Altered Collagen Fibril Formation in the Sclera of Lumican-Deficient Mice

Bobbie A. Austin,1 Christopher Coulon,2 Chia-Yang Liu,3 Winston W.-Y. Kao,3 and Jody A. Rada1

Purpose. To better understand the role of lumican (corneal keratan sulfate proteoglycan) in the scleral extracellular matrix, collagen fibril size, shape, and organization were evaluated in the sclera of wild-type mice and in mice homozygous or heterozygous for a null mutation in the lumican gene.

Methods. Anterior and posterior sclera from 6-month-old wild-type (lum+/lum+) and lumican-deficient mice (lum−/lum− and lum+/lum−) were analyzed by transmission electron microscopy. In addition, lumican was characterized in the sclera of wild-type and lumican-deficient mice by Western blot analyses.

Results. Lumican was present in the mouse sclera as an approximately 48kDa core protein containing short glycosaminoglycan side chains consisting of moderate- to low-sulfated keratan sulfate. The wild-type mouse sclera consisted of irregularly aranged lamellae of collagen fibrils with an average diameter of 47.37 ± 0.648 nm in the anterior sclera and 54.68 ± 0.342 nm the posterior sclera. Collagen fibrils in the sclera of lumican mutant mice (lum−/lum− and lum−/lum+) were significantly larger in diameter in anterior (72.61 ± 0.445 and 84.47 ± 0.394 nm, respectively) and posterior (75.92 ± 0.561 and 80.90 ± 0.490 nm, respectively) scleral regions compared with wild-type mice (P < 0.001).

Conclusions. The results of the present study indicate that null mutations in one or both alleles of the lumican gene result in significant defects in scleral collagen fibril formation that could lead to alterations in ocular shape and size and severely affect vision. (Invest Ophthalmol Vis Sci. 2002;43:1695–1701)

The sclera is a dense viscoelastic connective tissue that determines the shape and axial length of the eye and therefore plays a major role in influencing the refractive state. In mammals, the sclera is composed of interwoven lamellae consisting of collagen and elastic fibrils interspersed with non-collagenous glycoproteins and proteoglycans.1-7 Located between the lamellae are the scleral fibroblasts, which are responsible for the synthesis and degradation of the scleral matrix components. As in other connective tissues, it is accepted that collagen fibrils provide the sclera with its tensile strength, whereas the proteoglycans and glycoproteins serve to regulate collagen fibril formation and organization in the extracellular matrix, as well as participate in a variety of cellular processes, such as growth factor modulation, cell adhesion, and cell migration.8

The human sclera has been shown to contain the proteoglycans decorin, biglycan, and aggrecan, all of which contain sulfated glycosaminoglycan side chains.9 Additionally, the human sclera has been shown to contain the lumican (corneal keratan sulfate proteoglycan) core protein, where it is present in similar concentrations as in cornea, and exists as a glycoprotein form with varying amounts of tyrosine sulfation.9,10

Significant changes in proteoglycan synthesis have been shown to correlate with changes in the rate of axial elongation during postnatal ocular growth and during the development of myopia in a variety of animal models.11-15 suggesting that proteoglycans play a critical role in determining the biomechanical properties of the sclera. Moreover, two genetic loci associated with familial high myopia (MYP1 and MYP3) have been mapped to Xq28 and 12q21-23, respectively,14,15 which include or are near the loci for biglycan (Xq27ter),16 decorin (12q21-q22),17 and lumican (12q21.3-q22)18,19 genes, suggesting that mutations in these extracellular matrix components may be involved in some forms of human myopia.

Lumican was originally characterized in the cornea as a proteoglycan containing two to three keratan sulfate chains attached to a 51 kDa core protein.20 Using antibodies, cDNA probes and gene-specific primers specific for the regions of the lumican core protein, lumican has since been identified in many tissues, including arterial walls, heart, muscle, tendon, and sclera where it exists as a glycoprotein form containing only short chains of unsulfated keratan sulfate (lactosaminoglycan).9,21,22

Lumican is a member of the small leucine-rich proteoglycan (SLRP) family that includes decorin, biglycan, fibromodulin, proline arginine-rich end leucine rich repeat protein (PRELP), keratocan, chondroadherin, osteoglycin, and dermatan sulfate proteoglycan (DSPG)-3.23,24 All SLRPs contain a common central domain, consisting of approximately 10 leucine-rich repeats, which have been shown to be involved in strong protein-protein interactions.25 Decorin, fibromodulin, and lumican have been shown to inhibit the lateral assembly of collagen molecules from spontaneously forming collagen fibrils in vitro, resulting in the production of collagen fibrils with significantly smaller diameters.26-28 The inhibitory activity of lumican and decorin on collagen fibrillogenesis has been shown to reside in the core protein, not in the glycosaminoglycan side chains, and is dependent on the presence of disulfide bridges within the protein core.20,29

From the 1Department of Anatomy & Cell Biology, School of Medicine & Health Sciences University of North Dakota, Grand Forks, North Dakota; The GAIA Group, Novato, California; and the 3Department of Ophthalmology, University of Cincinnati Medical Center, Cincinnati, Ohio.

