Increased Elastin Expression in Astrocytes of the Lamina Cribrosa in Response to Elevated Intraocular Pressure

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PURPOSE. To determine whether abnormal elastin synthesis in the glaucomatous optic nerve head and lamina cribrosa is due to elevated intraocular pressure (IOP) or secondary to axonal injury, monkeys with elevated IOP and with optic nerve transection were compared.

METHODS. Unilateral, chronic elevated IOP was induced in 11 rhesus monkeys by laser scarification of the trabecular meshwork. IOP was monitored weekly and maintained within 25 to 45 mm Hg for 7 to 56 weeks. In 6 monkeys, unilateral, optic nerve transection was performed, and monkeys were killed after 4 weeks. Optic nerve damage was assessed by stereoscopic slit-lamp biomicroscopy and fundus photography and by confocal scanning laser ophthalmoscopy. The eyes were enucleated and processed for immunohistochemistry and in situ hybridization and for electron microscopic immunogold detection of elastin. Axonal loss was evaluated in cross sections of the optic nerve stained with phenylenediamine.

RESULTS. Compared with normal contralateral controls, the lamina cribrosa of eyes with elevated IOP exhibited markedly increased elastin and the presence of elastic aggregates in the extracellular matrix and upregulation of elastin mRNA in the astrocytes. In transected eyes, elastin appeared as fine fibers in the lamina cribrosa, without elastotic aggregates, and without new synthesis or abnormal deposition of elastin. At the transected site, new synthesis of elastin was present in the pia mater but not in astrocytes in the glial scar.

CONCLUSIONS. This study demonstrates that abnormal elastin synthesis in experimental glaucomatous optic neuropathy in the monkey is specific to elevated IOP and not secondary to axonal loss. The mechanisms by which elevated IOP induces enhanced elastin synthesis in laminar astrocytes are unknown but differ from those involved in acute axonal injury such as transection, where inflammation and breakdown of the blood-


Primary open angle glaucoma (POAG), a common form of optic neuropathy, is characterized by irreversible and progressive loss of axons from the retinal ganglion cells (RGCs), usually in response to abnormally elevated intraocular pressure (IOP). Because studies in human tissues are limited by their lack of a temporal sequence required to establish the relationship between cause and effect, different animal models have been developed to study the pathophysiological mechanisms of glaucoma. Although there is not a perfect animal model for glaucoma, monkeys with glaucoma induced by laser scarification of the trabecular meshwork have been of especial interest.1–4 There is substantial evidence that damage to the optic nerve axons occurs at the level of the lamina cribrosa in the optic nerve head (for comprehensive reviews, see Refs. 5–9). In the glaucomatous optic nerve, cupping of the optic disc and compression, stretching, and rearrangement of the cribiform plates of the lamina cribrosa occur in response to elevated IOP.1 Remodeling of the optic nerve head in glaucoma involves astrocyte responses and changes in the extracellular matrix (ECM) composition and distribution.10–22 Elastic fibers are a major component of the ECM of the lamina cribrosa of humans and nonhuman primates as demonstrated previously by our laboratory and by others.2 Elastic fibers provide the lamina with elasticity and resiliency to adapt to changes in IOP. Elastic fibers are composed of an amorphous core containing elastin, an insoluble polymer of 70 kDa, tropoelastin monomers, and a microfibrillar component, located primarily around the periphery and interspersed within the amorphous core. Elastin is secreted by elastogenic cells as tropoelastin, a soluble precursor, which is assembled into insoluble elastin by cross-linking in the extracellular space.3 Type 1B astrocytes are the major cell type in the human optic nerve head and are responsible for the synthesis of ECM macromolecules in the lamina cribrosa.4–6 Type 1B astrocytes express elastin perinatally and in early childhood, but elastin mRNA is not detectable in normal adult optic nerve heads by in situ hybridization (ISH) or by RT-PCR.7,8 In glaucomatous optic neuropathy, expression of elastin mRNA reappears, and abnormal deposition of elastin and elastosis follows.7,9 Elastotic degeneration of the ECM of the lamina cribrosa in glaucoma is characterized by a significant increase in the area occupied by elastin, which is organized in large amorphous aggregates of irregular and varied shapes.7,9,10 Elastic fiber degeneration or elastosis is apparent in early stages of POAG, with evidence of redistribution and disorganization of the elastic component, accompanied by upregulation of tropoelastin mRNA expression. These changes in elastic fibers may contribute to the changes in compliance and resiliency observed in the glaucomatous lamina cribrosa.11–15 After any type of insult to the central nervous system, astrocytes exhibit a vigorous response, specific to the region or

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Supported by National Eye Institute, Bethesda, Maryland, Grants EY06416, EY02687, and EY02698 and unrestricted grants from Research to Prevent Blindness to the Departments of Ophthalmology and Visual Sciences at Washington University and University of Wisconsin-Madison.

Submitted for publication September 20, 2000; revised January 8, 2001, and April 25, 2001; accepted June 7, 2001.

Commercial relationships: N.

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to the nature of the insult involved, and become reactive.\textsuperscript{14} In the optic nerve head, mature astrocytes become reactive in response to elevated IOP. Reactive astrocytes exhibit a characteristic phenotype and actively synthesize a variety of molecules in response to stress.\textsuperscript{15} Reactive astrocytes adopt many of the properties of immature astrocytes, such as motility and expression of developmentally regulated molecules, for instance, NCAM-180 and elastin in the case of glaucoma.\textsuperscript{6,16} In addition to participating in the remodeling of the ECM, reactive astrocytes also synthesize cytokines and mediators that are highly neurotoxic for RGCs.\textsuperscript{14,17,18}

It has been suggested that the neurodegeneration that follows optic nerve transection has similarities to glaucomatous optic nerve degeneration, and thus optic nerve crush and/or transection animal models have been used to develop strategies for neuroprotection in glaucoma.\textsuperscript{19–22} Elevated IOP is the most prominent risk factor in the development and progression of glaucoma, yet the mechanisms by which IOP damages the optic nerve head remain unknown. It is possible that the molecular mechanisms responsible for remodeling of the ECM in the optic nerve head are specific to mechanical stress associated with elevated IOP in glaucoma and not a secondary event due to loss of axons.

We designed this study to compare the expression of elastin, a marker of ECM remodeling in the glaucomatous ONH, in monkeys with laser-induced experimental glaucoma and in monkeys with optic nerve transection. To detect qualitative and quantitative changes in synthesis and deposition of elastin by type 1B astrocytes, we used immunohistochemistry at the light and electron microscopic level. To determine whether new elastin is synthesized in the ONH, we used ISH for detection and localization of elastin mRNA to specific cell types.

