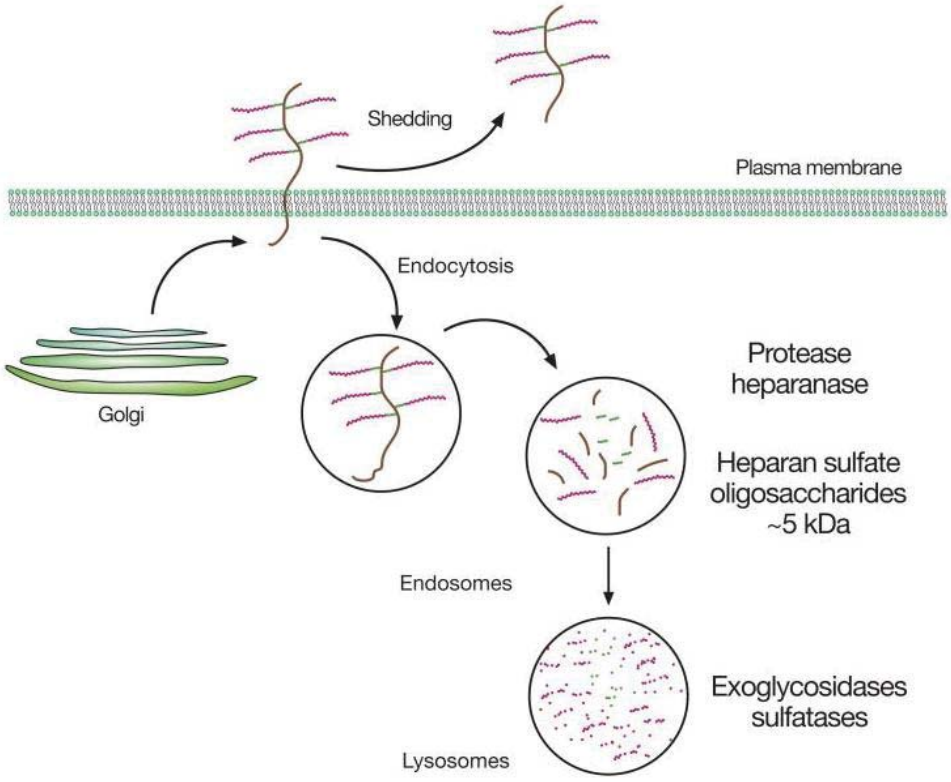


Figure 1.11 Heparan sulfate proteoglycans turn over by proteolytic shedding from the cell surface and endocytosis, as well as step-wise degradation inside lysosomes. (*Brown*) Core protein; (*magenta*) polysaccharide chains. (Adapted, with permission, from (111), ©The American Society for Biochemistry & Molecular Biology.)

1.2.3 Cartilage extracellular matrix

Cartilage matrix, Figure 1.12, consists of fibrillar networks, primarily of collagen II (112, 113), collagen VI, and highly negatively charged molecules of aggrecan (81, 114) providing fixed-charged density and therefore as osmotic environment that created a swelling pressure (115, 116). Also there are a number of noncollagenous glycoproteins that apparently contribute to the regulation of tissue assembly and properties. In some case these constitute small proteoglycans.

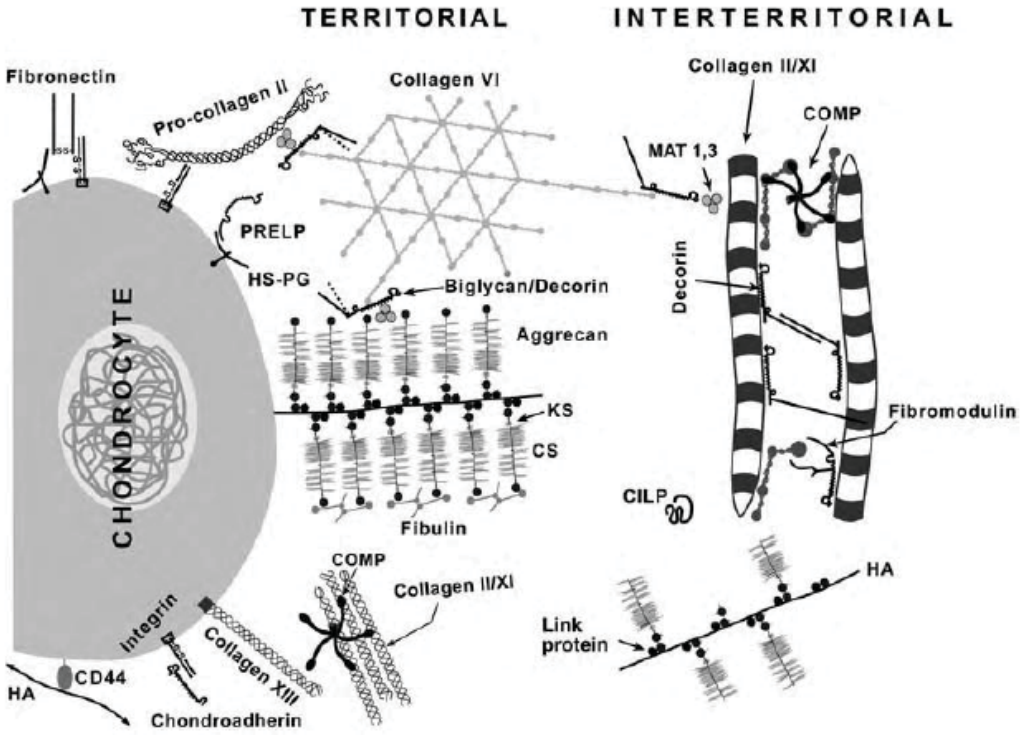


Figure 1.12 Illustration of components in the cartilage extracellular matrix. The different molecular organization of territorial (close to cells) and interterritorial (distant from cells) matrix is depicted. Major constituents are the networks based on collagen II and VI, respectively, where collagen fibers contain numerous bound molecules that have roles in regulating assembly and maintaining function of the network. Interactions at the surface of the chondrocyte are likely to have roles in providing cells with information on matrix properties. MAT1,3: Matrilin 1 and/or 3. PRELP: proline, arginine-rich end leucine-rich repeat protein. HS-PG: heparan sulfate proteoglycan. KS: keratan sulfate. CS: chondroitin sulfate. COMP: catilage oligomeric matrix protein. CILP: cartilage intermediate layer protein. HA: hyaluronic acid. CD44: cluster differentiation 44, hyaluronan receptor (36).

1.2.3.1 Aggrecan

Aggrecan has a major role on providing fixed-charged groups creating as osmotic environment in the cartilage, thus immobilizing water and restricting water flow. This is essence for the function of aggrecan in taking up and distributing load over the cartilage tissue. In fact, the charge density of aggrecan is so extreme that there is a very high swelling pressure in the tissue (115, 116) which is resisted by the collagen network. The tissue can thus be reviewed as a composite of aggrecan containing more than 100 negatively charged chondroitin sulfate side chains, each with some 50 carboxyl and 50 sulfate groups. The other component then is the fibrillar network consisting of collagen and attached crossbridging and linking noncollagenous as well as collagenous matrix protein.

1.2.3.2 The collagen network

The primary functional property of the major network in cartilage based on collagen II is to provide tensional stability. To achieve this, the tissue contains collagen fibers apparently linked together by glycoproteins/proteoglycans. Such linking molecules may function to extend the collagen fibrillar network throughout the tissue. They may also serve to regulate fibrillogenesis by preventing accretion of new collagen molecules to a pre-existing or forming fibril. The collagen fibers themselves contain two types of molecules (e.g. type II collagen and a few percent of type XI collagen), that are closely related and that form the actual fibers (117).

Collagen IX, which represents a more complex collagen consisting of classical triple-helical domains interrupted by globular domains, is bound along the surface of

the fibrils (117, 118). This collagen is largely covalently cross-linked to the collagen II fiber (52).

