Introduction: proteoglycans and vision

The remarkable physical properties of the cornea are a direct result of the unique biochemical composition and exquisite ultrastructural organization of the corneal stroma, a layer of connective tissue comprising 90% of the cornea. The stroma consists of multiple layers (lamellae) of parallel bundles of collagen fibrils of a small and highly uniform diameter, embedded in a hydrated-proteoglycan matrix. The stromal cells (keratocytes) comprise only 4% of the stromal volume and lie sandwiched between stromal lamellae. A number of physical properties have been identified as important in light transmission by the corneal stroma, particularly the small, uniform diameter and tight packing of the stromal collagen fibrils [1]. The stroma is strongly hydrophilic, and free access to water results in swelling of the tissue and increased light scattering owing to disruption of fibril spacing and a decrease in the extrabifrillar index of refraction [2, 3].

Stromal hydration is controlled by active water transport by the corneal endothelium and epithelium, cell layers bordering the stroma.

The hydrophilic properties of the stroma result from stromal proteoglycans. These compounds constitute the second most abundant biological material in the stroma, after collagen. The major corneal proteoglycans contain keratan sulphate or dermatan sulphate glycosaminoglycan chains. Dermatan sulphate in cornea is present as decorin, a small, widely distributed proteoglycan also known as PGII, PG40 or PG-S2 [4]. Corneal decorin is unusually low in sulphate and iduronic acid and was originally thought to be a chondroitin sulphate. Corneal keratan sulphate proteoglycan (KSPG) is a small proteoglycan, widely distributed in other tissues as an unsulphated glycoprotein (discussed below), occurring in cornea with a unique abundance and sulphation. The unique combination of highly sulphated KSPGs and of under-sulphated dermatan sulphate proteoglycan (DSPG) may be important in maintenance of corneal transparency because of the hydration properties of the two molecules. Water binding by KSPG is high, but reversible; however, DSPG exhibits a tight, essen-
tially non-reversible water binding [5]. Several metabolic-storage diseases, in which highly sulphated dermatan sulphate accumulates in tissues throughout the body, result in corneal opacity at an early age [6]. Macular-corneal dystrophy, a condition in which the carbohydrate moiety of KSPG is undersulphated or lacks sulphation, also results in corneal opacities [7]. Thus a proper amount and balance of stromal proteoglycans is essential for maintenance of corneal transparency.

**Structure of KSPG**

**Linkage to protein**

Keratan sulphate is a linear polymer of N-acetyl-lactosamine [GlcNAc(β1-4)Gal] which can be sulphated at the 6 position of either glucosamine or galactose. Keratan sulphate is present in most tissues in small amounts [8], and is a side-chain in several proteoglycans in addition to corneal KSPG [9]. The keratan sulphate of cornea is unique in its abundance, high degree of polymerization, and high sulphation. Unlike other glycosaminoglycans, keratan sulphate contains no uronic acids, so its anionic charge depends on sulphation. Proteins carrying poly-N-acetyl-lactosamine chains with little or no sulphation do not exhibit the characteristic size, acidity, and hydrophilic properties typical of proteoglycans, behaving rather like glycoproteins. Unsulphated poly-N-acetyl-lactosamine, known as lactosaminoglycan (LAG), is found in a number of extracellular glycoproteins [10].

Keratan sulphate in KSPG is attached to asparagine in the core protein via a mannose-containing linkage oligosaccharide. This N-linked oligosaccharide, shown in Fig. 1, is identical to biantennary hybrid N-linked oligosaccharides found in many glycoproteins [11]. Sialic acid, present in KSPG, can cap one branch of the oligosaccharide leaving keratan sulphate extending the other branch [12]. The N-linkage of corneal keratan sulphate differentiates it from all other glycosaminoglycans, including keratan sulphate of cartilage (aggrecan) and brain, which are all O-linked to serine or threonine [9, 13]. LAGs, like corneal keratan sulphate, are mostly N-linked though bi- or tri-antennary hybrid oligosaccharides [14]. Corneal KSPG, structure, therefore, is more representative of a highly sulphated N-linked glycoprotein rather than of a typical proteoglycan.