Supported by National Institutes of Health Grants EY09391 (JAR), EY11845 (WW-YK), and EY12486 (CYL), the Macula Vision Research Foundation (JAR), and OLERF (WW-YK).

Submitted for publication November 26, 2001; accepted January 17, 2002.

Commercial relationships policy: N.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked ‘advertisement’ in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Jody A. Rada, Department of Anatomy & Cell Biology, University of North Dakota School of Medicine & Health Sciences, 501 North Columbia Road, Grand Forks, ND 58202-9037; jarada@medicine.nodak.edu.

Investigative Ophthalmology & Visual Science, June 2002, Vol. 43, No. 6
Copyright © Association for Research in Vision and Ophthalmology
Two independent laboratories have generated lumican-deficient mice and have begun characterizing extracellular matrix defects in these mutants. Chakravarti et al. identified significant collagen fibril abnormalities in the posterior corneal stroma of 7.5-month-old lumican-deficient mice, including increased collagen fibril diameter, altered fibril packing and poor lamellar organization. In contrast, tendons of these lumican-deficient mice were not as dramatically affected as the cornea. Lumican-deficient tendons contained fibrils with slightly irregular borders at 1 and 3 months, but collagen fibril distributions were comparable with those of the wild-type tendons from 4 days to more than 3 months of age. Saika et al. generated a second lumican-null mouse and demonstrated that corneal epithelial wound healing was significantly delayed in mutant (lum-/−) mice compared with heterozygous (lum+/−) mice. These results suggest that lumican not only plays a role in the assembly and organization of collagen, but also may modulate cell behavior such as adhesion or migration.

The presence of a relatively high concentration of lumican core protein in the human sclera suggests an additional function for lumican in the scleral matrix. To understand the role of lumican in the organization and assembly of the scleral extracellular matrix, we examined scleral collagen fibrils in wild-type mice and in mice heterozygous and homozygous for a lumican-null mutation.

Materials and Methods

Lumican-null mice were generated by targeted gene disruption, as has been described. Briefly, a targeted embryonic stem cell clone derived from mouse strain 129/Sv was used to generate chimeric mice, which were mated with C57BL/6J mice. The resultant offspring were tested for the presence of the targeted locus by polymerase chain reaction (PCR) and Southern hybridization, as described previously. The mice were maintained and treated in accordance with National Institutes of Health guidelines and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Western Blot Analysis

To characterize lumican in wild-type mice, both eyes were enucleated from three normal BALB mice (6 months old), and sclera were isolated from cornea and cleaned of all vitreous, retina, choroid, muscle, and adnexa. The six sclera were then minced and extracted in 500 μL of 4 M guanidine-HCl containing 0.01 M sodium acetate, 0.01 M sodium EDTA, 0.005 M benzamidine HCl, and 0.1 M e-aminoo- caproic acid at 4°C overnight, followed by re-extraction in the same solvent for 2 to 4 hours at 4°C. The two extracts were combined and dialyzed exhaustively in distilled water and lyophilized. The scleral extract was reconstituted in distilled water, and aliquots of the scleral extract were digested with keratanase I, keratanase II, or endo-β-galactosidase in 0.1 M Tris (pH 7.4) containing 500 mM phenylmethylsulfonyl fluoride, 100 mM N-vanillylalaninamide, 100 mM EDTA, and 36 mM pepstatin A at 37°C for 3 hours. Digested and undigested samples were applied to a 10% SDS-PAGE gel, transferred to nitrocellulose, reacted with antisera against a C-terminal peptide of human lumican (Fig. 1). Lumican migrates as a 70- to 80-kDa band in undigested samples (Und). The lumican core protein was detected in 6-month-old BALB mice (6 months old), and sclera were isolated from cornea and cleaned of all vitreous, retina, choroid, muscle, and adnexa. The six sclera were then minced and extracted in 500 μL of 4 M guanidine-HCl containing 0.01 M sodium acetate, 0.01 M sodium EDTA, 0.005 M benzamidine HCl, and 0.1 M e-aminoo- caproic acid at 4°C overnight, followed by re-extraction in the same solvent for 2 to 4 hours at 4°C. The two extracts were combined and dialyzed exhaustively in distilled water and lyophilized. The scleral extract was reconstituted in distilled water, and aliquots of the scleral extract were digested with keratanase I, keratanase II, or endo-β-galactosidase in 0.1 M Tris (pH 7.4) containing 500 mM phenylmethylsulfonyl fluoride, 100 mM N-vanillylalaninamide, 100 mM EDTA, and 36 mM pepstatin A at 37°C for 3 hours. Digested and undigested samples were applied to a 10% SDS-PAGE gel, transferred to nitrocellulose, reacted with antisera against a C-terminal peptide of the human lumican core protein (generously supplied by Peter Roughley, Shriners Hospitals for Children, Montreal, Quebec, Canada), and detected with a chemiluminescent substrate (Western Star; Tropix, Bedford, MA). To determine the relative concentrations of lumican in the sclera of wild-type (lum+/+), heterozygous (lum+/−), and mutant (lum−/−) mice, eyes were obtained from 6-month-old wild-type 129/C57CL/B mice and lum+/+ and lum−/− littermates, and sclera was extracted as described. The protein concentration in scleral extracts was determined by using a microbichinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Scleral extracts containing 8 to 12.9 μg total protein were digested with endo-β-galactosidase as described earlier, electrophoresed on a 10% SDS-PAGE gel, and transferred to nitrocellulose.

