LOXL1 Deficiency in the Lamina Cribrosa as Candidate Susceptibility Factor for a Pseudoexfoliation-Specific Risk of Glaucoma

Ursula Schlötzer-Schrehardt, PhD,[1] Christian M. Hammer, PhD,[1] Anita W. Krysta,[1] Carmen Hofmann-Rummelt,[1] Francesca Pasutto, PhD,[2] Takako Sasaki, PhD,[3] Friedrich E. Kruse, MD,[1] Matthias Zenkel, PhD[1]

Purpose: To test the hypothesis that a primary disturbance in lysyl oxidase-like 1 (LOXL1) and elastin metabolism in the lamina cribrosa of eyes with pseudoexfoliation syndrome constitutes an independent risk factor for glaucoma development and progression.

Design: Observational, consecutive case series.

Participants: Posterior segment tissues obtained from 37 donors with early and late stages of pseudoexfoliation syndrome without glaucoma, 37 normal age-matched control subjects, 5 eyes with pseudoexfoliation-associated open-angle glaucoma, and 5 eyes with primary open-angle glaucoma (POAG).

Methods: Protein and mRNA expression of major elastic fiber components (elastin, fibrillin-1, fibulin-4), collagens (types I, III, and IV), and lysyl oxidase crosslinking enzymes (LOX, LOXL1, LOXL2) were assessed in situ by quantitative real-time polymerase chain reaction, (immuno)histochemistry, and light and electron microscopy. Lysyl oxidase-dependent elastin fiber assembly was assessed by primary optic nerve head astrocytes in vitro.

Main Outcome Measures: Expression levels of elastic proteins, collagens, and lysyl oxidases in the lamina cribrosa.

Results: Lysyl oxidase-like 1 proved to be the major lysyl oxidase isoform in the normal lamina cribrosa in association with a complex elastic fiber network. Compared with normal and POAG specimens, lamina cribrosa tissues obtained from early and late stages of pseudoexfoliation syndrome without and with glaucoma consistently revealed a significant coordinated downregulation of LOXL1 and elastic fiber constituents on mRNA and protein level. In contrast, expression levels of collagens and other lysyl oxidase isoforms were not affected. Dysregulated expression of LOXL1 and elastic proteins was associated with pronounced (ultra)structural alterations of the elastic fiber network in the laminar beams of pseudoexfoliation syndrome eyes. Inhibition of LOXL1 interfered with elastic fiber assembly by optic nerve head astrocytes in vitro.

Conclusions: The findings provide evidence for a pseudoexfoliation-specific elastinopathy of the lamina cribrosa resulting from a primary disturbance in LOXL1 regulation and elastic fiber homeostasis, possibly rendering pseudoexfoliation syndrome eyes more vulnerable to pressure-induced optic nerve damage and glaucoma development and progression.

Financial Disclosure(s): The author(s) have no proprietary or commercial interest in any materials discussed in this article. *Ophthalmology 2012;119:1832–1843 © 2012 by the American Academy of Ophthalmology.*

Secondary glaucoma associated with pseudoexfoliation syndrome represents a common and severe type of open-angle glaucoma accounting for approximately 20% to 25% of all cases of open-angle glaucoma worldwide.[1] The underlying disease has been characterized as a generalized elastosis, associated with the excessive production and abnormal aggregation of elastic proteins, such as fibrillin-1 and elastin, into typical pseudoexfoliation fibrils accumulating in the aqueous outflow pathways and other tissues inside and outside the eye.[2] Recent genetic studies in multiple populations convincingly have identified the *LOXL1* gene as a principal contributor to the risk of developing both pseudoexfoliation syndrome and pseudoexfoliation glaucoma.[3,4] Lysyl oxidase-like 1 (LOXL1), a member of the lysyl oxidase family of cross-linking matrix enzymes, has been shown to be required specifically for elastic fiber formation and stabilization by means of cross-linking tropoelastin monomers into elastin polymers.[5] Although the causative functional role of the missense changes caused by the disease-associated *LOXL1* variants remains unclear, previous studies have provided evidence for a dysregulated expression and involvement of LOXL1 in the formation of the aberrant fibrillar aggregates in ocular tissues of pseudoexfoliation syndrome eyes.[6,7]

Compared with primary open-angle glaucoma (POAG), pseudoexfoliation glaucoma has a more serious clinical course and worse prognosis.[8] It typically is associated with higher mean intraocular pressure (IOP) levels, greater diur-

ISSN 0161-6420/12/$–see front matter
http://dx.doi.org/10.1016/j.ophtha.2012.03.015

nal IOP fluctuations, higher frequency and severity of optic nerve damage, and more rapid visual field loss. Hence, IOP has been established as a major risk factor for glaucomatous damage in pseudoexfoliation syndrome patients, underlined by the observation that untreated IOP levels were correlated directly with mean visual field defects in patients with pseudoexfoliation glaucoma but not in patients with POAG.[9] However, the pseudoexfoliation process itself also has been shown to be a significant independent risk factor for glaucomatous optic nerve damage and progression. First, glaucomatous damage was reported to occur in a significant proportion of normotensive pseudoexfoliation syndrome eyes, particularly if these showed IOP levels of 18 mmHg or more and IOP fluctuations of 6 mmHg or more.[10–12] Moreover, despite similar mean baseline IOP values, a greater rate of conversion of ocular hypertensive patients to pseudoexfoliation glaucoma than to POAG has been reported.[13,14] At a given level of IOP, the probability of having glaucomatous damage was shown to be higher in eyes with pseudoexfoliation syndrome than in those without,[15,16] and patients with untreated pseudoexfoliation glaucoma progressed considerably faster than those with untreated POAG or normal-tension glaucoma.[17] Finally, the presence of pseudoexfoliation syndrome was the most important independent risk factor for glaucoma progression in the Early Manifest Glaucoma Trial.[18] The conclusions from these clinical studies were that the joint effect of IOP elevation and the pseudoexfoliation process itself, which may involve an increased vulnerability of the optic nerve head (ONH) to increased IOP, confers a greater risk for glaucomatous optic nerve damage in pseudoexfoliation syndrome patients compared with those without pseudoexfoliation syndrome.

Pronounced abnormalities of elastic components of the lamina cribrosa (LC) have been suggested to represent 1 important IOP-independent risk factor in pseudoexfoliation glaucoma patients. Previous studies have found a marked and site-specific elastosis of the LC in eyes with pseudoexfoliation glaucoma that was significantly more severe than in POAG and other types of glaucoma.[19,20] However, it is not known whether these elastotic alterations represent primary anomalies or secondary alterations in response to elevated IOP levels in glaucoma eyes. The authors' working hypothesis suggests the existence of a primary defect in LOXL1 regulation and elastin metabolism, which negatively affects the structural and biomechanical properties of the LC in early stages of pseudoexfoliation syndrome and increases the susceptibility of the ONH to IOP-induced damage. To test this hypothesis, this study investigated the expression and localization of major elastic proteins involved in elastic fiber assembly (elastin, fibrillin-1, fibulin-4)[21] and lysyl oxidase cross-linking enzymes (LOX, LOXL1, LOXL2), which are required for elastic fiber formation and maintenance,[22] as well as collagens (types I, III, and IV) in the LC of pseudoexfoliation syndrome and glaucoma eyes in various stages of the disease in comparison with normal controls. A small number of LC specimens obtained from eyes with POAG also served as a control group.

Materials and Methods

Tissue Specimens

Donor eyes used for corneal transplantation were obtained and processed within 15 hours after death. For RNA extractions, 10 donor eyes with early pseudoexfoliation syndrome without glaucoma (mean age, 79.5±9.2 years; 5 female, 5 male), 10 donor eyes with late pseudoexfoliation syndrome without glaucoma (mean age, 79.8±8.0 years; 5 female, 5 male), and 20 normal-appearing age-matched control eyes (mean age, 75.3±8.5 years; 7 female, 13 male) without any known ocular disease were used. Donors were classified as having early- or late-stage pseudoexfoliation syndrome according to the amount of macroscopically visible pseudoexfoliation material deposits on anterior segment structures. As described previously,[23] early stages were defined by a frosted appearance of the zonules, whereas late stages revealed prominent pseudoexfoliation material deposits on the lens, iris, ciliary processes, and zonules. The presence of pseudoexfoliation material was confirmed by electron microscopic analysis of small tissue sectors, and the absence of glaucoma was confirmed by microscopic analysis of optic nerve cross-sections. In addition, tissues of 5 eyes with a documented history of pseudoexfoliation-associated open-angle glaucoma (mean age, 74.3±11.2 years; 2 female, 3 male), which had to be enucleated surgically because of painful absolute glaucoma, as well as tissues of 5 donor eyes with a documented history of POAG (mean age, 82.3±11.7 years; 2 female, 3 male) were obtained for comparative analyses. Posterior segment tissues (retina, choroid, LC, sclera, and retrobulbar optic nerve) were prepared under a dissecting microscope and shock frozen in liquid nitrogen. The scleral specimens were subdivided into peripapillary sclera (2 mm surrounding the LC) and sclera from the equatorial region of the globe.

