Three-Dimensional Reconstruction of the Cornea by Electron Tomography

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**ABSTRACT**

Corneal transparency is fundamental to vision and is attributed to the organised collagen fibril architecture the tissue maintains, which is regulated by proteoglycans. To understand the inter-relationships between collagen and proteoglycans in the cornea, three-dimensional reconstructions were obtained of mouse and bovine corneas which allow the conformations that proteoglycans adopt three-dimensionally to be observed for the first time. The reconstructions highlight the complexity of collagen-proteoglycan interactions, showing a non-symmetrical arrangement of proteoglycans around collagen fibrils and distinct variations in their morphology. From structural information revealed through electron tomography, we postulate mechanisms through which proteoglycans may organise collagen fibril architecture in the cornea.

**KEYWORDS**

Transmission electron microscopy, electron tomography, three-dimensional reconstruction, cornea, collagen fibrils, proteoglycans

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**INTRODUCTION**

The cornea is the transparent window of the eye that is responsible for the refraction of light onto the lens and retina. Collagen is the major constituent of the cornea, where it forms fibrils that are highly tensile, rope-like structures, aligned parallel to each other in layers which exhibit a close-to-hexagonal distribution laterally [1]. When light enters the cornea, it is scattered by the collagen fibrils but experiences mutual interference that results in light transmission. The uniform diameter and spacing of these fibrils, which are required for corneal transparency, are regulated by the proteoglycan biopolymers [2]. As the ordered structure of the cornea is necessary for light transmission, the cornea is subject to relatively strict positional regulation of its components, in contrast to the fibrous tendon or the opaque sclera, which contain comparatively fewer proteoglycans [3].

The corneal proteoglycans are composed of a protein core covalently bound to one or more linear sugar side-chains. Two types of proteoglycans exist in the corneal stroma: keratan sulphate and a chondroitin sulphate/chondroitin sulphate hybrid. The protein cores attach to collagen fibrils, whereas the sugar side-chains extend into the interfibrillar space and look like filaments in the electron microscope when stained with a cationic dye (Figure 1, red arrowheads). The side-chains have a dense negative charge which influences both their hydration and interactions with other proteoglycans. Evidence from proteoglycan-knockout mice has shown that proteoglycans contribute to the control of collagen fibril assembly in the cornea. However, the mechanisms through which proteoglycans regulate collagen fibril architecture are currently not well understood.

Elucidation of proteoglycan function can be furthered by three-dimensional electron tomography investigation of bovine corneas [4], as well as genetically normal [5] and proteoglycan-knockout mice corneas. Bovine corneas are physiologically similar to human corneas, but are about a third thicker. In contrast, mouse corneas differ from other mammalian corneas as they are thin (80 µm compared to 550 µm in man) and their proteoglycans are not as negatively charged. Also, only 18% of total proteoglycan content is keratan sulphate, which is relatively low when compared with other mammals [6].

Conventional transmission electron microscopy (TEM) investigations of corneal ultrastructure have visualised proteoglycan-collagen interactions two-dimensionally. We have used electron tomography techniques to obtain three-dimensional reconstructions of collagen-proteoglycan interactions from a series of two-dimensional projections collected using TEM. Three-dimensional reconstructions enable a greater understanding of the native ultrastructure of biological systems, providing a foundation to interpret structure-function relationships. Initially, the specimen is tilted around an axis perpendicular to the electron beam, in one degree increments, to cap-
Buffer containing 0.1M MgCl₂ and 0.05% glutaraldehyde in 25 mM sodium acetate. Mouse corneas were excised and fixed in 2.5% Specimen Preparation. MATERIALS AND METHODS. Fibril assembly and homeostasis. into the mechanisms that regulate collagen and proteoglycans and thereby gain insights three-dimensional arrangement of collagen and proteoglycans and thereby gain insights into the mechanisms that regulate collagen fibril assembly and homeostasis.

