Population Differences in Elastin Maturation in Optic Nerve Head Tissue and Astrocytes

Zsolt Urban,1,2 Olga Agapova,3 Vishwanathan Huchbagowder,2 Ping Yang,2 Barry C. Starcher,3 and M. Rosario Hernandez5

PURPOSE. Glaucomatous optic neuropathy is characterized by remodeling of the extracellular matrix with disorganization of elastic fibers in the optic nerve head (ONH). There are significant differences in prevalence of glaucomatous optic neuropathy between African Americans (AAs) and Caucasian Americans (CAs). The goal of this study was to evaluate differences in elastin synthesis and maturation in ONH tissue and cells of AA and CA donors with no eye disease, to provide a basis for underlying racial differences in susceptibility to elevated intraocular pressure.

METHODS. The amount of mature elastin in ONHs from each group of donors was evaluated by desmosine radioimmunoassay. The distribution of elastic fibers in ONH tissue was investigated by immunofluorescent staining. Elastin and lysyl oxidase mRNA levels and alternative splicing of elastin in ONH astrocytes were investigated by quantitative PCR. Tropoelastin protein expression was assessed by immunoblot analysis.

RESULTS. ONHs from AA donors had significantly reduced levels of desmosine compared with those of CAs. The inclusion of exon 23 in elastin mRNA and tropoelastin synthesis were elevated in ONH astrocytes from AA individuals. The exclusion of exon 23 in elastin mRNA and lysyl oxidase-like 2 mRNA levels was significantly reduced in astrocytes from AA compared with CA donors.

CONCLUSIONS. A reduced number of cross-linking domains in elastin and decreased lysyl oxidase-like 2 expression leads to decreased amount of mature elastin in ONHs from healthy AA individuals compared with CA donors. These results suggest ELN and LOXL2 as candidate susceptibility genes for population-specific genetic risk of primary open-angle glaucoma (POAG). (Invest Ophthalmol Vis Sci. 2007;48:3209–3215) DOI:10.1167/iovs.07-0107

Primary open-angle glaucoma (POAG) is more prevalent in black Americans of African ancestry (AA) than in Americans of white ancestry (CA), with reported frequencies of 4% in AA individuals over the age of 40 years, compared with approximately 1% in CAs.1–3 The disease’s incidence is particularly frequent in Afro-Caribbean persons, with a prevalence of 7% in Barbados4 and 8.8% in St. Lucia.5 In addition to racial differences, a positive family history of POAG is a major risk factor for the disease in AA persons.2,6 More recently the Barbados Family Study of Open-Angle Glaucoma reported evidence for linkage of POAG to chromosomes 2 and 10 in this population.7 The Advanced Glaucoma Intervention Study (AGIS)8 in which the glaucoma outcomes were compared in AA and CA patients, concluded that after failure of medical therapy, surgical trabeculectomy delays progression of glaucoma more effectively in CA than in AA patients.9 Despite significant progress in this area, the metabolic and genetic factors contributing population differences in the risk of POAG remain incompletely understood.

The extracellular matrix (ECM) within the lamina cribrosa, and especially the elastic fibers, protect the optic nerve head (ONH) by buffering intraocular pressure changes.10 Differences in the expression and synthesis of elastin, the main component of the elastic fibers, may alter the responses of the tissue to elevated intraocular pressure (IOP). Elastin is synthesized by cells as a soluble precursor, tropoelastin, a modular protein with alternating hydrophobic and cross-link domains, encoded by the elastin gene. The human elastin gene (ELN, Ensembl gene ID ENSG00000049540 [http://wwwensembl.org]; Fig. 1) consists of 34 exons, each encoding a separate domain of tropoelastin (Uniprot ID P15502). Several of these exons are subject to alternative splicing.11 Specifically, exon 23, encoding a cross-link domain, and exon 52, coding for a hydrophobic domain, have been shown to be subject to alternative splicing both in ONH tissue and in cultured ONH astrocytes.12 Altered splicing frequency of these exons may result in population differences in tropoelastin isoform patterns the impact elastin maturation. Elastin maturation is dependent on lysyl oxidase enzymes, which are responsible for oxidation of the epsilon amino groups of lysyl side chains leading to the formation of desmosine and isodesmosine cross-links in elastin.13,14