The *LOXL1* genotypes and haplotypes, formed by the 2 nonsynonymous polymorphisms, rs1048661 and rs3825942 in exon 1 of the *LOXL1* gene, were determined through direct sequencing of cDNA from most of the pseudoexfoliation (n = 21) and control (n = 24) tissues as previously described.[6]

For immunohistochemistry analysis, posterior segments from 7 donor eyes with pseudoexfoliation syndrome without glaucoma (mean age, 78.7±9.7 years; 5 female, 2 male) and 7 normal-appearing age-matched control eyes (mean age, 76.0±6.2 years; 3 female, 4 male) were cryofixed in OCT compound (TissueTek O.C.T. compound; Sakura Finetek, Staufen, Germany). Three donor eyes with pseudoexfoliation syndrome (mean age, 72.0±10.5 years; 1 female, 2 male) and 3 normal donor eyes (mean age, 70.0±7.5 years; 1 female, 2 male) were used for transmission electron microscopy and electron microscopic immunogold labeling. Another set of fixed archived donor eyes with pseudoexfoliation syndrome (n = 7; mean age, 81.3±3.8 years, 4 female, 3 male) and without (n = 7; mean age, 78.2±7.1 years; 4 female, 3 male) was used for histochemical staining of elastin and collagen fibers on cross-sections of the LC.

Informed consent for tissue donation was obtained from the patients or their relatives, and the protocol of the study was approved by the local ethics committee and adhered to the tenets of the Declaration of Helsinki for experiments involving human tissues and samples.

Real-Time Polymerase Chain Reaction

Total RNA was extracted from ocular tissues using the RNeasy kit (Quiagen, Hilden, Germany), including an on-column DNase I digestion step. First-strand cDNA synthesis from 0.1 μg total RNA and quantitative real-time polymerase chain reaction were performed using the MyIQ thermal cycler and software (Bio-Rad

Table 1. Primers Used for Quantitative Real-Time Polymerase Chain Reaction Analysis

Gene	Accession No.	Product (bp)	Annealing Temperature (°C)	$MgCl_2$ (mM)	Sequence (5′–3′)
LOX	NM_002317	104	62	3.5	CAGATTTCTTACCCAGCCGACC
					GGCATCAAGCAGGTCATAGTGG
LOXL1	NM_005576	112	62	3.5	TGGCTGAACTCGTCCATGCTGTG
					ACTACGATGTGCGGGTGCTACTG
LOXL2	NM_002318	73	61	3.0	CATCCACCTCAACGAGATCCAG
					AGACTCGGCATTGAACTTGCAG
Elastin	NM_000501	197	62	3.5	AGGCAAACCTCTTAAGCCAGTTCC
					CAGACACTCCTAAGCCACCAACTC
Fibrillin-1	NM_000138	184	64	3.5	GAATGCAAGAACCTCATTGGCAC
					TGGCGGTAAACCCATCATTACAC
Fibulin-4	NM_016938	221	62	3.5	TGCCGTCATCAACGACCTAC
					CGATCTTGCGGTAACCATCAG
Col1A1	NM_000088	127	62	3.0	CGAAGACATCCCACCAATCACC
					GATCACGTCATCGCACAACACC
Col3A1	NM_000090	239	64	3.0	GGCTACTTCTCGCTCTGCTTCATC
					TGGGCAAACTGCACAACATTCTCC
Col4A1	NM_001845	116	63	3.0	CAGAGATGGTGTTGCAGGAGTG
					TGTCACCTTTGAGCCGCAAGTC
GAPDH	NM_002046	117	64	3.5	AGCTCACTGGCATGGCCTTC
					ACGCCTGCTTCACCACCTTC

Col = collagen; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; LOX = lysyl oxidase; LOXL = lysyl oxidase-like.

Laboratories, München, Germany). Polymerase chain reactions (25 μl) were run in duplicate and contained 2 μl first-strand cDNA, 0.4 μM each of upstream and downstream primer, and IQ SYBR Green Supermix (Bio-Rad). Exon-spanning primers (MWG Biotech, Anzing, Germany), designed by means of Primer 3 software (available at: http://fokker.wi.mit.edu/primer3/input.htm; accessed January 17, 2011), and polymerase chain reaction conditions are summarized in Table 1. For quantification, serially diluted standard curves of plasmid-cloned cDNA were run in parallel, and amplification specificity was checked using melt curve and sequence analyses using the Prism 3100 DNA-sequencer (Applied Biosystems, Foster City, CA). For normalization of gene expression levels, mRNA ratios relative to the housekeeping gene *GAPDH* were calculated.

Immunohistochemistry

Indirect immunofluorescence single- or double-labeling was performed on cryosections of posterior segment tissues using primary antibodies against LOX (generous gift from Ian Hornstra, St. Louis, MO; 1:100), LOXL1 (Ian Hornstra; 1:200; MaxPab B01P, Abnova, Taiwan; 1:100), LOXL2 (Ian Hornstra; 1:100), elastin (clone 10B8; Millipore, Schwalbach, Germany; 1:20), tropoelastin (Abcam, Cambridge, UK; 1:500), fibrillin-1 (clone 26; Millipore; 1:150; generous gift from Dieter Reinhardt, Montreal, Canada; 1:2000), fibulin-4 (Takako Sasaki, Erlangen, Germany; 1:500), and glial fibrillary acidic protein (Sigma-Aldrich, München, Germany; 1:100). Cryostat-cut sections (5 μm) were fixed in cold acetone, blocked with 10% normal goat serum, and incubated in primary antibodies diluted in phosphate-buffered saline overnight at 4°C. Antibody binding was detected by Alexa 488- and Alexa 555-conjugated secondary antibodies (Molecular Probes, Eugene, OR), and nuclear counterstaining was performed with DAPI (4',6-diamidino-2-phenylindole) or propidium iodide (Sigma-Aldrich). In negative control samples, the primary antibody was replaced by phosphate-buffered saline or equimolar concentrations of nonimmune rabbit immunoglobulin G or an irrelevant primary antibody.

Histomorphometric Analysis

Posterior segment tissues preserved in 4% buffered paraformaldehyde were processed for paraffin embedding. Using Weigert's resorcin-fuchsin and picrosirius red histochemistry, 5-μm–thick cross-sections of the LC were stained for elastin and collagen, respectively, according to standard protocols.[24] Sections were analyzed with a light microscope (BX51; Olympus Optical Co., Hamburg, Germany) and digital images were obtained from 6 different areas around the entire circumference of each LC with a charge-coupled device camera (F-View II; Soft Imaging System, Münster, Germany) at a magnification of ×40 covering an area of 0.095 mm^2. The percentage area occupied by elastin and collagen fibers in relation to the entire area analyzed was detected with an image analyzing software (Cell^F; Olympus) by threshold analysis of gray values; elastin and collagen fibers were detected by standardized threshold setting, including the dark fibers and excluding the bright background.

Transmission Electron Microscopy and Immunogold Labeling

For transmission electron microscopy, specimens were fixed in 4% paraformaldehyde/1% glutaraldehyde in 0.1 M phosphate buffer, postfixed in 2% buffered osmium tetroxide, dehydrated in graded alcohol concentrations, and embedded in epoxy resin according to standard protocols. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined with a transmission electron microscope (EM 906E; Carl Zeiss NTS GmbH, Oberkochen, Germany). For postembedding immunogold labeling, specimens were fixed in 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M cacodylate buffer for 5 hours at 4°C, dehydrated serially to 70% ethanol at −20°C, and embedded in LR White resin (Electron Microscopy Sciences, Hatfield, PA). Ultrathin sections were incubated successively in Tris-buffered saline (TBS), 0.05 M glycine in TBS, 0.5% ovalbumin and 0.5% fish gelatin in TBS, anti-elastin monoclonal antibody (clone 10B8)

diluted 1:10 in TBS-ovalbumin overnight at 4°C, and finally in 10 nm gold-conjugated secondary antibody (BioCell; Cardiff, Wales, UK) diluted 1:30 in TBS-ovalbumin for 1 hour.

In Vitro Elastogenesis Assay

To assess elastic fiber assembly in vitro, primary ONH astrocyte cultures were generated from LC tissue of 3 normal donors (mean age, 67±2.2 years; 2 male, 1 female) and were used at passages 2 to 3. The LC tissue was dissected into 4 to 6 explants, placed into laminin-coated culture plates (BD Biosciences, Heidelberg, Germany), capped with a coverslip, maintained in DMEM/Ham's F12 with 10% fetal bovine serum and 1% antibiotic-antimycotic mix (Invitrogen, Karlsruhe, Germany), and supplemented with 5 ng/ml basic fibroblast growth factor (bFGF) and 5 ng/ml platelet-derived growth factor (PDGF-AA) (Sigma-Aldrich) in a 95% air–5% CO_2 humidified atmosphere at 37°C. After outgrowth, astrocytes were separated from LC fibroblasts as described previously,[25] subcultivated, and immunohistochemically characterized using antibodies against glial fibrillary acidic protein.

Primary ONH astrocytes were seeded in 2-well Lab-Tek chamber slides (Thermo Scientific/Nunc, Langenselbold, Germany) at a density of 0.5×10^5 cells/well, grown to confluence, and treated with 5 ng/ml transforming growth factor β1 (TGF-β1; R&D Systems, Wiesbaden, Germany) to stimulate elastogenesis. In addition, 50 μg/ml β-aminopropionitrile (BAPN; Sigma), a specific inhibitor of lysyl oxidase activity,[26] was added to postconfluent control wells. After incubation with or without inhibitor for 2 to 14 days, the presence of elastin incorporated into the extracellular matrix was assessed by immunofluorescence, as described above.