There is a distinct and apparently separate collagen fibrillar system in cartilage make up of type VI collagen molecules. The character of these molecules is quite distinct, with globular domains capping the triple-helical structures (119, 120). They form thinner fibrils that occur predominantly closer to the cells (121). The function of this network is not clear, but in the tissue it interacts both with the type II collagen fibrillar system and with aggrecan (122). It may thus have a role in organizing and connecting the various networks in the tissue.

1.2.3.3 Collagen associated molecules

There are a number of noncollagenous molecules bound to the collagen fibrils in the tissue. These include decorin, fibromodulin, and lumican, all binding to fibers of collagen (123, 124), but in general not showing a great deal of specificity for a particular type of collagen. These molecules have structures of protein core, suitable for binding to other matrix components. A related molecule, biglycan, appears not to primarily bind type II collagen but binds tightly to the N-terminal domain of type V collagen (125).

1.2.3.4 Other cartilage extracellular matrix constituents

A prominent component in cartilage is COMP (Cartilage Oligomeric Matrix Protein). This protein is a homopentamer of five subunits, each with a molecular mass of 87 kDa. These are joined via a coiled-coil domain near the N-terminal (126) and the interaction is further stabilized by disulfide bonds. It has been shown that COMP

interacts with triple helical collagen with a K_D of 10^{-9} in a Zn^{2+}-dependent manner (127). Each of five subunits contains one binding site in the C-terminal globular domain, thus potentially providing the protein with five interaction sites. Therefore, COMP may have a role in stabilizing the collagen network and/or in promoting the collagen fibril assembly. Moreover, it may have a role in cell interactions. Its synthesis in the tissue is up-regulated in the early stages of osteoarthritis (128) indicating the role for the protein in repair attempts. However, its function in the tissue is little known.

Cartilage matrix protein (CMP, Matrilin-1) is the member of the matrilin family (129). These proteins contain von Willebrand Factor A (vWFA), in the case of matrilin-1, two such domains in each of the three identical subunits with a molecular mass of around 50 kDa. Matrilin-1 and -3 are generally restricted to cartilage (130, 131), while matrilin-2 has a more general distribution. Its role in the cartilage is not clear, but its two vWFA homology domains may mediate the ability of the protein to bind to collagen (122). Moreover, it was found that matrilin-1 has ability to bind to aggrecan (132).

An important filamentous network present in many weight-bearing tissue is the one containing collagen VI as the basic unit. The unit secreted from the cell is a tetramer, where each molecule contains a trimer of three different α-chains. The central part of the unit is a triple helical region, with globular structures at each end. The N-terminal extension of the alpha 3 chain contains 10 vWFA domains forming the major part of this globule of the subunit, while each of the three chains contains 2 vWFA domains in their C-terminal extension. These subunits are arranged in pairs in an antiparallel fashion (133). These are forming long filaments and orthogonal

structures by interactions via the N-terminal domains in both end-to-end and side-to-side interactions (134). These interactions may have a central role in networking the various structural elements in cartilage and thereby in forming an assembly suitable for sustaining high load.

1.2.3.5 Other proteins in cartilage

There are a number of other proteins in cartilage where the information on functional roles is scare. These include a 39-kDa protein (GP-39), also referred to as YKL-40, found predominantly in the more superficial parts of articular cartilage (135). Others are lubricin, which its function appears to relate to lubrication at soft tissue interfaces, fibronectin that is interesting up-regulated in early and late osteoarthritis (128, 136).

1.2.4 Cartilage matrix metabolism

Degradation and synthesis of cartilage macromolecules under normal physiological conditions is kept in equilibrium and can therefore be viewed as a balance (Figure 1.13). Chondrocytes and synovial cell response to a variety of cytokines and growth factors that stimulate the production of destructive proteinases. All four major classes of proteolytic enzymes (aspartic, cysteine, serine and metallo) are involved in normal turnover and pathological destruction, and the pathway that predominates will alter depending on the reproductive circumstance (137). These pathways are not usually exclusive and it is highly probable that total degradation of matrix components involves several pathways and classes of proteinases.

Chondrocytes maintain articular cartilage by replacing degraded components via local synthesis, which is balanced with degradation to prevent over-depositon of matrix. In arthritic diseases, this equilibrium is shifted towards degradation. As illustrated in Figure 1.13, chondrocyte and synovial cells are stimulated by both (a) anti-inflammatory and (b) pro-inflammatory cytokines, as well as mechanical stress, and cell-cell and cell-matrix contacts, via a variety of cell-surface receptors. These stimuli are transferred to the nucleus via intracellular signaling and mechanotransduction pathways, resulting in activation of gene transcription. Synthesis and secretion of matrix components, including aggrecan and collagen occurs, as well as enzymes such as aggrecanases (e.g. ADAMTS-5), pro-matrix metalloproteinase (pro-MMPs)(including procollagenases) and activating enzyme (e.g. MMP-3, membrane-type MMPs and plasmin). Aggrecanases promote rapid aggrecan loss, and this might be inhibited by tissue inhibitor of metalloproteinase 3 (TIMP-3). Induction of TIMPs, and other inhibitors, via anti-inflammatory agent can block some activating enzymes the otherwise covert pro-enzymes to their active forms. These activating enzymes might in turn specific cleavage of triple-helical collagen via collagenases as well as further nonspecific collagen hydrolysis by other MMPs; aggrecan degradation by MMPs might also occur.