MATERIALS AND METHODS
Specimen Preparation
Mouse corneas were excised and fixed in 2.5% glutaraldehyde in 25 mM sodium acetate buffer containing 0.1M MgCl₂ and 0.05% cuproinilc blue, a cationic dye for visualisation of proteoglycans [8]. Cuproinilc blue complexes with the sugar extension of proteoglycans because of the presence of negatively charged sulphate and carboxylate residues in the sequence of repeating disaccharides. Bovine corneas were also fixed using the same method, although another proteoglycan staining dye, cupromeronic blue, was used. The samples were then washed with sodium acetate buffer to remove unbound dye, and immersed in aqueous 0.5% sodium tungstate, then 0.5% sodium tungstate in 50% ethanol, to enhance the electron density of the stained complexes. After dehydration using an incremental ethanol series, mouse and bovine corneas were embedded in Araldite resin and regular collagen fibril order. Enzyme treatment shows that the chondroitin sulphate/dermatan sulphate proteoglycans exist as large, electron dense structures, where as keratan sulphate proteoglycans are small filaments that sit between adjacent fibrils [11].

Three-dimensional reconstructions illustrate the regular arrangement of collagen fibrils and the conformations that proteoglycans adopt threedimensionally (Figure 2). Neighbouring collagen fibrils are frequently connected at near-regular distances, primarily by small proteoglycans along the length of the fibril, whereas up to three collagen fibrils may be inter-connected by the largest proteoglycans. Interestingly, mouse corneal proteoglycans appear to show no specific ordered arrangement around collagen fibrils. Measurement of proteoglycan dimensions, using only whole structures contained in the three-dimensional reconstructions, revealed that three distinct proteoglycan sub-populations exist in the mouse cornea. No distinct structural differences in proteoglycan content were observed in reconstructions derived from sites in the anterior, mid or posterior stroma and the distribution of the three proteoglycan types was also relatively similar across the stromal depth. Small proteoglycans (31 ± 3.5 nm in length measured over the three sites; blue arrowheads in Figure 2) were seen connecting only neighbouring fibrils, and were frequently positioned perpendicular to the fibril. Lengths of these proteoglycans were relatively consistent across the stromal depth. Large proteoglycans varied in thickness and were of two distinct lengths. Of this type, intermediate sized proteoglycans (64 ± 3.8 nm long; yellow arrowheads in Figure 2) usually connected two fibrils, but sometimes could be seen to run alongside a collagen fibril. These proteoglycans var-

Figure 2: Three-dimensional reconstructions of the anterior region in the mouse cornea. Stereo-pairs of segmented tomograms showing collagen fibrils sectioned in (a) longitudinal and (b) transverse views. Proteoglycans are white and collagen fibrils green. Arrowheads show: small proteoglycans (blue); intermediate proteoglycans (yellow) and largest proteoglycans (red). Scale bars = 50 nm.

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Figure 3: Three-dimensional reconstructions of the anterior region in the bovine cornea. Stereo-pairs of segmented tomograms showing collagen fibrils sectioned in (a) longitudinal and (b) transverse views. Proteoglycans are white and collagen fibrils green. Arrowheads show: proteoglycans that tether neighbouring fibrils (blue); proteoglycans that connect more than two fibrils (yellow). Scale bars = 50 nm.
ied in thickness considerably. The largest proteoglycans linked three fibrils (123 ± 0.8 nm long, red arrowheads in Figure 2) and were seen less frequently than the other types.

**Bovine Cornea**

Three-dimensional reconstructions of bovine cornea also show no evidence of an ordered arrangement of proteoglycans around collagen fibrils (Figure 3). Large proteoglycans are in contact with two or more collagen fibrils, whilst shorter proteoglycans occupy the space between two adjacent fibrils. Proteoglycans in the reconstruction are often seen extending between more than two fibrils whilst some also lie along the fibril axis, similar to the situation in mouse cornea. Enzyme treatment suggests that chondroitin sulphate/dermatan sulphate proteoglycans account for the larger proteoglycans that extend between more than two fibrils [4]. Keratan sulphate proteoglycans extend between adjacent fibrils regularly, but many are randomly tilted with respect to the fibril axis. Also, proteoglycans do not always form bridges between the closest points on the surface of two adjacent fibrils.