**Core-protein isoforms**

Endo-β-galactosidase from Escherichia freundii cleaves keratan sulphate leaving the linkage oligosaccharide attached to the core protein (Fig. 1). SDS/PAGE analysis after enzymic removal of keratan sulphate showed multiple core proteins present in KSPG of human, rabbit and bovine corneas [15-17]. The core proteins from bovine KSPG had molecular masses of 48 and 36 kDa. Treatment of bovine KSPG with N-glycanase to remove both keratan sulphate and N-linked oligosaccharides reduced the size of core proteins to 37 kDa and 25 kDa. Core proteins of the same size were obtained by complete deglycosylation of KSPG using trifluoromethansulphonic acid [18]. The 37 and 25 kDa-proteins had different N-terminal amino-acid sequences, and differences in tryptic peptide maps demonstrated unique, primary amino acid sequences in each [18].

More recently, separation of the core proteins by ion-exchange chromatography followed by SDS/PAGE was able to identify two fractions of the 37 kDa-core (designated 37A and 37B). These two fractions differed in size by about 1 kDa. Examination of tryptic fragments of these proteins by reversed-phase h.p.l.c. identified differences in total peptides, in peptides containing cysteine, and in peptides recognized by antibodies [19]. As shown in Fig. 2, antibody made to a peptide duplicating the N-terminal sequence obtained from the mixture of the 37 kDa-cores bound only one of the three core proteins, the core designated 37B. Bovine KSPG, therefore, is a mixture of three unique proteins each substituted with keratan sulphate. Three KSPG proteins have been identified by translation in vitro of both bovine and chick mRNA, suggesting that each core protein is a product of a separate mRNA [20].

Recent studies (J. Funderburgh, J. Hassell, J.-P. Vergnes & G. W. Conrad, unpublished work) of a cloned bovine corneal cDNA determined an amino-
KSPG core proteins were released by endo-β-galactosidase and separated into fractions A (lanes 1 & 3) and B (lanes 2 & 4) by chromatography on DEAE-Sephacel as described [19]. The proteins were separated by SDS/PAGE and stained with Coomassie blue (lanes 1 & 2) or transferred to nitrocellulose and reacted with an affinity-purified antibody against a synthetic peptide duplicating the N-terminal amino-acid sequence obtained from the 37 kDa core proteins (lanes 2 & 4) [20]. The antibody reacted exclusively with the 37B isoform.

Acid sequence highly similar to that reported for lumican, a chicken KSPG core protein [21]. The deduced sequence of bovine lumican contained the sequence determined for the N-terminus of the 37B core protein, identifying that protein as lumican. The other KSPG proteins may also be products of the lumican gene, resulting from RNA splicing, or they could be products of related unique genes.

KSPG glycosylation

Several studies have noted that KSPG is heterogeneous in size and perhaps in the number of keratan sulphate chains per protein [22-24]. Work in our laboratory has provided information on the nature of this heterogeneity. Comparison of sizes of KSPG containing the smaller (25 kDa) and larger (37 kDa) cores suggested that KSPG with the larger core proteins contained 2-3-fold more keratan sulphate than KSPG with the smaller core protein [18]. Specific information on the number of chains linked per core was obtained by labelling sites on the core protein bearing keratan sulphate chains. After removal of the keratan sulphate chain with endo-β-galactosidase, the non-reducing terminal GlcNAc remaining on the linkage oligosaccharide (see Fig. 1) was labelled using galactosyltransferase and UDP-[3H]Gal [19]. The core proteins thus labelled at sites of keratan sulphate attachment were digested with trypsin, and the fragments separated by reversed-phase h.p.l.c. Three peptides bearing labelled linkage sites in the 37A core protein were identified, and one site each in 37B (lumican) and in the 25 kDa-cores. Sialic acid, labelled via periodate/NaBH₄ treatment of the core proteins [19], was found associated with the keratan sulphate linkage-containing peptides in all cases except one, opening the possibility that in one case (37A), both arms of the biantennary linkage region are extended by keratan sulphate. A diagram of the structures of corneal KSPG isoforms is shown in Fig. 3. The core proteins are present in purified KSPG in an approximate ratio of 3:6:2 (37A:37B:25 kDa), but owing to the larger number of keratan sulphate chains on the 37A protein, that isoform may contain 50–60% of the keratan sulphate in the cornea [19].