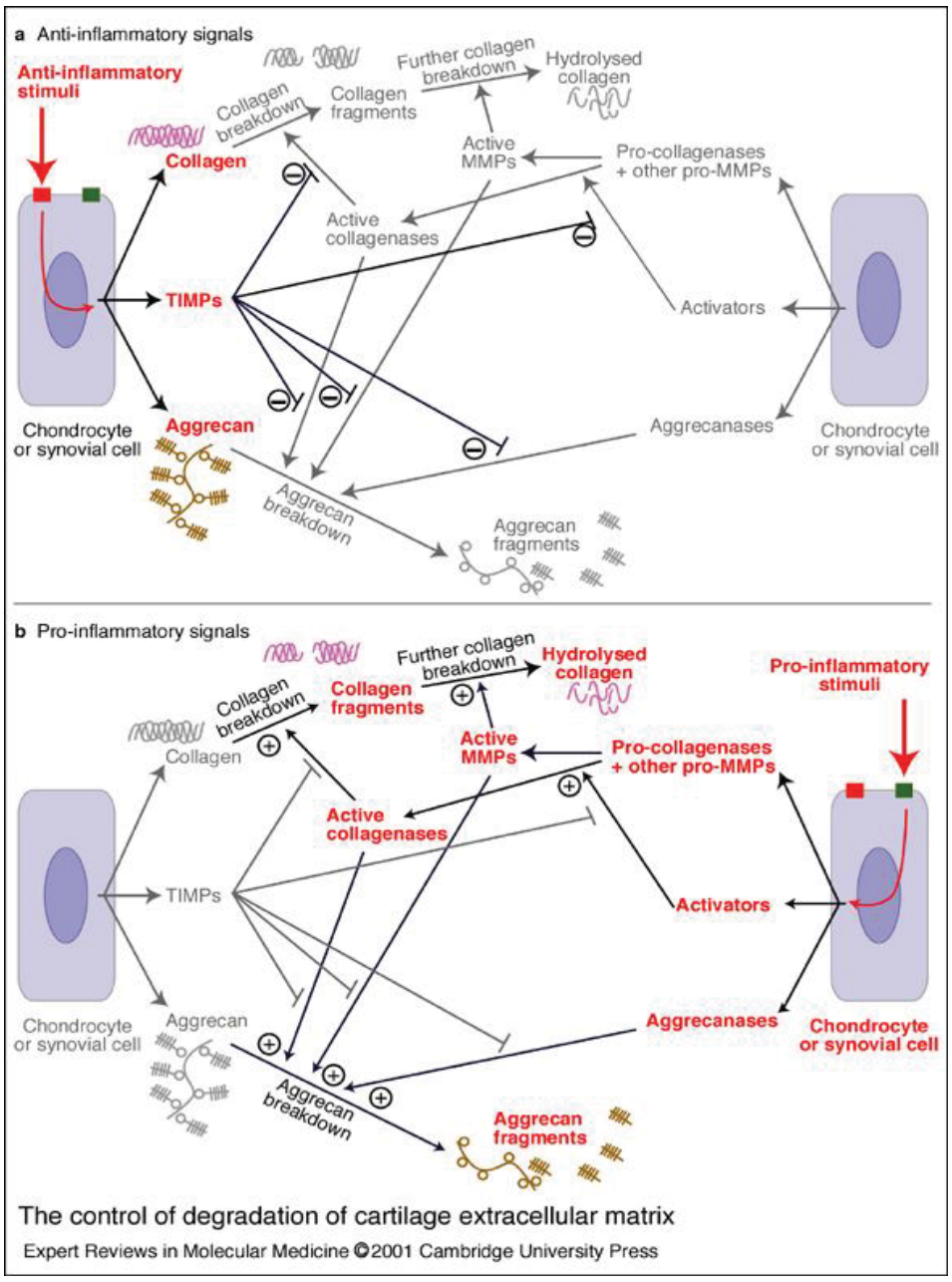


Figure 1.13 The control of degradation of cartilage extracellular matrix (138).

1.2.5 Osteoarthritis

Osteoarthritis (OA) is characterized by a progressive loss of articular cartilage accompanied by new bone formation and, often, synovial proliferation that may culminate in pain, loss of joint function, and disability. A variety of etiologic risk factors and pathophysiogogic processes contribute to the progressive nature of the disease and serve as targets of behavioral and pharmacologic interventions. Risk factors such as age, sex, trauma, overuse, genetics, and obesity can each make contributions to the process of injury in different compartments of the joint. Such risk factors can serve as initiators that promote abnormal biochemical processes involving the cartilage, bone, and synovium, which over a period of years result in the characteristic features of OA: degradation of articular cartilage, osteophyte formation, subchondral sclerosis, meniscal degeneration, bone marrow lesions, and synovial proliferation (140).

1.2.5.1 Pathobiology of osteoarthritis

Chondrocytes embedded within the negatively charge cartilaginous extracellular matrix, are subjected to mechanical and osmotic stresses (139-141). One of the most exciting emerging areas is that chondrocytes, like osteocytes in bone, serve as mechano-sensors and osmo-sensors, altering their metabolism in response to local physiochemistry changes in the microenvironment. Therefore, while obesity and joint misalignment are risk factors for OA in specific joints, the mechanism by which these risk factors initiate and perpetuate OA is largely by biochemical pathways. Several group have identified osmo-sensors and mechano-sensors in chondrocytes in the form of several ion channels, sulfate transporters and integrins (139-141). In

response to mechanical stress, changes in gene expression and an increase in production of inflammatory cytokines and matrix-degrading enzymes have been noted (Figure 1.14) (142). The recognition that chondrocytes act as mechano-sensors and osmo-sensors has opened up the possibility that these proteins could serve as novel targets for disease-modifying OA drugs.

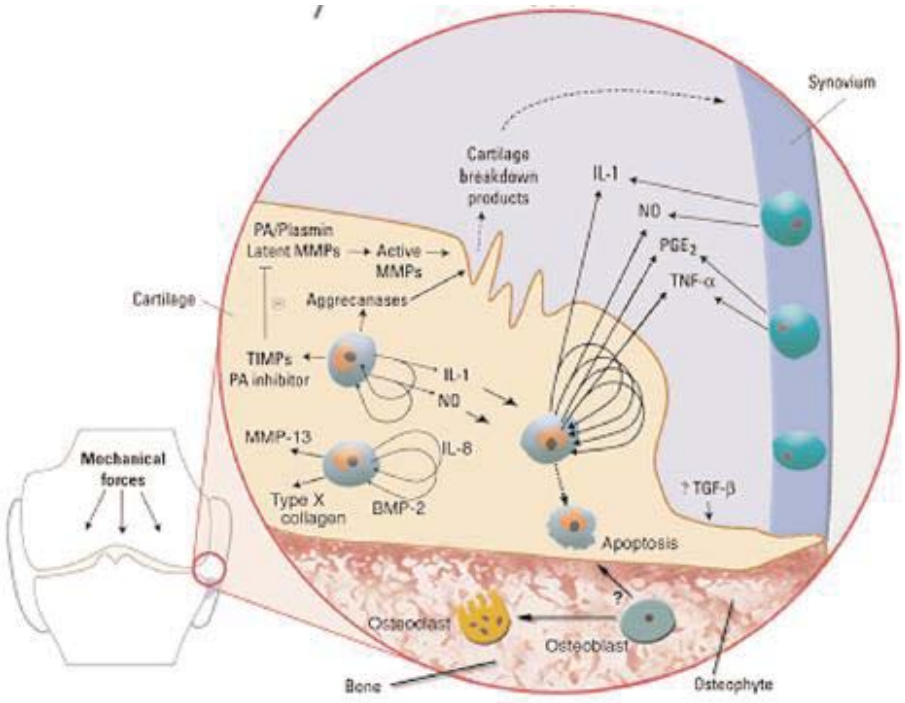


Figure 1.14 Molecular and cellular mechanisms that perpetuate osteoarthritis. BMP, bone morphogenetic protein; MMP, matrix metalloproteinase; NO, nitric oxide; PA, plasminogen activator; PG, prostaglandin; TGF, transforming growth factor; TIMP, tissue inhibitor of MMP; TNF, tumor necrosis factor. (Adapted from Abramson and coworkers (143).

1.2.5.2 Degeneration of articular cartilage in osteoarthritis

1.2.5.2.1 Cartilage degradation

Osteoarthritis is characterized by a loss of articular cartilage matrix, which is the result of the action of proteolytic enzymes that degrade both proteoglycans (aggrecanases) and collagen (collagenases).

Aggrecanase

Degradation of aggrecan is an important manifestation of OA. Aggrecan depletion in OA cartilage can be ascribed to increased proteolytic cleavage of the core protein and is mediated by various matrix proteinases. *In vitro* aggrecanolysis by MMPs has been widely studied; however, it is now well recognized that aggrecanases are the principal proteinases responsible for aggrecan degradation *in situ* in articular cartilage. Two recently identified aggrecanase isoforms (ADAMTS-4 and ADAMTS-5) are members of the 'A Disintegrin And Metalloproteinase with ThromboSpondin motifs' (ADAMTS) gene family, and there has been much interest in the possible role of these isoforms as therapeutic targets in OA (144).

Although aggrecanase was identified over a decade ago because of its unique cleavage site, only two aggrecanase proteins, aggrecanase-1 and -2, have been identified (145, 146). Both enzymes were purified from IL-1-stimulated bovine nasal cartilage and were identified by following their activity with an assay using the neoepitope antibody BC-3, which detects the new N-terminus, ARGS, formed by specific cleavage at the Glu^{373}-Ala^{374} bond in the IGD of the aggrecan core protein (147).

