CMLS, Cell. Mol. Life Sci. 62 (2005) 2241–2256
1420-682X/05/202241-16
DOI 10.1007/s00018-005-5217-x

CMLS Cellular and Molecular Life Sciences

Review

Aggrecan, aging and assembly in articular cartilage

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Received 19 May 2005; received after revision 17 June 2005; accepted 23 June 2005
Online First 7 September 2005

Abstract. The primary function of articular cartilage to act as a self-renewing, low frictional material that can distribute load efficiently at joints is critically dependent upon the composition and organisation of the extracellular matrix. Aggrecan is a major component of the extracellular matrix, forming high molecular weight aggregates necessary for the hydration of cartilage and to meet its weight-bearing mechanical demands. Aggregate assembly is a highly ordered process requiring the formation of a ternary complex between aggrecan, link protein and hyaluronan. There is extensive age-associated heterogeneity in the structure and molecular stoichiometry of these components in adult human articular cartilage, resulting in diverse populations of complexes with a range of stabilities that have implications for cartilage mechanobiology and integrity. Recent findings have demonstrated that aggrecan can form ligands with other matrix proteins. These findings provide new insights into mechanisms for aggregate assembly and functional protein networks in different cartilage compartments with maturation and aging.

Key words. Aggrecan; cartilage; proteoglycan; aging; assembly; extracellular matrix; glycosaminoglycans; collagen network.

Introduction

Aggrecan is the major proteoglycan of hyaline cartilage where it is present at very high concentrations in the form of aggregates, but it is also present in significant concentrations in the extracellular matrices of other tissues, including meniscus, tendon, brain and muscle. In cartilage it associates with hyaluronan (HA) and a small glycoprotein, link protein, to form aggregates. These multimolecular structures can contain over 100 molecules of both aggrecan and link protein associated with a single HA chain, and give rise to aggregates with molecular masses of 10^8–10^9 Da. The aggregates are hydrated due to their high fixed negative charge resulting from the very large numbers of polyanionic glycosaminoglycan chains on aggrecan, providing cartilage with a high water content (about 70% by wet weight of the tissue) [1, 2]. They constitute a space-filling gel and are therefore responsible for the compressive resilience of articular cartilage during joint loading and are a major contributor in the role of cartilage as an efficient load distributor. Cartilage is predominantly composed of extracellular matrix (ECM), whose constituents are synthesised by the resident chondrocytes which are also responsible for its maintenance. Aggrecan forms some 35% (dry weight) of the proteins found in cartilage, although the most abundant protein is collagen type II, which is present at up to 60% by dry weight of the tissue. The collagen forms a highly organised fibrillar network, serving as a tensile element, within which the aggregates are immobilised due to their large size. Many other structural proteins (collagens and non-collagenous proteins) enmeshed within the network are present at varying but low concentrations, some of which have important roles in the assembly of a complex three-dimensional collagen network.

In addition to the structural role of aggrecan, it also shapes nutrient and solute transport in cartilage due to its hydration property. At the molecular level, therefore, the assembly of aggregates is critical for the functional organisation of both aggrecan and other constituents of the ECM as well as the metabolic activity of the chondrocytes which synthesise and maintain the ECM constituents. A caveat in this role as an ECM molecular organiser is that aggrecan isolated from articular cartilage of increasing age exhibits extensive heterogeneity both in terms of molecular size and chemical modifications, which can affect its aggregation properties. Although there have been numerous biochemical studies on aggrecan and aggregates in cartilage, more recent studies have provided an exciting insight into new ligand binding capabilities of aggrecan that suggest there may be additional roles it plays. The interactions of aggrecan with ECM ligands suggest they are essential for the functional assembly of aggrecan and in the organisation of other ECM components within different compartments of the ECM. This review focuses on articular cartilage where most studies of this multifaceted molecule have been described.

From a historical perspective proteoglycans were first described from the study of the extracellular matrices of cartilage from which chondromucoid preparations were isolated. These early studies were confined to describing the nature of the polysaccharide components, for example that of chondroitin sulphate (CS) and hyaluronic acid [3]. These long, unbranched carbohydrates consisting of repeating disaccharide units were subsequently termed glycosaminoglycans (GAGs) to indicate the presence of amino sugars, but it was the work of Muir [4] which demonstrated the covalent linkage of CS to a protein core through serine residues. The nature of the linkages for other GAGs was later identified [5], and it became clear that the GAG chains were covalently linked to serine residues via a tetrasaccharide linker region consisting of xylose, galactose, galactose and uronic acid. Further studies showed that the large cartilage proteoglycans could interact non-covalently with HA [6, 7] to form multimeric aggregates and that this interaction was stabilised by a small glycoprotein termed link protein [8, 9]. This strengthened the concept of an organised ECM in which a hierarchical assembly of macromolecules was present and necessary for tissue function. The application of molecular approaches established that the large CS proteoglycan (aggrecan) from cartilage was encoded by a single gene and interestingly, that it had a modular structure having similarities to many other protein families. A series of seminal papers by Bayliss and co-workers [10–13] and by others [14, 15] detailed the profound age-related heterogeneity that aggrecan exhibits with respect to both core protein and GAG size, and also to the modification of the sulphation pattern of the CS sulphate chains. More recent findings challenge the established models of aggregation in mature cartilage and reveal new interactions with other ECM macromolecules.