Results

Expression of Lysyl Oxidases and Elastic Fiber Proteins in the Normal Lamina Cribrosa

In normal human elderly donor eyes (n = 7; mean age, 78.7±7.2 years), highest mRNA levels of lysyl oxidase isoenzymes were measured in the choroid, followed by sclera, retina, LC, and optic nerve (Fig 1A). In choroid, retina, and sclera tissues, LOX was the predominantly expressed isoform, followed by LOXL1 and LOXL2. However, LOXL1 was the only isoform distinctly expressed in LC tissue, although with considerable interindividual variability, whereas LOX and LOXL2 were just at the limit of detection. Highest mRNA levels of elastic proteins, that is, elastin, fibrillin-1, and fibulin-4, also were found in the choroid, followed by LC, retina, sclera, and optic nerve (Fig 1B). In LC tissue, the highest expression levels were observed for fibulin-4, followed by fibrillin-1 and elastin. Expression levels of lysyl oxidases and elastic proteins were not significantly different in peripapillary and normal sclera.

By immunolabeling, connective tissue septa of normal LC tissue revealed a complex, radially oriented elastic fiber network consisting of elastin-containing fibers (Fig 2A′) and prominent bundles of elastic microfibrils positive for fibrillin-1 and fibulin-4; they originated from the circularly arranged, dense fiber network in the peripapillary sclera and connected with the perivascular connective tissue of the central retinal vessels. Fibrillin-1 and fibulin-4 colocalized only partially with elastin (Fig 2A″, A‴), suggesting the presence of isolated elastic microfibrillar bundles independent of elastin fibers.

This complex elastic fiber network appeared in close association with LOXL1-positive cells in the prelaminar, laminar, and immediate retrolaminar regions (Fig 2B). Lysyl oxidase-like 1

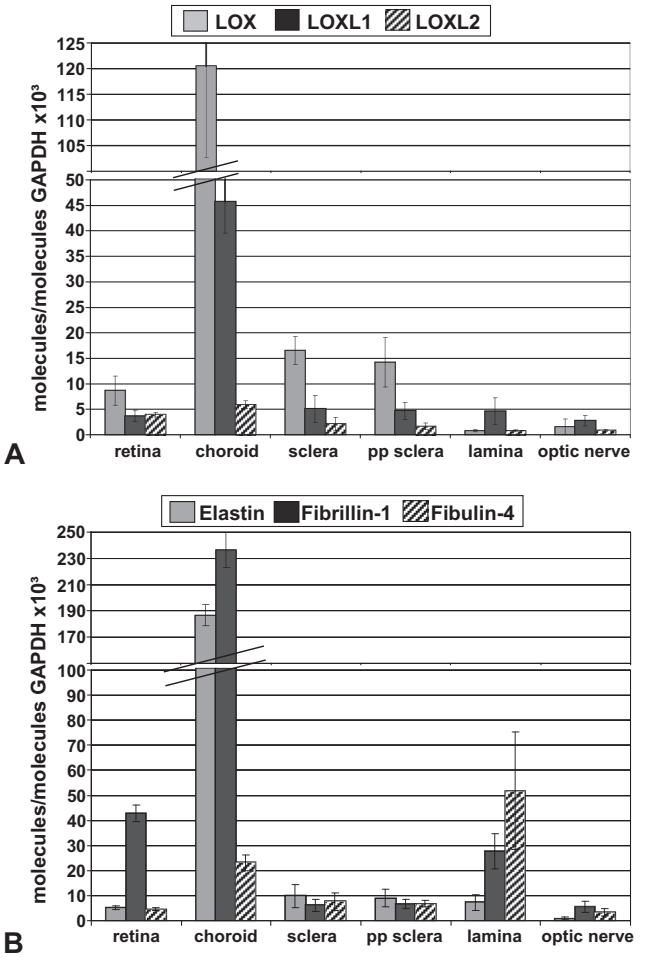

Figure 1. Expression of (**A**) lysyl oxidase isoenzymes LOX, LOXL1, and LOXL2, and (**B**) elastic fiber components in posterior segment tissues from normal human donors (n = 7; mean age, 78.7±7.2 years) using real-time polymerase chain reaction technology. The expression levels were normalized against glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), and the results are expressed as molecules of gene of interest per molecules GAPDH. The values represent mean values±standard deviation of 6 separate experiments. pp sclera = peripapillary sclera.

mainly was localized to the cytoplasm of cells located at the interface between axon bundles and connective tissue presumed to represent glial astrocytes by positive staining for glial fibrillary acidic protein (not shown). Cells localized within the laminar beams, presumably LC cells, mostly were negative. Lysyl oxidase-like 1–positive astrocytes were associated with few elastic fibers and microfibrillar bundles within glial columns in the prelaminar region (Fig 2B′), with prominent radially oriented elastic networks in the laminar region (Fig 2B″), and with predominantly sagittally oriented fibers and microfibrils in the connective tissue septa of the immediate retrolaminar portion of the ONH (Fig 2B‴). In addition, cells in the peripapillary sclera, optic nerve sheaths, and blood vessel walls (central retinal vessels, intrascleral vessels, prelaminar capillaries) also demonstrated positive staining for LOXL1 (not shown). Occasionally, LOXL1 was present along extracellular fibers in clear colocalization with tropoelastin (Fig 2C), both within the laminar beams (Fig 2C′) and the peripapillary sclera (Fig 2C″). In contrast, immunolabeling for LOX and LOXL2

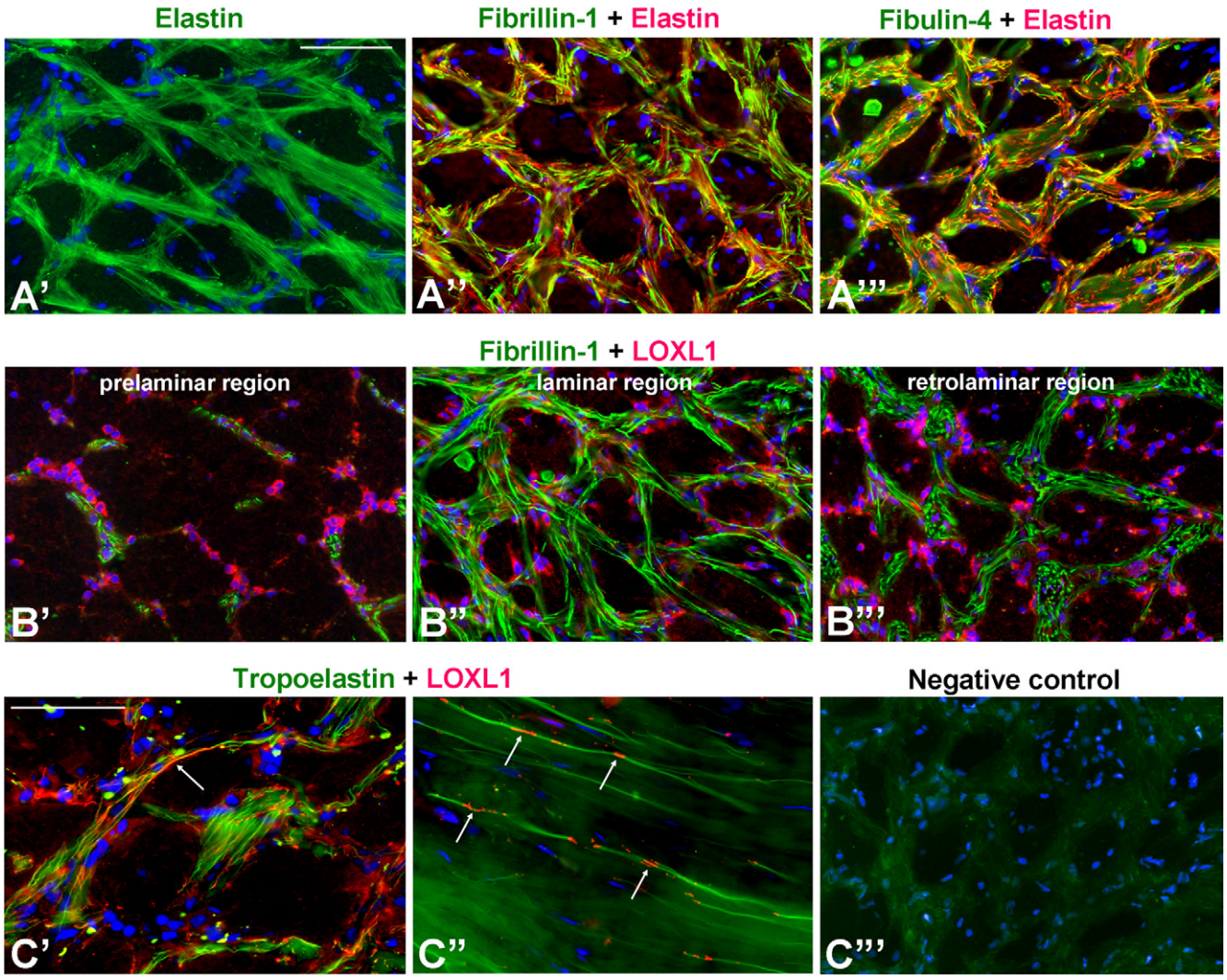

Figure 2. Immunofluorescence labeling of elastic proteins and LOXL1 in cross-sections of the lamina cribrosa of a normal human donor eye (age, 63 years). Positive signals are indicated by green or red fluorescence in contrast to blue fluorescence of nuclei stained with 4,′6-diamidino-2-phenylindole. **A**, Single staining for (**A′**) elastin and double staining for (**A′′**) elastin and fibrillin-1 or (**A′′′**) elastin and fibulin-4 showing distinct populations of elastic fibers and elastic microfibrils. **B**, Double staining for LOXL1 and fibrillin-1 in the (**B′**) prelaminar, (**B′′**) laminar, and (**B′′′**) immediate retrolaminar regions showing a predominant location of LOXL1 within astrocytes. **C**, Double staining for LOXL1 and tropoelastin showing colocalization along extracellular elastic fibers (arrows) within the (**C′**) laminar beams and the (**C′′**) peripapillary sclera. No staining reactions were seen after omission of primary antibodies (**C′′′**). Magnification bars = 100 μm in (**A**) and (**B**) and 50 μm in (**C′**) and (**C′′**).

demonstrated negative staining in normal LC tissue (not shown). Antibody binding was abolished when an irrelevant primary antibody or phosphate-buffered saline was used instead of the primary antibodies (Fig 2C′′′). Together, these findings indicate that LOXL1 is the major lysyl oxidase isoform in the human LC, where it seems to be involved in elastin fiber homeostasis.