There are racial differences in the normal optic disc. AA persons have significantly larger disc areas, larger cup areas, larger cup-to-disc ratios, and smaller neural rim area-to-disc area ratios than do CA persons.2 A morphometric study determined that in AAs the ONH has a larger total area of the lamina cribrosa and a greater number of pores than in CAs.15

In patients with glaucomatous optic neuropathy, elastic fibers become disorganized in the lamina cribrosa.16,17 In vivo, elevated IOP induces the expression of the elastin gene18 as does increased hydrostatic pressure in cultured ONH astrocytes.19 Based on these results, we hypothesized that population differences in glaucomatous optic neuropathy due to elevated IOP may in part be associated with altered elastic fiber synthesis and metabolism. To test this hypothesis, we compared the amount of mature elastin in ONHs from healthy AA and CA donors with no eye disease. We also investigated the expression of the ELN and lysyl oxidase (LOX) genes in ONH astrocytes from AA and CA donors.

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MATERIALS AND METHODS

Tissue Samples
Twelve pairs of normal human eyes from AA donors (age, 60 ± 10 years) and 12 pairs from CA donors (age, 58 ± 12 years) with no history of chronic central nervous system (CNS) or eye disease were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA) and from the Mid-America Eye Bank (St. Louis, MO) within 2 to 8 hours of death (Supplementary Table S1, online at http://www.iovs.org/cgi/content/full/48/7/3209/DC1). There was no significant difference between the two groups in age (t-test) and sex (Fisher’s exact test). Optic nerves were dissected and processed within 24 hours of death, to generate ONH astrocyte cultures. For each donor, a sample of the myelinated optic nerve was processed and stained for myelin degeneration to exclude any optic nerves with undiagnosed nerve disease. Assignment of donors to AA and CA groups was based on previously published guidelines.

Astrocyte Cultures
Primary cultures of human ONH astrocytes were established as previously described in detail. Briefly, explants from the human lamina cribrosa were carefully dissected, placed in T25 flasks, and maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 with 10% fetal bovine serum (FBS) and PSFM (10,000 U/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL amphotericin B; Invitrogen-Gibco BRL, Grand Island, NY). Primary confluent cultures were established by immunopanning as described. Only cell cultures that were at least 95% pure and positive for both glial fibrillary acidic protein (GFAP) and neural cell adhesion molecules (NCAM) characterized by immunostaining were used in this study. ONH astrocytes were cultured at 37°C in

Table 1. Oligonucleotide Sequences Used for RT-PCR Analyses

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FIGURE 1. The structure of the elastin gene. Exons (boxes) and introns (lines) are drawn to different scales as indicated by the scale bars. The domain encoded by each exon is indicated by shading as explained in the illustration. Asterisks: exons subject to alternative splicing (13, 22, 23, 26A, and 32). The numbering of every fifth exon as well as alternatively spliced exons 23 and 32, subjects of this study, are shown above the diagram.
DMEM/F-12 containing 5% FBS and PSFM under a humidified atmosphere of 95% air and 5% CO2. Second-passage cells were stored in liquid nitrogen until use. Then, for each set of experiments, the cells were thawed and cultured for one more passage, so that sufficient cells from the same batch were available to use in each set of experiments. In these studies, all astrocytes were used at the third passage.