**Materials and Methods**

**Subjects**

All studies were performed following the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Seventeen male and female rhesus monkeys (*Macaca mulatta*), ages 3 to 23 years, were separated into two groups as follows:

Ten animals had experimental glaucoma (referred to as experimental glaucoma group [ExpG]) induced in one eye by argon laser scarification of the trabecular meshwork (ALTS).\textsuperscript{21–24} A standard clinical argon laser (model 900; Coherent Radiation, Palo Alto, CA) and slit-lamp delivery system was used to produce 50 to 250 spots of 50-\(\mu\)m spot diameter, 1 to 1.5 W energy, and 0.5-second duration, over 270° of the angle circumference. This procedure was repeated at approximately 3- to 4-week intervals, when ocular inflammation had subsided, each time leaving a different quadrant untreated, until an elevation in IOP was achieved. In no case did IOP decrease, requiring additional ALTS, once it became elevated. One additional animal (11) did not undergo laser surgery but developed elevated IOP after an intracapsular lens extraction procedure as part of another protocol. In this animal, the vitreous in the ExpG eye moved forward, possibly blocking the anterior chamber angle, and biomicroscopic evidence of inflammation (anterior chamber cells and flare) was present for 1 or 2 weeks postoperatively. Anesthesia for lasering was ketamine (10 mg/kg IM) + acepromazine (0.2–1 mg/kg IM). Frequently the animals also received IM methohexital anesthesia, if photography and scanning laser ophthalmoscopy was done immediately before laser treatment (see below). In five of these animals, IOP was maintained at \(\sim 40\) to 50 mm Hg for 8 to 12 weeks, whereas in the other four animals IOP was kept at \(\sim 20\) to 30 mm Hg for 27 to 36 weeks. If IOP was higher than desired, the monkeys were treated topically once or twice daily with a single drop of one or more of the following until the desired IOP was achieved: Timoptic-XE (0.5% timolol maleate in gel-forming vehicle; Merck & Co, West Point, PA), Alphagan (0.2% brimonidine tartrate; Allergan, Irvine, CA), Trusopt (2% dorzolamide hydrochloride; Merck), PGF2a-1-isopropylester (2 µg in 5 µl saline; donated by Pharmacia Corp, Pepack, NJ). If necessary, acetazolamide (5 mg/kg, Ben Venue Laboratories, Bedford, OH) IM was also given once or twice daily.

Monkey 14 had the IOP in the lasered eye reduced to control levels with Timoptic, Alphagan, Trusopt, and PGF2a-1-isopropylester 1 week before being killed to determine whether cupping was due to a permanent structural change or merely a consequence of elevated IOP.

Six animals underwent transection of the optic nerve (ONT) in one eye, preserving the central vessels as verified by the absence of hemorrhage by indirect ophthalmoscopy at the completion of the transection and again several days later.\textsuperscript{25} Briefly, an oculoplastic surgeon performed a lateral orbitotomy under pentobarbital anesthesia (15 mg/kg IV or 35 mg/kg IM). The intracranal space was entered by gentle dissection between the lateral and superior recti muscles, under 2.5× loupe magnification. A malleable retractor was used to gently retract the globe medially. At all times pressure on the globe was kept as light as possible, and pressure was released for a few seconds every 2 to 3 minutes. Under visualization with an operating microscope, the optic nerve was exposed, and a sickle knife was used to make a 3-mm linear incision in the dura parallel to the nerve, as far posteriorly as practical (at least 15 mm posterior to the globe) to avoid damage to the central retinal artery. Dural vessels and use of cautery were avoided. Neurosurgical angled fine scissors were then used to extend the incision posteriorly several millimeters. The scissors were then inserted within the dural sheath, and the nerve transected (2 cuts each, two thirds through the nerve) under direct visualization. The retina was then observed by direct and indirect ophthalmoscopy, to ensure that no central retinal artery occlusion occurred. The wounds were closed, and the animals treated with systemic benzathine and procaine penicillin (30,000 U/kg; Phoenix Pharmaceutical, Inc., St. Joseph, MO) for 5 days and systemic methylprednisolone acetate (Depo-Medrol, 1 mg/kg IM; Pharmacia Corp., Pepack, NJ) for 3 weeks, tapering to 0.1 mg/kg for 1 more week until killing. The ONT monkeys were followed for 4 weeks by slit-lamp biomicroscopic examination of the anterior and posterior segments and indirect ophthalmoscopy of the posterior segment and then killed.

In all animals IOP was measured under ketamine anesthesia with a minified Goldmann (Haag-Streit, Koniz, Switzerland) applanation tonometer,\textsuperscript{26} occasionally backed up by measurements with a Tono-pen XL (Mentor O&O, Norwell, MA) if corneal edema or neovascularization, or head and eye movements under ketamine anesthesia prevented readings with the Goldmann.\textsuperscript{27} The IOP was measured before ALTS or ONT and then weekly thereafter, with the monkey lying prone in a head holder and with the head \(\sim 4\) cm above the heart.

Stereoscopic fundus photographic (Topcon TRC 50IA fundus camera; Topcon America Corporation, Paramus, NJ) and confocal scanning laser ophthalmoscopic (TopSS Topographic Scanning System; Laser Diagnostic Technologies, Inc., San Diego, CA) images were obtained in all animals before ALTS or ONT and before killing, and in some cases also at intermediate times during the period of pressure elevation. Pups were diluted with 2.5% phenylephrine HCl (Mydfrin; Alcon, Ft. Worth, TX) and 1% tropicamide (Mydriacyl; Alcon). Anesthesia for these procedures was ketamine (10 mg/kg, IM) + acempromazine (0.2–1 mg/kg IM), + methohexital sodium (15 mg/kg, IM) if needed to eliminate eye movements.

A summary of the clinical data on the animals is shown in Table 1.