Distribution of KSPG

Keratan sulphate, originally identified in and named for the cornea [25], had been extracted and purified from only cornea and cartilage in earlier studies using chemical means of identification. Availability of antibodies to sulphated regions of the keratan sulphate chain [16, 26] allowed more sensitive detection in tissues. A survey of bovine and chick tissues using two such monoclonal antibodies was
able to detect sulphated keratan sulphate epitopes in extracts of many tissues [8], and subsequent studies have confirmed a wide distribution of keratan sulphate [27-30]. The concentration of keratan sulphate in cornea is, however, 20-1000-fold greater than that in other tissues except cartilage [8].

Antibodies which bind the core protein of bovine KSPG also detected the presence of this antigen in many tissues [8]. Unlike keratan sulphate, however, the core protein was found to be quite abundant in several tissues. Cartilage, skeletal muscle, intestine, and dermis all contained 17-24% as much KSPG protein as cornea, based on tissue wet weight [8]. Immunoblotting after SDS/PAGE showed these antigens to be much smaller than corneal KSPG (60-80 kDa), but treatment with endo-β-galactosidase released 48 kDa- and 36 kDa-proteins analogous to those of cornea [31]. Recently, one of these non-corneal KSPG antigens was purified to homogeneity from bovine aorta (J. Funderburgh, M. Funderburgh, M. Mann & G. Conrad, unpublished work). This material had tryptic maps and an N-terminal amino-acid sequence identical to bovine lumican. Unlike corneal KSPG, the arterial KSPG contained short (8-9 disaccharides), unsulphated, LAG chains. This finding demonstrated that at least one of the KSPG isoforms is present in both corneal and in non-corneal tissues. Although it is found in cornea at 4-5-fold greater concentration than in other tissues, the presence of KSPG in massive tissues such as muscle and dermis means that corneal KSPG makes up less than 1% of the total KSPG in the body.

Corneal KSPG appears to be a specialized form of a common glycoprotein. The specialization consists of modification of the glycosylation of the molecule, in particular, lengthening of the LAG chain from 8 to over 40 disaccharide units and addition of sulphate. In making this modification, the cornea produces a molecule with the size and hydrophilic properties typical of proteoglycans. KSPG, therefore, represents a molecule which spans the gap between glycoprotein and proteoglycan. At this time, there is no data supporting the presence of the 37A isofrom outside of the cornea. This leaves open the possibility that some KSPG molecules may be limited to the cornea.

**KSPG during development and disease**

In early corneal development of the chicken, KSPG protein can be detected in the primary stroma and in peripheral cells before the corneal stroma is populated with cells (D. Schulz & G. Conrad, unpublished work). Highly sulphated keratan sulphate, on the other hand, appears to be synthesized only by the neural-crest-derived keratocytes, beginning about twelve hours after their migration into the primary stroma [32]. Both core protein and highly sulphated keratan sulphate accumulate exponentially during the period of embryonic development, during which the cornea becomes transparent and innervated. During this period, the relative sulphation of the keratan sulphate increases markedly [32, 33] in association with an increase in PAPS [34]. These observations suggest that stroma provides an environment which stimulates secretion of the highly sulphated, corneal form of KSPG.

The importance of the stromal environment is confirmed by studies in vitro. Keratocytes removed from the stroma and maintained in cell culture secrete little or no highly sulphated keratan sulphate, but do secrete a highly sulphated dermatan sulphate [35, 36]. Mammalian corneas in organ culture continue keratan sulphate synthesis at a reduced level, but this synthesis is eliminated after brief treatment of the tissue with collagenase [37]. The keratocytes, therefore, appear to be highly responsive to their extracellular environment, secreting proteoglycans characteristic of the normal cornea under conditions which approximate to those of a normal stroma.