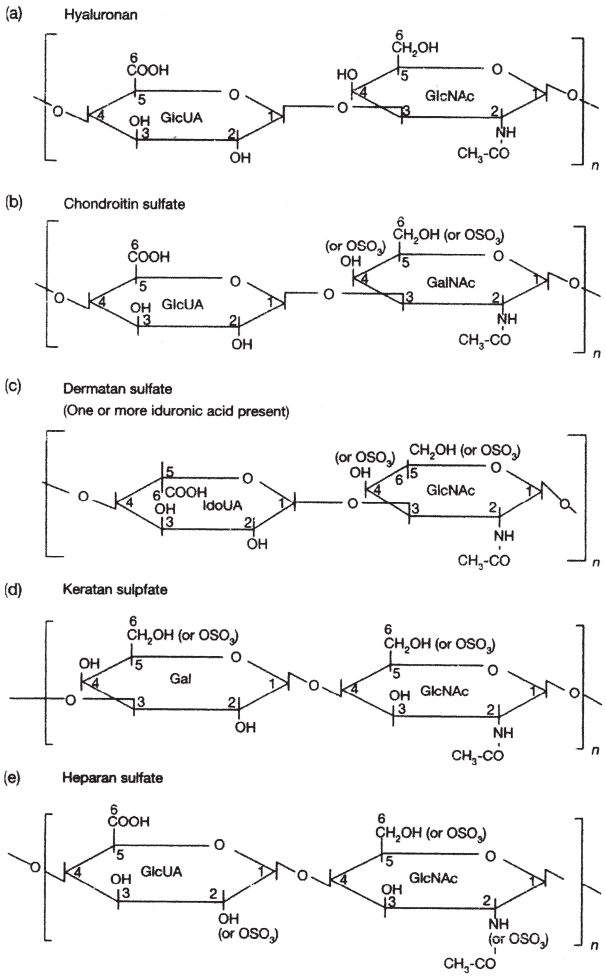

Figure 1. The repeating disaccharide units of glycosaminoglycans. All except hyaluronan (HA) are modified to contain sulphate groups and are found attached to protein cores, although covalent associations of HA with proteins have recently been described. The disaccharides may be substituted with ester or amino-linked sulphate groups shown in brackets (-OSO_3) at carbon positions where they occur. Disaccharides can be unsulphated (no substitutions) or mono- (e. g. chodroitin 4-sulphate or chondroitin 6-sulphate) and disulphated (saturated dissacharides). GlcUA, glucouronic acid; GlcNAc -D-*N*-acetylgluosamine; GalNAc, -D-*N*-acetlygalactosamine; IdoUA, iduronic acid; Gal, galactose.

Proteoglycan families and conserved modular structures

Proteoglycans are widely distributed in the body where they show enormous diversity in size, structure and function, attributable largely to the heterogeneity of the attached GAGs side chains. The attachment of GAGs to

Table 1. Proteoglycan families.

Proteoglycan family	Typical features
Hyaltectin (Lecticans)	contain lectin binding domains
Small leucine-rich proteoglycans (SLRPs)	contain a domain with repeats rich in leucine residues
Heparan sulphate proteoglycans	located on the cell surface and can bind many growth factors on their heparan sulphate chains
Part-time proteoglycans	located on the cell surface and in the extracellular matrix, and include CD44 and some collagens (IX, XII, XIV and XVIII)
Phosphocans	receptor-type protein-tyrosine phosphatases, synthesized as CS proteoglycans; expressed by neuronal cells

The core protein of these can be found substituted with one or more GAGs, and are grouped according to size, shared domains or function. Some, such as CD44 and the part-time proteoglycans, are only occasionally substituted with GAG, and both glycosylated and non-glycoslylated forms can be present.

the protein core is at peptide consensus sequence sites at serine or threonine residues via an *O*-glycosidic group, while oligosaccharides attached to core protein asparagines via *N*-glycosidic groups are also present. The GAGs are extended polysaccharides containing repeating disaccharide units which are either of two modified sugars: *N*-acetylgalactosamine (GalNAc) or *N*-acetylglucosamine (GlcNAc) and a uronic acid such as glucuronate (GlcUA) and iduronate or galactose (Gal). There are five types of GAGs described in mammalian tissue depending on the disaccharide unit (fig. 1). All except HA are modified to contain sulphate groups and are found attached to cores of proteins. GAGs are polyanionic because of their sulphate and acidic substitutions, and to a large extent are responsible for the physiological properties of the proteoglycan they reside on. For a high molecular weight polymer such as HA (although unmodified with sulphate) the effect is to attract a large volume of water; indeed, HA is found in most body fluids and new functions for this GAG continue to be described [16, 17], some of which may contribute to ECM assembly and organisation.

There are currently some 30 genetically distinct proteoglycans that have been identified which can be grouped into five families (table 1) loosely based on core protein size, type of GAG side chains and cellular localisation. Aggrecan belongs to the large aggregating proteoglycan family, the lecticans, which also form part of the larger family of proteins termed hyaladherins, so called because they can form non-covalent associations with HA [18]. The molecular structure of aggrecan from cartilage was the first to be described in several species (rat, human, bovine), revealing a complex modular organisation common to these large proteoglycans, the most notable feature of which was an extended central region dedicated to the substitution of GAGs (fig. 2). Although many consensus sequence GAG modification sites are present on these proteoglycans, significant differences exist between the family members. For example, versican [19] has an extended GAG attachment sequence which is subject to alternative splicing [20–23], whereas this region in the nervous tissue proteoglycans brevican [24] and neurocan [25] is much shortened. Aggrecan is heavily substituted with GAGs and *N*- and *O*-linked oligosaccharides, having approximately 110 CS and 30 or more keratan sulphate (KS) chains attached in distinct GAG attachment regions. Because the average chain weight of CS is approximately 20 000, the core protein generally comprises only 10–20% of the weight of the proteoglycan. By comparison, versican carries 12–15 CS chains. Thus, while the genomic organisation of the aggrecan family of proteoglycans is nearly identical, having probably arisen from a common ancestral origin, it is clear that protein-specific divergence exists in the carbohydrate bearing exon while the globular structures are highly conserved. These characteristics indicate that the globular domains likely perform common functions, whereas divergence in the carbohydrate-bearing region has lead to functional specialisation within this family.

Functional studies of aggrecan

N-terminal structures

The core protein of aggrecan has a modular structure (fig. 2) containing three disulphide-bonded globular domains, G1, G2 and G3, and an extended central portion dedicated to GAG modification [26, 27]. The N-terminal of the protein is formed by the G1 domain, also referred to as the HA-binding region (HABR). As the name indicates, the G1 domain interacts with high specificity to a decasaccharide length of HA, thus facilitating the formation of multimolecular aggregates [7, 28] (fig. 3A). Within the G1 domain there are three modules critical to effect maximal binding to HA: two tandem repeat sequences or link modules and a module which has structural similarity with the immunoglobulin fold (Ig fold) [29]. An *O*-linked carbohydrate side chain is present which is important in fortifying the interaction between HA and the G1 domain [28]. Interestingly, these carbohydrate side chains become elongated in preference to KS chains with age [30–32]; whether this influences the G1-HA binding affinity and, therefore, modifies the aggregate size or stability is not known, although carbohydrates appear essential for high-affinity binding of aggrecan to HA [33]. Each aggrecan: HA interaction is stabilised by the binding of one molecule of link protein [7, 34–35], a 41–48 kDa glycoprotein with close sequence similar-

