An X-Ray Diffraction Study of Corneal Structure in Mimecan-Deficient Mice

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Purpose. Keratan sulfate proteoglycans (KSPGs) in the corneal stroma are believed to influence collagen fibrillar arrangement. This study was performed to investigate the fibrillar architecture of the corneal stroma in mice homozygous for a null mutation in the corneal KSPG, mimecan.

Methods. Wild-type ($n = 9$) and mimecan-deficient ($n = 10$) mouse corneas were investigated by low-angle synchrotron x-ray diffraction to establish the average collagen fibrillar spacing, average collagen fibril diameter, and level of fibrillar organization in the stromal array.

Results. The mean collagen fibril diameter in the corneas of mimecan-null mice, as an average throughout the whole thickness of the tissue, was not appreciably different from normal (35.6 ± 1.1 nm vs. 35.9 ± 1.0 nm). Average center-to-center collagen fibrillar spacing in the mutant corneas measured 52.6 ± 2.6 nm, similar to the 53.3 ± 4.0 nm found in wild-type mice. The degree of local order in the collagen fibrillar array, as indicated by the height-width (H:W) ratio of the background-subtracted interfibrillar x-ray reflection, was also not significantly changed in mimecan-null corneas (23.4 ± 5.6), when compared with the corneas of wild-types (28.2 ± 4.8).

Conclusions. On average, throughout the whole depth of the corneal stroma, collagen fibrils in mimecan-null mice, unlike collagen fibrils in lumican-null mice and keratocan-null mice, are of a normal diameter and are normally spaced and arranged. This indicates that, compared with lumican and keratocan, mimecan has a lesser role in the control of stromal architecture in mouse cornea. (*Invest Ophthalmol Vis Sci.* 2005;46: 4046–4049) DOI:10.1167/iovs.05-0325

Corneal clarity relies on the maintenance of a stromal matrix consisting of uniformly small-diameter collagen fibrils, equally spaced and fairly well ordered, to satisfy the requirements for light transmittance.[1,2] Small leucine-rich proteoglycans (PGs) within the matrix are believed to be necessary elements for the development and maintenance of a well-ordered corneal stroma. Within the cornea, PGs carry either keratan sulfate (KS) or chondroitin sulfate/dermatan sulfate (CS/DS) side-chains, the former population comprising the KSPGs lumican,[3] keratocan,[4] and mimecan.[5] Sulfation of corneal GAGs lends them an overall negative charge and high water-binding capacity,[6] and evidence from several animal models suggests that KS plays a pivotal role in the regulation of corneal stromal structure. For example, the accumulation of KS after developmental day 12 in chick cornea coincides with the onset of corneal transparency from day 14 onward.[7,8] Also, the wound area and surrounding tissue of rabbit corneas contains high levels of CS and DS and low levels of KS.[9] These scars, which display large interfibrillar spaces, later regain normal fibrillar spacing along with an increase in KS levels.[10]

The generation of mice deficient in one or more PGs has allowed researchers to investigate the role of these molecules in corneal ultrastructure, and several studies have been undertaken in an attempt to determine the function of all three KSPGs. These research efforts have indicated that lumican-null murine corneas display a severe phenotype. They are significantly thinner than normal and develop bilateral corneal opacification and a disrupted stromal matrix.[11–15] The corneas of keratocan-deficient mice, in contrast, are virtually indistinguishable from the wild-type in corneal clarity. Nevertheless, they have a thinner corneal stroma,[16] with x-ray scattering studies disclosing collagen fibrils that have a larger-than-normal average diameter and increased interfibrillar spacing.[17] A recent investigation of mimecan-deficient mice disclosed that mutant corneas also appear clinically normal, with no obvious changes in clarity, even though alterations in collagen fibril size are indicated by electron microscopy.[18] The purpose of this investigation was to use x-ray fiber diffraction methodologies to determine to what extent mimecan regulates the overall collagen fibrillar architecture in the mouse cornea.

