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Proteoglycans: An Overview

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Proteoglycans are a diverse group of heterogeneous macromolecules that are ubiquitous in the body and most abundant in the extracellular matrix of connective tissues. Their selective distribution and arrangement in distinct anatomic sites and their widely different structures lead to the conclusion that they have dissimilar and as yet incompletely understood functions in different tissues. Proteoglycans contain core proteins to which one or more glycosaminoglycan side chains are covalently attached. Glycosaminoglycans are linear anionic polysaccharides with repeating disaccharide units containing a hexosamine residue and usually, but not always, a hexuronic acid residue. Except for hyaluronic acid, they are almost always sulfated.

The earliest-reported and most extensively studied proteoglycans are those from mammalian cartilages; they were first described as components of the ground substance [1]. Progress in elucidating the structure of cartilage proteoglycan paralleled the development of techniques permitting the extraction and subsequent purification of the intact macromolecule. Early studies focused primarily on the examination of the glycosaminoglycan chains that could be extracted from cartilage in high yields following alkali degradation of the parent proteoglycan molecule. One of the earliest studies was in 1884, when Krukenberg [2] extracted cartilage with alkali and subsequently ethanol precipitated an acidic substance with an elementary composition similar to that of what was later termed *chondroitin sulfate*. In 1891, Schmiedenberg [3] gave the name *chondroitin sulfuric acid* to the substance isolated from alkaline extracts of cartilage. After degradation by acid hydrolysis, the substance yielded hexosamine and hexuronic acid. These were later identified as galactosamine (at the time called *chondrosamine*) and glucuronic acid by Levene and his collaborators in the 1920s [4]. It was not until the 1950s that the detailed structure of chondroitin sulfate was determined largely through the work of Meyer and collaborators [5–8].

During the same period, evidence was accumulating that the glycosaminoglycan chains were covalently linked to protein. Prior to this time, research proceeded on the presumption that the polysaccharides, by analogy to nucleic acids, were bound in the tissue to proteins as a complex via salt linkages [9]. It is interesting to note, however, that suggestions of a covalent linkage were present as early as 1889. Mörner

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[10] isolated a sulfate-containing preparation, *chondromucoid*, from cartilage after several days of autolysis. This material had a much higher content of nitrogen than the corresponding preparation isolated from alkaline extracts. Jorpes in 1929 [11], utilizing techniques previously applied to the isolation of nucleic acids [12], extracted cartilage with a weak salt solution and found that only small amounts of the chondroitin sulfate could be solubilized without alkali treatment. Furthermore, Partridge [13] showed that this putative complex was stable over a large pH range and that increasing temperature induced a reduction in viscosity of the complex, suggesting that salt linkages were not the primary bonds linking the complex. Studies by Shatton and Schubert in 1954 [14], Mathews and Lozaityte in 1958 [15], and Malawista and Schubert in 1958 [16] all indicated that protein was firmly attached to chondroitin sulfate chains.

However, it was not until 1958 that Muir [17] made the important observation that the chondroitin sulfate-protein complex isolated with 10% $CaCl_2$ and purified via precipitation with 5-amino-acridine contained after papain digestion nearly half the serine present in the original preparation. She deduced from these data that serine was involved in a covalent linkage between chondroitin sulfate and protein. Serine was shown to be the direct site of glycosaminoglycan attachment by two groups of investigators; Rodén and coworkers [18–20] and Anderson et al [21]. The former workers used hyaluronidase and proteolytic enzymes on a proteoglycan preparation from nasal cartilage and isolated a fraction that consisted of small peptides with oligosaccharides attached. Structural work on this fraction indicated that the attachment between protein and chondroitin sulfate consisted of a glycosidic bond between the hydroxyl group of a serine residue in the core protein and a xylose residue at the reducing end of the glycosaminoglycan chain. Two galactose residues are attached to the xylose through β-glycosidic bonds. The second galactose is then attached to the repeating sequence of chondroitin sulfate through a β 1–3 linkage with glucuronic acid. The data for this structure were summarized by Rodén in 1968 [20].