**Tissue Desmosine Assay**

Analysis of desmosine was used to determine the abundance of cross-linked elastin in ONH tissues from the right and left eye of 10 AA donors (mean age, 54 ± 18 years) and 10 CA donors (mean age, 61 ± 11 years; Supplementary Table S2, http://www.iovs.org/cgi/content/full/48/7/3209/DC1). The optic nerve heads were dissected from nor-
mal human eyes. The wet weight of the samples was 9.69 ± 1.96 mg. A sample of the posterior sclera was obtained 2 mm away from the optic disc and used for comparison. The tissues were hydrolyzed in 6 N HCl at 100°C for 24 hours, evaporated to dryness, and redissolved in water. Desmosine was quantified by radioimmunoassay (RIA) as previously described.24 Hydroxyproline was determined by amino acid analysis. For comparisons, posterior sclera from selected samples was also included in these determinations. For statistical analysis of group means Student’s t-test was used. The values for desmosine and hydroxyproline were similar in left and right eye; thus, we used the mean between eyes for the calculation.

Immunofluorescence Staining

Cells grown on coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and processed for standard indirect immunofluorescence. The Fixed coverslips were washed in PBS with 0.5% BSA (0.5% BSA/PBS) and permeabilized with 0.1% Triton X-100 in distilled water. Coverslips were blocked with 10% donkey serum (Sigma-Aldrich, St. Louis, MO) in 0.5% BSA/PBS for 30 minutes. The astrocytes were stained for single or double immunofluorescence using a polyclonal antibody against human tropoelastin, which reacts strongly with human tropoelastin and less strongly with insoluble elastin (1:100; Elastin Products Company, Owensville, MO) and a monoclonal antibody against human glial fibrillary acidic protein (GFAP, 1:400; Sigma-Aldrich) diluted in blocking solution for 2 hours at room temperature. After repeated washes with PBS, the coverslips were incubated with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen-Molecular Probes, Eugene, OR) secondary antibodies diluted in 0.5% BSA/PBS. Control samples were incubated with nonimmune serum. After they were washed with 0.5% BSA/PBS, the cells were rinsed with PBS and mounted on slides in mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) with or without DAPI (4′,6′-diamino-2-phenylindole).

Ten normal ONHs from AA (mean age, 59.8 ± 12 years) and CA (mean age, 65.1 ± 11.8 years) donors were fixed in buffered 10% formaldehyde at enucleation and embedded in paraffin. Six-micrometer cross-sections of the ONH at the level of the lamina cribrosa were used for elastin and GFAP immunodetection. Sections were processed for single or double immunofluorescence staining using the same primary and secondary antibodies used for cell culture staining. For negative control, the primary antibody was replaced for nonimmune serum. To control for cross-reactivity in double immunofluorescence, sections were incubated with primary antibody followed by the wrong-species secondary antibody. Serial sections of normal AA and CA eyes were stained simultaneously to control for variations in immunostaining.

SDS-PAGE and Western Blot Analysis

For protein extraction, ONH astrocytes were grown on 35-mm plates to confluence, washed twice in cold 1× PBS and incubated for 15
RNA Isolation and Reverse Transcription

Total RNA was isolated from human ONH astrocytes cultured from 12 AA and 12 CA donors (TRizol; Invitrogen-Life Technologies, Carlsbad, CA). After isolation, RNA was precipitated and resuspended in 10 μL nuclease-free water. RNA absorbance at 260 nm and absorbance ratios at 260/280 nm were measured. Random-primed cDNA was synthesized by the appropriate secondary antibody conjugated to horseradish peroxidase for 1.5 hours. For the detection of membrane-bound antibodies, we used the enhanced chemiluminescence (ECL) Western blot detection system (GE Healthcare). The membranes were reprobed with phase-dense footprinting (DPD) for the detection of bands with exon 23 or with exon 23 and primers that recognize total elastin

Analysis of Elastin mRNA Splicing Variants by Real-Time Quantitative PCR

For elastin mRNA, relative quantification of gene expression was performed using the standard curve method (1:2.5, 1:10, 1:40, and 1:160; according to the manufacturer’s instructions; 7700 Prism, Applied Biosystems) of a mix of all samples were used for standard curves. Relative amounts of elastin mRNA were calculated from the standard curve and normalized to the relative amounts of 18S RNA, which was obtained from a similar standard curve. The results were expressed as the mean ± SE of the relative amount of normalized mRNA. Significant differences between the means were set at P < 0.05 (Student’s t-test).

