Structure
Article

Structural Interactions between Collagen and Proteoglycans Are Elucidated by Three-Dimensional Electron Tomography of Bovine Cornea

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SUMMARY

Interactions between collagens and proteoglycans help define the structure and function of extracellular matrices. The cornea, which contains proteoglycans with keratan sulphate or chondroitin/dermatan sulphate glycosaminoglycan chains, is an excellent model system in which to study collagen-proteoglycan structures and interactions. Here, we present the first three-dimensional electron microscopic reconstructions of the cornea, and these include corneas from which glycosaminoglycans have been selectively removed by enzymatic digestion. Our reconstructions show that narrow collagen fibrils associate with sulphated proteoglycans that appear as extended, variable-length linear structures. The proteoglycan network appears to tether two or more collagen fibrils, and thus organize the matrix with enough spatial specificity to fulfill the requirements for corneal transparency. Based on the data, we propose that the characteristic pseudohexagonal fibril arrangement in cornea is controlled by the balance of a repulsive force arising from osmotic pressure and an attractive force due to the thermal motion of the proteoglycans.

INTRODUCTION

Extracellular matrices are complex biological systems, in which collagens and proteoglycans are the major components. Comprehending the interactions and controlling influences of these macromolecules is key if we are to fully understand the structure-function relationships in the extracellular matrix, and the cornea is an excellent model system in which to investigate this. The cornea is a uniquely transparent connective tissue, its light transmissive properties arising via optical interference effects as a consequence of structural order in the array of uniformly thin collagen fibrils that form the bulk of the corneal stroma. This structural order—often described as pseudohexagonal or lattice-like—is believed to be governed by macromolecular interactions between collagen fibrils and sulphated proteoglycan molecules that occupy the extrafibrillar space.

The main constituent of the cornea is collagen, a tough fibrous protein that is employed repeatedly in biological systems to make structures resistant to deformations and external stresses (Knupp and Squire, 1998; Humzah and Soames, 1998; Holzapfel, 2008). In cornea, the predominant fibril-forming collagens are types I and V, in which 300-nm-long molecules are arranged into hybrid fibrils of remarkably regular diameter (Birk et al., 1988; Birk et al., 1990). Uniaxially aligned fibrils pack together to form lamellae that are stacked and often interwoven, especially in the anterior stroma (Komai and Ushiki, 1991). The fibril axis of adjacent lamellae changes, generally by up to 90°, between successive lamellae in the central areas of large mammalian corneas such as human and bovine (Meek and Boote, 2004). Such a three-dimensional arrangement accounts for corneal toughness and resistance to deformations. Crucially, corneas need to be transparent and current explanations for corneal transparency are largely based on the fact that the collagen fibril arrangement within corneal lamellae is on a pseudohexagonal lattice with short-range spatial order—i.e., the distribution of distances between adjacent fibrils is narrow (Maurice, 1957; Hart and Farrell, 1969; Benedek, 1971; Farrell et al., 1973). To understand the basis of corneal transparency, we need to appreciate that the light entering the cornea is scattered by each and every fibril it encounters, and that for the cornea to be transparent, the scattered rays must interfere destructively everywhere except in the forward direction. For this to happen, the arrangement of the collagen fibrils cannot be random, and adjacent fibrils must obey fairly precise positional rules so that the light scattered by a fibril can interfere appropriately with that scattered by adjacent fibrils. In the cornea, the collagen fibrils are arranged in a pseudohexagonal lattice with ~60-nm-long sides. It has long been believed that this lattice-like structure is the result of structural interactions between collagen fibrils and a family of hybrid carbohydrate-protein macromolecules called proteoglycans (PGs) (Bettelheim and Plessy, 1975; Borcherding et al., 1975).