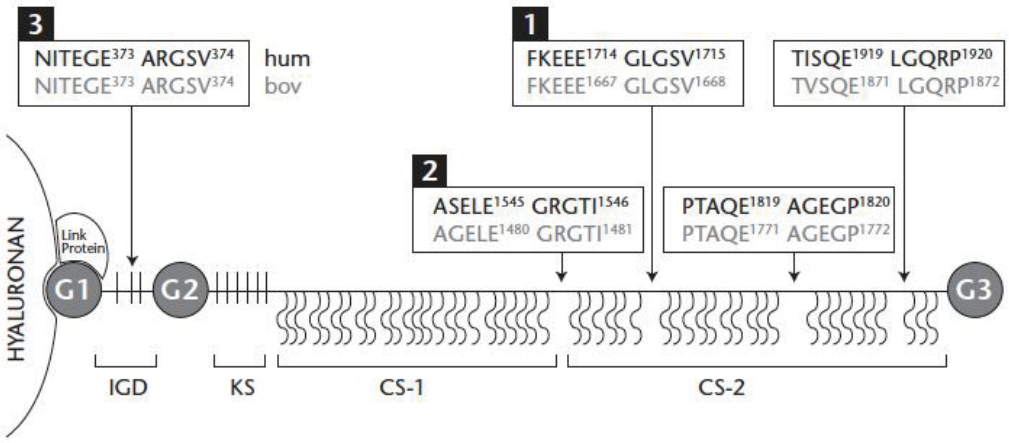


Figure 1.15 Aggrecanase cleavage sites in the aggrecan core protein. The schematic diagram shows the aggrecan core protein with globular G1, G2 and G3 domains. The core protein is substituted with chondroitin sulphate (CS) (wavy lines) and keratan sulphate (KS) (straight lines) chains. Aligned aggrecanase cleavage sites are shown for human (hum, black) and bovine (bov, grey). Numbered flags above the boxed sequences denote the preferred order of enzymatic cleavage (IGD, Interglobulardomain) (148).

Aggrecanase-1 and -2 are members of the ADAMTS family of zinc metalloproteinase's and have been designed ADAMTS-4 and -5, respectively. They are both multidomain MMPs that are secreted into the extracellular space as furin-active proteases (135). They also both consist of a signal sequence, prodomain, catalytic domain, disintegrin-like domain, spacer region, and thrombospondin motifs (TSP) and submotifs (Figure 1.16), which help to regulate their activity and substrate specific (149).

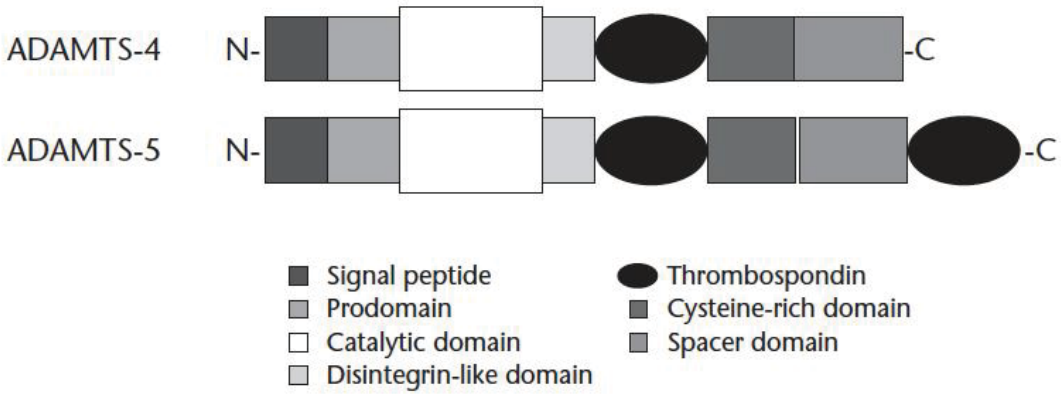


Figure 1.16 The structure of ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2). Both proteinases consist of a signal sequence peptide, prodomain, catalytic domain, disintegrin-like domain, spacer domain, and thrombospondin motifs and submotifs, which play a role in regulating their activity and substrate specificity (150).

Although there are multiple sites of cleavage along the protein core, the critical site of cleavage occurs within the IGD located between the G1 and G2 to release the GAG-rich C-terminus, which is important for the mechanical properties of cartilage. Cleavage at the Glu^{373}-Ala^{374} bond in the IGD is the signature activity of the aggrecanases and is widely reported in humans and in animals as an activity marker (151, 152). Early research using N-terminal sequence analysis demonstrated that the GAG-containing aggrecan fragments found in the synovial fluid of patients with RA and other types of inflammatory arthritis, OA and joint injury, all had the N-terminus sequence Ala-Arg-Gly-Ser (153). This idicates that they had been claved by aggrecanase of the aggrecanase cleavage site, Glu^{373}-Ala^{374}, within the IGD, which is a classic cleavage site for aggrecanase-mediated aggrecan degradation. Further evidence is provided by the fact that C-terminal fragments with the new N-terminus, ^{374}ARGSVIL, formed by cleavage between the amino acid residues Glu^{373}-Ala^{374}, have been identified in the synovial fluid of patient with OA (154), and in chondrocyte cultures stimulated with IL-1 or retinoic acid (155), suggesting that cleavage at this site plays a crucial role in cartilage degradation. Cleavage at Glu^{373}-Ala^{374} is not the preferred site of action of these enzymes. ADAMTS-4 and -5 cleave aggrecan preferentially at four additional sites located in the CS-rich region, between G2 and G3 domain as shown in Figure 1.15.

To examine the activity of aggrecanase in cartilage matrix degradation in OA, one of initial approaches was to identify and characterize specific enzyme-generated cleavage products *in vitro*. To define the identify, distribution and quantity of such fragments, monospecific antibodies were produced that recognized the enzyme-generated neoepitopes that were not present in the native, uncleaved molecules.

Antibody BC-3 recognized the new N-terminus (ARGSV) on aggrecan degradtion products, produced by the action of the uncharacterized proteolytic activity, aggrecanase (156-158). This antibody was first used in the detection of aggrecan degradation products in culture medium obtained from two *in vitro* culture systems: bovine cartilage explants treated with either retinoic acid and IL-1, and rat chondrosarcoma cells treated with retinoic acid (147). Both IL-1 and retinoic acid treatment caused an increase in aggrecan catabolism. This, in turn, increased the release into the medium of specific aggrecan degradation products containing the ASGSV neoepitope generated by the action of aggrecanase (147). Further study on human cartilage, using new epitope antibodies to G1 aggrecan fragment, has shown that aggrecan generated G1-Asn-Ile-Thy-Glu-Gly-Glu fragments are present in normal articular cartilage, and that these fragments appear to accumulate with age (159, 160). This neoepitope was also detected by immunohistochemistry in articular cartilage from patients undergoing joint replacement for OA and RA: cartilage in these specimens was significantly more degraded and high levels of staining for the NITEGE neoepitope were always seen in areas with extensive cartilage damage (159). The demonstration of these two aggrecanase-derived fragments again supports the conclusion that cleavage at the Glu^{373}-Ala^{374} site within the IGD plays a critical role in cartilage breakdown. A schematic representation of the structure and generation of the ARGSV and NITEGE neoepitopes is present in Figure 1.17.

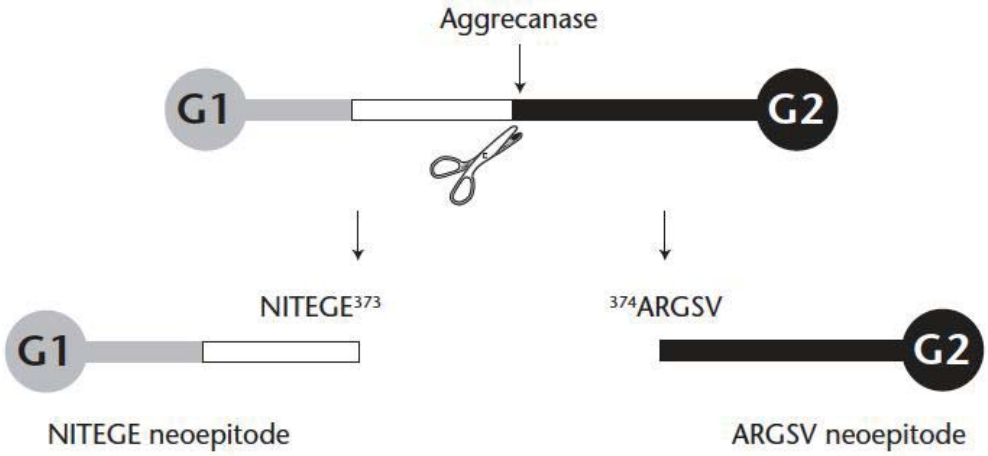


Figure 1.17 Schematic representation of the two main aggrecanase-generated aggrecan cleavage fragments, NITEGE neoepitope and ARGSV neoepitope (G1 and G2 represent globular domains) (148).