For lysyl oxidases, relative quantification of gene expression was performed using the ΔCt method. Cycle thresholds (Ct) were determined using the thermocycler software for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and each of the lysyl oxidase isoforms. The expression level of each lysyl oxidase was determined relative to GAPDH by subtracting the average Ct, C for GAPDH from each of the Ct levels for lysyl oxidase measurements to obtain ΔCt, raising 2 to the power of −ΔCt. For statistical analysis of group means, Student’s t-test was used.

Analysis of Elastin mRNA Splicing Variants by Real-Time Quantitative PCR

To detect elastin mRNA splicing variants, we designed primers or primers and probes (Table 1) that specifically recognize elastin cDNA with exon 32 or with exon 23 and primers that recognize total elastin (all splicing variants; Primer Express software; Applied Biosystems). For the detection of ELN splicing variant with exon 32, 5 μL of cDNA diluted 1:20 were amplified in 25 μL of reaction mixture with SYBR Green (Bio-Rad) with specific primers, and quantitative PCR was performed by monitoring the increase of green fluorescence in real time (MiyQ; Bio-Rad). For the detection of ELN splicing variant with exon 23, 5 μL of cDNA diluted 1:20 were amplified in 25 μL of reaction mixture with SYBR Green (Bio-Rad) with specific primers and probe. We used a labeled probe for the detection elastin with exon 25 to avoid nonspecific amplification. The ratio of splicing variations detected with exons 23 and 25 to total elastin was calculated using the standard curve method. Tenfold serial dilutions of human elastin cDNA with exon 23 (clone D) or with exon 32 (clone E) cloned into pb8-5K were used for standard curves. All determinations were performed in duplicate. Significant differences between the means were set at P < 0.05 (Student’s t-test).

Figure 5. Tropoelastin expression in primary ONH astrocytes. (A) Quantitative RT-PCR analysis of elastin mRNA expression normalized to 18S RNA showed significantly higher elastin mRNA level in astrocytes from AA donors compared to CA. (B) Immunoblotting for tropoelastin (ELN) demonstrated consistently increased expression of tropoelastin in AA samples compared with CA; β-actin was used as a loading control.
RESULTS

Reduced Mature Elastin in AA ONH Tissue

We first investigated the amount of mature elastin in ONH tissue isolated from healthy AA and CA donors by desmosine radioimmunoassays of protein hydrolysates. Results were normalized to the total protein content of the samples. AA ONHs contained significantly less desmosine than did CA samples (Fig. 2A). In contrast, the levels of hydroxyproline, a measure for collagen content were the same (Fig. 2B). As an additional control, we investigated sclera samples from the same donor eyes for both desmosine and hydroxyproline. There was no significant difference between the AA and CA groups of sclera samples for these amino acids (data not shown).

Distribution of Elastin in ONH Tissues and Astrocytes

To study the amount and distribution of elastin, we stained sections of ONHs with an antibody that reacts with both elastin and its soluble precursor, tropoelastin. To examine the localization of astrocytes, the same sections were stained for GFAP. Elastic fibers and astrocyte cell bodies were found in the cribriform plates (Figs. 3A, 3B). In contrast, nerve bundles lacked elastic fibers but contained astrocyte processes. Cultured ONH astrocytes maintained the expression of both tropoelastin and GFAP (Figs. 3C–F). However, most of the elastin staining was intracellular in these cultures, suggesting that ONH astrocytes had limited ability to assemble elastic fibers in vitro. Despite our initial observations of reduced amounts of mature elastin in ONH tissue, immunofluorescent staining of samples from age-matched AA and CA donors did not show consistent group differences in the amount or distribution of elastin (Fig. 4).