PGs are characterized by a protein core to which one or more anionic glycosaminoglycan (GAG) chains are attached. In cornea, there are two families of PG: the chondroitin sulphates/dermatan sulphates (CS/DS) and the keratan sulphates (KS) (Anseth, 1961; Axelsson and Heinegard, 1978, 1980; Gregory et al., 1982). Electron microscopy measurements on bovine corneas showed that stained CS/DS PG filaments are ~70 nm long, whereas KS PG filaments are ~40 nm long (Scott, 1992). Protein cores of corneal PGs likely attach to collagen fibrils, with their GAG chains free to interact with other components

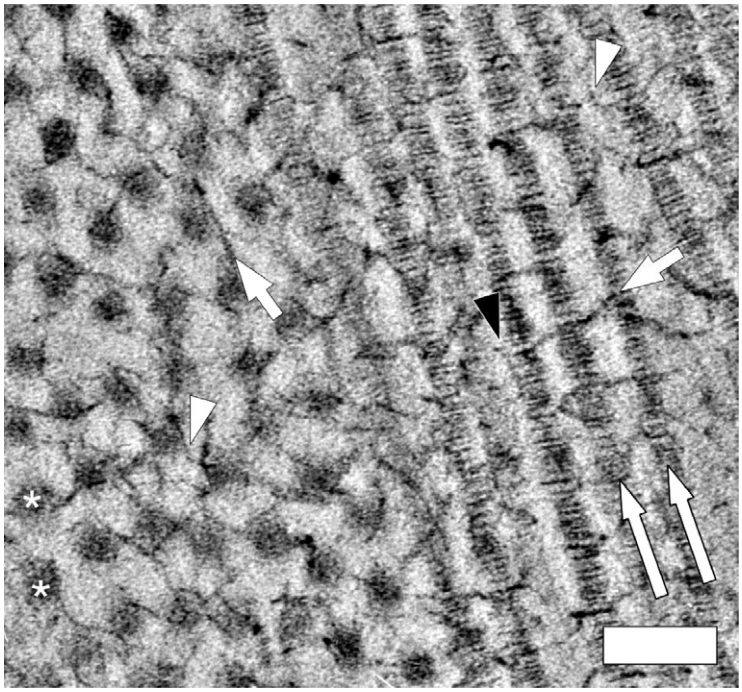

Figure 1. Appearance of a Bovine Cornea as Imaged in a Transmission Electron Microscope

The imaged region is at the interface between two lamellae, and shows a transverse view on the left-hand side, where some of the collagen fibrils are marked by asterisks. On the right-hand side there is a longitudinal view where some collagen fibrils are highlighted by long arrows. Short arrows indicate large, stained proteoglycan filaments that connect two or more adjacent collagen fibrils. A white arrowhead points to a smaller proteoglycan between fibrils. A black arrowhead indicates proteoglycans running axially, not bridging adjacent fibrils. Scale bar represents 100 nm.

of the extrafibrillar matrix. It was suggested by Scott that GAG-GAG interactions might lead to the formation of "yardsticks" that keep the collagen fibrils at defined distances (Scott, 1995).

Specific structural models on how the PG-collagen interactions occur were put forward by Maurice (1962), Farrell and Hart (1969), and Muller et al. (2004). All these models are based on a systematic six-fold arrangement of PGs around the collagen fibrils. The simplest models by Maurice and by Farrell and Hart consisted essentially of six PG complexes extending at regular axial intervals from one collagen fibril to six adjacent fibrils. Muller and collaborators proposed a modified version of this based on an electron microscopy study of human corneas in which the PG complexes do not form bridges between adjacent fibrils, but between next nearest neighboring fibrils, thus accounting for the size of some PG complexes that are seen to be longer than the average interfibrillar spacing.

In this paper, we use electron tomography to study the cornea and image for the first time the interactions between PGs and collagen fibrils in three-dimensions. These reconstructions, unlike previous thinking, do not show any systematic six-fold arrangement of PGs around the collagen fibrils, nor a pattern of links between next-nearest neighbors. Rather, our data lead us to propose a new model of collagen-PG interactions in cornea, and a possible mechanism of how positional order in the cornea is maintained.