The keratocytes also alter proteoglycan secretion in response to pathological conditions of the cornea. A low-sulphated KSPG and a highly sulphated DSPG accumulate in healing corneal wounds, in those which penetrate the stroma and form scar tissue on a fibrin clot, as well as in partial thickness wounds similar to incisions made by keratotomy surgery [38, 39]. In keratoconus, a disease which results in progressive thinning and scarring of the central cornea without external trauma, a similar phenomenon of reduction in KSPG sulphation and accumulation of a highly sulphated dermatan sulphate takes place [40-42]. These observations suggest that keratocytes react to various types of stress with a 'wound healing' response. This involves loss of the highly sulphated KSPG and low-sulphated decorin (characteristic of normal cornea), and secretion of low-sulphated KSPG and highly sulphated DSPG typical of non-corneal connective tissues. Considering the hydration properties of the two corneal proteoglycans, it would appear likely that loss of highly sulphated keratan sulphate and buildup of highly sulphated DSPG, typical of pathological conditions of the cornea, may have a role in the formation of opaque
scar tissue. Corneal scar tissue results in visual impairment or blindness for hundreds of millions of individuals [43]; thus, understanding the factors which induce the secretion of scar tissue by keratocytes and the role of the proteoglycans in light scattering by scar tissue remains an important subject for future research.

Other biological roles for KSPG

There is little doubt that corneal KSPG fulfills the space-filling hydrophilic structural role which has been proposed for it. Yet there is good reason to believe that this molecule may be involved in more specific interactions with other matrix components or with cells. In cornea, KSPG is associated with collagen fibrils [44], and recent work showed that the lumican core protein altered collagen fibrillogenesis in vitro [45]. The primary structure of chicken lumican, determined from cDNA, is similar to decorin and fibromodulin [45], both of which are known to interact with collagen. Together, these three form a family of small interstitial proteoglycans (SIPGs) widely distributed in connective tissues throughout the body. The abundance and glycosylation of different members of this group, in a given tissue, may play a role in determining the ultrastructure of the extracellular matrix of that tissue.

In addition to protein interactions, the unsulfated LAG chains on KSPG present in early corneal development and healing corneal wounds and in non-corneal KSPG may mediate interactions between KSPG and cells. Extracellular LAGs have been implicated in numerous events including cell-matrix recognition, cell adhesion, and cell motility [10]. Neural adhesion is reported to rely on extracellular LAGs [10], and keratan sulphate (a sulfated LAG) was found to inhibit neural outgrowth [46]. Thus corneal innervation may be mediated by increases in KSPG sulphation during development and decreases in KSPG sulphation in healing wounds. LAGs are also involved in the attachment of activated macrophages [47], providing a second possible role for the low-sulfated KSPG in healing-corneal wounds. These are two of many potential interactions in which cellular activity might be affected by KSPG.

Conclusions

Much understanding of the chemistry of the KSPG family of molecules has been gained in the half century since the initial characterization of keratan sulphate [25, 48]. The carbohydrate structures are well characterized. We know that there are several protein components, that KSPG is present in most tissues, and that KSPG can vary greatly in glycosylation and sulphation, leading to a remarkable variation in the physical and chemical properties of the molecules. Many biological questions, on the other hand, are still unanswered: the control of KSPG secretion by keratocytes, the importance of KSPG to corneal transparency, and the interactions of KSPG with other extracellular matrix components and cells. Understanding of these and other aspects of KSPG biology represents the future for research on this important and interesting family of compounds.

The authors thank Drew Schultz for technical assistance. This work was supported by American Heart Association Kansas Affiliate Grant-in-Aid: KS-89-12 (to J.L.F.) and National Eye Institute Grant EY 00952 (to G.W.C.).


Received 22 July 1991