Elastin Expression in ONH Astrocytes

Elastin expression was evaluated by quantitative RT-PCR assays using RNA isolated from ONH astrocytes. Surprisingly, cells from AA donors showed higher steady state levels of elastin mRNA than did the CA cells (Fig. 5A). Immunoblot analysis, furthermore, demonstrated correspondingly increased levels of tropoelastin in AA astrocytes (Fig. 5B).

Elastin mRNA Splicing

We hypothesized that altered splicing frequency of exons 23 and 32 may result in population differences in tropoelastin isoform patterns impacting elastin maturation. Therefore, we used quantitative PCR to measure the abundance of elastin mRNA isoforms containing exon 23 or 32 and normalized the results to total elastin mRNA levels in ONH astrocytes. Exon 23 was included in elastin mRNA significantly less frequently in AA than in CA astrocytes (Fig. 6A). In contrast, there were no significant group differences in the splicing of exon 32 (Fig. 6B).

Lysyl Oxidase Expression

To test whether variation in lysyl oxidase expression contributes to population differences in the amount of mature elastin

![Figure 6](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933444/) Alternative splicing of elastin mRNA and lysyl oxidase expression in ONH astrocytes. Quantitative RT-PCR analysis of alternative splicing of exons 23 (A) and 32 (B) in elastin mRNA from cultured ONH astrocytes. The fraction of elastin mRNA with exon 23 was significantly lower in AA than in CA ONH astrocytes. No significant (NS) difference was found in the splicing of exon 32. (C) The expression of lysyl oxidases relative to GAPDH was determined by quantitative RT-PCR, by the \( \Delta C_t \) method. The average of triplicate measurements of each of 12 CA and 10 AA samples are presented. Error bars, SEM. LOXL2 was most highly expressed among the lysyl oxidases and showed significantly lower levels in AA than in CA samples. Expression differences in other lysyl oxidases by race were not significant.
in ONHs, we analyzed steady state mRNA levels for lysyl oxidase enzymes LOX and LOXL1, -2, -3, and -4 in ONH astrocytes. We found that LOXL2 was expressed at the highest levels in astrocytes. Moreover, astrocytes from AA donors expressed significantly lower levels of LOXL2 than did cells from CA donors (Fig. 6C).

**Discussion**

Our studies showed significantly lower amounts of mature elastin in the optic nerve heads of AA individuals than in CA donors, despite increased expression of tropoelastin in ONH astrocytes isolated from AA tissues. We identified two likely causes of reduced elastin maturation in AA individuals. First, ONH astrocytes from AA donors showed reduced inclusion of alternatively spliced exon 23 into elastin mRNA. Exon 23 encodes a cross-linking domain; therefore, its absence from tropoelastin is expected to reduce the efficiency of elastin cross-linking.

The second potential cause of reduced elastin maturation in AA individuals is reduced lysyl oxidase expression. Indeed, our studies showed significantly lower LOXL2 mRNA levels in AA astrocytes than in CA astrocytes. LOXL2 has recently been shown to have lysyl oxidase activity, similar to LOX and LOXL1, and to cross-link extracellular matrix molecules such as elastin and collagen. We also demonstrated that LOXL2 is the major lysyl oxidase expressed by ONH astrocytes, and therefore its expression is an important determinant of the total lysyl oxidase activity in the cribiform plates.

Limitations of this study include a relatively low number of subjects, which did not provide sufficient power to conduct multivariate analysis to control for confounding variables such as the age and the sex of the donors. However, the mean age and the sex distribution were not significantly different between the two test groups, suggesting that these variables did not have major effects on our results.

Taken together, our studies uncovered significant differences in elastin synthesis and maturation between the studied population groups. Based on the observation of degenerative changes in the elastic fibers of the lamina cribrosa in patients with POAG and the increased prevalence of POAG in AA individuals, we propose that such differences in elastin maturation may contribute to the population-specific genetic risks of POAG. Finally, our results implicate ELN and LOXL2 as candidate susceptibility genes for POAG.

**References**