Anderson et al, in 1965 [21], used different methods to deduce that serines were involved in the attachment region. They observed a loss of serine when proteoglycans were treated with 0.5 M NaOH at room temperature for 19 hr. They then showed that this loss was the result of a β-elimination reaction in which the α hydrogen from the serine is removed and the chondroitin sulfate chain is released with the formation of a dehydroalanine residue in the polypeptide. Unsubstituted serines are not affected by the base treatment. The same authors presented evidence for the elimination mechanism by catalytically reducing the dehydroalanine residues and observing an increase in alanine content in subsequent amino acid analyses. Their experiments provided a chemical mechanism for explaining one of the oldest observations known for the properties of cartilages, namely, that extraction of the tissue with mild base readily solubilizes most of the tissue chondroitin sulfate [2,22].

The elucidation of the current structural model for the cartilage proteoglycan was slow to develop. Progress to a large extent was hampered due to the lack of an effective extraction procedure that would yield an intact macromolecule. However, indications of the main structural features of proteoglycans were present in many early studies. Shatton and Schubert in 1954 [14] were one of the first to isolate the cartilage proteoglycans by prolonged water extraction. The yield with this type of extraction, however, was low and the possibility of significant autolysis could not be

excluded. Mathews and Lozaityte in 1958 [15] subsequently presented data that suggested that the structure of a proteoglycan macromolecule consisted of a protein core with a number of chondroitin sulfate chains attached to it. The model presented by these investigators was similar to the one currently accepted. They used a proteoglycan preparation that contained about 16% protein to determine physical properties before and after degradation of the chondroitin sulfate side chains with hyaluronidase. Light scattering measurements gave a MW value for intact proteoglycans of 4×10^6. From their data they concluded that the molecules were rod-shaped and consisted of a central protein of 3,700 Å to which some 60 chondroitin sulfate chains were attached by one or more bonds to produce a comblike structure. They also proposed that aggregates of MW 50×10^6 could be formed by association of basic units.

The results described by Partridge, Davis, and Adair in 1961 [23] supported this model, and on the basis of reduction studies these investigators proposed that the chondroitin sulfate chains were unbranched and had molecular weights of approximately 28,000. Their evidence seemed to indicate that the reducing termini of the polysaccharide chains were free. Partridge and Elsden in 1961 [24] treated proteoglycans with dilute base, which, as indicated above, breaks the bond between protein and chondroitin sulfate. They then separated the protein fraction from chondroitin sulfate using ion-exchange chromatography. They found that glucosamine, characteristic of the repeating disaccharide of keratan sulfate, copurified with the protein fraction, leading them to suggest that proteoglycan macromolecules contained keratan sulfate linked to the same protein core. They further proposed that each keratan sulfate chain had only one attachment point to the protein core. It is now known that cartilage proteoglycan has keratan sulfate attached to the protein core via an O-glycosidic bond between N-acetylgalactosamine and serine or threonine.

All the isolation procedures for the proteoglycans from cartilage described above yielded molecules that were significantly altered either through extraction with alkali or by autolysis. The introduction of high-speed homogenization of the cartilage prior to extraction in water by Malawista and Schubert [16] in 1958 allowed about 80% of the total proteoglycans within the tissue to be extracted without alkaline treatment. The proteoglycans were precipitated with ethanol and separated by ultracentrifugation into fractions differing in molecular size and composition [25–27]. The extraction technique did not prevent possible degradation by lysosomal enzymes during the extraction. In addition, the shear forces generated during tissue homogenization possibly induced further degradation of the molecules.

A very effective method for the separation of cartilage proteoglycans from contaminating proteins, equilibrium CsCl density gradient centrifugation, was introduced by Franek and Dunstone in 1967 [28]. The technique exploited differences in the buoyant density of glycosaminoglycans and proteins in CsCl. Highly charged glycosaminoglycans, which have a high buoyant density, accumulate at the bottom of the centrifuge tube, whereas proteins having low buoyant densities float to the top of the gradient. The technique was used to purify proteoglycans found to contain two components with different sedimentation rates in the analytical ultracentrifuge [28], one of 20–25 S and the other of 75–85 S unit [29]. These improvements in extraction and purification techniques still did not prevent degradation of the proteoglycan molecules. Degradation could arise in at least three ways: first from proteolytic degradation during extraction and isolation, second from mechanical degradation

from the high shear forces introduced during homogenization, and third from cleavage in situ followed by selective extraction of degradation products because of their higher solubility.