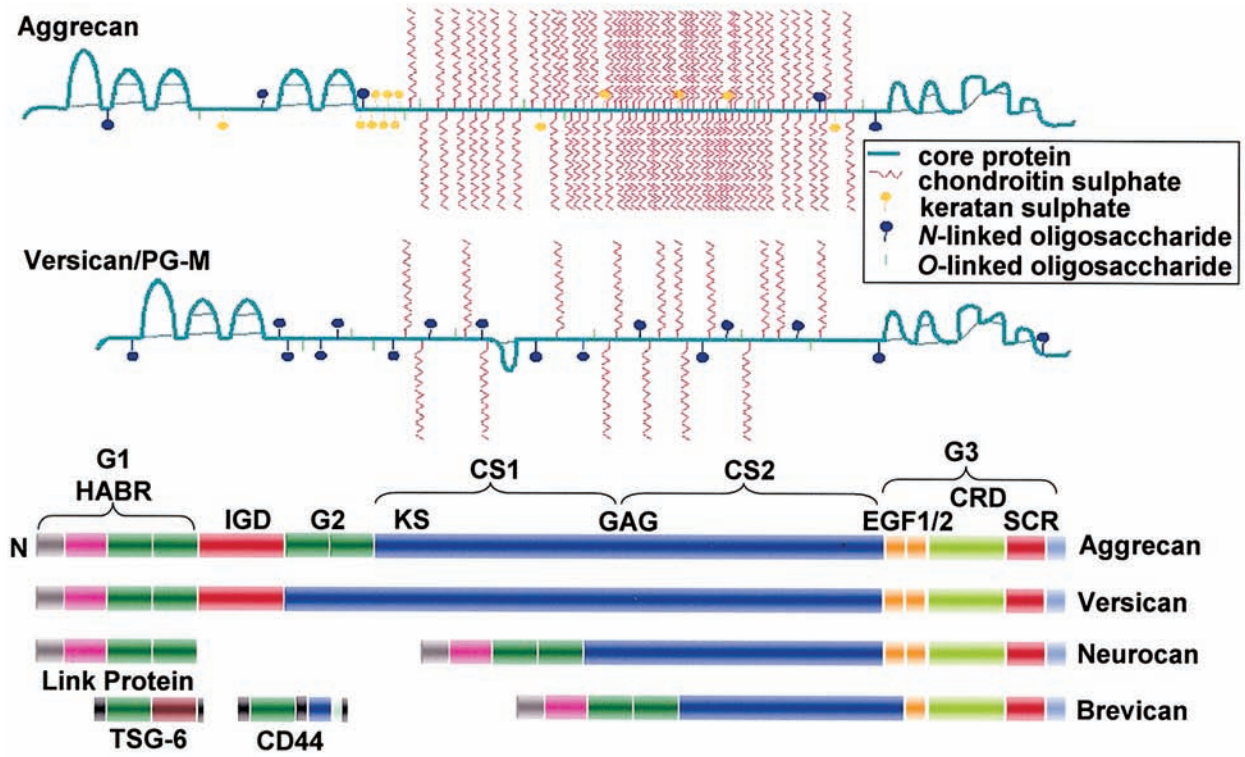

Figure 2. Modular structures of the hyaladherins. The upper schematic depicts the relative size and positions of the various modules found on aggrecan and versican. The size of the core proteins of these proteoglycans is over 2000 amino acid residues. The lack of the G2 domain and the fewer CS chains on versican are the most obvious features. The GAG attachment region is subdivided into the CS1 (evenly spaced CS attachment consensus sites) and CS2 (clusters of consensus sites) segments. The EGF1/2, CRD and the SCR modules together make up the G3 domain. Similar organisation of one or more of these modules is found on other proteins as shown on the lower schematic. Link protein belongs to a family of four members. N, N-terminal of the core protein; HABR G1, HA binding region (G1 domain); IGD, interglobular domain; G2, G2 domain; GAG, glycosaminoglycan attachment region; KS, keratan sulphate attachment segment; CS1 and CS2, chondroitin sulphate regions 1 and 2; EFG1/2, epidermal fibroblast growth factor-like modules 1 and 2; CRD, carbohydrate recognition module; SCR, short complement-recognition module.

ity to the G1 domain of aggrecan, and the two interact via the Ig-fold sequence [33, 36–37]. The association between aggrecan G1 and HA is tight, with an apparent K_D of approximately 0.226 μM, whilst that between link protein and HA is higher still, with a K_D of 0.089 μM, thereby greatly increasing the binding of the ternary complex [34]. The stability of aggregates is therefore critically dependent upon the interaction between these three components in the extracellular matrix.

The G2 domain shares some of the structural features of the G1 domain, containing the link modules but not the Ig-fold sequence [38]. However, it has no HA-binding properties [39, 40] despite sharing considerable sequence homology with the G1 link modules. It has been suggested that the presence of an extra *N*-linked oligosaccharide on G2 may disrupt the folding of this region and prevent binding to HA [41]. Proteins of this family exist which, despite having only one link module (fig. 3), are capable of binding HA. These include CD-44 and TSG-6, which can bind HA with high affinity [42, 43]. The reasons are as yet unclear why these proteins only require one module for binding to HA whilst aggrecan and versican G1 domains require both, whereas the G2 domain cannot. Although the exact function of the G2 domain is still unknown, it appears to function by inhibiting aggrecan secretion prior to GAG modification in the Golgi [44]. It has been shown that both G1 and G2 domains work in concert with each other inhibiting secretion of aggrecan, whilst the G3 domain and the chondroitin sulphate-glycosylated core protein region promote secretion. Therefore, G2 acts as a secretion retardant, ensuring that only fully glycosylated aggrecan monomer is secreted [45]. In addition to inhibiting secretion, the G1 link modules of aggrecan and versican may also have a regulatory function in aggrecan biosynthesis by inhibiting GAG chain attachment to core protein [46]. It is intriguing that in this family of large proteoglycans aggrecan is unique in having a G2 domain as well as being heavily substituted with chondroitin and keratan sulphate chains, which lends support to the hypothesis that the G2 domain may influence GAG attachment. Its presence also results in an interglobular domain (IGD) spanning the G1 and G2 do-