Matrix metalloproteineases (MMPs)

Current data indicate a major role for the MMP family in the OA and RA disease processes. Other enzymes from the serine- and cysteine-dependent protease families, such as the plasminogen activator (PA)/plasmin system and cathepsin B, respectively, also play roles but they appear to act primarily as activators of MMPs. Another MMP activity, aggrecanase, appears specifically responsible for aggrecan cleavage.

Metalloproteinases belong to a superfamily of zinc-dependent proteases known as metzincins. They are often grouped into four distinct subfamilies in which the MMPs and the adamalysins are the most relevant for arthritis.

MMPs are a family of zinc endopeptidases that are structurally and functionally related. The MMPs are capable of degrading a variety of ECM protein components including the collagens, proteoglycans, fibronectin and laminin (Table 1) (161).

Table 1.1 Classification of matrix metalloproteinases (continuted).

MMP	Enzyme name	Latent/active (kDa)	Preferred substrates	Cytokine/growth factor inducers	Cytokine/growth factor inhibitors
MMP-1	Collagenase-1 or interstitial collagenase	52/43	Collagen type I, II, III, VII, X, gelatins, aggrecan, tenascin, link protein	EGF, FGF-a, -b, -7, -9, GM-CSF, HGF, IL-1α, -1β, -5, -6, -8, -10, PDGF, TGF-α, -β1, TNF-α, -β	BMP-2, IL1Ra, -11, -13, TGF-β1, -β2, -β3
MMP-2	Gelatinase A or gelatinase 72 kDa	72/62	Gelatin, collagens I, II, III, IV, V, VII, X, XI, type IV collagenase, fibronectin, laminin, aggrecan, elastin, tenascin, vitronectin	EGF, endothelin-1, FGF-b, -3, G-CFS, GM-CFS, HGF, IGF-1, IL-1α, -1β, -3, -6, -8, -13, LIF, M-CFS, oncostatin M, PDGF, TGF-α, -β1, TNF-α	INF-β, -γ, IL-4, -10
MMP-3	Stromelysin-1	52/43	Aggrecan, gelatin, fibronectin, laminin, link protein, elastin, collagen types I, III, IV, V, VIII, IX, X, procollagenase-1, vitronectin, tenascin, decorin	EGF, FGF-b, HGF, INF-β, -γ, IGF-1, IL-1α, -1β, -6, -8, -10, -17, -18, oncostatin M, PDGF, TGF-α, TNF-α	INF-γ, IL-4, -11, -13, TGF-β1
MMP-4	Identified as MMP-3				
MMP-5	Identified as MMP-2				
MMP-6	Identified as MMP-3				

MMP-7	Matrilysin	28/19	Aggrecan, fibronectin, vitronectin, tenascin, laminin, gelatin, collagen type IV, elastin, procollagenease-1, link protein	EGF, FGF-a, -b, IL-1α, 1β, -4, -10, FGF-9, -10, INF-γ, TGF-β1, TNF-α	TGF-β1
MMP-8	Neutrophil collagenase	75/55	Collagen types I, II, II, VIII, X, aggrecan, link protein	IL-1β, TNF-α	IL-1Ra, -4, -10. INF-β, -γ, TGF-β1, -β2
MMP-9	Gelatinase B or gealatinase 92 kDa	92/82	Gelaltin, collagen types I, III, IV, V, VII, X, XI, XIV	EGF, FGF-b, -3, G-CSF, GM-CSF, HGF, INF-α, -γ, IGF-1, IL-1Ra, IL-1β, -3, -6, -8, -13, -17, M-CSF, oncostatin M, PDGF, TGF-α, -β1, TNF-α, -β	
		/65	Aggrecan, elastin, vitronectin		
MMP-10	Stromelysin-2	52/44	Aggrecan, fibronectin, laminin, collagen types I, III, VI, V, VIII, IX, gelatin, elastin, laminin	EGF, FGF-7, TGF-α, -β1, TNF-α	
MMP-11	Stromelysis-3	51/46	Fibronectin, laminin, collagen type IV, aggrecan, gelatin, α1-	EGF, FGF-b, IGF-II, IL-6, PDGF	

MMP-12	Metalloelastase	52/20	antitrypsin, serpin Elastin	GM-CSF, IL-1β, -13, M-CSF, PDGF, TNF-α	INF-γ, TGF-β1
MMP-13	Collagenase-3	52/42	Collagen types I, II, III, VII, X, aggrecan, gelatins	EGF, FGF-b, -7, IL-1β, -6, LIF oncostatin M, PDGF, TGF-α, -β1, -β2, TNF-α	BMP-2, -4, -6 INF-γ, IL-4, -13, TGF-β1
MMP-14	MT1-MMP	64/54	Pro-MMP-2, collagen types I, II, III, dermatan sulfate, laminin, fibronectin, gelatin, vitronectin	GM-CSF, HGF, IL-1α, -1β, TNF-α	
MMP-15	MT2-MMP	71/61		*	*
MMP-16	MT3-MMP	66/56	Pro-MMP2		
MMP-17	MT4-MMP	62/51			
MMP-18[a]	Collagenase-4	53/42		*	*
MMP-19	RASI-1	54/45		*	*
MMP-20	Enamelysin	54/22	Enamel matrix	*	*
MMP-21	X-MMP	70/53		*	*
MMP-22	C-MMP	52/42		*	*
MMP-23	CA-MMP or MIFR-1	56/65		*	*
MMP-24	MT5-MMP	63/45/28		*	*
MMP-25	MT6-MMP	63/58		*	*
MMP-26	Endometase	28/19		*	*
MMP-27	Epilysin	56/45		*	*
MMP-28	(Newly identified Gene Bank AAG41981.1)				

* Indicates no link with cytokines/ growth factors.

[a] These MMPs might be identified as other known MMPs.

To date, at least 25 different MMPs have been identified that share significant sequence homolog and a common multi-domain organization. According to their structural and functional properties, the MMP family can be subdivided into five major groups: (1) the collagenases (MMP-1, 8, -13), (2) the gelatinases (MMP-2, -9), (3) the stromelysins (MMP-3, -10, -11), (4) a heterogeneous subgroup including matrilysin (MMP-7), enamelysin (MMP-20), macrophage metalloelaftase (MMP12) and MMP19, and (5) the membrane-type MMPs (MMP-14 to -17 and -24, -25 or MT1-6-MMP). Other MMPs have been identified but their functions have not yet been completely elucidated. The first four groups are considered to be the 'classical MMPs (162).