Expression of Lysyl Oxidases and Elastic Fiber Proteins in the Lamina Cribrosa of Pseudoexfoliation Syndrome Eyes

Next, expression levels of lysyl oxidases and elastic proteins in normal LC tissue (n = 20) were compared with those of eyes with POAG (n = 5) and those of eyes with pseudoexfoliation syndrome without glaucoma (n = 20). Compared with normal age-matched controls, expression of LOXL1 was reduced significantly in pseudoexfoliation specimens (40% of control levels; $P = 0.001$), accompanied by a significant reduction of elastin (53%; $P = 0.001$), fibrillin-1 (43%; $P = 0.001$), and fibulin-4 (55%; $P = 0.001$; Fig 3A). In contrast, expression of LOX and LOXL2, which were at the limit of detection, showed no differences between pseudoexfoliation syndrome and control specimens. Differences in expression levels of collagen types I, III, and IV between pseudoexfoliation syndrome and control samples were statistically not significant. However, POAG specimens revealed significantly increased expression levels of collagen type IV (230%; $P = 0.05$), but otherwise were not significantly different from the control group.

After subdividing the pseudoexfoliation syndrome group into early stages (n = 10) and late stages (n = 10), both early and late stages showed a significant decline in expression of LOXL1 (34% and 48% of control levels, respectively) and the elastic proteins elastin (63% and 43%, respectively), fibrillin-1 (45% and 40%,

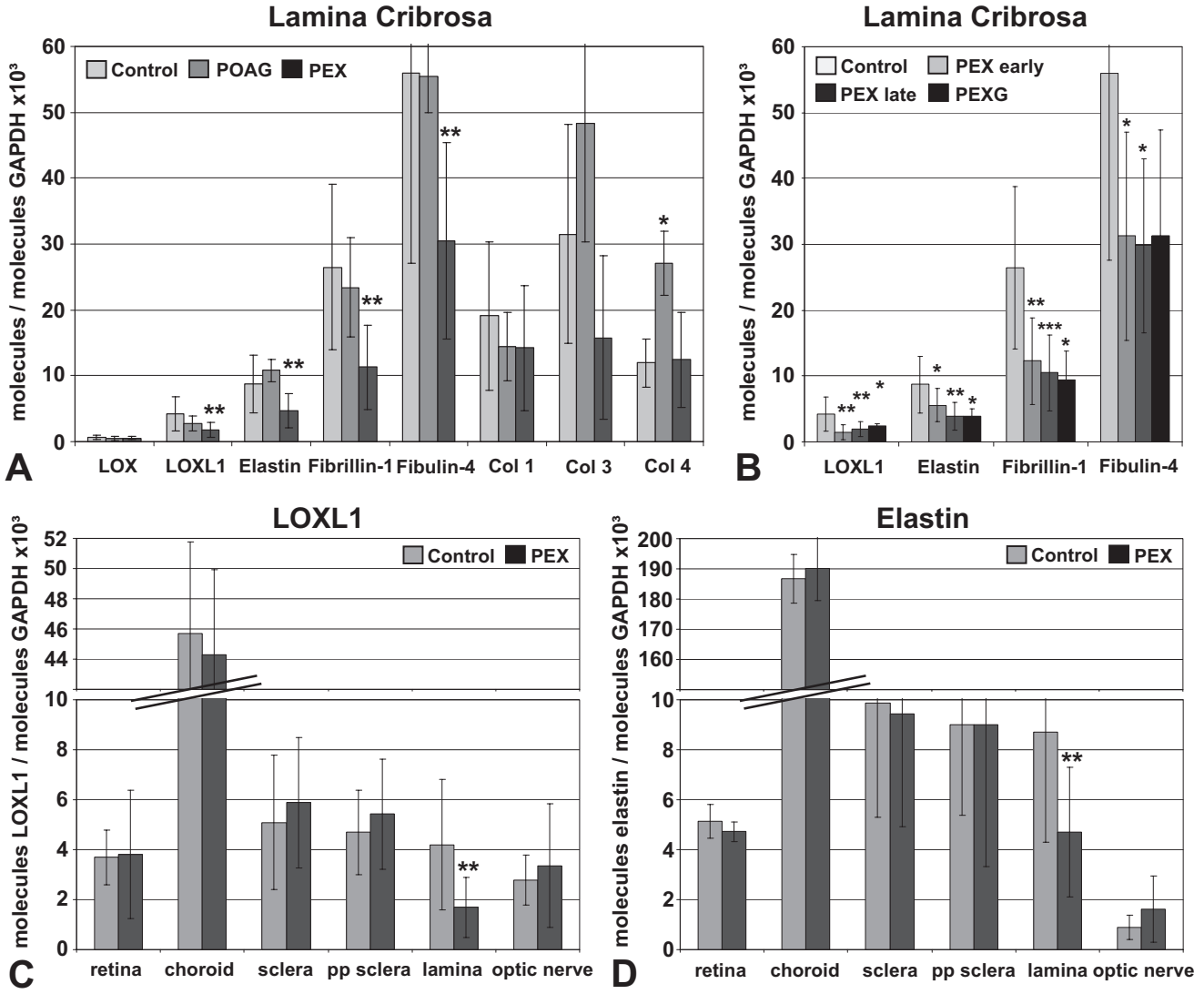

Figure 3. **A**, Expression of LOX, LOXL1, elastin, fibrillin-1, fibulin-4, collagen type I, collagen type III, and collagen type IV in the lamina cribrosa of normal human donor eyes (control; n = 20), eyes with primary open-angle glaucoma (POAG; n = 5), and eyes with pseudoexfoliation (PEX) syndrome (n = 20) using real-time polymerase chain reaction technology. **B**, Expression of LOXL1, elastin, fibrillin-1, and fibulin-4 in the lamina cribrosa of normal human donor eyes (control; n = 20), eyes with early stages of pseudoexfoliation (PEX) syndrome (PEX early; n = 10), eyes with late stages of PEX syndrome (PEX late; n = 10), and eyes with PEX-associated open-angle glaucoma (PEXG; n = 5). Expression of (**C**) LOXL1 and (**D**) elastin in the retina, choroid, sclera, peripapillary (pp) sclera, lamina cribrosa, and optic nerve of normal human control eyes compared with eyes from patients with PEX syndrome (n = 20 for each patient group). The expression levels were normalized against glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), and the results are expressed as molecules of gene of interest per molecules GAPDH (**P* = 0.05, ***P* = 0.001, ****P* = 0.0001).

respectively), and fibulin-4 (55% and 53%, respectively) compared with normal age-matched controls (Fig 3B). A similar, statistically significant reduction in expression levels of LOXL1 (55%; $P = 0.004$), elastin (45%; $P = 0.03$), and fibrillin-1 (36%; $P = 0.03$) also was found in pseudoexfoliation eyes with end-stage glaucoma (n = 5) compared with controls (Fig 3B). The pseudoexfoliation-associated significant decrease in LOXL1 and elastic protein expression was found to be specific for the LC, whereas other posterior segment tissues, such as choroid, retina, sclera, and optic nerve, showed no significant differences in expression levels between pseudoexfoliation syndrome and control eyes (Fig 3C, D).