RESULTS

Conventional two-dimensional (2D) micrographs of bovine cornea prepared with cupromeronic blue stain show PGs as filaments of electron-dense material both in transverse and longitudinal sections (Figure 1). Some PGs extend between, and are in contact with, two or more collagen fibrils (short arrows). Other PGs are shorter and occupy the space between adjacent fibrils (white arrowheads). Between 5 and 8 PG filaments per D-period were found to form bridges between collagen fibrils (see Figure S1 available online). Images such as this, however, provide us with no 3D information about collagen-PG interactions, so to address this deficiency we generated stereo pairs from two separate real space 3D reconstructions of transverse and longitudinal views of the central anterior region of a bovine cornea (Figure 2). Unlike models that have been proposed previously, these reconstructions show no evidence of a systematic arrangement of PGs around the collagen fibrils. In particular, PGs in transverse view do not show an intrinsic six-fold symmetry around any collagen fibril (Figure 2A). This type of symmetry would be expected if there was a systematic PG-mediated structural interaction between adjacent collagen fibrils that gave rise to the close-to-hexagonal arrangement of the fibrils themselves. It is also evident from the 3D reconstructions that PGs that form bridges between fibrils do not always do so between the closest points on the surface of two adjacent fibrils, but demonstrate a tendency to bridge adjacent fibrils only tangentially, so that a PG chain often extends between more than two collagen fibrils (Figures 2A and 2B, arrowheads). In addition to the PGs described, some stained filaments remain axially close to a single fibril (such as that highlighted with a red arrowhead in Figure 2).

To clarify the structural roles played by PGs with CS/DS and KS GAG chains, some sections from a cornea were treated either with keratanase or with chondroitinase ABC. These are enzymes that specifically digest the KS and the CS/DS chains, respectively (Scott and Haigh, 1985; Scott and Bosworth, 1990). Enzyme treatment appeared to generate some fibril coalescence in the past (Kühn and von der Mark, 1978), so care should be used in interpreting the results obtained by this method. Figures 3A, 3C, 3E, and 3G show typical longitudinal and transverse views of what is seen in the electron microscope after treating some of the sections. Longitudinal and transverse views of a keratanase-treated section from which KS chains had been removed indicate CS/DS PG bridges between adjacent collagen fibrils (Figures 3A and 3C). Most bridges extend over two or more fibrils, and repeat axially every ~65 nm. In transverse view, the collagen fibrils have lost their pseudohexagonal arrangement, and are seen to associate linearly forming a necklace-like structure in which one or more CS/DS PG holds

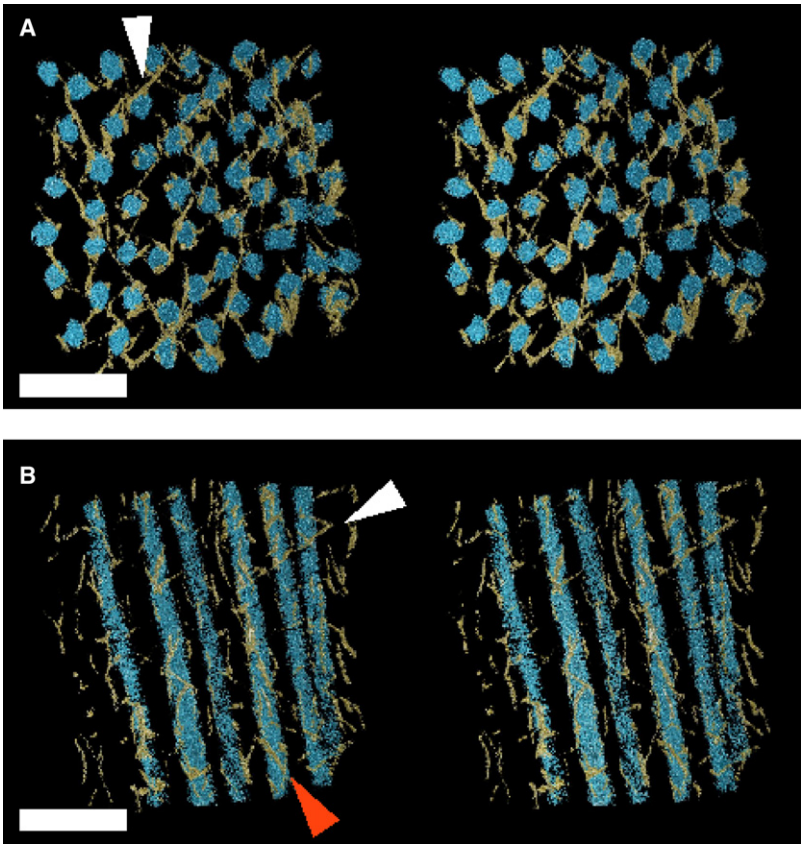

Figure 2. Stereo Pairs of Two Separate 3D Reconstructions of Bovine Cornea

In both panels the collagen fibrils are colored blue and the proteoglycans gold.