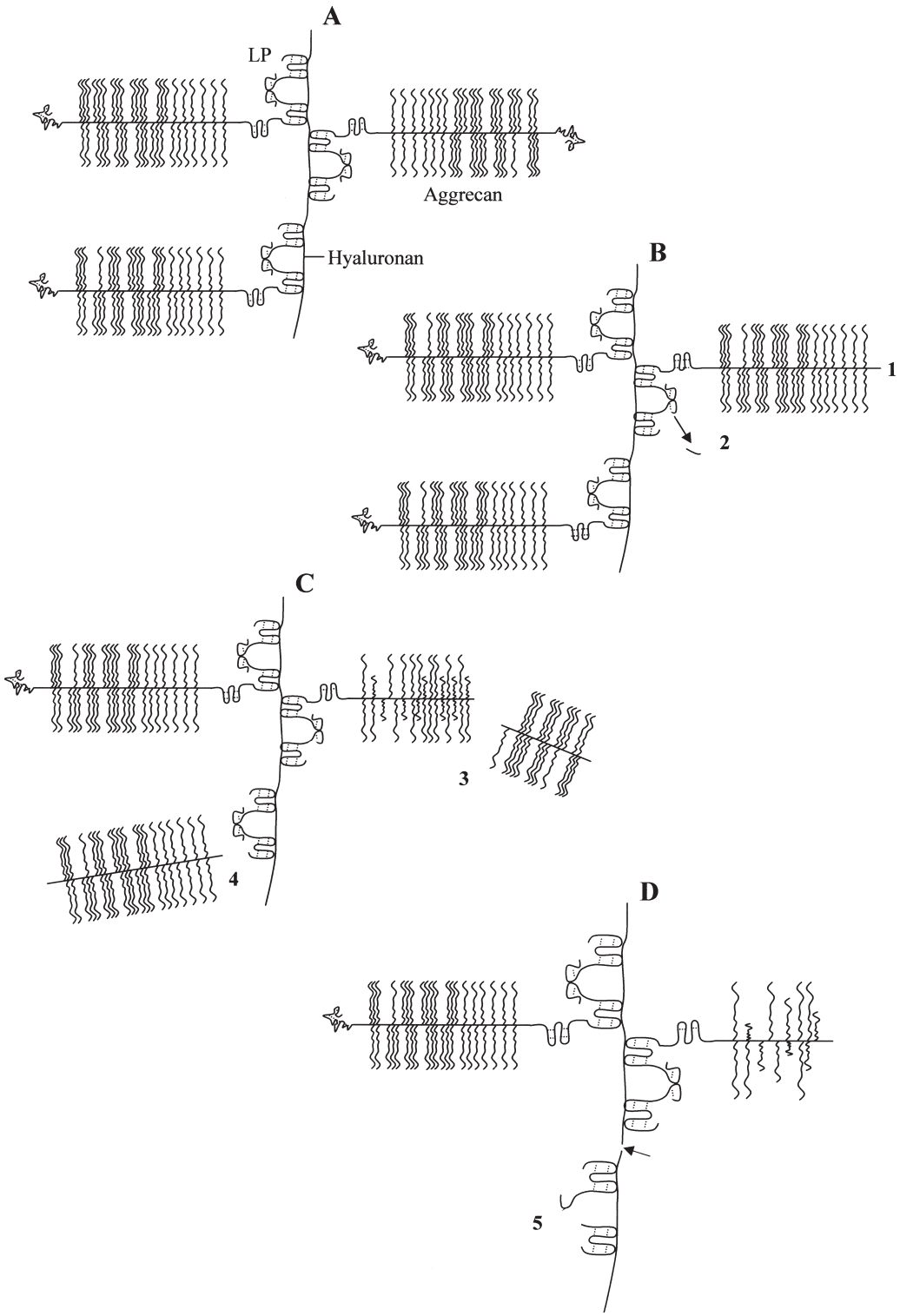

Figure 3. Processing of aggregates leading to an increasing polydisperse population with advancing age in articular cartilage. **(A)** A ternary complex between HA, aggrecan and link protein results in the formation of aggregates. Over a hundred aggrecan and link protein molecules may be arranged on a single filament of HA. (**B**) An early processing event is the proteolytic cleavage of the G3 domain (1) and the N-terminal of the link protein (2). (**C**) Further proteolytic cleavage shortens the CS attachment region, and some trimming of CS chains can also occur (3). Heterogeneous-sized aggrecan molecules can accumulate with aging. The IGD region is particularly susceptible to proteolytic attack, resulting in the detachment of the CS region, which can be lost by diffusion from the ECM into the synovial fluid (4). The residual G1 domain remains bound to the HA and accumulates in the matrix. (**D**) Further proteolytic events can degrade G1 and the link protein (5). Residual peptides of G1 (30–69 kDa) and link protein (27–30 kDa) can be identified in extracts derived from mature and old cartilage. HA is also reduced in molecular size by catabolic action of hyaluronidases (arrow). The KS chains on aggrecan have been omitted for clarity.

mains which imparts several key properties to aggrecan. The IGD is a short protein region [(127 residues in human aggrecan) [47]] which by electron microscopic techniques appears of constant length of 25 nm [48, 49], suggesting it to be a stiff structure. It has been the focus of intense research because of its susceptibility to proteolytic cleavage both in experimental systems studying proteoglycan catabolism and in cartilage degenerative disease. In particular, cytokines such as interleukin-1 and tumor necrosis factor target the degradation of aggrecan via cleavage at clusters of proteolytic sites along the protein core [50, 51]. The cleavage sites within the IGD are the most significant as they lead to the detachment and subsequent loss of the entire CS attachment region from the ECM by diffusion [52–54], and impact the viscoelastic properties of cartilage. Furthermore, the liberated G1 domain following such cleavage remains attached to the HA: link protein ternary complex and can accumulate with age, thereby competing for binding sites on HA with newly synthesized proteoglycan during turnover and remodeling of the cartilage ECM. There are two major cleavage sites on the IGD initially identified by peptide analysis of cartilage proteoglycan fragments to occur at Asn^{341}-Phe^{342} and Glu^{373}-Ala^{374} [50, 55–57]. The former site can be generated in vitro by a number of metalloproteinases [56, 58–62], whereas the latter is generated by the proteolytic activity of so-called aggrecanases [57, 63], which have relatively recently been identified to be the disintegrin-metalloproteinases ADAMTS-4 and ADAMTS-5 [64, 65]. Aggrecan cleaved at both Asn^{341}-Phe^{342} and Glu^{373}-Ala^{374} is present in normal and osteoarthritic cartilage [57, 61, 63], in experimentally induced arthritis and in cartilage stimulated to degrade by proinflammatory cytokines, including interleukin-1 and tumor necrosis factor [50–51, 66–70]. The cytokine-stimulated release of proteoglycan in normal and OA cartilage appears to be due to a primary cleavage mediated by ADAMTS-4 and -5 whereas MMP activity is a secondary event [71, 72], while gene knockout studies suggest that ADAMTS-5 may be the pivotal proteinase in cartilage in experimental OA [73, 74]. Altered glycosylation patterns within the IGD during aging or in degenerative changes may also influence the susceptibility of the IGD to proteolytic cleavage [32, 75]. The IGD thus may have an important function in regulation of the proteolytic activity, leading to the degradation of aggrecan during normal turnover and the progression of OA, although this involves complex proteolytic mechanisms as detailed in many reviews [76–79].