Materials and Methods

Mimecan-null mice and wild-type counterparts were housed at the Division of Biology, Kansas State University, and handled at all times in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The production of the mutants has been reported previously.[18] Ten four-month-old animals were killed by cervical dislocation and the corneas excised at the limbus. Each cornea was individually wrapped in plastic film to limit dehydration, frozen immediately in liquid nitrogen, stored at −80°C, and shipped on dry ice to the United Kingdom. Freezing is an accepted way of storing corneas for investigation of extracellular matrix structure by synchrotron x-ray scattering,[19] and tissues remained wrapped at −80°C before examination of 9 wild-type and 10 mimecan-null corneas. Experiments were performed at the Synchrotron Radiation Source ([SRS], Daresbury Laboratory, Cheshire, UK).

For data collection, corneas were secured in a sealed specimen holder between two sheets of Mylar, where they were allowed to thaw. Each specimen was then placed in turn in the path of a focused (1.5 × 1.0 mm), monochromatic ($\lambda = 0.154$ nm) x-ray beam on SRS

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Station 2.1. The shutters were opened for a 3-minute exposure of each cornea. X-ray fiber diffraction patterns were recorded on a multiwire, gas proportional area detector situated directly behind the cornea at a distance of approximately 9 m. An evacuated tube with polyester film windows separated the specimen from the detector to reduce air scatter. The window nearest the detector contained a small lead beam-stop that purposely blocked the direct x-ray beam, which passed through the cornea undeviated, and thus allowed x-ray diffraction patterns to be recorded. The analytical procedures used to calculate the average collagen interfibrillar spacing, average collagen fibril diameter, and level of the local order in the fibrillar array (i.e., the H:W ratio) have been described previously.[14,20,21] All data were calculated after calibration of the system according to the meridional reflections arising from the 67 nm axial D-periodic collagen repeat in hydrated rat tail tendon.

Results

Low-angle x-ray fiber diffraction patterns obtained from wild-type and mimecan-null mouse corneas, in both cases, consisted of well-defined interfibrillar reflections formed by the interference of x-rays scattered by collagen fibrils with some degree of lateral order (Fig. 1). Vertical transects across these diffraction patterns generated x-ray scattering–intensity plots for wild-type and mimecan-null corneas with similar profiles (Fig. 2). The major scattering elements in the cornea are the stromal collagen fibrils, but a degree of background scatter is produced by nonfibrillar elements in the tissue. After subtracting a power-law background from the experimental data and measuring the position of the background-subtracted interfibrillar reflection (Fig. 2), we obtained data for the mean center-to-center collagen interfibrillar Bragg spacing in each of 9 wild-type and 10 mimecan-null corneas (Table 1). Statistical analysis ($\pm$SD) shows that this value in wild-type (53.3 $\pm$ 4.0 nm) and mimecan-null (52.6 $\pm$ 2.6 nm) corneas is not significantly different (independent samples *t*-test; $P = 0.684$). The collagen interfibrillar Bragg value is quoted for consistency with other structural x-ray studies. Bragg spacing differs from the actual center-to-center collagen interfibrillar spacing in the cornea because the mode of fibrillar packing is not taken into account. If, for example, pseudohexagonal packing of collagen fibrils is assumed, the interfibrillar Bragg spacing must be increased by a 1.12 multiplication factor.[22]

The angular width of a collagen interfibrillar x-ray reflection is an indicator of the degree of local order in the fibrillar array, with narrower reflections formed by more well-ordered spatial arrangements. Narrower reflections give rise to sharper peaks in the x-ray intensity profile of the interfibrillar reflection, and in this study we used the H-W ratio (i.e., height of the background-subtracted peak divided by peak width at half height) to represent a reflection's sharpness.[20,21] Accordingly, a higher H:W ratio is indicative of a matrix arrangement with more local order. The data (Table 1) show that the extent of local order in the collagen fibrillar array was less in mimecan-null corneas (H:W ratio = 23.4 $\pm$ 5.6 vs. 28.2 $\pm$ 4.8 in wild-types), but not significantly so (independent samples *t*-test; $P = 0.063$).