Antibodies specific for the lumican core protein were used to detect the lumican in scleral samples. Digitized images of the Western blots were obtained using a flatbed scanner, and densitometry was performed using NIH Image version 1.61 (provided in the public domain by the National Institutes Health, Bethesda, MD, at http://rsb.info.nih.gov/nih-image/).

Transmission Electron Microscopy

Lumican-null homozygotes (lum−/−), heterozygotes (lum+/−), and wild-type 129/C57CL/B mice (lum+/+) were killed at 6 months of age, and whole eyes were enucleated in the Department of Ophthalmology, University of Cincinnati Medical Center, immersed in Karnovsky fixative, and sent to the Department of Anatomy and Cell Biology (Grand Forks, ND) on wet ice by express mail. With the aid of a dissection microscope, each eye was divided at the equator into anterior and posterior hemispheres, and corneas were cut away from the anterior scleral rim. Anterior and posterior scleral regions were divided into four equal portions and postfixed for 90 minutes in 1% osmium tetroxide in 0.1 M sodium cacodylate-HCl buffer (pH 7.3). Sclera were then rinsed with distilled water and stained en bloc with 0.5% aqueous uranyl acetate for 90 minutes at 4°C. Tissues were then dehydrated through a series of graded ethanol and propylene oxide and embedded in Epon 812-Araldite (Tousimis Research Corp., Rockville, MD). Thin sections were obtained using an ultramicrotome (model MT2-B Dupont-Sorvall, Newtown, CT), stained with uranyl acetate and lead citrate. Thin sections were examined with a transmission electron microscope (model H7500; Hitashi, Tokyo, Japan) and regions containing cross-sections of collagen fibrils were photographed at 80 kV for subsequent image analysis.

Image Analysis

Electron microscope negatives were digitized on a flatbed scanner at a resolution of 1200 dpi. Collagen fibril diameters were measured with a customized macro (Christopher Coulon, the GAIA Group, Novato, CA) in Object Image, ver. 2.07. Anterior and posterior sclera from three homozygous mutants, two heterozygous mutants, and two wild-type animals were analyzed in the present study. Fifteen to 32 areas of collagen fibrils were analyzed for each scleral region of each animal, generating 857 to 2799 measurements for each condition. Fibril measurements were analyzed on computer (Excel; Microsoft, Redmond, WA), using Student’s t-tests assuming unequal variances.

Results

Lumican in the Mouse Sclera

The lumican core protein was detected in 6-month-old BALB mouse sclera by Western blot analyses with antisera against a C-terminal peptide of human lumican (Fig. 1). Lumican migrated as a 70- to 80-kDa band in undigested samples (Und). Digestion of the scleral extract with keratanase I and keratanase II (KI/II) reduced a portion (~47%) of the lumican immunoreactive band ~50 kDa, indicating that this portion of scleral lumican contained short keratan sulfate side chains, which were moderately sulfated. Digestion of the scleral extract with endo-β-galactosidase (β) reduced all scleral lumican to ~48 kDa, suggesting that the remaining 53% of lumican not reduced by keratanase digestion consisted of short chains of unsulfated keratan sulfate (lactosaminoglycan) chains. No bands were detected in samples containing enzymes only (Enz).