CS region

The KS and CS attachment regions form the most dominant feature of aggrecan, making up some 80% of the protein molecular mass. The KS attachment region adjacent to the G2 domain is where many of the 30 or so KS chains on human aggrecan are located, and consists of a series of tandemly repeating hexapeptides which have the consensus sequence Glu-Glu/Lys-Pro-Phe-Pro-Ser [47, 80], with the KS chains attached to the serine side chains. The number of such consensus sequence motifs varies between species from 4 to 23 repeats [31], although mouse or rat aggrecans with only 4 repeats do not possess KS chains. KS chains are also present on the G1 domain via *N*- and *O*-linked substitutions that can vary between immature and mature cartilage [30, 32] and on the adjacent CS-attachment region via *O*-linked substitutions on threonine residues. The proline-rich repeats in the KS attachment region can interact with moderate affinity with collagen fibrils [81], probably through the formation of a polyproline coil which, together with the rigid and extended conformation of the KS region, is suggested to enable a collagen fibril to traverse the centre of the aggregate and may also aid the assembly and integrity of aggregates [81].

The CS attachment region is essential for aggrecan to perform its major function of maintaining the hydration of cartilage. It consists of 120 repeating concensus sequence motifs of Ser-Gly-X-Gly or (Asp/Glu)-X-Ser-Gly interspersed along is length [38, 47] which serve as sites for xylosylation on the serine residues [82, 83]. These repeats form two distinct patterns designated as CS1- and CS2-repeat regions [84] with the number of Ser-Gly dipeptides much lower in the CS2 segment (fig. 3). Not all sites are utilized, as typically there are about 100 CS chains present on aggrecan each of about 40–50 disaccharide units. Post-translational modifications of the CS chains (and KS chains) result in sulphate ester substitutions at the carbon-4-*O*-hydroxyl or carbon-6-*O*-hydroxyl position of many of the GalNAc residues, resulting in unsulphated, monosulphated and disulphated residues. These two domains contribute some 8 000–10 000 negatively-charged groups to the molecules, all fixed to the protein core. The high charge density imparted by the sulphated glycosaminoglycan chains results in a high degree of hydration for the proteoglycan aggregate and is essential for the ability of cartilage to imbibe water in keeping with the Donnan equilibrium. The protein is therefore osmotically active, and the resultant swelling of the cartilage is resisted by the stiff collagen network, creating a swelling pressure which enables the tissue to deform reversibly under mechanical load. The CS region is less well conserved between species than the globular domains and suggests that the precise pattern and number of Ser-Gly sequences is not critical to its function in bearing large numbers of CS chains. Indeed, uniquely in humans allelic variations give rise to a variable number of tandem repeats [84], potentially resulting in a range of substitutions.

C-terminal region

The G3 domain comprised of two epidermal growth factor-like domains (EGF1 and 2), a carbohydrate recognition domain (CRD) similar to the mammalian type C lectins and a complement regulatory protein B component (short complement repeat, SCR). The individual functions of these modules are unclear, but it is readily lost from the mature aggrecan molecule soon after synthesis, and the number of aggrecan molecules with an intact G3 structure declines with age in human cartilage [85]. This would suggest it provides a function in newly secreted aggrecan during the assembly of the proteoglycan ternary complex in the ECM, but has a lesser role subsequently. It may also have an important intracellular role as it appears to provide the core protein with a correctly folded C-terminal cap, which is essential for intracellular trafficking of aggrecan [44, 86–89] and promoting glycosaminoglycosylation of the core protein [44, 86, 90]. The presence of any one of the four modules of the G3 domain is sufficient to prevent core protein degradation [91], although other studies have reported that the CRD was necessary for the processing, trafficking and secretion of the protein [92]. A requirement for a heat shock protein of 25 kDa (HSP25) has also been implicated in G3-mediated aggrecan secretion. These modules may therefore have varied functions, and it is therefore interesting that the aggrecan gene encodes alternatively spliced forms [38, 47, 85, 93, 94]. Thus, both the EGF-like and the SCR modules can undergo alternative splicing independently, resulting in the presence of multiple variants of aggrecan messenger RNA (mRNA). The CRD module is not known to be alternatively spliced, whereas in human cartilage the EGF1 module is present at only a low abundance. In contrast, versican mRNA does not undergo alternative splicing of its C-terminal globular region [93]. Malignant chondrosarcomas also exhibit altered splicing of aggrecan mRNA in this region [95]. In our studies, the SCR- and EGF2-carrying mRNA variants are both preferentially retained in aggrecan during mouse embryonic development and not in young or mature animals where they are expressed at low abundance (table 2). The expression level observed in fetal mice is similar to levels in early OA in the STR/ort strain (not shown), which spontaneously develops OA with age [96]. The expression pattern suggests a similarity in the chondrocyte phenotype between developing cartilage and osteoarthritic cartilage prior to the appearance of lesions, lending support to the hypothesis that chondrocytes in osteoarthritic cartilage start to re-express many molecular markers characteristic of chondrocytes in fetal skeletal development [97–99]. The implication of differentially expressed aggrecan mRNA variants in osteoarthritic degeneration is not clear, although it may indicate an anabolic response to initiate a repair process where these modules are needed for the assembly of aggrecan aggregates or for the formation of a functional collagen network. Our previous findings showing that the G3 domain is associated mainly with immature, newly synthesised aggrecan [85] support this hypothesis. Indeed, more recent studies provide fresh insights into the role of this globular structure in its ability to bind to other matrix components that may permit essential cross-links during ECM formation during cartilage development and turnover as discussed below.