**Tissue Processing**

Immediately before killing, a few of the glaucomatous and ONT monkeys underwent aspiration of \(\sim 100\) µl of vitreous from each eye. After induction with IM ketamine (10 mg/kg), the monkeys were placed under deep pentobarbital anesthesia (35 mg/kg IM or 15 mg/kg IV), and the pupils were dilated with phelyphrine and tropicamide. A 23-gauge needle was inserted through the pars plana 12 to 4 mm toward the papillo-macular nerve fiber bundle under direct visualization with an operating microscope. By using ocular geometry, we
Pharmacological treatments were used to control IOP in some of the animals. In one experiment, MOPC monoclonal antibody (mAb) and antiserum against human elastin were used to estimate the needle tip to have been 4 mm from the retinal surface. The vitreous sample was quickly frozen in liquid nitrogen for future biochemical assay, in conjunction with samples from other animals from other protocols, to be reported elsewhere, and the vitreous cavity volume was restored with sodium hyaluronate (Healon, Pharmacia). The monkeys were then perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). After enucleation the eyes were immersed in 4% paraformaldehyde for 12 to 24 hours, after which optic nerve heads were dissected free from the ciliary body and brain. The optic nerves were then postfixed in 0.5% glutaraldehyde/2% paraformaldehyde for 1 hour and processed for embedding in LR White resin (Electron Microscopy Sciences, Fort Washington, PA). Samples for light microscopy and ISH were extensively washed in 0.2% glycine in PBS and processed for paraffin embedding. Slides for ISH were handled using sterile techniques to avoid RNase contamination of the sections. Samples of myelinated nerve for evaluation of axonal damage were osmicated and stained with tannic acid and lead citrate and examined with a Zeiss EM 902A electron microscope (Oberkochen, Germany).

Electron Microscopy and Image Analysis

After embedding, ultrathin random sections were made of the lamina cribrosa and placed onto nickel grids, as previously described.7,9 For immunogold localization of elastin, grids were first blocked with 5% nonfat milk and then with an antiserum against human aortic aorta elastin (work dilution 1:100; Elastin Products, Owensville, MO). Colloidal gold-labeled IgG (Jansen Biotech, Olen, Belgium) was diluted 1:12 in 0.05 M Tris buffer containing 1.5% bovine serum albumin (BSA), pH 8.0. An antiserum against human aortic aorta elastin (work dilution 1:100; Elastin Products, Owensville, MO). Colloidal gold-labeled IgG (Jansen Biotech, Olen, Belgium) was diluted 1:12 in 0.05 M Tris buffer containing 1.5% bovine serum albumin (BSA), pH 8.0. All specimens were counterstained with uranyl acetate and lead citrate and examined with a Zeiss EM 902A electron microscope (Oberkochen, Germany).

Table 1: Clinical Data

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Age (y), Sex</th>
<th>Group</th>
<th>Duration* (weeks)</th>
<th>Drug Treatment and Duration†</th>
<th>IOP (mm Hg)‡§</th>
<th>C/D Ratio</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23, F</td>
<td>ONT</td>
<td>4</td>
<td>D: 4 weeks</td>
<td>E: 19.8 ± 3.1</td>
<td>E: 0.3</td>
<td>EM</td>
</tr>
<tr>
<td>2</td>
<td>19, M</td>
<td>ONT</td>
<td>4</td>
<td>D: 4 weeks</td>
<td>E: 15.6 ± 1.8</td>
<td>E: 0.4</td>
<td>EM</td>
</tr>
<tr>
<td>3</td>
<td>10, M</td>
<td>ONT</td>
<td>4</td>
<td>D: 4 weeks</td>
<td>E: 24.6 ± 6.8</td>
<td>E: 0.3</td>
<td>LM/ISH</td>
</tr>
<tr>
<td>4</td>
<td>7, M</td>
<td>ONT</td>
<td>4</td>
<td>D: 4 weeks</td>
<td>E: 30§</td>
<td>E: 0.3</td>
<td>LM/ISH</td>
</tr>
<tr>
<td>5</td>
<td>6, M</td>
<td>ONT</td>
<td>4</td>
<td>D: 4 weeks</td>
<td>E: 10§</td>
<td>E: 0.3</td>
<td>LM/ISH</td>
</tr>
<tr>
<td>6</td>
<td>5, M</td>
<td>ONT</td>
<td>4</td>
<td>D: 4 weeks</td>
<td>E: 13.1 ± 2.1</td>
<td>E: 0.2</td>
<td>LM/ISH</td>
</tr>
<tr>
<td>7</td>
<td>14, M</td>
<td>ExpG</td>
<td>12</td>
<td>None</td>
<td>E: 45.5 ± 9.2</td>
<td>E: 0.8</td>
<td>EM</td>
</tr>
<tr>
<td>8</td>
<td>9, M</td>
<td>ExpG</td>
<td>8</td>
<td>None</td>
<td>E: 15.8 ± 1.7</td>
<td>E: 0.3</td>
<td>EM</td>
</tr>
<tr>
<td>9</td>
<td>12, M</td>
<td>ExpG</td>
<td>12</td>
<td>T: wk 3–5.5</td>
<td>E: 41.5 ± 2.8</td>
<td>E: 1.0</td>
<td>EM</td>
</tr>
<tr>
<td>10</td>
<td>24, M</td>
<td>ExpG</td>
<td>12</td>
<td>T: wk 0–9.3</td>
<td>E: 53.3 ± 3.6</td>
<td>ND‡</td>
<td>LM/ISH</td>
</tr>
<tr>
<td>11</td>
<td>23, F</td>
<td>ExpG</td>
<td>12</td>
<td>None</td>
<td>E: 46.1 ± 5.8</td>
<td>E: 1.0</td>
<td>LM/ISH</td>
</tr>
<tr>
<td>12</td>
<td>22, M</td>
<td>ExpG</td>
<td>27</td>
<td>None</td>
<td>E: 29.9 ± 4.8</td>
<td>E: 0.6</td>
<td>EM</td>
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<tr>
<td>13</td>
<td>3, M</td>
<td>ExpG</td>
<td>24</td>
<td>None</td>
<td>E: 19.7 ± 0.4</td>
<td>E: 0.2</td>
<td>EM</td>
</tr>
<tr>
<td>14</td>
<td>17, F</td>
<td>ExpG</td>
<td>26</td>
<td>T: wk 7.3–26</td>
<td>E: 42.0 ± 1.7</td>
<td>E: 0.4</td>
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<tr>
<td>15</td>
<td>5, F</td>
<td>ExpG</td>
<td>31</td>
<td>AG: wk 24.7–26</td>
<td>E: 30.8 ± 13.0</td>
<td>E: 0.9</td>
<td>LM/ISH</td>
</tr>
<tr>
<td>16</td>
<td>20, F</td>
<td>ExpG</td>
<td>18</td>
<td>AG: wk 2.4–18</td>
<td>E: 18.1 ± 0.5</td>
<td>E: 0.2</td>
<td>LM/ISH</td>
</tr>
<tr>
<td>17</td>
<td>20, F</td>
<td>ExpG</td>
<td>18</td>
<td>AG: wk 2.4–18</td>
<td>E: 20.6 ± 0.6</td>
<td>E: 0.0</td>
<td>LM/ISH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PG: wk 3.5–18</td>
<td>E: 38.7 ± 2.3</td>
<td>E: NV</td>
<td>LM/ISH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T: wk 0.3–18</td>
<td>E: 17.7 ± 0.5</td>
<td>C: 0.2</td>
<td></td>
</tr>
<tr>
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</table>

EM, electron microscopy; LM, light microscopy; ISH, in situ hybridization; ExpG, experimental glaucoma; IOP, intraocular pressure; ONT, optic nerve transection; E, experimental; C, control; NV, not visible.