It was not until the introduction of dissociative extraction conditions in combination with CsCl density gradient centrifugation introduced by Sajdera and Hascall in 1969 [30–32] that chemical characterization of intact proteoglycans could be done. These investigators found that the proteoglycans could be isolated from slices of nasal cartilage in very good yields (85% of the total) by gently shaking the tissue in concentrated salt solutions such as 3 M $MgCl_2$, 2 M $CaCl_2$, or 4 M guanidine hydrochloride. The proteoglycans were subsequently purified by CsCl equilibrium density gradient centrifugation in 0.4 M guanidine hydrochloride (associative gradient), to yield a preparation referred to as the *proteoglycan aggregate fraction.* This fraction showed a bimodal distribution in the analytical ultracentrifuge, with major, fast-sedimenting and minor, slow-sedimenting components. It was also shown that in 4 M guanidine hydrochloride only the slow-sedimenting component could be demonstrated. By CsCl gradient centrifugation in 4 M guanidine hydrochloride (dissociative gradient) the slow-sedimenting component now referred to as the *proteoglycan monomer* was separated from a fraction rich in protein referred to as the *glycoprotein link fraction*. Hascall and Sajdera [31] concluded that aggregates were formed from monomer and link protein. It was suggested that denaturing salt solutions such as 4 M guanidine hydrochloride dissociated the aggregates present in vivo, thereby facilitating extraction of proteoglycans from the matrix. Reaggregation occurred whenever the salt concentration was reduced to associative conditions. Aggregation was dependent on solvent pH and on intact disulfide bonds in the macromolecules.

On the basis of these and further experiments, Hascall and Sajdera devised a two-step purification method. After dissociative extraction, the extract was dialyzed to associative conditions and then subjected to centrifugation in CsCl under associative conditions to yield a bottom fraction enriched in proteoglycan complexes. This fraction was recentrifuged in CsCl under dissociative conditions to give proteoglycan monomer and glycoprotein link. The proteoglycan subunit had an average S_0 of 25 S and a MW of 2.5×10^6 but showed a considerable variation in weight, ranging from 1.3 to 3.7×10^6. These investigators suggested that the polydispersity in the population occurred because different core proteins had different numbers of chondroitin sulfate chains attached, averaging about 90 chains per core protein [30–32].

Using the isolation and purification technique of Hascall and Sajdera [30], Tsiganos et al [33] demonstrated by gel chromatography, on Sepharose 2B, that the individual proteoglycan fractions obtained from the dissociative CsCl density gradient centrifugation differed in their glucuronic acid to protein ratios, thereby suggesting that proteoglycans were polydisperse and heterogeneous in chemical composition. These investigators interpreted their results as indicating that there were several core proteins differing in length and in the type and number of glycosaminoglycan chains attached.

Further fractionation of the components of the aggregate revealed the presence of two factors separable by density gradient centrifugation, which were both required to permit aggregation to occur [34,35]. Subsequently, the additional link component was identified as hyaluronic acid, which accounted for about 1% of the glycosaminoglycan in bovine nasal cartilage and was shown to bind to proteoglycan. This interaction was shown to be highly specific, and the stoichiometry suggested that

many proteoglycans were binding to each hyaluronate chain [36]. In addition to hyaluronic acid, a glycoprotein component was released from the aggregate in the density gradient. This link protein component was shown to bind to the aggregate and stabilize the proteoglycan-hyaluronate bond.

The work on the cartilage proteoglycan structure during the last decade has followed two main lines: 1) determination of the chemical structure and physical properties of the proteoglycan subunit (monomer) and 2) determination of the macromolecular structure and organization of the proteoglycan aggregate. As a source of proteoglycans, many investigators have used bovine nasal septum, bovine tracheal cartilage, or pig laryngeal cartilage. For other, mostly biosynthetic studies, rat chondrosarcoma or embryonic chick chondrocytes were used.