(A) Transverse view.

(B) Longitudinal view.

Some stained proteoglycan filaments form bridges between two or more collagen fibrils (white arrowhead), whereas others remain axially close to a collagen fibril (red arrowhead). Scale bar, 100 nm. See supporting videos for 3D animations.

the collagen fibrils together. When regions of these enzyme-digested tissues were reconstructed by electron tomography, it was apparent that CS/DS-stained filaments are relatively long (about 300 nm) and hold several collagen fibrils together, mainly interacting in a tangential fashion (Figures 3B and 3D). Longitudinal and transverse views of a chondroitinase ABC-treated sample in which the KS subpopulation remains highlight some KS bridges between the collagen fibrils (Figures 3E and 3G). Most of these KSPG filaments extend between adjacent fibrils with a ~65 nm axial periodicity, but many are randomly tilted with respect to the collagen fibril axis. In transverse view (Figure 3G), the collagen fibrils are seen again to arrange linearly as necklaces and are held together by KS chains. The 3D reconstructions from these specimens (Figures 3F and 3H) indicate that the stained KSPGs are shorter than the CS/DS ones, and appear to connect the collagen by means of several bridges between adjacent fibrils.

DISCUSSION

From the 3D reconstructions (Figures 2 and 3), it is apparent that stained PG filaments can extend between two or more fibrils. In bovine cornea, PGs contrasted with cupromeronic blue are estimated from ultrathin sections to be 40–70 nm long (Scott, 1992), thus individual PG molecules are not long enough to span the distance between more than two fibrils. A possible explanation is that GAG chains from separate PGs might join together to form the long staining complexes we see in 3D.

Existing evidence points to a direct contact between collagen fibrils and PG protein cores (Scott, 1992), and the simplest way to explain the bridges seen in the reconstruction is if the GAG chains of the PGs join together in an antiparallel fashion (Scott, 1995). Structural data alone cannot indicate what type of bonds might exist between GAG chains, and it cannot be excluded that other proteins or molecules present in the cornea may mediate some PG associations (Cooper et al., 2006). Nevertheless, our findings are consistent with antiparallel associations arising, for example, by means of hydrogen bonds between the GAG chains, hydrophobic interactions (Scott 2003), or by means of antiparallel supercoiling as in the case of hyaluronic acid (Arnott et al., 1983). The existence of tilted PG bridges along the collagen fibrils (Figures 1 and 2) could possibly be explained by the presence of a large number of PGs within the relative small volume between fibrils. Because antiparallel GAG interactions are not covalent, they can conceivably break and reform repeatedly, and because of the high PG density, the probability of the formation of antiparallel interactions between chains at different axial positions on adjacent fibrils is increased. Tilted bridges will then arise if the interacting PGs are not at the same axial positions or they belong to a different PG family. This noncovalent GAG chain bonding concept would lead to the idea of a "fluid" cornea, in which the relative positions between collagen fibrils are not fixed, and neither are the interactions between the extrafibrillar PG molecules. This may facilitate the transport of nutrients and molecules across the cornea, and reduce the possibility of permanent structural damage in case of corneal deformation due to external stresses. Detailed mechanisms explaining reversible deformation of extracellular matrices have been proposed previously (Scott 2003), and are essentially based on the capability of the GAG chains to form antiparallel associations that can be broken and reformed with different overlap lengths.

The 3D reconstructions suggest that CS/DS form longer multimeric chains that extend among several collagen fibrils, whereas the KS are probably the shorter chains seen to connect adjacent fibrils. Based on the reconstructions, we hypothesize a new model of collagen-PG interactions in cornea (Figures 4A and 4B). In this, PG bridges are not arranged with six-fold symmetry around collagen fibrils, as has been proposed previously.