Analyses of the first subsidiary equatorial reflections in the x-ray intensity profiles (Fig. 2) disclosed that the average collagen fibril diameter in mimecan-null corneas (35.6 $\pm$ 1.1 nm) was not appreciably different from normal (35.9 $\pm$ 1.0 nm; independent samples *t*-test; $P = 0.476$; Table 1). Thus, for all parameters measured, the fibrillar architecture of the mimecan-deficient mouse cornea, taken as an average throughout the whole corneal thickness, did not differ appreciably from that of wild-type corneas.

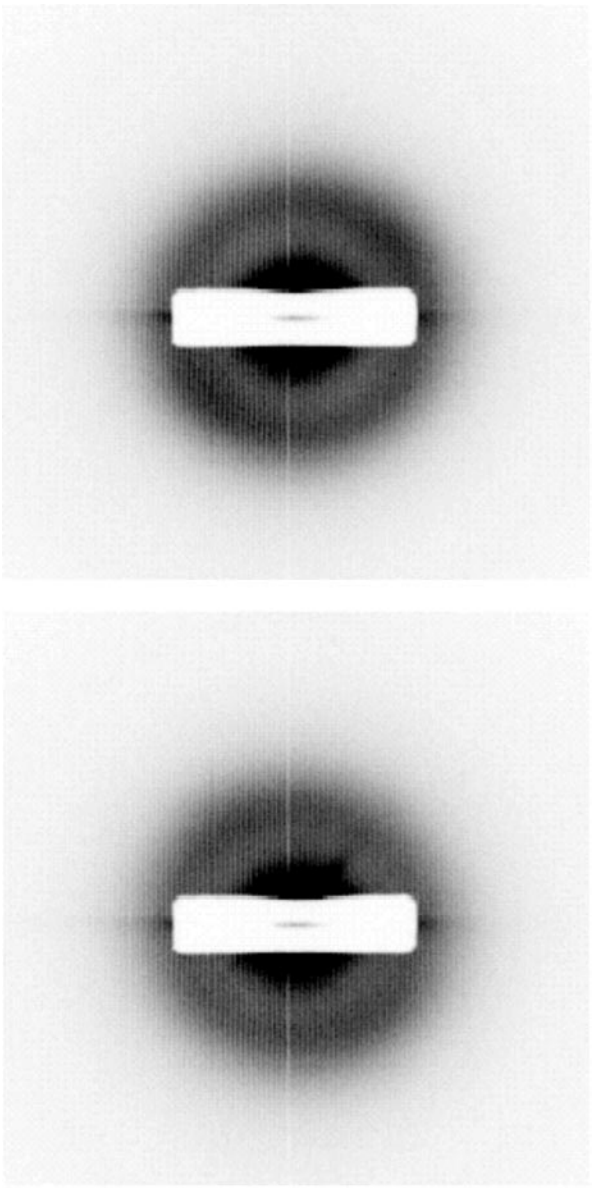

Figure 1. *Top*: a low-angle x-ray diffraction pattern from wild-type mouse cornea (Table 1, specimen 4) containing a dark, circular interfibrillar x-ray reflection produced by x-rays scattered by regularly arranged stromal collagen fibrils. *Bottom*: corresponding diffraction pattern from a mimecan-null cornea (Table 1, specimen 12). The two were not appreciably different.