MMPs, although the product of different genes, share structural and functional properties, including optimal activity at neutral pH and the requirement for calcium and zinc for biological activity. In general, MMP enzymes are composed of three distinct domains: as amino-terminal propeptide domain that is involved in the maintenance of enzyme latency; a catalytic domain that binds zinc and calcium ions; and a haemopexin-link domain at the carboxy terminal (162). In addition to the zinc ion required for enzymatic catalysis, the catalytic domains of matrixin contain additional structural metal ions, which are required for the stability and expression of enzymatic activity. The 72 kDa (MMP-2) and 92 kDa (MMP-9) gelatinases contain three repeats of fibronectin-like type II domains inserted within the catalytic domain. These domains have been shown to interact with various collagens and gelatins (163). Additionally, the haemopexin-like domains seem to play a role in substrate binding. These domains are an absolute requirement for collageneases to cleave triple helical interstitial collagens, although the catalytic domains alone retain proteolytic activity

toward other substrates. The haemopexin domain of the 72 kDa gelatinase (MMP-2) is also required for the cell surface activation of pro-MMP by membrane type I MMP (MT-1MMP) (164).

MMPs are synthesized as pro-enzymes and must be activated by proteolytic cleavage. Generally, they are present as soluble forms, but some are membrane bound. The activation of the latent secreted enzymes results from the proteolytic cleavage of the propeptide domain from the N-terminus of the enzyme. Due to their specificity and enzymatic turnover, MMPs are controlled at multiple levels. Synthesis, activation and inhibition of the active enzymes are controlled by both physiological and pathological factors such as pro-inflammatory cytokines, hormones, growth factors and proteases.

The collagenouses are secreted as latent enzymes and, once activated, are capable of degrading native collagens. Three collagenases have been identified in human cartilage and their levels shown to be increased in OA: collagenase-1 (MMP-1), collaganase-2 (MMP-8), and collagenase-3 (MMP-13). Although all three collagenases are active on collagen fibrils, they are biochemically distinct role for each (165). Collagenases-3 preferentially cleaves type II collagen and is 5-10 times more active on this collagen type than collagenase-1; collagenase-2 has a higher specific for type I collagen, and colagenase-1 for type III collagen. There is also a striking difference in the distribution of collagenase-1 and collagenase-3 within arthritic cartilage (166, 167). In OA, collagenase-1 and collagenase-2 are located preferentially in the superficial zone of the cartilage, whereas collagenase-3 is found mainly in the lower intermediate and deep layers (deep zone). Moreover, results from an immunohistochemical study using the dog anterior cruciate ligament transection

OA model (166) demonstrated that the level of collagenase-1 in chondrocytes increased steadily in the superficial zone of the cartilage in association with the progression of the lesions, whereas the levels of collagenase-3 in the deep zone reached a plateau at the moderate stage of the disease. Taken together, data on these collagenases suggest that, in OA tissue, collagenase-1 is involved in tissue destruction during the inflammation process and collagenase-3 has a role in the remodeling phase of the disease.

The gelatinases, which are secreted as pro-enzymes, have a substrate preference for denatured collagen, gelatin, and type IV and V collagens. Both gelatinases (MMP-2 and -9) have been identified in the articular tissues and have been shown to be increased in human OA cartilage.

The stromelysins show broader substrate specificity that includes proteoglycans, fibronectin, elastin and laminin. Stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), and stromelysin-3 (MMP-11) have been described in arthritic tissues. The expression of stromelysin-1 has been found to be increased in OA tissues and stromelysin-2 and stromelysin-3 were detected in the synovial fibroblasts of RA patients. Histochemical studies have demonstrated a relationship between the level of stromelysin-1 and the severity of proteoglycan degradation. This enzyme is also implicated in the enzymatic cascade responsible for the activation of procollagenase-1 (MMP-1). Matrilysin (MMP-7) has not been assigned to any of the previous groups. This enzyme is highly active against various melecules of the extracellular matrix, especially proteoglycan. Matrilysin was found to be overexpressed in human OA cartilage and its expression enhanced by treatment with the proinflammatory cytokines IL-1β and TNF-α.

1.2.5.2.2 Cartilage synthesis

The metabolic imbalance in OA includes both an increase in cartilage degradation and an insufficient reparative or anabolic response. The identification of anabolic agents that can be utilized to restore cartilage is an area of significant investigation. Molecules of interest include cartilage anabolic factors such as bone morphogenetic proteins (BMP), insulin-like growth factor-1 (IGF-1), transforming growth factor (TGF-β), and fibroblast growth factors (FGFs). Growth factors such as BMPs have the ability to reverse catabolic responses by IL-1 (168). Conversely, normal chondrocytes exposed to IL-1 or chondrocytes from OA patients exhibit decreased responsiveness to growth factors (169). An understanding of the interaction between catabolic cytokines and anabolic growth factors could lead to the identification of molecules that restore the responsiveness of diseased chondrocytes to anabolic growth factors or inhibitions of inflammatory cytokines.

1.2.5.2.3 Inflammation

The role played by inflammatory cytokines and mediators produced by joint tissues in the pathogenesis of OA is attracting increased attention. Among the many biochemical pathways that are activated within joint tissues during the course of OA are mediators classically associated with inflammation, notably IL-1β and tumor necrosis factor (TNF)-α. These cytokines, in an autocrine/paracrine manner, stimulate their own production and induce chondrocytes to produce proteases, chemokines, nitric oxide, and eicosanoids such as prostaglandins and leukotrienes. The action of these inflammatory mediators within cartilage is predominantly to drive catabolic pathways, inhibit matrix synthesis, and promote cellular apoptosis. Thus, although

OA is not conventionally considered an inflammatory arthritis, that concept – based histologically on the numbers of leukocytes in synovial fluid – should be reconsidered. Indeed, "inflammatory" mediators perpetuate disease progression and therefore represent potential targets for disease modification.

As noted above, a characteristic feature of established OA is increased production of pro-inflammatory cytokines, such as IL-1β and TNF-α, by articular chondrocytes. Both IL-1β and TNF-α exert comparable catabolic effects on chondrocyte metabolism, decreasing proteoglycan collagen synthesis and increasing aggrecan release via the induction of degradative proteases (12). IL-1β and TNF-α also induce chondrocytes and synovial cells to produce other inflammatory mediators, such as IL-8, IL-6, nitric oxide, and prostaglandin E_2. The actions of both cytokines are in part mediated by activation of the transcription factor nuclear factor-κB, which further increase their own expression and that of other catabolic proteins such as inducible nitric oxide synthase (iNOS) and COX-2, thus creating an autocatalytic cascade that promotes self-destruction of articular cartilage (170).

Interleukin-1β, which was used in this study, was described in details as follow. The IL-1 family of cytokines comprises 11 different ligands that share some animo-acid sequence homolog: IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-18, IL-1F5, IL-1F6, IL-1F7, IL-1F9, IL-1F9, IL-1F10 and IL-33. IL-1β is synthesized as 31 kDa precursor peptide (pro- IL-1β) and is cleaved to generate 17 kDa mature IL-1β. IL-1β is primarily produced by macrophage and is secreted after cleavage of its pro-form by the cysteine protease caspase-1 or IL-1-converting enzyme (ICE) (171). IL-1Ra binds to the same receptors as IL-1β but does not induce any

intracellular response. IL-Ra prevents the interaction between IL-1 and its cell-surface receptors, thus acting as a naturally occurring inhibitor. IL-1Ra has 26% amino acid sequence homology with IL-1β.

IL-1 binds to three different receptors, IL-1 Receptor 1 (IL-1RI), IL-1RII and IL-1 Receptor Associated protein (IL-1RAcP), which are expressed either as membrane-bound or soluble proteins. Binding of IL-1 to IL-1RI induces the recruitment of the IL-1RAcP and downstream cell signaling. IL-1RII has a short cytoplasmic domain (29 amino acids) and does not transduce any intracellular signal (172). IL-1RII may thus exist only as a decoy receptor either on the cell surface or in the cell microenvironment as a soluble form after enzymatic cleavage of the extracellular portion (172). IL-1Ra has the greatest binding affinity for IL-1RI. The off-rate is slow and binding of IL-1Ra to the cell surface IL-1RI is nearly irreversible. By contrast, IL-1β binds to IL-1RII with a greater affinity that does IL-1Ra. Moreover, the binding of IL-1β to soluble IL-1RII is nearly irreversible due to a long dissociation rate (2 h). Thus, both membrane-bound and soluble IL-1RII functions as natural inhibitors of IL-1β signaling. Soluble IL-1RAcP increases 100-fold the affinity of binding of IL-1β to sIL-1RII, while failing to alter the low binding affinity of IL-1Ra (173). This may explain why, in the human system, the inhibitory activity of IL-1Ra is enhanced by sIL-1RII, but hindered by the soluble, recombinant form of IL-1RI (174).