* Length of time between ONT or initial IOP elevation and sacrifice.
† Treatment dosages (also see Methods) and duration (ONT), or timespan of treatment from initial IOP elevation (ExpG): D, Depomedrol (methylprednisolone acetate, 1 mg/kg IM, once daily for 3 weeks; dose then tapered gradually for 1 week, to 0.1 mg/kg at killing, except for monkey 11, which then survived for another 8 weeks); T, Timoptic XE (0.5% timolol maleate in gel-forming vehicle); AG, Alphanagen (0.2% brimonidine tartrate); TS, Trusopt (2% dorzolamide hydrochloride); each given as one ~30 µl drop from commercial bottle once or twice daily.
‡ Values are means ± SEM.
§ IOP measured only prior to ONT.
¶ Not determined due to severe corneal neovascularization.

The indicated % of Tonopen measurements taken for the following monkeys: 9 (11%), 10 (33%); 11 (100%), 16 (7.7%), 17 (10.3%) were corrected with a factor intermediate to that required for humans (1) and cynomolgus monkeys (0.692) because of the intermediate size of the rhesus monkey eye.41 All other IOP measurements were made with the mini-Goldmann applanation tonometer.

The monkeys were then perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). After enucleation the eyes were immersed in 4% paraformaldehyde for 12 to 24 hours, after which optic nerve heads were dissected free from the sclera and surrounding tissues. Samples for electron microscopy were postfixed in 0.5% glutaraldehyde/2% paraformaldehyde for 1 hour and processed for embedding in LR White resin (Electron Microscopy Sciences, Fort Washington, PA). Samples for light microscopy and ISH were extensively washed in 0.2% glycine in PBS and processed for paraffin embedding. Slides for ISH were handled using sterile techniques to avoid RNase contamination of the sections. Samples of myelinated nerve for evaluation of axonal damage were osmicated and embedded in epoxy resin.

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Sagittal sections taken from the lamina cribrosa were examined, and areas of ECM containing cross-sectioned elastic and collagen fibers were examined. Twelve micrographs randomly taken from the lamina cribrosa at 26000× magnification from each eye were scanned with a HP Scanjet 4c (Hewlett-Packard, Palo Alto, CA) connected to a PC computer. Using Optimas image analysis software (Bothel, WA) gold grains were counted per 46.5-μm² area and plotted for statistical analysis. A code number given by the electron microscope identified the photographs so that the investigator performing the measurements did not know which eye, control or experimental, and to which group each animal belonged. The coded photographs were given to the investigator in random order as the electron microscopy technician printed them.

Statistical Analysis

Statistical evaluations were performed at the Biostatistics Core Module of the Department of Ophthalmology and Visual Sciences, Washington University. To standardize the elastin count between monkeys, the elastin count of the experimental eye was expressed as a fraction of the elastin count in the control eye of the same monkey. Descriptive statistics were computed for the elastin count of ONT and ExpG experimental and control eyes and their respective elastin ratios. To determine whether the mean elastin ratio of the ONT eyes was different from the mean elastin ratio of ExpG eyes, we performed a repeated-measures analysis of variance using SAS software (Cary, NC). The repeated-measures analysis was designed to assess the effects of using different methods to induce axonal degeneration (ONT vs. ExpG), using multiple measurements, and the possible interaction of methods for inducing axonal degeneration with multiple measurements. A significant interaction would indicate a difference between the ONT and ExpG methods in the elastin ratio of samples with high elastin counts compared with samples with low elastin counts.

Evaluation of Nerve Damage

To evaluate optic nerve damage in both ExpG and ONT groups, cross sections were taken from the myelinated optic nerves and stained with p-phenylenediamine.28-30 Digital images were taken with 2× magnification, so that the entire circumference of the nerve was within the lens field. Images were imported into Optimas software, where the total circumference area as well as the areas with axonal degeneration was measured. Results were expressed as a ratio between area of axon degeneration and total area. “Mild” axon loss was defined as a loss of up to one third of myelinated axon area. “Moderate” axon loss when there was loss between one and two thirds of myelinated axon area, and “marked” axon loss when the loss in axon area surpassed two thirds of the total myelinated area.9

Light Microscopy Immunohistochemistry

Antibodies. A rabbit polyclonal antibody anti-human aortic α-elastin (Elastin Products), at 1:400 dilution were used for double immunofluorescence. Rhodamine-Red-labeled goat anti-rabbit IgG and Oregon-Green-labeled goat anti-mouse IgG were obtained from Molecular Probes (Eugene, OR).

Immunofluorescence Staining. Sections (6 μm) were deparaffinized using xylene and rehydrated ethanol (100%, 90%, and 70%). To reduce unspecific labeling, sections were preincubated in Tris-glycine buffer (0.1 M glycine, brought to pH 7.4 using 1 M Tris buffer) for 30 minutes and washed twice in PBS, 5 minutes each. Sections were then incubated for 30 minutes in 10% nonimmune calf serum in PBS, followed by incubation with the primary antibody mixture diluted in 1%BSA/PBS for 30 minutes. After extensive washes in 1%BSA/PBS, sections were incubated with the second antibody mixture for another 30 minutes, washed, and mounted with ProLong medium (Molecular Probes). Negative controls were performed by replacing the primary antibodies with nonimmune serum or by incubating sections with inappropriate secondary antibody.

In Situ Hybridization

Probe Preparation. Antisense and sense riboprobes were prepared from human tropoelastin clone HDE-1 (gift from Jeffrey Davidson, Vanderbilt University, Nashville, TN)30 subcloned into a PGM-3Z vector (Promega, Madison, WI). Antisense digoxigenin-labeled probe was transcribed by SP6 RNA polymerase from NdeI-linearized DNA template, whereas sense digoxigenin-labeled probe was transcribed by T7 RNA polymerase from HindIII-linearized DNA template. In vitro transcription was carried out according to the manufacturer’s instructions (Dig-RNA labeling system; Roche Molecular Biochemicals, Indianapolis, IN).