To investigate a potential effect of the *LOXL1* genotype, LOXL1 expression levels in the LC were correlated with the *LOXL1* genotypes and haplotypes formed by the 2 pseudoexfoliation-associated nonsynonymous risk variants, rs1048661 (R141L) and rs3825942 (G153D). The distribution of *LOXL1* genotypes and haplotypes in the study samples is summarized in Figure 4A (available at http://aaojournal.org). In brief, the risk genotype GG of rs1048661 was distributed equally among pseudoexfoliation cases (approximately 52%) and control cases (approximately 46%), whereas the risk genotype GG of rs3825942 was highly overrepresented in pseudoexfoliation cases (100%) compared with controls (50%). Approximately 76% of pseudoexfoliation cases and 37% of control cases carried the high-risk haplotype (G-G), whereas 2 copies of the high-risk haplotype (G-G/G-G) were present in 52% of pseudoexfoliation cases and 17% of control cases. In both pseudoexfoliation and control cases, expression of LOXL1 was affected by rs1048661 alleles leading to a reduction of approximately 18% per

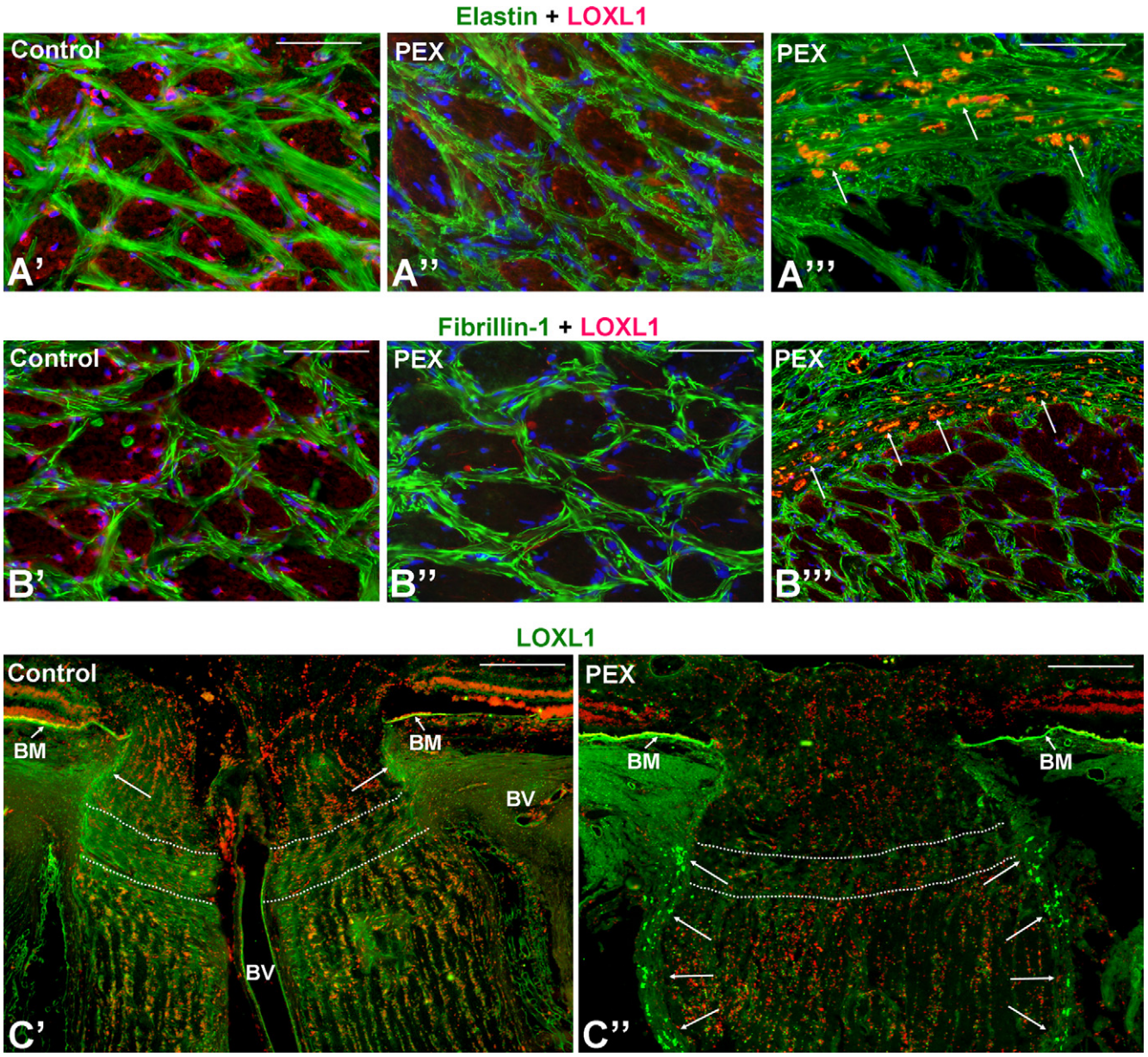

Figure 5. Immunofluorescence labeling of elastic proteins and LOXL1 in optic nerve head tissue of normal human donor eyes and eyes with pseudoexfoliation (PEX) syndrome. Nuclei are stained with 4,′6-diamidino-2-phenylindole (blue fluorescence) in (**A**) and (**B**) or with propidium iodide (red fluorescence) in (**C**). **A**, Double staining for LOXL1 (red) and elastin (green) showing decreased LOXL1 immunoreactivity and irregular elastin staining in cross-sections of the lamina cribrosa of (**A**′′) PEX syndrome eyes compared with (**A**′) normal eyes. Pseudoexfoliation material aggregates demonstrating immunopositive labeling for both LOXL1 and elastin (orange, arrows) accumulate in the peripapillary sclera of (**A**′′′) PEX syndrome eyes. **B**, Double staining for LOXL1 (red) and fibrillin-1 (green) showing decreased LOXL1 immunoreactivity but regular fibrillin staining in the lamina cribrosa of (**B**′′) PEX syndrome eyes as compared with (**B**′) normal eyes. Pseudoexfoliation material aggregates immunopositive for both LOXL1 and fibrillin-1 (orange, arrows) accumulate in the peripapillary sclera of (**B**′′′) PEX syndrome eyes. **C**, Sagittal section through the optic nerve head of a (**C**′) normal eye showing widespread immunostaining for LOXL1 (green) in Bruch's membrane (BM), lamina cribrosa (dotted lines), peripapillary sclera (arrows), and blood vessel (BV) walls. Optic nerve head tissue of a (**C**′′) PEX syndrome eye shows reduced LOXL1 immunostaining within the lamina cribrosa (dotted lines), but accumulation of LOXL1-positive PEX material aggregates (arrows) within the peripapillary sclera and optic nerve sheaths. Magnification bars = 100 μm in (**A**′), (**A**′′), (**B**′), and (**B**′′); 50 μm in (**A**′′′); 200 μm in (**B**′′′); and 1000 μm in (**C**′) and (**C**′′).

risk G allele, whereas rs3825942 alleles and the haplotype did not affect LOXL1 expression levels in the LC (Fig 4B, C, available at http://aaojournal.org).

By immunolabeling, the LC of nonglaucomatous pseudoexfoliation syndrome eyes revealed a disorganized and fragmented elastic fiber system, which was particularly distinct for elastin-positive elastic fibers (Fig 5A′, A′′) and less prominent for elastic microfibrils demonstrating positive staining for fibrillin-1 (Fig 5B′, B′′) and fibulin-4 (not shown) compared with controls. In contrast to control eyes, LOXL1 was hardly detected in LC astrocytes of pseudoexfoliation syndrome eyes (Fig 5A′′, B′′), but rather, accumulated within extracellular pseudoexfoliation mate-

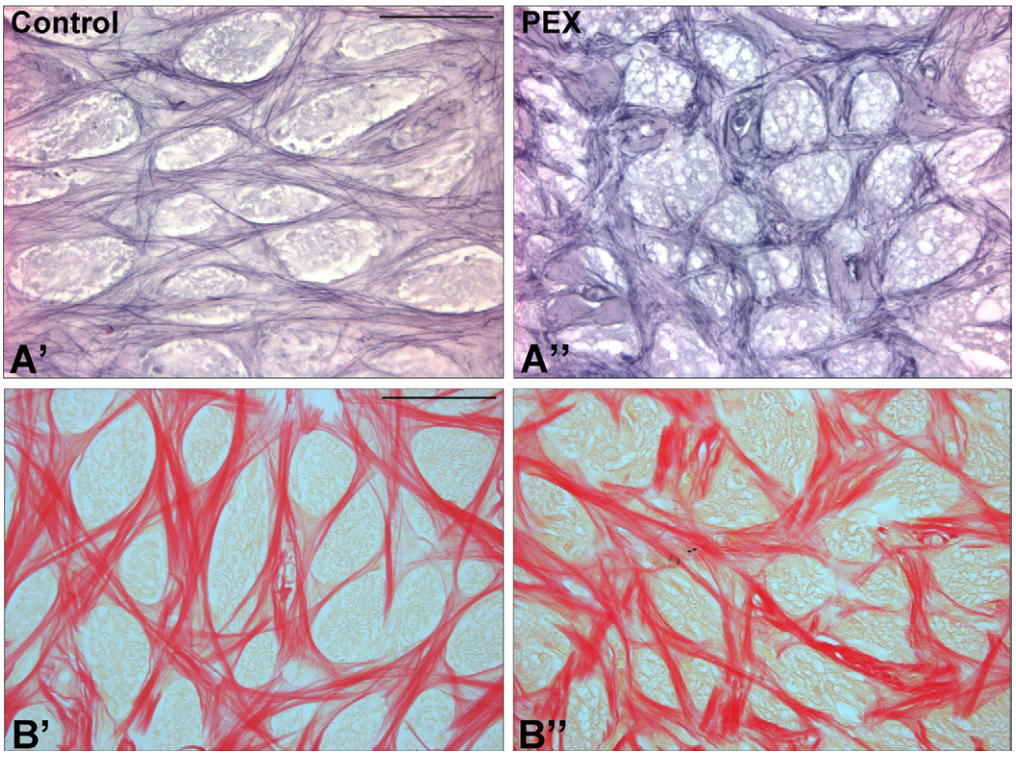

Figure 6. Histomorphometric analysis of elastic fibers and collagen fibers in paraffin cross-sections of the lamina cribrosa of a normal human donor eye (age, 78 years) and an eye with pseudoexfoliation (PEX) syndrome (age, 77 years). **A**, Light microscopic appearance of representative areas of the lamina cribrosa analyzed for purple-stained elastin fibers using Weigert's resorcin-fuchsin in (**A′**) normal and (**A′′**) PEX syndrome tissue specimens. **B**, Light microscopic appearance of representative areas of the lamina cribrosa analyzed for red-stained collagen fibers using picrosirius red in (**B′**) normal and (**B′′**) PEX syndrome tissue specimens. Magnification bars = 100 μm.

rial aggregates in clear colocalization with elastin, fibrillin-1, and fibulin-4 (Fig 5A′′′, B′′′). Such LOXL1-positive pseudoexfoliation aggregates were observed in all pseudoexfoliation syndrome specimens, but not in controls, and accumulated within the optic nerve sheaths and the posterior regions of the peripapillary sclera (Fig 5C′, C′′). In addition, blood vessel walls of prelaminar capillaries and retrobulbar short posterior ciliary arteries showed focal accumulations of LOXL1-positive pseudoexfoliation deposits (not shown).