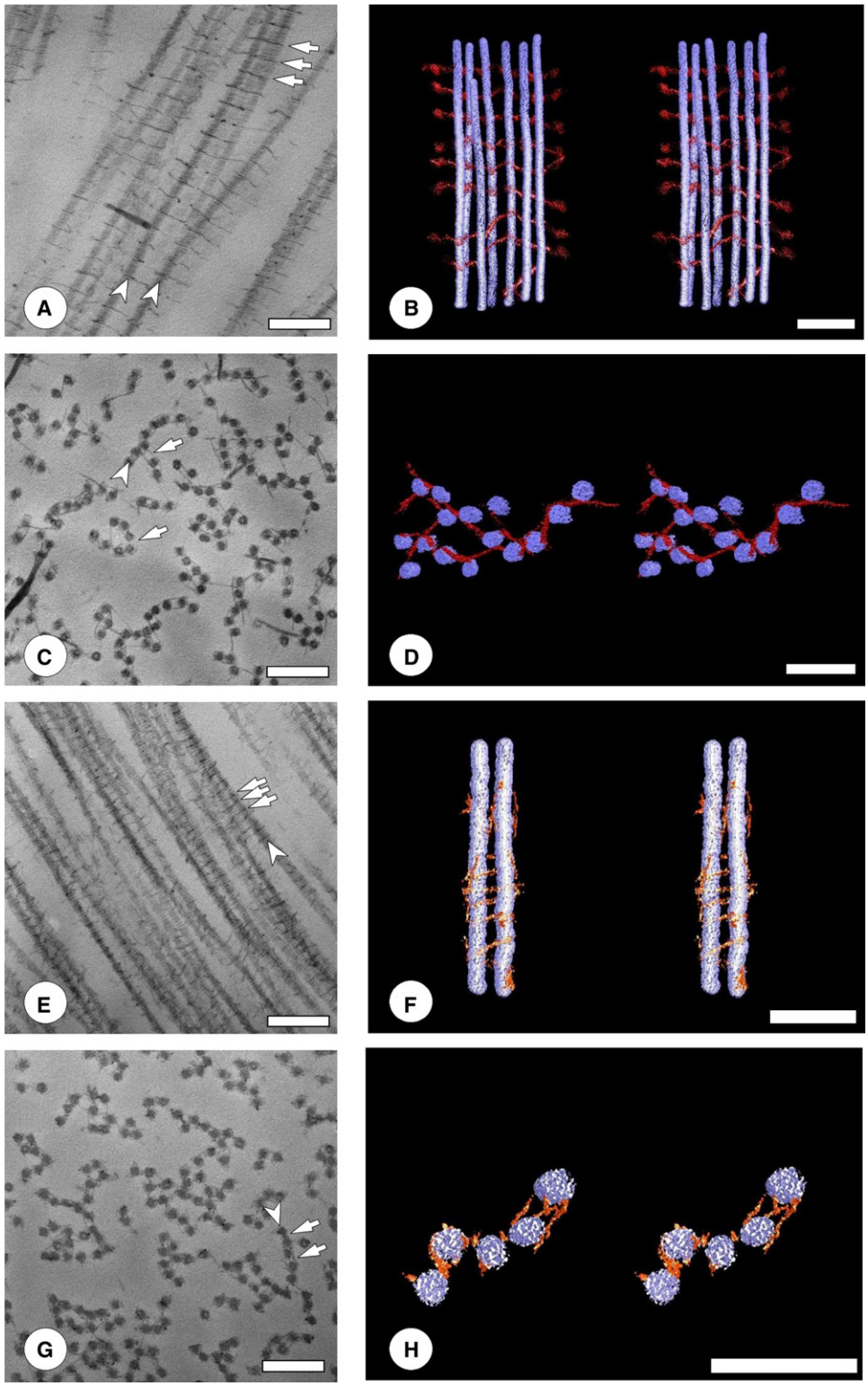

Figure 3. Enzyme-Treated Samples from which the CS/DS or KS GAG Chains Were Removed