The latest advances in this field will only be highlighted here; the reader is referred to some recent reviews for detailed information [37–43]. A major improvement in the methods of extraction with guanidine hydrochloride came through the studies of Oegema et al [44] and Pearson and Mason [45], who introduced protease inhibitors in all extraction and subsequent purification procedures. These modifications decreased the proteolytic degradation of the proteoglycans. These methods are currently used in most studies on proteoglycans. With regard to the further elaboration of the aggregate structure, Heinegard and Hascall [46] showed that the binding of the proteoglycan core to hyaluronic acid was mediated by a specific domain of the proteoglycan core protein that does not contain chondroitin sulfate chains. This region is referred to as the *hyaluronic acid-binding region* and is probably located at or near the N-terminal of the core protein [47,48]. It is rich in glutamic acid (or glutamine) and arginine, and has a MW of 60,000–80,000, which represents approximately one-fourth of the core protein.

In 1977, Heinegard and Axelsson [49] provided evidence that most of the keratan sulfate is located in a specific domain of the core protein. This led to the current model for the chondroitin sulfate proteoglycan of bovine cartilage in which three regions have been defined. The largest, chondroitin sulfate-rich region contains most of the approximately 100 chondroitin sulfate chains and 20–30% of the 50–80 keratan sulfate chains. This portion of the core contains more than half of the total protein in the proteoglycan. Serine, glycine, and glutamic acid (or glutamine) represent about 60% of the amino acid residues. The second region of the proteoglycan is the keratan sulfate-rich region, located between the chondroitin sulfate-rich region and the third region, the hyaluronic acid–binding region described above. It contains the majority of the keratan sulfate chains. The protein portion of this region has a MW of about 30,000 [49]. It is enriched in glutamic acid (or glutamine) and proline, which represent about 50% of the total amino acids; serine constitutes another 10–15%. Recent evidence has also shown that there are many smaller oligosaccharides attached to the core protein of the proteoglycan [50,51]. These are both O-glycosidically linked oligosaccharides that are found predominantly in the chondroitin sulfate-rich region of the molecule, and N-glycosylamine-linked (glycoprotein-type) oligosaccharides that are mainly on the hyaluronate-binding region [52]. The mucin type O-linked oligosaccharides have structures similar to that of the linkage region of keratan sulfate. These may be primers for synthesizing keratan sulfate chains on proteoglycans in mature tissues.

Most recently, it was shown that proteoglycans contain phosphate esters [53–55] located primarily on the xylose residues in chondroitin sulfate chains [55] but also

on serine residues in the core protein [54,55]. The function of the phosphate residues is currently unknown.

Since most of the proteoglycans within the cartilage matrix seem to be present as aggregates, a significant research effort has focused on determining how aggregates form and are stabilized [for reviews see 37,40,42,43]. Specific proteoglycans can selectively interact with a small segment of a single molecule of hyaluronic acid. This interaction is noncovalent in nature, specifically for hyaluronic acid, and occurs in the hyaluronic acid-binding region of the core protein. This hyaluronic acid-binding region, according to studies with low-angle neutron scattering, seems to be a globular protein of elongated ellipsoid shape containing N-linked oligosaccharides [56]. As many as 200 proteoglycans can bind to one single hyaluronic acid molecule of 1×10^6 MW to form aggregates of MW 5×10^7 to 5×10^8 [40] that by electronmicroscopy are more than 2 μm long [57]. A link protein [41,58] stabilizes each monomer by interacting with both the hyaluronic acid and the hyaluronic acid-binding region of the core protein. Such link-stabilized aggregates do not readily dissociate at physiological pH and in nondenaturing conditions. The stabilized aggregate protects the hyaluronic acid-binding region of the core protein from degradation by proteases. Link stabilization occurs extracellularly [59–61], allowing the assembly of the aggregates in an extracellular matrix consisting of a collagenous network surrounding the chondrocyte. Recent results indicate that the assembly processes in certain circumstances may be more complex [62,63]. It appears that newly made proteoglycan from some sources is less able to bind to hyaluronic acid than older material synthesized earlier in the tissue.