Table 2. Age-related splicing of aggrecan mRNA in CBA mice.

Age	E14.5	E17.5	12–18 weeks	25–35 weeks
G1 (total aggrecan)	100	100	100	100
SCR (± s.e.m.)	59 (7.5)	47 (5.5)	22 (3.5)	28 (6.2)
EGF2 (± s.e.m.)	63 (9.0)	55 (7.3)	8 (3.5)	14 (6.1)

Total RNA prepared from the knee joint cartilage of mice was assayed for the expression of aggrecan and the SCR- and EGF2-like containing variants using a competitive RT-PCR procedure as described earlier [129]. The two variants are expressed as a percentage of the total aggrecan population measured using primers to the G1 domain. Fetal mice are shown at E14.5 and E17.5 days of development, and adult mice are shown grouped into 12–18 and 25–35 weeks of age. Each column represents pooled tissue from three animals, i.e. six limbs. Mean (± standard error) of four measurements are shown. These data show that the SCR and EGF2 modules are preferentially spliced out of the aggrecan mRNA in adult tissue compared with fetal stages.

Age-related changes

Articular cartilage, often regarded by some as a homogeneous tissue, is composed of a complex and highly ordered ECM designed to provide near-frictionless articulation and withstand repetitive load-induced compression. The matrix has many remarkable features: (i) it is sparsely populated by a single-cell type, chondrocytes, which are responsible for the co-ordinated synthesis and turnover of the matrix. Their number and activity varies with the depth of the tissue; (ii) there are three distinct zones through the depth of the tissue such that the surface is different in composition to the middle and deep zones of the tissue; (iii) it is avascular and aneural; and it has a low pH with an oxygen gradient from the synovial surface to deep zone where the pO_2 may be as low as 2–5% [100, 101]. In the absence of vasculature, nutrients including oxygen diffuse in predominantly from the synovial fluid and from the capillary beds of the underlying bone. There is also a decrease in the thickness and cellularity of articular cartilage with age [102, 103]. Furthermore, the two major extracellular matrix proteins in articular cartilage, collagen type II and aggrecan, are relatively long-lived in the tissue and as a consequence undergo considerable non-enzymatic modification by re-

ducing sugars resulting in the accumulation of advanced glycation end products (AGEs) via the Maillard reaction [104, 105]. Measurements of AGEs indicate the half-life of collagen in healthy human cartilage to be over 100 years [106, 107] whereas that for aggrecan is about 3.5 years, although the proteolytically cleaved 'free G1' domain (see above) has a resident time in the matrix of between 19 and 25 years [108, 109]. Interestingly, link protein has a half-life similar to that of the free G1 domain, suggesting that it, too, has a slow turnover rate in articular cartilage [109].

In addition to these non-enzymatic modifications, aging of human articular cartilage is characterised by changes in the structure of aggrecan and the multimolecular aggregates that it forms with HA and link protein (fig. 3 B–D). Both biosynthetic and catabolic processes bring about these molecular changes, regulated by many cellular and extracellular mechanisms. These changes do not occur uniformly but are dependent upon factors including species, joint, cartilage zone, tissue compartment (pericellular, territorial and interterritorial) and topographical region of the joint surface which all determine specific qualitative and quantitative changes in aggrecan structure. However, it is the age of the individual that has the most profound effect on the composition, stoichiometry and stability of aggregates. Not only does the biosynthetic capability of human cartilage decline after skeletal maturity [110], but also aggrecan undergoes extensive post-translational modifications during normal aging, particularly in the pattern of glycosylation and sulphation of GAG chains and to its protein core. Proteoglycans extracted from cartilage form distinct subpopulations based on differences in size and composition of the constituent molecules which can be resolved into two or three species as first demonstrated by McDevitt and Muir [111]. A number of studies have shown that in human articular cartilage there is an increase in the KS content and a concomitant decrease in CS with age [2, 11, 112–114]. There is also a decrease in CS chain length, whereas KS chains exhibit an increase in their length [115–117] although their numbers appear to decrease with age [117]. Significant changes also occur in the content of 6-sulphated disaccharides, which predominate in early life and continue to increase with maturation of cartilage [110] such that the chondroitin 6-sulphate: chondroitin 4-sulphate ratio is 9:1 in adult cartilage [110, 118]. The 6-sulphation is largely on chain internal disaccharides, although a significant proportion is also found on terminal GalNAc residues which are disulphated (4- and 6-sulphation) [119]. The rapid disulphation of terminal GalNAc residues has been proposed to control chain length by terminating biosynthetic activity [120, 121]. The terminal GalNAc caps in fetal and postnatal growth plate cartilage tend to be only chondroitin 4-sulphate, suggesting age-associated changes occur on aggrecan CS after skeletal maturity [119].

These observations indicate that two distinct and independent mechanisms contribute to produce the changes. The first of these involves various glycosyltransferases and sulfotransferases which function in a complex but co-ordinated manner to bring about elongation and sulphation of GAG chains (reviewed in [122–124]). This area of glycobiology continues to expand as regulation of the enzymes (synthetic and degradative) is elucidated to provide biosynthetic mechanisms for the observed changes in chain length heterogeneity and sulphation during development, growth and maturation. This is a major factor in determining the size and composition of aggregates [125]. The second mechanism implicates selective proteolytic degradation of the glycosaminoglycan-rich region of the molecule and the accumulation of partially degraded aggrecan in the ECM during normal turnover. In this model of aggrecan turnover the length of aggrecan monomers shortens with age due to proteolytic trimming at both ends of the molecule (fig. 3), a process that is dependent on metabolically functioning chondrocytes [126]. The loss of the C-terminal G3 domain is an early event, and as its abundance in aging cartilage declines [85, 127], further proteolytic activity within the CS region results in a heterogeneous sized aggrecan population. Shortening of the CS region exaggerates the increase in the KS content associated with aging as KS-rich fragments accumulate. Degradation at the N-terminal is concentrated within the IGD segment where ADAMTS-4/5 and MMP activity releases the CS-rich region from aggregates which is then able to exit the ECM by diffusion (see above), leaving the freed G1 domain bound to the HA filament. Proteoglycan aggregates thus have fewer aggrecan monomers in aging cartilage. Partially degraded aggrecan products are known to accumulate with age in the matrix, but their relationship within the aggregating complex has only recently been fully explored [127]. Thus, the molar ratio of aggrecan:LP in the neonate and up to 5 years is close to 1:1, in marked contrast to mature cartilage where this ratio reaches 4:1. Proteolytic cleavage within the IGD region leads to an increase in the concentration of free G1 with age such that in mature cartilage there is a large excess of free G1 domain over LP (5-7:1 ratio) [127]. Furthermore, the de novo synthesis of link protein falls dramatically compared with aggrecan both at the transcriptional and translational levels soon after skeletal maturity [128, 129]. Taken together, this signifies a relative deficiency in the LP content of aggregates in mature cartilage which can lead to a less stable interaction between aggrecan and HA in aggregates which are not fully LP stabilised [130]. The stability of aggregates in mature cartilage is likely to be further compromised by the age-related increase in degradative fragments of the free G1, LP [127, 131–133] and HA [134], which all can compete with intact aggrecan for binding sites on HA. The findings of Wells et al.