Upon binding of IL-1β to IL-1RI, MyD88, IRAK (IL-1 receptor-associated kinase), and TRAF-6 (TNF receptor-associated factor 6) form a complex. TAB 2 (TAK1 binding protein) binds to TRAF6 via K63-linked polyubiquitination of TRAF6 (175), and recruits TAK1 and TAB1 (TAK1 binding protein (176-178).

TAB1 is important for functional activity of TAK1 (179). TAK1 phosphorylates and activates MEK3/6, and MEK4/7 MAP2Ks, which in turn can activate p38 and JNK (180-182) and finally AP1. Furthermore, TAK1 activates IKK (inhibitor of κB kinase) (183) leading to NFκB translocation into the nucleus. Both transcription factors, AP1 and NFκB, are of major importance for OA, as they are increasingly activated under pathological conditions (184), and the expression of a number of genes important for the progression of OA.

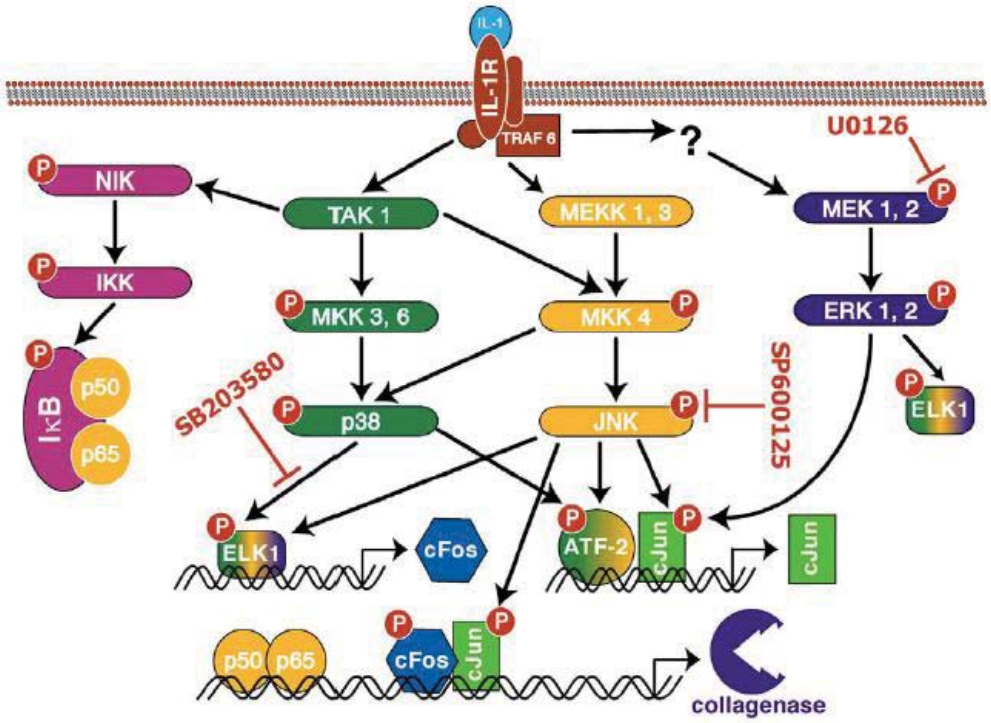


Figure 1.18 Major signaling pathways for IL-1 beta in chondrocytes and synovial cells. U0126 and SP600125 are chemical inhibitors that prevent the phosphorylation of MEK1, 2 and JNK, respectively. SB203580 is a chemical inhibitor known to block the kinase activity of p38. (adapted from (185))

Destructive effects of IL-1β include both elevation of cartilage catabolism and suppression of cartilage anabolism. For increasing of cartilage catabolism, IL-1β induces of proteolytic molecules involved in cartilage degradation and, moreover, induces inflammatory mediator and cell infiltration.

IL-1β upregulates the major extracellular proteolytic enzymes in cartilage degradation such as MMPs amd ADAMTSs. MMP-1, -3 and -13, but not MMP-2, were upregulated in both normal human and OA chondrocytes following IL-1β treatment (186) and upregulation of gene expression of MMP-1, -3 and -13 in articular cartilage from patients with OA, along with an increase in collagen and proteoglycan degradation, with all these effects being significantly suppressed by IL-1Ra treatment (187). Many documents reported the upregulation of ADAMTS-4 in normal human and OA chondrocytes by IL-1β (186) and, moreover, both ADAMTS-4 and -5 were upregulated by IL-1β in human OA synovial fibroblasts (188). Not only MMPs and ADAMTSs were upregulated by IL-1β, but cathepsin B (189) and urokinase-type plasminogen activator receptor (uPAR) (190), which are involved in cartilage degradation by serine proteinases and is upregulated in OA, were also stimulated in chondrocytes in a dose-dependent manner by IL-1β.

In addition, IL-1β has the capacity to induce several proinflammatory mediator including cytokines, chemokine, angiogenic factors, and proteolytic enzymes involved in the increase of local hematopoietic cells during OA. Hence, one generated, IL-1β can induce several molecules that act as cell chemoattractants. Thus, an increase in articular hematopoietic cells can induce thickening of the synovial membrane, increase in oxidative burst activity and decease in O_2 concentration, and

generation of additional inflammatory and proteolytic enzymes that can lead to progression of OA.

For the effect of IL-1β on the suppression of cartilage anabolism, IL-1β can decrease ECM synthesis by decreasing the anabolic activities of chondrocytes and/or the cell densities of articular chondrocytes. It is well known that IL-1β downregulates proteoglycan and collagen biosynthesis such as the suppression of GlcAT-1 mRNA (38%) following treatment of rat artilar chondrocytes with IL-1β, which correlated with 32% inhibition of preteoglycan synthesis (191) and the downregulation of type II collagen in human chondrocytes treated with IL-1β (192). IL-1β, moreover, may also induce apoptosis of chondrocytes. Several investigators have reported reduced numbers of chondrocytes due to an increase in apoptotic chondrocytes in patients with OA (193), with a potential link to IL-1β as the possible culprit in these processes.

1.2.5.3 Symptoms and signs

The cardinal symptom of OA is pain, which occurs with joint use and is relieved by rest. It is usually aching in character and poorly located (194). There is no strict correlation between joint symptoms and the extent or degree of pathological or radiographic changes (195). Only 30% of the patients with radiographic evidence of OA complain of pain at relevant sites (196). In advanced cases, pain may awaken the patient from sleep because of the loss of protective muscular joint splinting, which limits painful motion during the day. OA is sometimes associated with acute or subacute inflammation. Short-lasting morning stiffness is a common complaint. Articular gelling, a transient stiffness lasting only for several flexion-extension cycles, is extremely common in elderly patients, especially in the lower extremity joints.

Limited motion develops as the disease progresses because of joint-surface incongruity, muscle spasm and contracture, capsular contracture, and mechanical block due to osteophytes or loose bodies. Crepitus, a cracking or grating sound as the joint is moved, may be due to cartilage loss and joint-surface irregularity. Joint enlargement may be caused by secondary synovitis, an increase in synovial fluid, or marginal proliferative changes in cartilage or bone (osteophytes). Late stages of the diseases are associated with gross deformity and subluxation due to cartilage loss, the collapse of subchondral bone, the formation of bone cysts, and gross bony over growth (197).