Aside from the major cartilage proteoglycan described above, several distinct proteoglycans have been discovered in cartilaginous tissues. For example, using agarose-polyacrylamide composite gel electrophoresis introduced by McDevitt and Muir [64], Stanescu et al [65] were able to separate for the first time several distinct bands representing different proteoglycans. Within the articular cartilage, Sweet et al [66] were able to show that proteoglycans from the nonweight-bearing area had a somewhat higher relative content of chondroitin sulfate than those isolated from areas of maximum articulating contact. In distinct but minor populations of proteoglycans, first described in bovine nasal cartilage [67], the proteoglycans are large but cannot interact with hyaluronic acid. These nonaggregating proteoglycans have an amino acid composition that seems to preclude their being derived directly from the aggregating proteoglycan. In addition, the large aggregating proteoglycan described so far can be separated into at least two immunologically different populations. Only one of these proteoglycans contains a prominent keratan sulfate-rich region in addition to the chondroitin sulfate-rich region. This population is therefore called the *keratan sulfate-rich proteoglycan*. The other proteoglycan has only a chondroitin sulfate-rich region and by analogy is called the *chondroitin sulfate-rich proteoglycan* [68]. Future studies on cartilage proteoglycan composition, polydispersity, and biosynthesis will have to take into account this recent evidence of proteoglycan heterogeneity.

The cartilage-type proteoglycans vary with age and the source of the cartilage tissue. When isolated and studied in vitro the major class of cartilage proteoglycan can occupy about 50 times its dry weight in the solvent. Within the tissue, however, the proteoglycans are highly concentrated (about 100 mg/ml) so that the macromolecules are compressed to about 20% of their extended structure in solution. During the biosynthetic phase, when still within the cell, the proteoglycans are thought to be

even more compressed, eg, within the Golgi and secretory vesicles, before secretion into the extracellular space against a concentration gradient.

Proteoglycans are central to the proper functioning of cartilage, which is to be able to reversibly absorb loading forces [40–42]. Proteoglycans have a high negative-charge density and are present in high concentration largely in aggregated form within the tissue, where they fill the interstices between collagen fibers. Thus, proteoglycans are not freely mobile in the extracellular matrix of cartilage, being restrained by the collagenous network. Within the tissue, these highly charged molecules occupy only a fraction of the hydrodynamic domain they would if free in solution. They therefore attract water, providing a swelling pressure of several atmospheres within the tissue. This turgor pressure provides stiffness to the tissue and serves to maintain its shape. Upon loading, the cartilage matrix can deform by the expulsion of water from the loaded region. Movement of water is retarded by the attractive forces of the proteoglycan, thus energy is being absorbed by the tissue as water is displaced. Concomitantly, as the tissue deforms, the proteoglycans are forced closer together, effectively increasing the negative charge density, which in turn increases the resistance of the tissue to further deformation. Ultimately the deformation reaches an equilibrium in which the loading force is balanced by the swelling pressure of the proteoglycan. As the load is removed, the tissue regains its original form by imbibing water. The unloaded form of the tissue will be reached when the swelling pressure of the proteoglycan is balanced by the resistance of the collagen network to further expansion.

In addition to the physical properties ascribed to the proteoglycans, proteoglycans in cartilage also express another function due to their high concentration within the tissue: They retard the diffusion of certain macromolecules through connective tissues, and they enhance the diffusion of small molecules [69]. This accelerated transport is considered to be due to excluded-volume effects.

The role of proteoglycans in providing form is important in embryonic development [41,42]. Cartilage in the epiphyseal growth plate provides a scaffolding for new bone formation. Although there have been many experimental data suggesting that proteoglycans play an active role in the mineralization process most of the evidence is still circumstantial [70].