Tissue Preparation and Hybridization. ISH was carried out as described previously.16 Briefly, 6-μm sagittal sections of monkey ONH and transected optic nerves were deparaffinized and washed in ethanol. Slides were then incubated for 15 minutes in PBS containing 0.3% Triton X-100, followed by 5 μg/ml proteinase K (Roche) treatment in PBS for 30 minutes at 37°C. Slides were fixed in 4% paraformaldehyde, followed by a 0.2% glycine/PBS wash. Slides were then immersed in freshly prepared 0.5% acetic anhydride in 0.1 M triethanolamine buffer, pH 8.0, for 10 minutes at room temperature in a slow shaker. Slides were dehydrated in ethanol and baked for 30 minutes before addition of probes. Hybridization was performed in a buffer containing 50% deionized formamide, 2× SSC, 20 mM Tris-HCl, pH 8, 1× Denhardt’s, 1 mM EDTA, 10% dextran sulfate, 0.5 μg/ml yeast tRNA, and 10 mM DTT. Fifty microliters of hybridization mixture containing 100 ng of labeled probe was applied to each section and let to hybridize overnight at 42°C. Slides were washed in 2× SSC twice for 15 minutes each at 37°C, followed by another wash in RNase buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 8, 1 mM EDTA) for 15 minutes at 37°C. Slides were treated with 20 μg/ml RNase A (Roche) for 30 minutes at 37°C, followed by washes in 2×, 1×, 0.5×, and 0.1× SSC, 15 minutes each at 45°C. Slides were rinsed with Tris buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl) and blocked with 2% calf serum in the same buffer for 30 minutes at room temperature. Slides were then reacted with an alkaline phosphatase-conjugated antibody against digoxigenin (Roche) for 1 hour at 37°C. After washes in Tris buffer, slides were rinsed in development buffer (0.1 M Tris, pH 9.5, 0.1 M NaCl, 50 mM MgCl₂, 0.1% Tween 20, 5 mM levamisole) followed by color development with NBT/BCIP solution (Roche) for 3 hours at room temperature. Slides were rinsed in distilled water and mounted with AquaMount (Fisher Scientific, Piscataway, NJ) without additional counterstaining.

Visualization and Photography

Slides were examined in a Nikon Optiphot-2 microscope equipped with epiluorescent illumination (Tokyo, Japan), and images were recorded using a digital camera (Spot Diagnostic Instruments, Sterling Heights, MI) and stored as a computer file. Color images of the blue-stained tissues processed for ISH were converted to grayscale using Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA).

RESULTS

Clinical Findings in Experimental Eyes

An example of glaucomatous damage is shown (Fig. 1A) in monkey 16 at 4 months into its elevated pressure protocol; the entire optic disc surface is excavated, the disc margin is undermined, there is substantial peripapillary atrophy, and the retinal nerve fiber layer is substantially attenuated; Figure 1B corresponds to the contralateral intact eye. The axonal loss for monkey 16 was advanced (0.89).

An example of ONT is shown in Figure 1C in monkey 4; Figure 1D corresponds to the contralateral intact eye. Although
disc pallor and loss/attenuation of the nerve fiber layer was evident in this animal, in most cases, indirect ophthalmoscopy, slit-lamp biomicroscopic fundoscopy, and stereoscopic fundus photography could detect no clear-cut changes in the fundus of eyes approximately 1 month after ONT vs. their presurgical baseline or vs. their contralateral control. Monkeys 2, 3, and 6 had a slightly pale disc with normal appearing vessels at 4 weeks after ONT. Changes in the clinical appearance of the optic disc begin to be visible 5 weeks after experimental optic nerve trauma. Consistent with ONT transection, all ONT animals exhibited an afferent pupillary defect in the ONT eye. The pupil in the transected eye was generally larger than that in the control eye, and the consensual response to light was weak in some ONT eyes throughout the 4 weeks. The dilated pupil and absent or weak consensual response to light may have resulted from damage to the ciliary ganglion or the parasympathetic efferent fibers traveling with the ciliary nerves during the dissection, so that the iris sphincter muscle received reduced innervation. There was no systematic alteration in IOP in these ONT eyes, although we have seen transient elevations in other ONT animals, presumably related to orbital swelling and pressure on the globe, as can occur after orbitotomy in humans. The presurgical IOP elevation in monkey 4 was fortuitous; one occasionally sees this in ketamine-anesthetized monkeys before they have “settled down.”

The color fundus photographs were not taken with the filters, film, and wide field/low magnification required to optimize visualization of the retinal nerve fiber layer, but rather with the standard white light and color film use for clinical evaluation of the nerve head. Nonetheless, because of the high

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933590/...)

**Figure 1.** (A) Fundus photographs of experimental glaucoma (ExpG) and control (B) eyes of monkey 16 at 4.5 months after unilateral laser-induced IOP elevation (ExpG = 40 mm Hg; control = 20 mm Hg). The entire ExpG disc surface is excavated, the disc margins are undermined (black arrowhead), there is substantial peripapillary atrophy (white arrowhead), and the retinal nerve fiber layer (asterisk in control eye) is substantially attenuated (white star), allowing clear visualization of the choroidal vasculature. The animal was killed 1 week later, after 18 weeks of IOP elevation, at which time the disc appearance was similar. (C) Fundus photographs of optic nerve transection (ONT) and of control (D) eyes of monkey 4, 3.5 weeks after transection, 1 week before killing. Note normal retinal vasculature, absence of retinal or vitreous hemorrhage, presence of pallor but absence of disc cupping and very early, mild attenuation of nerve fiber layer (absence of striations emanating from the temporal disc margin) as a result of ONT. (E) IOPs for monkey 14. This monkey was unusual in that only one laser session was required to elevate the IOP; usually two to three sessions are required. Topical Timoptic-XE 0.5% was given once daily or every other day where indicated to maintain IOP at the desired level. Before killing additional pharmacologic treatments (see Methods) were given to lower the IOP to control levels to assess disc cupping. The axonal loss for ExpG was mild (0.28).
reflectivity of the nerve fiber layer in the monkey, attenuation of the nerve fiber layer was easily discernible in ocular hypertensive eyes with advanced disc cupping (Fig. 1A vs. 1B: monkey 16) and in the ONT eye with advanced pallor (monkey 4) and was more subtly/equivocally present in several of the other ONT eyes, all compared with their contralateral controls.