Discussion

An appreciation of the respective structural roles of the three stromal matrix KSPGs in the cornea is of fundamental importance for a fuller understanding of corneal ultrastructure and transparency. X-ray fiber diffraction studies of the corneas of lumican-null mice and keratocan-null mice have disclosed clear matrix changes, though to different extents.[14,17] In the current investigation, we used similar methodologies to probe the fine structure of the cornea in mice lacking the other main corneal KSPG, mimecan. The results showed that (1) the average collagen interfibrillar spacing in mimecan null corneas was not significantly different from that in wild-type corneas, (2) the level of local order in the fibrillar array was similar in the two tissues, and (3) the mean collagen fibrillar diameter in the corneas of mimecan-null mice was essentially unchanged. The

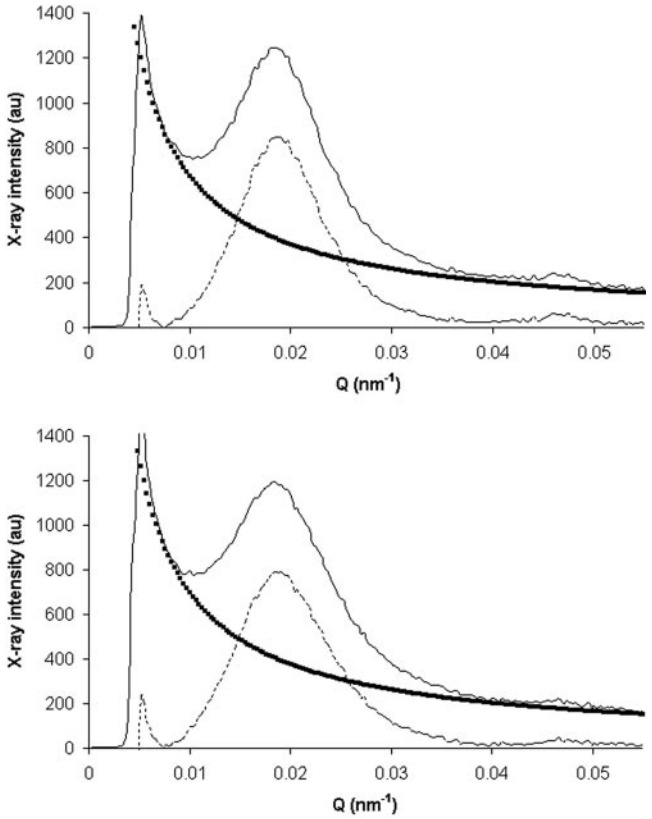

FIGURE 2. X-ray scattering–intensity plots from the wild-type (*top*) and mimecan-null (*bottom*) corneas shown in Figure 1. *Solid line*: x-ray-intensity scan radiating outward from the center of the pattern. A power-law background function (▪) is fitted to each experimental data set. *Dasbed lines*: x-ray intensity after background removal, with the interfibrillar peak visible at approximately $Q = 0.02$ nm^{-1} and the first subsidiary equatorial reflection used to calculate fibril diameter seen at approximately $Q = 0.047$ nm^{-1}.

data we report were obtained from unfixed corneas maintained at close to physiologic hydration. Moreover, all collagen fibrils throughout the whole of the tissue volume through which the x-ray beam passes contribute to the diffraction pattern, and in these experiments this is a volume measuring 1.5 mm^2 at the cornea's surface extended throughout the whole of the cornea's thickness. Thus, we sampled an extensive number of the collagen fibrils in the mouse cornea and generated highly representative, quantitative measurements of fibrillar architecture.

Previous work has indicated that, in vitro, mimecan has the ability to regulate the fibrillogenesis of type I collagen.[23] In the mimecan-null cornea, manual measurements from electron micrographs of glutaraldehyde-fixed tissue have reported the existence of larger than normal collagen fibrils, although it was not ascertained quantitatively how widespread these changes were.[18] The large sampling achieved by x-ray diffraction provided for an excellent evaluation of the overall structural dimensions of the stromal matrix, and whereas our analysis did not entirely rule out the existence of some larger than normal fibrils in the corneas of mimecan-null mice, it clearly indicated that, throughout the whole tissue thickness, the collagen fibrillar diameter was, on average, essentially unchanged. This is not the case in keratocan-null corneas where x-ray scattering investigations have shown a small but consistent increase in average fibril diameter throughout the tissue.[17] Nor is it the case in lumican-null corneas where the marked variation in collagen fibril diameter seen on electron microscopy[12] was so extensive that it precluded the formation of a measurable x-ray reflection that would have allowed us to calculate average collagen fibril diameter.[14] Recent work has indicated that the severity of phenotype in lumican-null corneas might be because this molecule, as well as serving as a structural regulator in its own right, also modulates keratocan gene expression.[24]