Panels (A) and (C) were treated with keratanase to remove KS GAG chains, and panels (E) and (G) with chondroitinase ABC to remove CS/DS chains. Collagen fibrils are indicated by arrowheads and proteoglycans by arrows. Panels (B) and (D) are stereo pairs from longitudinal and transverse 3D reconstructions of keratanase-treated specimens. The collagen fibrils are colored blue, the CS/DS chains red. Long CS/DS chains are seen to interact with a 65 nm axial periodicity with several collagen fibrils. Panels (F) and (H) are reconstructions from chondroitinase ABC-treated specimens, with collagen depicted in blue and KS chains in orange. Here, KS chains form axially periodic bridges between collagen fibrils. Scale bars, 100 nm. See supporting videos for 3D animations.

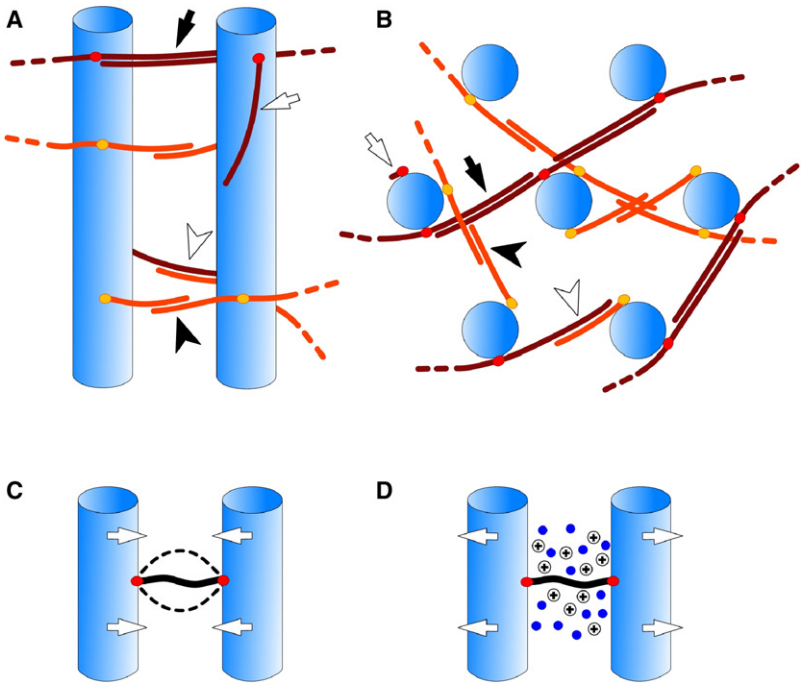

Figure 4. Model of the Interactions between Collagen and Proteoglycans

(A) Longitudinal view.

(B) Transverse view. Black arrows indicate antiparallel interactions between CS/DS GAG chains (painted in red). Black arrowheads point to an antiparallel interactions between KS chains (in orange). Axially running proteoglycans are indicated by white arrows, whereas hybrid CS/DS–KS antiparallel interactions are labeled by white arrowheads.

(C) Attractive forces between collagen fibrils arise from GAG chain vibrations.

(D) Repulsive forces between collagen fibrils are due to the Donnan effect. See text for details.

Rather, the PGs attach to collagen fibrils via their protein cores at specific axial locations depending whether they are CS/DS or KS PGs, as has been proposed by a number of investigators (Scott and Haigh, 1985; Scott and Bosworth, 1990; Meek et al., 1986), but the azimuthal position is not predefined. One or two GAG chains then extend from the protein core, and can interact with other GAG chains. If long enough domains from two separate GAG chains make contact, relatively stable antiparallel connections can be formed by means of noncovalent bonds. In this model, these connections may be between the same type of PG or can be mixed, because CS/DS and KS GAG chains are essentially equivalent from a structural point of view (Scott, 1995). The directions of these hypothesized bridges are randomly distributed, so that a collagen fibril is connected directly to several others by different PG/GAG complexes. Although in this model there are no systematic six-fold interactions between collagen fibrils, a six-fold-like arrangement of collagen fibrils will result because of the action of the PGs (see below).