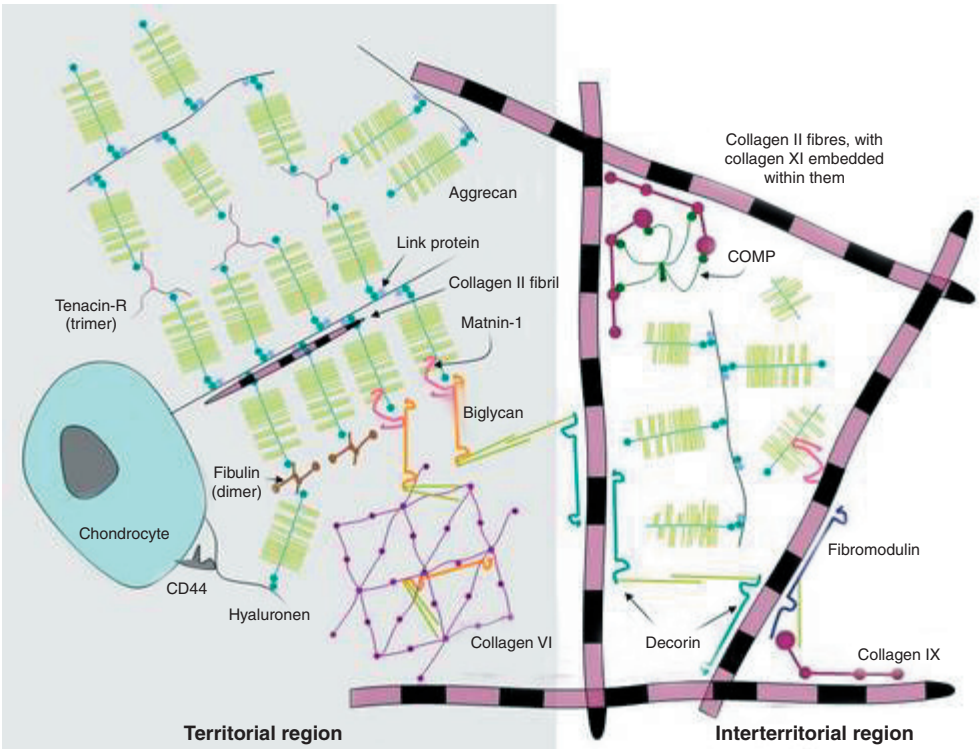

Figure 4. The assembly of aggregates and interactions with extracellular matrix components. The ECM of articular cartilage consists of a territorial (pericellular) region and an interterritorial region as depicted in this schematic, filled with many structural proteins not all of which are shown here. The pericellular space is rich in collagen type VI and in this model the initial ordered assembly of aggrecan may involve cooperative associations with other proteins, such as fibulin and tenacin-R, depending on their spatial and temporal expressions. The extended arrangement of aggrecan on a HA filament has been proposed to create a molecular channel that could enable the movement of proteins such as collagen type II fibrils, which can bind to the KS-rich segment of aggrecan to travel from the chondrocyte to the distant matrix [81]. Multimolecular bridges may further link aggrecan with the collagen type VI network. Aggregates enmeshed within collagen type II networks in the interterritorial matrix are of varying heterogeneity, with some molecules free to diffuse out into the synovial fluid, whereas others may remain attached via interactions with proteins such as matrilin-1. The organisation of the collagen fibres is strengthened by collagen-binding proteins, including decorin, fibromodulin and collagen type IX, and also indirectly with the non-collagenous protein COMP (cartilage oligomeric protein). *Modified from a schematic of Lorenzo and Heinegård, Lund University, Sweden.*

[127] demonstrate that the generally accepted model of aggregate formation in cartilage (one LP binding to both aggrecan and HA) is true for immature human cartilage but cannot be applied to adult cartilage, which has a heterogeneous population of complexes with a range of stabilities. The relatively slow turnover rate of these partially degraded components further lends support to the hypothesis that their accumulation may disturb the ordered assembly of aggregates and their stability with aging.

Models of aggregate assembly

The ternary complex formed between aggrecan, link protein and HA is essential in the formation of supramolecular structures. It is accepted that this is a highly ordered process controlled by the chondrocytes and requires the involvement of other proteins. It is less obvious what mechanisms control these processes during growth and turnover in mature tissue. Since HA is synthesised at the cell surface and extruded directly into the pericellular space [135], it is likely that assembly begins at or close to the cell surface, requiring the co-ordinated synthesis of the components. Indeed, the retention of a proportion of newly synthesised aggrecan at the cell surface requires the binding of hyaluronan to its cell surface receptor, CD44 [136, 137], suggesting that aggrecan can attach to a hyaluronan scaffold soon after secretion and remain in the pericellular environment (fig. 4). Although there are no similar reports for link protein, it may also be present on these new cell-surface-associated assemblies because it regulates the alignment of aggrecan along the central HA filament to form a continuous densely packed array [138]. This model of aggregate assembly may apply during fetal (developing/remodeling), juvenile (remodeling) and mature (turnover) phases, but in the latter case the decrease in link protein availability through decreased synthesis [128] may produce aggregates that are less ordered. It is unlikely that aggregates are retained at the cell surface for a sufficiently long period to allow accu-

mulation of link protein prior to release, as our studies show that aggregates derived from mature cartilage are deficient in link protein [127]. Not all newly synthesised aggrecan can immediately bind to HA because, remarkably, it can display delayed incorporation into aggregates [139, 140]. This is a small but transient pool of aggrecan with a half-life of about 24 h in vivo [141] which represents a metabolically active pool that is converted within the matrix into a form with a high affinity for HA binding into aggregates representing the major, but metabolically inactive pool ($t_{1/2} > 3$ years). The inference is that delayed aggregation enables these molecules to migrate freely away from the cell into the interterritorial matrix where they then get incorporated into aggregates. Studies based on transport and incorporation rates [142] suggest that the processing of newly synthesised aggrecan in the extracellular environment is different between immature and mature cartilages, giving rise to a number of intermediate structures. Distinct metabolic pools of aggregates varying in aggrecan numbers have been reported in other species [143], and it is noteworthy that chondrocytes isolated from immature cartilage assemble aggregates containing at least twice the number of aggrecan molecules compared with mature chondrocytes [144]. Processing of aggrecan thus involves age-dependent biological and environmental signals that 'mature' the molecule with time to promote aggregation, which may include structural changes in the G1 domain [145–148] to trigger HA binding.