Light-scattering from the extracellular stromal matrix of cornea, like x-ray scattering, is based on the combined scattering from all fibrils in the path of the radiation. Theories of corneal transparency[2] state that the fraction of light transmitted through a cornea, $F(\lambda)$, falls off exponentially with the

TABLE 1. Individual and Average Measurements in the Stroma of Wild-Type and Mimecan-Deficient Mouse Corneas

Genotype	Specimen	Collagen Interfibrillar Spacing (nm)	Collagen Fibril Diameter (nm)	Height/Width Ratio
Mimecan$^{+/+}$	1	52.4	36.3	35.4
	2	51.9	34.5	26.8
	3	50.8	35.2	31.2
	4	53.6	35.2	30.8
	5	54.9	36.6	26.9
	6	53.8	36.3	32.8
	7	56.5	36.3	24.7
	8	60.3	37.6	25.8
	9	45.7	35.4	19.5
	Mean	53.3 ± 4.0	35.9 ± 1.0	28.2 ± 4.8
Mimecan$^{-/-}$	10	51.9	34.3	33.2
	11	53.7	37.1	24.9
	12	53.5	35.4	26.1
	13	51.2	36.1	19.9
	14	49.5	36.1	19.0
	15	54.4	34.9	27.6
	16	49.4	35.4	18.7
	17	50.3	35.4	13.7
	18	57.2	37.4	23.7
	19	55.4	33.8	27.2
	Mean	52.6 ± 2.6	35.6 ± 1.1	23.4 ± 5.6

product of the total scattering cross-section (σ), the collagen fibril number density (ρ), and the thickness of the tissue (t):

$$F(\lambda) = e^{-\sigma\rho t}$$

Detailed calculations of corneal transparency are not trivial, particularly because σ is itself a complex function of the wavelength of light, the diameters of the collagen fibrils, their mode of packing, and the ratio of the refractive index of the hydrated fibrils to the refractive index of the extrafibrillar matrix.[2] Nevertheless, when considering the transparency of lumican-deficient corneas, one could reasonably argue that the increased average interfibrillar spacing seen by x-ray diffraction[14] is indicative of a lower ρ. The lumican-null cornea is considerably thinner than normal, and so t is also reduced.[12] Both of these changes point to an increase in $F(\lambda)$, the corollary being that the alterations in matrix structure must increase the value of σ sufficiently to bring about the three-fold increase in backscattered light from the corneal stroma that is seen in the lumican-null mouse.[12] Keratocan-null corneas are also thinner than normal, with a wider average interfibrillar spacing (and therefore lower t and ρ), but no detectable reduction in corneal clarity.[16,17] This can be explained if we accept that stromal matrix alterations in this mutant are less extensive than in the lumican-null animal, leading to less pronounced changes in σ. Normal corneal thickness (t) has been reported in mimecan-null mice.[18] Because the current investigation discloses no significant alterations in matrix architecture in mimecan-null corneas we can reasonably surmise that any changes in σ and ρ are small. Thus, it is not surprising that these corneas show no detectable loss of corneal clarity.[18]

Mouse cornea, unlike the corneas of most other species that have been investigated, contains KS that is predominantly undersulfated.[25,26] Nevertheless, these PGs are considered to be instrumental in the formation and maintenance of a structurally normal corneal stroma.[11,12,16,18] Based on the findings of this and previous[14,17] x-ray scattering experiments on all three KSPG-null mouse corneas, there appears to be a hierarchy within the KSPG population in terms of their relative influence as structural regulatory molecules: lumican, keratocan, and mimecan in decreasing order of importance.

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