These findings indicate a pseudoexfoliation-specific and site-specific downregulation of LOXL1 and elastic fiber constituents on both the mRNA and protein level in the LC of eyes with pseudoexfoliation syndrome in association with structural alterations of the laminar elastic fiber system. However, the dysregulated LOXL1 expression cannot be attributed clearly to the known LOXL1 risk genotypes.

Morphologic Alterations of Elastin in the Lamina Cribrosa of Pseudoexfoliation Syndrome Eyes

To characterize further the elastotic alterations observed in the laminar beams of pseudoexfoliation syndrome eyes, the elastic fiber system was analyzed qualitatively and quantitatively on the light and electron microscopic level in LC cross-sections of normal control (n = 7) and nonglaucomatous pseudoexfoliation syndrome eyes (n = 7). Whereas the collagen fibers stained with picrosirius red apparently were not altered in pseudoexfoliation syndrome tissues (Fig 6B′, B′′), the elastic fiber network stained with resorcin-fuchsin appeared markedly disorganized and fragmented in pseudoexfoliation syndrome specimens compared with controls (Fig 6A′, A′′). However, there was no quantitative difference in the percentage area of the laminar beams occupied by elastin (pseudoexfoliation, 40.3%; control, 39.5%), nor in the percentage area covered by collagen (pseudoexfoliation, 55.5%; control, 57.2%) between the groups (data not shown).

Transmission electron microscopy and elastin immunogold labeling revealed regularly structured collagen fibers, but strikingly abnormal elastic fibers, in the laminar beams of pseudoexfoliation syndrome specimens compared with controls (Fig 7A′, A′′). The abnormal elastic fibers had a moth-eaten, fragmented appearance and consisted of a diminished elastin core embedded in a prominent amorphous matrix (Fig 7B′, B′′). The elastic fiber morphologic features appeared normal in other locations within the optic nerve and sclera of the same eyes with pseudoexfoliation syndrome (not shown).

Effect of Lysyl Oxidase Inhibition on Elastogenesis In Vitro

In primary cultures of ONH astrocytes obtained from normal donor eyes (n = 3), basal expression levels of lysyl oxidases were highest for LOX, followed by LOXL1, and were lowest for LOXL2, whereas basal expression levels of elastic fiber proteins were similar for fibrillin-1, fibulin-4, and elastin. Expression levels of lysyl oxidases and elastic proteins could be increased significantly (LOXL1, 3-fold; elastin, 2-fold; fibrillin-1, 5-fold; fibulin-4, 2-fold) by TGF-β1 (5 ng/ml) after incubation for 2 to 14 days (data not shown). By immunocytochemistry, weak expression of LOX, LOXL1, and LOXL2 was observed in the cytoplasm of individual ONH astrocytes. Elastic proteins also were expressed mainly in-

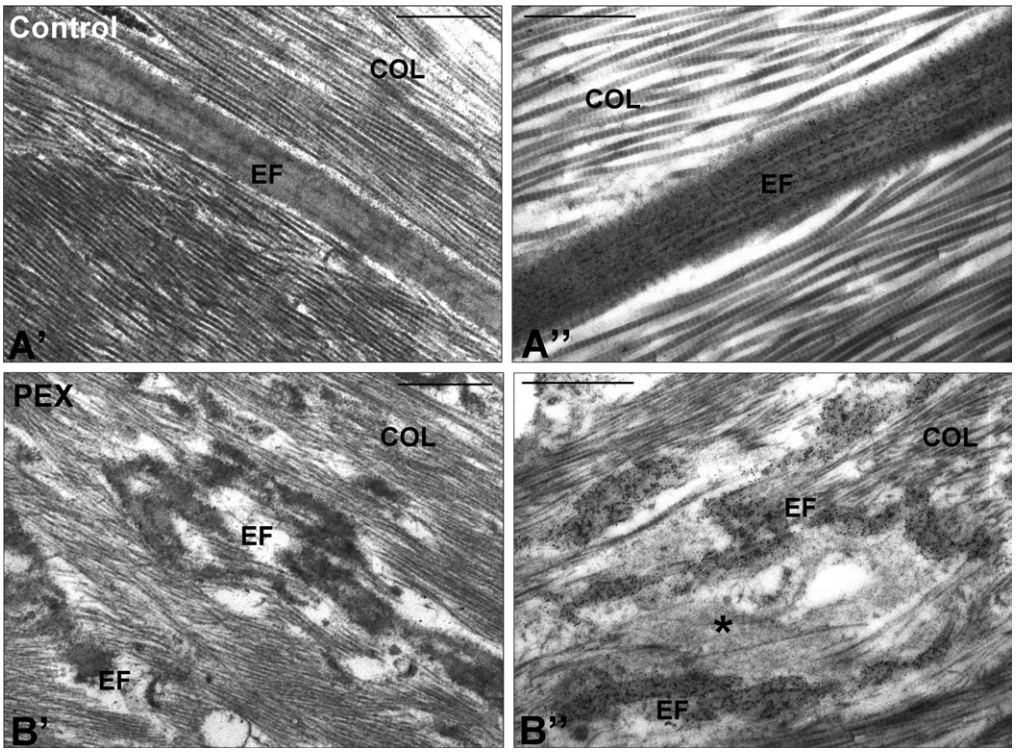

Figure 7. Transmission electron micrographs showing the ultrastructure and anti-elastin immunogold staining patterns of elastic fibers in the laminar beams of a normal human donor eye (age, 79 years) and an eye with pseudoexfoliation (PEX) syndrome (age, 83 years). **A**, Elastic fibers (EFs) with (**A′**) normal structure and (**A′′**) homogenous anti-elastin immunoreactivity in control tissue. **B**, Abnormal EFs showing a (**B′**) moth-eaten structure and consisting of (**B′′**) elastin-positive patches interspersed with amorphous material (asterisks) in PEX syndrome tissue. COL = collagen fibers. Magnification bars = 1 μm.

tracellularly and were immunolocalized to few extracellular fibrous structures (Fig 8A, available at http://aaojournal.org). On stimulation with TGF-β1 (5 ng/ml), intracellular expression levels of lysyl oxidases were increased markedly, whereas fibrillin-1 and fibulin-4 could be localized mainly to prominent microfibrillar networks in the extracellular space (Fig 8B, available at http://aaojournal.org). Deposition of insoluble elastin into the extracellular matrix was not observed before 10 days of culture.

To determine if LOXL1 is required for elastic fiber assembly, postconfluent cells were cotreated with TGF-β1 (5 ng/ml) and BAPN (50 μg/ml) up to 14 days to inhibit lysyl oxidase activity. β-Aminopropionitrile is a widely used small-molecule inhibitor of lysyl oxidase activity and is an efficient competitive inhibitor of LOXL1 in vitro.[26] The presence of BAPN markedly decreased immunostaining for elastin in the extracellular matrix, whereas fibrillin-1 and fibulin-4 immunostaining patterns appeared to be unchanged (Fig 8C, available at http://aaojournal.org). The treatment with BAPN had no effect on cell viability or elastin expression (not shown). These observations suggest that lysyl oxidase cross-linking is important for elastic fiber formation by ONH astrocytes in vitro.

Discussion

Alterations of the Extracellular Matrix in the Lamina Cribrosa of Pseudoexfoliation Syndrome Eyes

A growing body of evidence supports the concept that IOP-related stress and strain affecting the load-bearing connective tissues of LC and peripapillary sclera and subsequent damage to optic nerve axons at the level of the LC are central determinants in the pathophysiology of glaucoma.[27–29] The glaucomatous ONH shows a reduced compliance to IOP,[30] which is presumed to result from extracellular matrix changes including degeneration of elastic fibers, adversely affecting the structural and biomechanical properties of the ONH.[31–33] Elastic fibers are one of the major extracellular components providing the LC and peripapillary insertion zone with elasticity and resilience to adapt to changes and fluctuations in IOP. Elastic fiber formation, maintenance, and stability have been shown essentially to involve the cross-linking action of LOXL1.[5] Thus, LOXL1 generally has been considered a crucial factor in connective tissue remodeling during dynamic processes such as tissue injury and repair.