Based on this proposed model we can further postulate on the controlling mechanisms of corneal PGs. Between any two adjacent collagen fibrils two opposite forces are able to arise: an attractive force due to the thermal motion of the PG/GAG complex, and a repulsive force due to osmosis (Figures 4C and 4D). The attractive force arises from collisions between the molecules in the corneal stroma that are in thermal motion and the PGs themselves. These collisions cause the PGs to change shape and move from their fully extended conformation, and essentially we can view this change of shape as a vibration of the GAG chains with different modes. Because the extremities of the PG complexes—i.e., the respective protein cores—are anchored on collagen fibrils, any vibration of the GAG chains creates a retractive force that acts to bring the PG extremities, and thus the collagen fibrils, closer together. This process may take place alongside others. For example, the capability of L-iduronate present in DS GAG chains to vary in length as a consequence of a conformational change, or the possibility of dimerized chains to overlap by varying lengths (Scott, 2003), may also contribute to the retractive forces between fibrils. At the same time, positively charged ions are drawn by the charges on the GAG chains and create local concentration gradients in the corneal stroma that attract water molecules by osmosis and the Donnan effect (Elliott and Hodson, 1998). The excess water molecules occupy the volume between collagen fibrils and create a repulsive force by increasing the pressure between them. We propose that these two forces are balanced in the corneal stroma. In physiological conditions, any three adjacent fibrils would experience no net force and would maintain their relative positions at the vertices of a triangle whose sides are defined by the lengths of the PG/GAG complexes. In this way a collagen fibril becomes surrounded by the other six fibrils at the vertices of a hexagon, and structural order required for tissue transparency is attained. Although this model is based on observations on bovine corneas, similar mechanisms are likely to apply to all corneas. Thin corneas of small mammals such as the mouse have much reduced levels of sulphated KS (Scott and Bosworth, 1990; Young et al., 2005). However, as Scott and Bosworth (1990) have indicated, this can be compensated for by heightened CS/DS levels, such that the total sulphated polyanion content (relative to hydroxyproline) remains approximately constant, as does the polyanionic charge per unit volume of stroma, and thus osmotic pressure. This would give rise to comparable repulsive forces between fibrils across species, with different KS and CS/DS contributions. Attractive forces in the corneas of different animals would depend on the number and type of GAG bridges between fibrils. Because PG composition varies across at least some species (Scott and Bosworth, 1990), this would give rise to different GAG bridges and thus attractive forces of different magnitudes. Balance between attractive and repulsive forces in different animals may be reached at different fibril separation lengths, and could be a possible reason for the variation in interfibrillar spacing observed across species (Meek and Leonard 1993).

Predictions can be made from this model on the likely structure of the cornea with defective PGs. For example, we can hypothesize that if the ordinarily constant overall charge of the GAG

chains becomes reduced, fewer cations would be attracted to the interfibrillar space and the volume between the collagen fibrils would have to decrease for the fibrils to experience the same level of osmotic pressure. This would lead to attractive and repulsive forces being balanced when collagen fibrils are closer together than would normally be the case. Perhaps circumstantial support for such a system can be inferred from studies of both thick (human) and thin (mouse) corneas. In the inherited human disease, macular corneal dystrophy, for example, a mutation in a carbohydrate sulphotransferase gene (*CHST6*) leads to less sulphation of KS (Akama et al., 2000; Akama et al., 2001), and presumably less negatively charged KS GAGs in cornea. A reduced collagen fibril spacing is also seen in this disease (Quantock et al., 1990). Corneas of mice with a targeted gene deletion in *Chst5*, which encodes a sulphotransferase enzyme involved in the sulphation of KS chains, lack even the relatively low levels of sulphated KS present in normal mouse cornea (Scott and Bosworth, 1990; Young et al., 2005), and have a relatively closely spaced collagen fibril array (Hayashida et al., 2006). Our model may also be consistent with the findings of structural stromal changes in mice with gene-targeted deletions in KSPG core proteins. For example, the lumican-null cornea—which also lacks the KSPG core protein keratocan (Carlson et al., 2005) —contains pockets of abnormally large and irregularly shaped collagen fibrils (Chakravarti et al., 2000, 2006). These fibrils probably represent coalesced fibrils, and we can predict that, with the major KSPG core proteins affected, the distribution of PGs around the collagen fibrils would be markedly anisotropic, thus creating a force imbalance that in some regions of the tissue (especially deeper stromal regions, where KS is believed to play a more influential role) reduces the repulsive force leading to fibril aggregation. Of course, these examples do not provide definitive proof of the hypothesized model, and the situation is not straightforward given that we do not fully understand the possible compensatory role of CS/DS when KS is affected.