At the time the confocal scanning laser ophthalmoscopic images were obtained, the alignment system had not yet been optimized for the anesthetized monkey, which unlike the conscious human subject, cannot fixate on the target light. This made precise quantitative comparison between concurrent experimental and control eye scans or between baseline and postintervention scans difficult. Nonetheless, in some of the glaucomatous and ONT animals, flattening of the papillary/peripapillary superior temporal and inferior temporal “humps” could be seen in the experimental eyes (not shown).

Because the monkey lamina cribrosa is relatively elastic, we believed that it was necessary to demonstrate that mild cupping, such as in monkey 14, was the result of permanent damage and not merely pressure-induced mechanical backward bowing. Observing the nerve head at normal IOP addressed this potential uncertainty. An IOP graph for monkey 14 is shown in Figure 1E. Lowering IOP pharmacologically for 1 week before killing resulted in no change in the surprisingly modest disc cupping (OD C/D = 0.4; OS C/D = 0.2), thus suggesting that there was actual loss of tissue, rather than simply a pressure-induced mechanical backward bowing of the elastic lamina cribrosa. The axonal loss for monkey 14 was also mild (0.28), consistent with the clinical appearance. This procedure was not carried out in hypertensive eyes with clinically obvious advanced cupping, which was borne out histologically, as in monkeys 15 and 16, where the cupping was advanced (0.9) or total (1.0), and the axonal loss ratios were 0.58 and 0.89, respectively, compared with the contralateral eyes.

**Evaluation of Nerve Damage**

After p-phenylenediamine staining, degenerated axons stain in dark brownish-red color. The area occupied by degenerated axons was then measured and expressed as a ratio of the total cross-sectional area. ExpG eyes had ratios of 0.27 ± 0.02 for mild damage (n = 3), 0.51 ± 0.05 for moderate (n = 5), and 0.73 ± 0.08 (n = 3) for marked axonal loss, respectively. ONT eyes with optic nerve transection had a ratio of degenerated axons of 0.73 ± 0.05 (n = 6). In most animals there was good correlation between the clinical findings (Table 1) and the microscopic evaluation of axonal loss. However, one monkey (monkey 15 described above), exhibited a C/D of 0.9 at the time of killing and the axonal loss was borderline advanced (0.58).

**Electron Microscopy**

In cross sections of control eyes, elastic fibers were qualitatively normal, with the characteristic roughly round morphology, and were embedded in a collagenous matrix (Fig. 2B), as previously described for human eyes. Immunogold staining using an anti-human elastin antibody positively identified elastic fibers. In eyes of the ExpG group large confluent elastic fiber aggregates were observed (Fig. 2A), with the same characteristics of the elastic degeneration present in the human glaucomatous lamina cribrosa. However, no alterations in the morphology of elastic fibers were found in the lamina cribrosa of eyes from ONT group (Fig. 2C) compared with contralateral control eyes (Fig. 2D), which showed round elastic fibers throughout the ECM of the lamina cribrosa.

**Statistical Analysis**

Descriptive statistics are displayed in Table 2. In two ONT monkeys, the mean elastin count was 222.3 ± 158.6 (mean ± SD) for the experimental eyes and 267 ± 187.3 for the control eyes by EM. The mean ratio of the matched ONT experimental to control samples was 0.87 ± 0.17. In three ExpG monkeys, the mean elastin count was 507.3 ± 343.9 for experimental eyes and 153.1 ± 65.6 for the control eyes. The mean of ratio of experimental eyes to control eyes in the ExpG-matched samples was 3.28 ± 1.14.

The results of the repeated-measures analysis of variance are displayed in Table 3. The results indicated a significant difference in the mean elastin ratio between the ONT and ExpG groups (P < 0.02). There was no significant trend among the multiple measures (P = 0.23) nor any significant interaction between multiple measures and methods (P = 0.32). These results indicate that ExpG eyes produce significantly more elastin than do ONT eyes when compared with their respective contralateral control eyes. To rule out the possibility that statistical results may be due to computing a ratio based on matching the sorted elastin counts of the experimental to control eye, the same repeated-measures analysis of variance was rerun five times using random pairings of elastin samples to compute the ratios. All five analyses confirmed the results given above by producing statistically significant ONT vs. ExpG differences (all P < 0.05) and by reproducing no significant trends for multiple measures or interaction effects (all P > 0.05). To rule out the possibility that the statistical results may be due to the large disparity in variance between ONT (SD = 0.17) and ExpG (SD = 1.14) methods, additional analyses were performed using a natural log transformation of the ratios. The analyses using the natural log transformation were repeated for each of the several analyses performed on the untransformed ratios (matched pairs and five random analyses using random pairs) and confirmed the significant differences between ONT and ExpG methods (all P < 0.05) and the nonsignificant multiple measures and interaction effects (all P > 0.05).

**Light Microscopy Immunohistochemistry**

To localize elastin in the optic nerve heads of monkeys, double immunofluorescence was performed using antibodies against GFAP and α-elastin.

**Experimental Glaucoma.** In control eyes elastin staining was localized to the ECM in the cribiform plates and in blood vessel walls, as described previously (Figs. 3B, 3D, 3F). In optic nerve heads with mild glaucomatous damage, there was enhanced immunoreactivity for elastin and some remodeling of the ECM was observed, as well as few large round astrocytes (reactive astrocytes) at the edge of the cribiform plates and in the nerve bundles (Fig. 3A). In eyes with moderate glaucomatous damage (Fig. 3C) there was also enhanced elastin immunoreactivity when compared with contralateral control eyes (Fig. 3D). In addition, there was extensive remodeling of the ECM with many reactive astrocytes in the nerve bundles, where there was colocalization of elastin and GFAP staining in the cytoplasm of astrocytes. In eyes with advanced glaucomatous damage (Fig. 3E) there was extensive remodeling of the ECM and compression of the cribiform plates, with most reactive astrocytes located in the nerve bundles. There was also enhanced immunoreactivity for elastin when compared with control contralateral eyes (Fig. 3F). No differences in elastin immunoreactivity were apparent in the distal optic nerve between eyes with experimental glaucoma and contralateral controls. Elastic fibers follow longitudinally the pial septa (data not shown).