Certain genetic mutations, nanomelia in chickens [71] and cartilage matrix deficiency in mice [72], exhibit very low or no synthesis of cartilage proteoglycans. The consequences of such a lack are severe skeletal abnormalities, including a markedly decreased width of the growth plate. These mutations are lethal. Thus it is clear that proteoglycans in cartilage play an important structural role, vital to the proper function of the tissue.

This overview of the cartilage proteoglycans clearly demonstrates that these macromolecules are present in the tissues as both polydisperse and heterogeneous components. The heterogeneity within each population of proteoglycans is due to biosynthetic differences in the core protein, whereas proteoglycans with a single core protein type may show polydispersity due to differences in the size, charge, number, and chemical modification of the glycosaminoglycan side chains, in addition to degradative changes introduced in the core protein during the life of the proteoglycan in the extracellular matrix. Studies in this field have to take these observations into consideration, especially when relationships between physiological and pathologic conditions are to be considered.

Specific tissues also frequently contain different classes of proteoglycans. Again, this can be illustrated by the cartilage tissue, which shows aside from the chondroitin sulfate- and keratan sulfate-containing proteoglycans also a proteoglycan that yields upon chemical analysis dermatan sulfate as its component glycosaminoglycan. Dermatan sulfate, or chondroitin sulfate B, is composed of disaccharides of N-acetylgalactosamine and hexuronic acid with varying amounts of iduronic acid instead of glucuronic acid. The average sulfation of dermatan sulfate is close to one per disaccharide unit and the linkage region of the carbohydrate to the core protein is the same as in the chondroitin sulfate proteoglycans. Dermatan sulfate is synthesized as chondroitin sulfate in which the glucuronic acid is epimerized to iduronic acid during or after chain polymerization.

Recent studies by Rosenberg et al [73] indicate that a dermatan sulfate proteoglycan is present in low concentrations in bovine fetal epiphyseal cartilage following the advent of chondrogenesis but that it appears in increased concentration in aging bovine articular cartilage. Dermatan sulfate proteoglycans were also found in the developing chick limb, wherein two types were identified [74–76]. They had larger glycosaminoglycan chains than did the cartilage proteoglycan [74,75] and were found associated with discrete developmental stages in the chondrogenic process [76]. One of the dermatan sulfate proteoglycans was found to be disulfide bonded to collagenous polypeptide [75]. Immunologically, the cartilage-derived dermatan sulfate and the chondroitin sulfate proteoglycans are distinct. These data on the dermatan sulfate proteoglycans not only describe the presence of heterogeneous populations of this class similar to that of the chondroitin sulfate proteoglycan, but also point out another important facet: Tissues frequently contain different classes of proteoglycans.

There are no uniform classifications of proteoglycans. Historically, classes were established according to the predominant glycosaminoglycan side chains. In the future, classification will require that the structure of the core protein be used as part of the characterization. However, until this information is available, the proteoglycans will still have to be classified in several ways: eg, by their predominant glycosaminoglycan (eg, chondroitin sulfate), by their glycosaminoglycan content in hybrid molecules, (eg, chondroitin sulfate-keratan sulfate proteoglycans from cartilage), by their ability to interact with hyaluronic acid (aggregating proteoglycans), or by their source (cell, tissue). It is not possible in this brief overview to give examples for all these classes and to be comprehensive, but it should be taken into consideration that in most cases a chemical (carbohydrate and peptide)- as well as a tissue-specific description of a proteoglycan should be used to circumvent confusion. This becomes especially important in light of the more recent data on the heparan sulfate-heparin proteoglycans that seem to show quite different morphologic distributions as well as functions.

With regard to the postulated physiological function of proteoglycans, few experimental data are available that justify certain hypotheses. As outlined above, proteoglycans undoubtedly play prominent roles in the organization, structure, and function of extracellular matrices. The physical properties of the different connective tissues depend on the differences in the proportion of proteoglycans and structural proteins, collagen and elastin, and how these molecules are organized within the extracellular matrix. A general statement, however, can be made for many connective tissues: Proteoglycans are space-filling and show specific interactions within the extracellular milieu. The elucidation of the nature of these interactions in terms of their molecular mechanisms and physiological role must await future developments.

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