In this study, LOXL1 was found to be the most abundant lysyl oxidase isoform in the normal human LC, where it localized mainly to astrocytes in close association with a complex elastic fiber network comprising both elastic fibers and microfibrillar bundles. Occasionally, LOXL1 also could be observed along elastic fibers in colocalization with tropoelastin, supporting the notion of its functional role as a key enzyme in elastic fiber stabilization and maintenance. This assumption also is substantiated by in vitro inhibition studies showing that lysyl oxidase cross-linking is required for elastin deposition to the extracellular matrix and for

elastic fiber assembly by stimulated ONH astrocytes after prolonged time in culture.

Compared with age-matched controls, eyes with pseudoexfoliation syndrome revealed a statistically significant downregulation of LOXL1 and elastic proteins, both on the mRNA and protein levels, which seemed to be confined to the LC in the posterior segment of the eye. Expression levels of other lysyl oxidase isoforms and of collagen types I, III, and IV in pseudoexfoliation syndrome specimens showed no significant differences compared with controls. In contrast, POAG specimens showed no significant differences in expression levels of LOXL1 and elastic proteins compared with controls but confirmed a previously reported significant upregulation of collagen type IV despite low sample size.[34] To discover any potential effects of IOP, early and late stages of pseudoexfoliation syndrome as well as pseudoexfoliation glaucoma eyes were included in this study. In fact, coordinated downregulation of LOXL1 and elastic fiber constituents was observed in all pseudoexfoliation syndrome specimens with and without glaucoma, including very early stages of the disease, suggesting a pseudoexfoliation-specific primary dysregulation in LOXL1 and elastic protein expression.

Reduced expression levels of LOXL1 and elastic proteins obviously were associated with pronounced structural alterations of elastic fibers involving a disorganized and fragmented network of ultrastructurally abnormal elastin fibers in the laminar beams of pseudoexfoliation syndrome eyes. Fragmentation of elastic fibers without increase in elastin synthesis may be the result of impaired fiber stabilization by LOXL1 deficiency, increased proteolytic activity by elastin-degrading enzymes such as matrix metalloproteinases and elastase, or both, which remains to be analyzed in future studies. Nevertheless, the present findings in nonglaucomatous pseudoexfoliation syndrome eyes confirm and expand the result of previous studies showing a marked and site-specific elastosis in the LC of patients with pseudoexfoliation glaucoma, which was significantly more pronounced than in POAG and other types of glaucoma.[19,20] Rather, similar structural alterations in very early stages of the disease, which largely may exclude the effect of long-lasting and high levels of IOP, suggest basic alterations in elastic fiber homeostasis caused by *LOXL1* dysregulation in the LC of pseudoexfoliation eyes.

Potential Mechanisms of *LOXL1* Dysregulation in the Lamina Cribrosa of Pseudoexfoliation Syndrome Eyes

To date, multiple studies have confirmed variants in exon 1 of the *LOXL1* gene as principal genetic risk factors for pseudoexfoliation syndrome or pseudoexfoliation glaucoma, accounting for virtually all pseudoexfoliation cases within all geographical populations analyzed.[4] None of these studies reported any significant differences between pseudoexfoliation syndrome and pseudoexfoliation glaucoma, suggesting that the *LOXL1* gene may contribute to disease onset and may confer risk of glaucoma mainly through pseudoexfoliation. It therefore can be hypothesized that the *LOXL1* missense variants that increase the risk of pseudoexfoliation syndrome are associated with a reduction in LOXL1 expression and activity in the LC. Confirming previous reports on ocular[6] and extraocular[3] tissues, the expression level of LOXL1 in the LC was affected only by rs1048661 alleles in both pseudoexfoliation syndrome and control samples. However, the risk alleles of rs3825942 alleles, which were overrepresented in pseudoexfoliation syndrome cases, had no effect on LOXL1 expression levels. Because the risk genotype of rs1048661 was distributed equally among pseudoexfoliation syndrome and control cases, it cannot sufficiently explain the reduced LOXL1 expression levels in the LC of pseudoexfoliation syndrome eyes. Therefore, the findings support the notion that the pseudoexfoliation-specific dysregulation of LOXL1 expression, which previously also was demonstrated in various anterior segment tissues of pseudoexfoliation syndrome eyes,[6,7] may be modulated by additional pseudoexfoliation-associated genetic or external factors. Moreover, the pseudoexfoliation-associated missense variants are common in the normal population and show a different allele frequency in certain nonwhite populations of pseudoexfoliation syndrome patients, founding the quest for other causative variants possibly affecting *LOXL1* regulation.[35] Recently, novel polymorphisms in the promoter region of *LOXL1* have been identified to be associated significantly with pseudoexfoliation syndrome and pseudoexfoliation glaucoma in a United States white population and were suggested to influence *LOXL1* gene expression by causing a reduction in LOXL1 protein expression and activity.[36] Further functional analyses of the *LOXL1* promoter polymorphisms will shed some light on these unresolved issues.

In the posterior segment, dysregulated expression of LOXL1 apparently was confined to the LC of pseudoexfoliation syndrome eyes, indicating that other tissue-specific external factors may modulate local LOXL1 expression levels in addition to genetic predisposition. Expression of LOXL1 by ONH astrocytes may be sensitive to small fluctuations in IOP or locally active factors involved in pseudoexfoliation syndrome pathophysiology. Lysyl oxidases have been shown to be regulated by a variety of external factors, including mechanical stress[37] and TGF-β1,[38] a key mediator in the fibrotic pseudoexfoliation process.[39] In accordance, TGF-β1 was shown to induce expression of LOXL1 together with elastic fiber constituents by cultured ONH astrocytes in this study. However, the pseudoexfoliation-specific genetic background may alter the cellular response to these or other environmental stimuli in vivo, and future studies are planned to investigate the combined effect of genotype and external factors on matrix assembly by ONH astrocytes in vitro.

Potential Consequences of *LOXL1* Dysregulation in the Lamina Cribrosa of Pseudoexfoliation Syndrome Eyes

Lysyl oxidase-mediated cross-links determine the tensile and mechanical properties of the extracellular matrix and contribute to the stability of connective tissues.[22] Hence, dysregulation of lysyl oxidases was shown to underlie the

onset and progression of various pathologic conditions affecting connective tissues, such as fibrotic disorders and neurodegenerative and cardiovascular diseases.[40] Reduced expression of lysyl oxidase family members has been associated particularly with the pathophysiology of cardiovascular diseases, lung emphysema, pelvic organ prolapse, and cutis laxa and has been linked to structural alterations of elastin and collagen fibers together with altered biomechanical properties of tissues. For instance, perivascular matrix alterations secondary to LOX deficiency have been proposed as causes of abdominal aortic aneurysm and spontaneous coronary artery dissection.[41] Low expression levels of LOXL1, fibrillin-1, and elastin also were reported in fibroblast cultures derived from patients with cutis laxa,[42] suggesting the existence of common regulation mechanisms for these genes. Overall, these findings suggest that reduced expression and activity of lysyl oxidases result in mechanically weaker connective tissues.

Consistently, reduced expression of LOXL1, a key enzyme for maintenance of functional elastic fibers, in the LC of pseudoexfoliation syndrome eyes may account for the elastotic matrix changes, which are supposed to alter the biomechanical properties of this critical structure significantly. A recent study applied atomic force microscopy nanoindentation for probing the mechanical properties of unfixed cryosections of the LC in pseudoexfoliation syndrome and control eyes. The analyses revealed a statistically significant decrease in Young's modulus of elasticity, which is a measure of tissue stiffness, of LC specimens obtained from pseudoexfoliation syndrome eyes compared with control eyes.[43] This marked decrease in stiffness, implying an increased deformability of the ONH in response to IOP, may facilitate mechanical damage to optic nerve axons and may render pseudoexfoliation syndrome eyes more vulnerable to elevated IOP. Abnormally cross-linked LOXL1-positive pseudoexfoliation material aggregates, accumulating within the posterior regions of the peripapillary sclera, optic nerve sheaths, and blood vessel walls, may contribute further to altered biomechanical properties of the ONH in pseudoexfoliation syndrome eyes.

In conclusion, the present findings provide evidence for a pseudoexfoliation-specific elastinopathy of the LC resulting from a primary disturbance in LOXL1 regulation and elastic fiber homeostasis, possibly leading to alterations of biomechanical properties. Such an inherent weakness of the LC may render eyes with pseudoexfoliation syndrome more vulnerable to IOP-induced optic nerve damage, even at lower levels of stress, and may explain why the probability of having glaucomatous damage is higher in eyes with pseudoexfoliation than in those without for the same level of IOP.[16,17] Thus, LOXL1 likely represents a major IOP-independent susceptibility factor for a pseudoexfoliation-associated risk of glaucoma development. Interestingly, reduced levels of LOXL2 and elastin in ONH tissue of black donors compared with those of white American donors already have suggested LOXL2 previously as a candidate susceptibility gene for a population-specific risk of POAG.[44] The present findings substantiate a critical role of lysyl oxidases for the structure and stability of individual ONH tissues. The findings may have direct consequences for the clinical management of pseudoexfoliation patients, underlining the need for an exact diagnosis, a strict IOP-reducing therapy, and close and regular follow-up.