**Optic Nerve Transection.** In eyes where the optic nerve was transected (Fig. 4A) there was little or no remodeling of the ECM of the optic nerve head. Elastin staining was localized to the ECM of the cribiform plates and to blood vessels, without qualitative differences from contralateral control eyes.
However, there was extensive gliosis as evidenced by enhanced immunoreactivity for GFAP when compared with contralateral control eyes (Fig. 4B). Intense GFAP staining was observed in the nerve bundle area consistent with gliosis (Fig. 4A). No colocalization of elastin and GFAP immunoreactivity was observed in the lamina cribrosa of transected eyes. At the transected site, elastin immunoreactivity was localized primarily to the pia mater and to newly blood vessels. No colocalization of elastin to GFAP positive astrocytes was observed in the transected site. There was also elastin staining in the regenerating pia mater surrounding the glial scar (Fig. 4C). In contralateral control optic nerves (Fig. 4D), elastin was primarily localized to the pia mater.

**In Situ Hybridization**

To localize cells synthesizing elastin in the optic nerve head of monkeys, ISH was performed using a digoxigenin-labeled tropoelastin probe. In experimental glaucoma eyes with advanced damage (Fig. 5A), most astrocytes in the cribriform plates, at the edges of the plates and in the nerve bundles expressed tropoelastin mRNA. No hybridization was observed in contralateral control eyes (Fig. 5B). In eyes with mild glaucomatous damage, strong reaction for tropoelastin mRNA was localized to rounded astrocytes at the edges of the plates (Fig. 5C). No hybridization signal was detected in the contralateral un-

<table>
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<th>Method</th>
<th>N*</th>
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<th>Control Elastin Count †</th>
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<td>507.3 ± 343.9</td>
<td>153.1 ± 63.6</td>
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* N = the number of experimental glaucoma (ExpG), optic nerve transection (ONT), and control samples drawn from each group. Twelve samples were drawn from each eye, with the ONT group having 2 monkeys and the POAG group having 3.
† Values represent the mean elastin count (gold grains) per 46.35-μm² area ± SD.
‡ The mean ratio ± SD of ExpG and ONT to control eyes.

**Table 3. Repeated-Measures Analysis of Variance**

<table>
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<th>Effect</th>
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<td>0.23</td>
</tr>
<tr>
<td>Method–multiple measures</td>
<td>11</td>
<td>1.21</td>
<td>0.32</td>
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d.f., degrees of freedom; ExpG, experimental glaucoma; ONT, optic nerve transection.
treated eye (Fig. 5D) or in the distal optic nerves from both experimental and control eyes (data not shown).

In the optic nerve transection group no hybridization for tropoelastin was observed in the lamina cribrosa or adjacent myelinated nerve in either experimental eyes (Fig. 6A) or in the control, nontransected eyes (Fig. 6B). In contrast, fibroblast-like cells in the pia mater adjacent to the transection site expressed elastin mRNA (Fig. 6C). No hybridization was observed in the pia mater of the nontransected myelinated nerve (Fig. 6D).

No hybridization signal was detected in control sections hybridized with the sense probe or with the plasmid without the antisense insert (data not shown).

In conclusion, monkeys 11, 13, and 15 without topical treatments exhibited increased elastin deposition and mRNA levels in all eyes with surgically induced elevated IOP, but no new elastin deposition or expression in the contralateral control eyes was detected by immunohistochemistry and ISH. Monkeys 9, 10, 14, 16, and 17 received topical IOP-lowering drugs in the eyes with elevated IOP and exhibited results similar to monkeys 11, 13, and 15. Monkeys with optic nerve transection did not exhibit any differences in elastin deposition or elastin mRNA levels between the transected eye and the contralateral control.

**DISCUSSION**

In this study we demonstrate an increase in the expression of elastin in eyes of primates with experimental glaucoma as assessed by light and electron microscopy immunohistochemistry as well as by ISH. Our results indicate that elastin upregulation in experimental glaucoma is a specific response to elevated IOP-related stress and not a nonspecific secondary response to axonal degeneration, because there was no upregulation of elastin expression in monkey eyes with surgical transection of the optic nerve.

Elastin is an important component of the ECM of the lamina cribrosa that provides resiliency and deformability to the tissue. The elastic properties of the lamina cribrosa are important to buffer the constant fluctuations in IOP. Elastic fibers in the lamina cribrosa must undergo constant stretch and relaxation cycles in response to daily, normal variations in IOP. A recent report using a finite element theoretical model of the human ONH indicate that the IOP-related stress applied to the lamina...
cribrosa is substantial even at low levels of IOP. In human glaucomatous optic neuropathy, astrocytes express elastin mRNA and synthesize abnormal elastic fibers leading to elastotic degeneration of the ECM and loss of resiliency and deformability, most likely in response to elevated IOP. Recent studies on cultured astrocytes of the human lamina cribrosa demonstrate that gene expression and synthesis of elastin is increased when astrocytes are exposed to increased hydrostatic pressure, providing evidence that mechanical stress is involved in the regulation of elastin synthesis by laminar astrocytes.

Elastotic degeneration of the lamina cribrosa has been shown in human POAG and in glaucoma associated with pseudoexfoliation (PEX). Elastosis is characterized by in-

FIGURE 4. Double immunofluorescence localization of GFAP (green) and elastin (red) in optic nerve heads of monkeys with optic nerve transection. (A) In the optic nerve head of eyes with optic nerve transection (monkey 3, Table 1), elastin immunoreactivity is similar to that of control eye (B), but there is enhanced GFAP immunoreactivity in the experimental eye (A). At the site of transection (C), elastin staining localizes to the pia mater (PM) and to newly formed blood vessels. In the contralateral control optic nerve (D), elastin immunoreactivity is also restricted to the pia mater. LC, lamina cribrosa; NB, nerve bundles; V, blood vessel; PS, pial septum; Scar, glial scar.

FIGURE 5. In situ hybridization using a digoxigenin-labeled antisense tropoelastin probe in eyes with experimental glaucoma. (A) In eyes with advanced glaucoma (monkey 9, Table 1) tropoelastin mRNA was localized to large astrocytes at the edge and inside the cribriform plates in the nerve bundles (arrows). No hybridization was present in the contralateral control eye (B). (C) In eyes with mild glaucoma (monkey 14, Table 1) strong signal for tropoelastin mRNA was localized to rounded astrocytes in the cribriform plates. (D) No hybridization was present in the contralateral control eye. LC, lamina cribrosa; NB, nerve bundles; V, blood vessel. Magnification bars, (A) 20 μm; (B, C, and D) 10 μm.
increased deposition of elastin and elastin-associated microfibrils, which do not organize into typical elastic fibers but form large bizarre aggregates of disorganized matrix that label with elastin antibodies. Elastin mRNA was demonstrated increased tropoelastin mRNA levels in the lamina cribrosa. In the present study, elastin mRNA was detected in eyes with experimental glaucoma, large confluent elastic fiber aggregates were observed in the ECM of the lamina cribrosa with characteristics similar to the elastic fibers present in the human glaucomatous lamina. Moreover, similar to the observations in human glaucoma, the area of the ECM occupied by elastin-labeled material was significantly increased in eyes with experimental glaucoma when compared with the contralateral untreated eyes and to the eyes with optic nerve transection.