Abnormal Collagen Morphology

Low-power electron microscopy indicated that the normal (wild-type) mouse sclera consisted of irregularly arranged lamellae of collagen fibrils in both the anterior (Fig. 2A) and posterior (Fig. 2B) sclera. Between lamellae, scleral fibroblasts...
(Fig. 2A, F) could be identified throughout the scleral thickness and melanocytes (Fig. 2B, M) were present in the region of the sclera adjacent to the ocular choroid (Figs. 2A, 2B, C). The general organization of scleral collagen in heterozygous mice appeared similar to that of wild-type mice, with distinct lamellae observed in anterior (Fig. 2C) and posterior (Fig. 2D) scleral regions. Within each lamella, collagen fibrils ran parallel to each other; however, collagen fibrils within separate lamellae ran in a variety of orientations, without an apparent pattern.

The scleral lamellae in homozygous mutant (lum⁻/lum⁻) mice (Figs. 2E, 2F) appeared less distinct than that of wild-type (lum⁺/lum⁺) or heterozygous (lum⁺/lum⁻) mice. In some areas, adjacent collagen fibrils ran in several orientations (arrow) and in other areas, relatively large gaps appeared within the collagenous lamellae (Fig. E, ✽).

At higher magnifications, irregularities in collagen fibril size and organization were apparent (Fig. 3). Collagen fibrils from the anterior and posterior sclera from lum⁺/lum⁺ and lum⁻/lum⁻ mice (Figs. 3C–3F) appeared more variable in their diameters and interspacing and generally appeared larger and more loosely packed within the scleral lamellae than collagen fibrils in the anterior and posterior sclera of lum⁺/lum⁻ mice (Figs. 3A, 3B). Ultrastructural analyses of collagen fibrils from anterior and posterior scleral regions of wild-type (lum⁺/lum⁺) mice demonstrated mean cross-sectional diameters of 47.37 ± 0.648 and 54.68 ± 0.342 nm (SEM), respectively (Fig. 3A, 3B). Collagen fibrils from anterior and posterior scleral regions of heterozygous (lum⁺/lum⁻) mice were significantly wider in diameter than collagen fibrils from similar regions in wild-type mice (72.61 ± 0.445 nm in the anterior region, P < 0.001; 75.92 ± 0.361 nm in the posterior region, P < 0.001; Figs. 3C, 3D, 4C, 4D). Moreover, collagen fibrils in anterior and posterior scleral regions of homozygous mutant (lum⁻/lum⁻) mice were significantly wider in diameter than collagen fibrils from similar regions in heterozygous (lum⁺/lum⁻) mice (84.47 ± 0.394 nm in the anterior region, P < 0.001; 91.87 ± 0.578 nm in the posterior region, P < 0.001; Figs. 3E, 3F, 4E, 4F).
Examination of the frequency distributions of collagen fibril diameters from wild-type, heterozygous, and homozygous mutant mice suggests that mutant mice exhibit wider variation in collagen fibril diameter in the anterior and posterior sclera than did the wild-type or heterozygous mice (Fig. 4).

**Haploinsufficiency**

The finding that lum$^+$/lum$^-$ mice demonstrated significant abnormalities in collagen fibril diameter compared with lum$^-$/lum$^-$ mice suggests that mice heterozygous for the lumican mutation are haplotype deficient in lumican which results in significant alterations in scleral collagen. To verify a haplotype deficiency in scleral lumican expression, sclera from lum$^+$/lum$^+$, lum$^+$/lum$^-$, and lum$^-$/lum$^-$ mice were extracted with 4 M guanidine hydrochloride, and the relative lumican content was measured by Western blot analyses with lumican-specific antisera, after digestion of scleral extracts with endo-β-galactosidase (Fig. 5). Densitometric analyses of the 48-kDa lumican-immunoreactive bands from lum$^+$/lum$^+$ and lum$^+$/lum$^-$ mice indicated that lumican was reduced in the sclera of lum$^+$/lum$^-$ mice by ~70% compared with that in the wild-type (lum$^+$/lum$^+$) sclera (3309.787 ± 570.963 pixel density/µg protein vs. 944.651 ± 233.919 pixel density/µg protein in lum$^+$/lum$^+$ and lum$^+$/lum$^-$ mice, respectively, $P < 0.01$).

**DISCUSSION**

Similar to lumican previously described in the human sclera,9 mouse scleral lumican contained short glycosaminoglycan side chains consisting of moderate- to low-sulfated keratan sulfate.

The results of the present study indicate that the normal mouse sclera at 6 months of age consists of irregularly arranged lamellae of collagen fibrils with an average diameter of 47.37 nm in the anterior sclera and 54.68 nm the posterior sclera35 or may be due to regional differences in collagen fibril–regulating components of the scleral extracellular matrix.