References

1. Ritch R, Schlötzer-Schrehardt U. Exfoliation syndrome. Surv Ophthalmol 2001;45:265–315.
2. Schlötzer-Schrehardt U, Naumann GOH. Perspective—ocular and systemic pseudoexfoliation syndrome. Am J Ophthalmol 2006;141:921–37.
3. Thorleifsson G, Magnusson KP, Sulem P, et al. Common sequence variants in the *LOXL1* gene confer susceptibility to exfoliation glaucoma. Science 2007;317(5843):1397–400.
4. Chen H, Chen LJ, Zhang M, et al. Ethnicity-based subgroup meta-analysis of the association of *LOXL1* polymorphisms with glaucoma. Mol Vis 2010;16:167–77.
5. Liu X, Zhao Y, Gao J, et al. Elastic fiber homeostasis requires lysyl oxidase-like 1 protein. Nat Genet 2004;36:178–82.
6. Schlötzer-Schrehardt U, Pasutto F, Sommer P, et al. Genotype-correlated expression of LOXL1 in ocular tissues of patients with pseudoexfoliation syndrome/glaucoma and normal subjects. Am J Pathol 2008;173:1724–35.
7. Schlötzer-Schrehardt U. Molecular pathology of pseudoexfoliation syndrome/glaucoma—new insights from *LOXL1* gene associations. Exp Eye Res 2009;88:776–85.
8. Konstas AGP, Stewart WC, Stromann GA. Clinical presentation and initial treatment patterns in patients with exfoliation glaucoma versus primary open-angle glaucoma. Ophthamol Surg Lasers 1997;28:111–7.
9. Teus MA, Castejon MA, Calvo MA, et al. Intraocular pressure as a risk factor for visual field loss in pseudoexfoliative and in primary open-angle glaucoma. Ophthalmology 1998;105:2225–30.
10. Puska P, Vesti E, Tomita G, et al. Optic disc changes in normotensive persons with unilateral exfoliation syndrome: a 3-year follow-up study. Graefes Arch Clin Exp Ophthalmol 1999;237:457–62.
11. Yarangümeli A, Davutluoglu B, Köz ÖG, et al. Glaucomatous damage in normotensive fellow eyes of patients with unilateral hypertensive pseudoexfoliation glaucoma: normotensive pseudoexfoliation glaucoma? Clin Exp Ophthalmol 2006;34:15–9.
12. Koz OG, Turkcu MF, Yarangumeli A, et al. Normotensive glaucoma and risk factors in normotensive eyes with pseudoexfoliation syndrome. J Glaucoma 2009;18:684–8.
13. Pohjanpelto P. Influence of exfoliation syndrome on prognosis in ocular hypertension > 25 mmHg. A long-term follow-up. Acta Ophthalmol 1986:64:39–44.
14. Grodum K, Heijl A, Bengtsson B. Risk of glaucoma in ocular hypertension with and without pseudoexfoliation. Ophthalmology 2005;112:386–90.
15. Davanger M, Ringvold A, Blika S. Pseudo-exfoliation, IOP and glaucoma. Acta Ophthalmol 1991;69:569–73.
16. Topouzis F, Harris A, Wilson MR, et al. Increased likelihood of glaucoma at the same intraocular pressure in subjects with pseudoexfoliation: The Thessaloniki Eye Study. Am J Ophthalmol 2009;148:606–13.
17. Heijl A, Bengtsson B, Hyman L, et al. Natural history of open-angle glaucoma. Ophthalmology 2009;116:2271–6.
18. Leske MC, Heijl A, Hyman L, et al. Predictors of long-term progression in the early manifest glaucoma trial. Ophthalmology 2007;114:1965–72.

19. Netland PA, Ye H, Streeten BW, Hernandez MR. Elastosis of the lamina cribrosa in pseudoexfoliation syndrome with glaucoma. Ophthalmology 1995;102:878–86.
20. Pena JD, Netland PA, Vidal I, et al. Elastosis of the lamina cribrosa in glaucomatous optic neuropathy. Exp Eye Res 1998;67:517–24.
21. Wagenseil JE, Mecham RP. New insights into elastic fiber assembly. Birth Defects Res C Embryo Today 2007;81:229–40.
22. Csiszar K. Lysyl oxidases: a novel multifunctional amine oxidase family. Prog Nucleic Acid Res Mol Biol 2001;70:1–32.
23. Zenkel M, Lewczuk P, Jünemann A, et al. Pro-inflammatory cytokines are involved in initiation of the abnormal matrix process in pseudoexfoliation syndrome/glaucoma. Am J Pathol 2010;176:2868–79.
24. Clark, G. Staining Procedures. 3rd ed. Baltimore: Williams & Wikins; 1973: 50, 60.
25. Lambert W, Agarwal R, Howe W, et al. Neurotrophin and neurotrophin receptor expression by cells of the human lamina cribrosa. Invest Ophthalmol Vis Sci 2001;42:2315–23.
26. Tang SS, Trackman PC, Kagan HM. Reaction of aortic lysyl oxidase with beta-aminoproprionitrile. J Biol Chem 1983;258:4331–8.
27. Quigley HA, Addicks EM, Green WR, Maumenee AE. Optic nerve damage in human glaucoma. II. The site of injury and susceptibility to damage. Arch Ophthalmol 1981;99:635–49.
28. Burgoyne CF, Downs JC, Bellezza AJ, et al. The optic nerve head as a biomechanical structure: a new paradigm for understanding the role of IOP-related stress and strain in the pathophysiology of glaucomatous optic nerve head damage. Prog Ret Eye Res 2005;24:39–73.
29. Sigal IA, Ethier CR. Biomechanics of the optic nerve head. Exp Eye Res 2009;88:799–807.
30. Zeimer RC, Ogura Y. The relation between glaucomatous damage and optic nerve head mechanical compliance. Arch Ophthalmol 1989;107:1232–4.
31. Hernandez MR, Ye H. Glaucoma: changes in extracellular matrix in the optic nerve head. Ann Med 1993;25:309–15.
32. Burgoyne CF, Morrison JC. The anatomy and pathophysiology of the optic nerve head in glaucoma. J Glaucoma 2001; 10(Supp11):S16–8.
33. Quigley HA, Brown A, Dorman-Pease ME. Alterations in elastin of the optic nerve head in human and experimental glaucoma. Br J Ophthalmol 1991;75:552–7.
34. Hernandez MR, Ye H, Roy S. Collagen type IV gene expression in human optic nerve heads with primary open angle glaucoma. Exp Eye Res 1994;59:41–51.
35. Williams SE, Whigham BT, Liu Y, et al. Major LOXL1 risk allele is reversed in exfoliation glaucoma in a black South African population. Mol Vis 2010;16:705–12.
36. Fan BJ, Pasquale LR, Rhee D, et al. LOXL1 promoter haplotypes are associated with exfoliation syndrome in a US Caucasian population. Invest Ophthalmol Vis Sci 2011;52: 2372–8.
37. Kirwan RP, Fenerty CH, Crean J, et al. Influence of cyclical mechanical strain on extracellular matrix gene expression in human lamina cribrosa cells in vitro. Mol Vis 2005;11:798–810.
38. Sethi A, Mao W, Wordinger RJ, Clark AF. Transforming growth factor-beta induces extracellular matrix protein crosslinking lysyl oxidase (LOX) genes in human trabecular meshwork cells. Invest Ophthalmol Vis Sci 2011;52: 5240–50.
39. Schlötzer-Schrehardt U, Zenkel M, Küchle M, et al. Role of transforming growth factor-β1 and its latent form binding protein in pseudoexfoliation syndrome. Exp Eye Res 2001;73: 765–80.
40. Rodriguez C, Rodriguez-Sinovas A, Martinez-Gonzalez J. Lysyl oxidase as a potential therapeutic target. Drug News Perspect 2008;21:218–24.
41. Sibon I, Sommer P, Lamaziere JM, Bonnet J. Lysyl oxidase deficiency: a new cause of human arterial dissection. Heart 2005;91:e33.
42. Debret R, Cenizo V, Aimond G, et al. Epigenetic silencing of lysyl oxidase-like-1 through DNA hypermethylation in an autosomal recessive cutis laxa case. J Invest Dermatol 2010; 130:2594–601.
43. Braunsmann C, Hammer CM, Rheinlaender J, et al. Evaluation of lamina cribrosa and peripapillary sclera stiffness in pseudoexfoliation and normal eyes by atomic force microscopy. Invest Ophthalmol Vis Sci 2012;53:2960–7.
44. Urban Z, Agapova O, Hucthagowder V, et al. Population differences in elastin maturation in optic nerve head tissue and astrocytes. Invest Ophthalmol Vis Sci 2007;48:3209–15.

Footnotes and Financial Disclosures

Originally received: July 25, 2011.
Final revision: March 7, 2012.
Accepted: March 7, 2012.
Available online: May 24, 2012. Manuscript no. 2011-1092.

[1] Department of Ophthalmology, University of Erlangen-Nürnberg, Erlangen, Germany.

[2] Institute of Human Genetics, University of Erlangen-Nürnberg, Erlangen, Germany.

[3] Department of Experimental Medicine I, University of Erlangen-Nürnberg, Erlangen, Germany.

Financial Disclosure(s):
The author(s) have no proprietary or commercial interest in any materials discussed in this article.

Correspondence:
Ursula Schlötzer-Schrehardt, PhD, Department of Ophthalmology, University of Erlangen-Nürnberg, Schwabachanlage 6, D-91054 Erlangen, Germany. E-mail: ursula.schloetzer-schrehardt@uk-erlangen.de.