The increase in the amount of immunogold-labeled elastin material in the ECM of eyes with experimental glaucoma indicates that elastotic fibers may consist of newly synthesized elastin that does not organize into normal elastic fibers. Activation of tropoelastin mRNA expression indicates new synthesis of elastin. Previous studies in human glaucoma have demonstrated increased tropoelastin mRNA levels in the lamina cribrosa. In the present study, elastin mRNA was detected in eyes with glaucoma and not in contralateral controls or in eyes with optic nerve transection by ISH, further indicating that the increase in deposition of elastin is due to new synthesis, most likely in response to elevated IOP. In comparison, in eyes with transected optic nerves, elastin mRNA was detected in fibroblasts in the pia mater at the site of transection but not in the lamina cribrosa in the optic nerve head. New synthesis of elastin and other ECM proteins occurs normally in wound healing of connective tissues in response to inflammatory mediators released at the site of injury. These factors released distally to the lamina cribrosa, at the site of transection, did not stimulate synthesis of elastin in nearby optic nerve astrocytes, strongly supporting the concept that astrocytes of the lamina cribrosa are uniquely specialized to synthesize elastin.

Synthesis of elastin by astrocytes in situ and in vitro has not been reported in other regions of the normal central nervous system (CNS). In the lamina cribrosa, synthesis of elastin by astrocytes most likely represents a specialization of these cells to the mechanical function of the tissue. Recent reports indicate that several astrocytomas, the most common form of primary brain tumors, express elastin and elastin binding protein (EBP) in vivo and in vitro. These studies strongly suggest that synthesis and degradation of elastin may play a role in cell adhesion for migration and in the regulation of proliferation of the tumor cells. To date, there is no evidence of type 1B astrocyte proliferation or elastolytic activity in glaucoma; however, optic nerve head astrocytes become reactive in both human and in experimental glaucoma in monkey and rat (reviewed in Ref. 9). Reactive astrocytes migrate out of the lamina cribrosa into the nerve bundles in glaucoma. Increased synthesis of soluble tropoelastin by astrocytes in response to elevated IOP may play a role in cell adhesion and migration of astrocytes in glaucoma by providing a substrate for attachment. Perhaps tropoelastin secretion is involved in the transition from quiescent to reactive astrocytes in glaucoma.

Mechanical stress can regulate expression of a variety of ECM genes, growth factors, cell adhesion molecules etc. reviewed in Refs. 45–46. Astrocytes are attached to the ECM and to neighboring astrocytes by cell surface adhesion molecules, which are connected, to the cytoskeleton. Thus, changes in the ECM can be transmitted intracellularly through activation of ion channels or other membrane components. Because the cortical cytoskeleton is coupled to cell adhesion molecules, to membrane-bound enzymes, and to ion channels, biomechanical stress will cause intracellular responses in astrocytes (reviewed in Refs. 45–49). It is unknown how elastin connects with the cell membrane, but cell surface adhesion molecules or other binding proteins may bind elastin or elastin-associated microfibrils to the surface of astrocytes. In astrocytoma, elastin is bound to the cell surface by EBP, a 67-kDa nonintegrin receptor. It is possible that through cell surface adhesion molecules, EBP, or other unidentified receptors, the
extracellular signal generated by pressure-induced mechanical stress is transmitted intracellularly to stimulate elastin synthesis.

Synthesis of growth factors, cytokines, and other cellular mediators by astrocytes in response to abnormally elevated IOP may also stimulate elastin synthesis in vivo. Members of the TGF-β family of growth factors modulate tissue remodeling by upregulating synthesis of most ECM proteins, including elastin, collagen, fibronectin, and proteoglycans, and by decreasing the production of ECM-degradative enzymes. Of particular interest to the glaucomatous optic nerve head is TGF-β2, known to upregulate elastin synthesis in many organs and diseases. Previous work in our laboratory found that TGF-β2 is synthesized locally by the astrocytes and that TGF-β2 is released in large amounts in the glaucomatous optic nerve head. TGF-β1 and TGF-β2 control elastin expression in mature tissues at the posttranscriptional level by allowing steady state levels of elastin mRNA to build up and protein production to occur. Thus, accumulation of abnormal elastin in response to elevated IOP in the lamina cribrosa may be due at least in part to release of TGF-β2. Recently, the abnormal accumulation of ECM in experimental diabetic nephropathy, a hallmark of the disease, was suppressed by anti-TGF-β antibody therapy. One can speculate that interfering locally with TGF-β2 may prevent the extensive remodeling of the ECM in glaucoma.

In conclusion, glaucomatous optic neuropathy is a chronic disease that spans decades, in which structural changes in the ECM and loss of resiliency and compliance may occur throughout the disease process and contribute to its progression. Increased expression of abnormal elastin and elastosis may persist after the pressure is lowered, permanently changing the tissue biomechanical properties of the tissue and increasing susceptibility to further damage, even at lower levels of IOP-related stress. Although there are many differences between the primate model of experimental glaucoma and reported here and human glaucoma, the results of this study coupled with our recent observations in vitro indicate that optic nerve head astrocytes are mechanosensitive cells. Astrocytes of the lamina cribrosa respond to the stress generated by elevated IOP in a manner similar to that in human glaucoma. In contrast, the acute loss of axons that occurs in transection neither elicits responses in astrocytes of the optic nerve head nor alters the structure of the tissues. This evidence strongly suggests that abnormally elevated IOP or perhaps even IOP within generally normal levels in the presence of other susceptibility factors predisposing to increased expression of abnormal elastin and elastosis (e.g., decreased vascular perfusion or PEX) and perhaps race or unknown genetic factors are indeed a major factors in the development of the neuropathy. In addition, our results further indicate that the site of injury to the axons of the retinal ganglion cells is at the level of the optic nerve head.

Acknowledgments

The authors thank Belinda McMalan and Zelma Jones for excellent assistance with tissue processing and microscopy; Mac Gordon and Brad Wilson for statistical analysis; Kristin J. Tarbet for surgical assistance; Julie Kiland and Jennifer Seeman for expert clinical assistance with the monkeys; and John Peterson for fundus photography and scanning laser ophthalmoscopy.

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