The immediate observation from the three-dimensional reconstructions of both bovine and mouse corneas is that, whilst collagen fibril organisation is regular and characteristic of the corneal stroma, proteoglycans appear to have no specific ordered arrangement around collagen fibrils and show a distinct variation in size.

**DISCUSSION**

Complex three-dimensional relationships between proteoglycans and collagen fibrils are observed in mouse and bovine corneas, which exhibit no obvious structural regularity. It is evident that proteoglycans are in contact with two or more collagen fibrils. Keratan sulphate proteoglycans are seen bridging neighbouring fibrils at regular intervals whereas chondroitin sulphate/dermatan sulphate proteoglycans connect neighbour and next-nearest neighbour fibrils and in some cases lie along the fibril. It is possible that randomly tilted small proteoglycans and larger proteoglycans lying along the fibril may occur as a result of side-chain collapse when stained with cuprolinic blue, a possibility which has yet to be tested fully.

The variation in length of chondroitin sulphate/dermatan sulphate proteoglycans suggests anti-parallel linear aggregation, which is possible when hydrogen and hydrophobic attraction prevents the expected mutual repulsion [3]. It is also possible that positive ions (Na⁺, K⁺) present in the cornea may screen negative charges, negating mutual repulsion of the side-chains and allowing their assembly. The linear aggregation of proteoglycans as multimers may be aided by the dimerisation of their protein cores (Figure 4), however these proposed structural interactions are yet to be confirmed.

The observed differences in proteoglycan thickness in the mouse cornea could be accounted for either by a variation in side-chain stain density or by lateral aggregation of proteoglycans in the form of polymers (Figure 4). The relative sizes of cuprolinic blue (<0.5 nm) and tungstate ions used for the staining of proteoglycans is probably insufficient to explain the observed differences in chain thickness [12], and our findings support the idea of anti-parallel associations between proteoglycans. Indeed, a lateral association of several proteoglycans would confer structural integrity, and such a property could accommodate an ability of proteoglycans to readily disassociate and re-aggregate to enable fibril flexibility accordingly.

The non-covalent nature of this self-association would lead to the idea of a ‘fluid’ cornea, whereby collagen fibril positions are not fixed, and neither are the proteoglycan-proteoglycan interactions. This would allow for the passive movement of water and nutrients through the cornea and would also prevent irreversible deformation upon impact injury or external stresses.

To explain the regularity of collagen fibril spacing within the cornea, we propose a three-dimensional model in which two equal but opposite forces act simultaneously on collagen fibrils (Figure 4).

Attractive forces arise due to the thermal motion of proteoglycans whilst repulsion occurs as a result of osmosis. Proteoglycans that bridge neighbouring fibrils retract from their fully extended conformations because of their thermal motion, drawing fibrils together. Expansion of the interfibrillar space, on the other hand, occurs because of the positive ions drawn to the negatively charged sugar side-chains of the proteoglycans. Water molecules are subsequently driven into the stroma via osmosis (the Donnan effect) and occupy the volume between fibrils, increasing interfibrillar pressure. As a result, proteoglycans both contract and expand the space between collagen fibrils in order to optimise their distribution.

In our model these two forces operate in tandem and are balanced. The cornea, therefore, is proposed to be a flexible and adaptive biological system where proteoglycans can adopt varied, reversible conformations allowing the fibrillar array to remodel over time.

**CONCLUSIONS**

Collagen fibril architecture within the cornea is regular and it is maintained by the proteoglycans. We propose that proteoglycans aggregate laterally to account for the variable thicknesses observed and linearly to enable the connection of more than two fibrils. This arrangement of proteoglycans, along with the forces that are a consequence of the proteoglycan structure and charge distribution, regulate transparency, flexibility and resilience of the cornea. Investigation of mouse corneas with genetic alterations in proteoglycans will allow us to test these ideas more fully.

**REFERENCES**