1.2.5.4 Treatment of osteoarthritis

The principal objectives of treatment are to control pain adequately, improve function, and reduce disability. The status and requirements of patients often change over time, thus making it necessary to review and adjust treatment regularly rather than rigidly containing a single intervention. Education is one of the most important issues for patients with OA.

1.2.5.4.1 Pharmacologic treatment

The drugs used for OA management including analgesics, nonsteroidal anti-inflammatory drugs (NSAIDs), coxibs, opioid analgesics, topical analgesic, intraarticular steroids, hyaluronan and hylans, and potential disease-modifying osteoarthritis drugs (DMOADs). The detail of each group of drug was described below.

For analgesics, mild and moderate pain in OA patients can be relieved by using simple analgesics such as acetaminophen. However, acetaminophen-induced toxicity includes hepatotoxicity and potential renal damage. NSAIDs are widely used to reduce pain and inflammation and improve function in OA patients (198). The ability of different NSAIDs is to inhibit COX, the enzyme that catalyzes the synthesis of cyclic endoperoxide from arachidonic acid to proinflammatory and other forms of proteoglycan (13). Gastrointestinal (GI) intolerability problems, including dyspepsia, abdominal pain, and nausea, are the most frequent adverse events associated with nonselective NSAIDs (199). The discovery of COX-2, the second isoform of COX, led to the development of NSAIDs with the same analgesic and anti-inflammatory activity as nonselective NSAIDs but without the inherent risk of gastroduodenal mucosal damage and impaired platelet aggregation mediated by COX-1 inhibition (200). However, further placebo-controlled data relating to patients at high and low risk of cardiovascular events are warranted to clarity the cardiovascular effects of this class of agents. Opioid therapy may be considered in OA patients with severe pain when NSAIDs or tramadol are inefficacious or not tolerated. However, opioid use is limited by the tolerance, dependence, and adverse effects that may occur (201). The use of topical analgics, such as capsaicin cream or topical NSAIDs, is frequently prescribed as monotherapy or adjunctive treatment especially in hand or knee OA (202). The intraarticular administration of glucocorticoids (steroids) has been beneficial in treating acute episode of pain, especially when there is evidence of inflammation and joint effusion (203). Intraarticular treatment with HA and hyalans has recently become more widely accepted in the armamentarium for OA pain (14). As HA is reversible for the

viscoelastic properties of synovial fluid, the goal of intraarticular OA therapy is to help replace the synovial fluid that has lost its viscoelasticity. The efficacy and tolerability of intraarticular HA for the treatment of pain associated with knee OA have been demonstrated in several studies (204-206).

A number of agents are now referred as DMOADs, although none of them has as yet been reproducibly shown to alter the natural history of the disease (207). They include the following: (1) inhibitors of MMP, such as tetracycline and its semisynthetic forms (doxycyclin and minocyclin) (208); (2) growth factors and cytokine manipulation (17); (3) genetic therapy (18); and (4) sulfated and nonsulfated sugars (19).

1.2.5.4.2 Nonpharmacological treatment

There is a little evidence that most of them are efficient because of the paucity of research studies and fundamental methodological flaws in those that have been published (209). If evidence-based management guidelines were to be constructed solely in the basic of sound research studies, few could be recommended including exercise, bracing and footwear, physical rehabilitation, and behavioral interventions.

1.2.5.4.3 Alternative treatments

Nearly two-third of rheumatic patients use complementary or alternative therapies, and those with OA are the most frequent users (210). Alternative treatments include herbs, diets, homeopathy, mind-body interventions, manual healing, electromagnetic therapy, and acupuncture (211, 212).

1.2.5.4.4 Surgical treatment

Surgical treatment of OA is considered only after the feature of nonsurgical treatment (213). The main nonbiological procedures are osteotomy, arthroscopy, arthrodesis, and arthroplasty (214-217). The biological restoration of articular cartilage can be approached by stimulating resident hyaline cartilage to repair the defects by mechanical means (osteotomy), biologically enhancing bone marrow progenitors, or carrying our cartilage transplantation by means of osteochondral autografting, osteochondral allografting, or the use of tissue engineering techniques (218, 219).

1.2.6 *Sesamum indicum Linn.* and *Alpinia galanga*

1.2.6.1 *Sesamum indicum Linn.* And sesamin

Sesamum indicum is scientific classified in the order of Lamiales and the family of Pedaliaceae. Sesame is grown primarily for its oil-rich seeds, which come in a variety of colors, from cream-white to charcoal-black. Sesame lignans such as sesamin, episesamin, sesamolin, and γ-tocopherol from *S. indidum* seeds play important roles in plant defense, such as antifeedant as well as a potent antioxidants and insecticides [220-223]. Sesamin is the most abundant lignan in sesame seed [221, 222].

There are well documented of the health benefits of sesamin including the ability to reduce cholesterol concentration in serum [224], hypolipidemic effect [225], enhancement of vitamin E level [226], neuroprotective effect [227], antioxidative effect [28, 228] and anti-cancer effect [229].

Interestingly there were many reports of the anti-inflammatory activity of sesamin [25-28]. Sesamin inhibit delta-5 desaturase activity, resulting in an accumulation of dihomo-(-linolenic acid) (DGLA) that can displace arachidonic acid and decrease the formation of pro-inflammatory mediator, such as prostaglandin E_2 and leukotriene B_4 [25, 26]. Moreover, sesamin and the other active compound, sesamolin, suppressed LPS-induced NO production in microglia and macrophage through inhibition of signal transduction pathway or nuclear transcription factor [27, 28].

For the metabolism and the distribution of semamin, it was reported that sesamin may be, at first, incorporated into the liver and then transported to the other tissues (lung, heart, kidney, and brain) and it is lost from the body within 24 h after administration [230]. Enterolactone was found as the major metabolite of sesamin both *in vivo* and *in vitro* [231].

1.2.6.2 *Alpinia galanga*

Alpinia galanga is scientific classified in the order of Zingiberales, the family of Alpinioideae and the subfamily of Alpinieae. Normally *A. galanga* is known as galanga, galangale, Siamese ginger and is a spice used in cooking, especially in Indonesian and Thai cuisine.

In 2003, Matsuda *et al*. reported that Thai medicinal plant, *Alpinia galanga* Linn. (B.L. Burtt), has been categorized as an antimicrobial agent, and could also serve as carminative, stomachic, anti-rheumatic, anti-flatulent and anti-itching agents [21]. Its antirheumatic action resulted in suppression of synthesis of prostaglandin through inhibition of cyclooxygenase-1 and cyclooxygenase-2 [22]. In addition, the

aqueous acetone extract from rhizomes of this plant has possible inhibitory effects on lipopolysaccharide (LPS)-induced nitric-oxide production [23]. In 2001, highly pure extracts of *Zingiber officinale* and *A. galanga* were shown to significantly reduce symptoms of OA of the knee [24]. Moreover, extracts of *A. galanga* and *Z. officinale* could synergistically inhibit chemokine expression (TNF-α, MCP-1 and IP-10) [232]. In 2006, Pothacharoen *et al.* reported that *A. galanga* extract inhibited degradation of cartilage matrix stimulated by IL-1β in the chondrosarcoma and synovial fibroblast; and it also increased levels of anabolic gene expression but decreased catabolic gene expression levels in both cell types [233].

1.3 Objectives

1. To investigate the chondroprotective effect of *Alpinia galanga*'s active phytochemical and phytochemical of *Sesamum indicum,* sesamin, and compare their chondroprotective effect.

2. To investigate the molecular mechanisms of the phytochemical with highest chondroprotective effect.

3. To investigate the chondroprotective effect of phytochemical in papain-induced OA rat model.

4. To investigate the additive effect of phytochemical and drug used for arthritic treatment.