There is mounting evidence that aggrecan can form interactions in situ with other non-collagenous ECM proteins in modulating the assembly of aggregates and associated networks. Many of these interactions are mediated via the G3 sub-domains, and include fibulin-1 and fibulin-2, which act as non-covalent ligands for the CRD module [149, 150]. These fibulins can self-associate, enabling the formation of cross-bridges between neighboring aggrecan molecules [150] and therefore may contribute to the organisation of aggregates. This function probably is more important during growth and maturation of cartilage when fibulins are present in significant amounts to produce cross-linked networks of aggregates (fig. 4). Similar cross-linked networks also occur via non-covalent associations between the CRD module and the matrix glycoproteins tenacin-C and tenacin-R [151, 152]. In addition to these important and direct protein-protein interactions of the CRD module in aggrecan assembly, its other reported binding abilities to carbohydrate and cell surface sulphated glycolipids [153–155] could provide different roles. Differential splicing of the mRNA in this region occurs during development and aging in mouse (table 2) and human [93] articular cartilages, and may also be a mechanism for modulating such interactions [156]. Direct interactions between collagen type II and aggrecan can occur within the KS-rich region [81], whereas indirect association between these two major cartilage components is thought to occur via matrilin-1 at the CS-rich region [157]. Matrilin-1 associates with type II collagen and can regulate fibrillogenesis [158]. It also decorates the CS-rich region of aggrecan via non-covalent and covalent cross-links [157, 159]. Wiberg et al. [160] have demonstrated that matrilin can function as a bridge to link aggrecan with collagens type II and VI through its associations with biglycan and decorin, which bind to these collagens. There may also be additional age-related interactions of aggrecan directly with other small proteoglycans, as appears to be the case with lumican in the sclera [161]. Such associations provide models for the assembly of macromolecular networks in the cartilage ECM, which depending upon the spatial (cartilage zone) and temporal expression of specific protein family members could mediate key steps that determine the nature of aggregates in various cartilage compartments (territorial, interterritorial and depth), consequently affecting the biomechanical properties of the tissue. Elucidating the roles of minor proteins in the initial assembly of the ECM and their role in cartilage homeostasis would help resolve many of the age-related changes that occur on aggrecan and aggregates.

Conclusions: implications of age-related changes in the ECM

The transition from a fetal cartilage, where it functions as a template for bone formation, to a weight-bearing tissue in adults necessitates a programmed change. In contrast, the consequences of many of the changes that occur in mature cartilage with aging are less clear; some may be detrimental to the functional properties of the tissue, whereas others may be beneficial. Alterations in some structural parameters, such as changes in the size and number of CS chains likely influence the physicochemical properties of aggrecan. Alterations in the structure of aggregates may affect viscoelastic (compressive resilience) properties, while other changes may influence the metabolic activity of the chondrocytes. Peptide fragments of link protein [162–164], G3 domain [165–167] and HA oligosaccharides have been reported to act in this manner [168–171]. This may explain, at least in part, the age-related decline that is observed in the response of chondrocytes to growth factors [172, 173]. The consequence of the alterations in the sulphation pattern of CS remains unclear, but by inference may reflect important but as yet poorly understood changes in the properties of the ECM. This is supported by observations that neuronal growth can be regulated by CS chains of proteoglycans which bind to differentiating factors and that the pattern of 4- or 6-sulphation, including those on aggrecan, can differentially regulate neurite outgrowth [174, 175]. It

remains to be demonstrated whether chondrocyte activity can be modulated by modifications in the sulphation pattern of CS in a similar manner.

Alterations in cartilage with normal aging are independent of the changes that occur in degenerative joint disease or injury, where pathology-related changes in the ECM and cells accumulate over 2–3 decades. OA is characterised by focal lesions of articular cartilage which eventually result is extensive erosion of the tissue, an indication of dysregulation of cartilage homeostasis. The prevalence of OA increases with increasing age, and age is a major epidemiological characteristic in OA; however, other accepted major risk factors include environment and genetics. It is probable that some age-related changes predispose cartilage to the development of OA, but pertinent changes are not obvious, as most investigations of biochemical and molecular alterations in osteoarthritic cartilage have relied on residual, severely deteriorated cartilage obtained at resection, which is not necessarily indicative of the changes that occur in the early stages of the disease. A recent study by Lorenzo et al. [176] demonstrates that, in the rare cases where available, human cartilage in the very early stages of the disease process exhibits distinct processing of matrix proteins compared with normal cartilage, suggesting that alterations in chondrocyte metabolism occur before overt fibrillation is evident. These changes are similar to those in later stages of the disease, indicating that the early changes are indeed part of the OA process. The distribution of some of the collagen-binding proteins is also altered; cartilage oligomeric protein is expressed by cells in deeper layers of cartilage, decorin expression increases and fibronectin decreases. Such findings are consistent with an alteration in the surface of the collagen fibrils and by extrapolation an alteration of tissue tensile properties. It is possible that failure to provide the appropriate mechanical property in a particular compartment could be harmful to the chondrocyte during normal joint loading when the macromolecular composition and structure have been altered [177]. Further studies in the very early stages of OA should clarify to what extent age-related alterations in aggregate assembly and the collagen networks orchestrate the initiation of cartilage degeneration.

Acknowledgements. Funding from the Arthritis Research Campaign (UK) and the Biotechnology and Biological Sciences Research Council (BBSRC, UK) is gratefully acknowledged. Dedicated to the memory of Professor Michael T. Bayliss, mentor and friend, who contributed so enormously to this field.

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