The sclera of homozygous lumican mutant mice (lum$^-$/lum$^-$) demonstrated significant abnormalities in scleral collagen fibril size and arrangement compared with wild-type or heterozygous mice. The mean collagen fibril diameter almost
doubled in the anterior mutant sclera and increased 48% in the posterior sclera of mutant mice compared with wild-type mice. Moreover, collagen fibrils were observed to be more variable in diameter and were distributed irregularly throughout the scleral stroma in heterozygous and homozygous mutant mice compared with wild-type mice. Considerably more disorganization and a greater change in fibril diameter were observed in the anterior sclera than in the posterior sclera of lumican mutant mice (compare Figs. 2E, 2F with Figs. 3E, 3F). These differences between the anterior and posterior sclera of lumican mutant mice may be a reflection of variation in collagen-to-lumican ratios in the anterior sclera compared with the posterior sclera and/or differences in the developmental age of these scleral regions.

The distribution and average size of collagen fibril diameters in the anterior and posterior sclera of heterozygous mice were significantly altered compared with those in the wild-type mice, and Western blot analyses confirmed that scleral lumican

---

**Figure 4.** Morphometric analysis of scleral collagen fibrils. The distributions of collagen fibril diameters were analyzed in the anterior (A, C, E) and posterior (B, D, F) sclera of 6-month-old-wild-type (lum+/lum+, A, B), heterozygous (lum+/lum−, C, D), and homozygous mutant (lum−/lum−, E, F) mice. The average fibril diameter, the variance, and the total number (n) of fibrils measured are indicated in each histogram.
concentration was significantly reduced in heterozygous mice. Taken together, these data indicate that haploinsufficiency of the lumican gene in the sclera caused significant alterations in collagen fibril size and would likely lead to profound effects on the biomechanical properties of the sclera. No haploinsufficiency has been reported in other studies examining collagen fibril diameters in cornea or tendon of lumican knockout mice. It is likely that in cornea, the presence of keratocan compensates for the absence of lumican in heterozygous and homozygous mutant mice, resulting in little or no change in collagen fibril diameter in lumican-deficient mice. Although the sclera contains several members of the SLRP family, keratocan is absent in the adult mouse sclera and therefore a similar compensatory mechanism for modulating collagen fibril diameter would be absent in lumican-deficient sclera. This absence of a keratocan compensatory mechanism in the sclera is most likely responsible for the more dramatic defects in fibril diameter and organization observed in sclera compared with cornea of lumican-deficient mice. Interestingly, mice heterozygous for a mutation in the fibromodulin gene demonstrated an abnormal morphology of tendon collagen fiber bundles compared with wild-type mice, although the distributions of individual collagen fibril diameters were similar between the two groups. The defects observed in sclera collagen fibril diameter and organization in lumican-deficient mice in the present study would be expected to result in significant changes in the biomechanical properties of the sclera and to lead to severe defects in ocular shape and size. Volumetric estimations of eye size in wild-type and lumican-deficient mice suggest that eyes are larger in lumican-deficient mice. A large genetic locus for autosomal dominant high myopia has been identified at 12q21-23 (MYP3) that includes several SLRP genes, including DSPG-3, decorin, and lumican. Although heteroduplex and sequence analysis excluded lumican as the causative gene involved in the family with 12q21-23-linked high myopia, the results of the present study indicate that a mutation in one or both alleles of the lumican gene will lead to significant defects in scleral extracellular matrix which, in turn, could result in alterations in ocular shape and size and severely affect vision.

Acknowledgments

The authors thank Virginia Achen and Kim Young for assistance with the transmission electron microscopy used in this study.

References


ERRATUM

Erratum in: "Effect of Administration of CTLA-Ig as Protein or cDNA on Corneal Allograft Survival" by Comer et al. (Invest Ophthalmol Vis Sci. 2002;43:1095-1103).

In Fig. 6B, the hatched line (representing AdCTLA) was plotted for 10 graft recipients, and not 5 as the amended figure has been.

The online version of this article was corrected on May 17, 2002.

Figure 6. Corneal allograft survival after adenovirus-mediated gene transfer of CTLA4-Ig. (A) Ex vivo transduction of donor cornea with 5 × 10⁷ PFU/mL AdCTLA before transplantation resulted in longer survival than in Ad0-transduced grafts. (B) Greater prolongation of graft survival was found after intravenous injection of AdCTLA on day 1 after transplantation. MST in Ad0-treated recipients was identical with that in unmodified control animals (data not shown).