In summary, 3D reconstructions of corneal stroma show that the arrangement of PGs around the collagen fibrils is not fixed and six-fold. Instead, the PGs form an extended network that occupies the extrafibrillar space. Presumably, this acts to anchor collagen fibrils and give them spatial order, and it is predicted that the pseudohexagonal arrangement of fibrils is obtained through the balance of two opposing forces created by the PGs. It is further predicted that the GAG chains of the PGs interact transiently with one another in a manner that would allow the tissue to respond to external mechanical stresses (Scott 2003). It is anticipated that similar or related mechanisms might exist in other extracellular matrices, with the precise nature of the proteoglycan-collagen relationship tuned to the functional needs of the particular tissue.

EXPERIMENTAL PROCEDURES

Tissues

Bovine corneas were dissected from freshly slaughtered animals, and full-thickness biopsies (approximately 1 mm^3 in size) were taken from the center of each cornea.

Electron Microscopy

For the visualization of sulphated PGs, corneas were fixed for approximately 18 hr in 2.5% glutaraldehyde in 25 mM sodium acetate buffer (pH 5.7) with 0.1 M magnesium chloride and 0.05% cupromeronic blue. The samples were then briefly washed in buffer followed by 15 min incubations in aqueous 0.5% sodium tungstate, then 0.5% sodium tungstate in 50% ethanol, to enhance the electron density of the PG-cupromeronic blue complex (Scott and Haigh,1985; Scott and Bosworth, 1990). Specimens were dehydrated through an ascending ethanol series before being embedded in Araldite resin and polymerized at 60°C for 24 hr. Ultrathin sections (~110 nm thick) were cut with a diamond knife on a Leica Ultracut UC6 ultramicrotome. Sections were collected on formvar-coated slot grids, stained with saturated aqueous uranyl acetate for 30 min at room temperature, and examined at 80 kV in a JEOL 1010 transmission electron microscope (Japan). To differentiate between KS and CS/DS proteoglycan distribution within the corneal stroma, some samples were exposed to GAG specific degrading enzymes. Enzyme digestion was performed after 20 min fixation with 2.5% paraformaldehyde. Samples of cornea approximately 0.5 mm^3 were digested for 10 hr at 37°C with either keratanase—keratan sulphate endo-β-galactosidase E.C.3.2.1.103 (5 U/ml in Tris-acetate buffer pH 7.4) —containing 0.5 mg/ml bovine serum albumin, 0.3 M sodium acetate, and 0.5 M sodium chloride to degrade KS, or chondroitinase ABC (2.5 U/ml in Tris-acetate buffer pH 7.4) to degrade 0-, 4-, and 6-sulphated CS and DS. Both keratanase and chondroitinase ABC were used in the presence of proteolytic inhibitors EDTA, benzamidine, and soybean trypsin inhibitor (Scott and Haigh,1985). Controls included sham-enzyme digestion 10 hr at 37°C in Tris-acetate buffer alone. Corneal samples were fixed, stained for PGs, and processed for electron microscopy as described earlier.

EM Tomography

Electron microscopy tomography series were taken on a JEOL 1010 electron microscope at 80 kV equipped with a bottom-mount 14-bit CCD camera (Orius SC1000 Gatan, Pleasanton, CA). Tomographic data were collected from −60° to +60° in 1° increments. Single-tilt data sets were acquired. The image series were aligned and three dimensionally reconstructed with IMOD and EM3D tomography software (Kremer et al., 1996; Ress et al., 2004).

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and six movies and can be found with this article online at doi:10.1016/j.str.2009.11.013.

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