Localization of Elastin in the Normal and Glaucomatous Human Trabecular Meshwork

Junichi Umihira,* Seiji Nagata,* Masahiko Nohara,* Toru Hanai,† Nobuteru Usuda,† and Katsuzo Segawa*

Purpose. The extracellular materials (ECMs) in the trabecular meshwork (TM) are thought to play a crucial role in aqueous outflow resistance. Immunohistochemical localization of elastin, one of the major ECMs in the normal and glaucomatous human TM, was examined ultrastructurally.

Methods. Eight normal eye bank eyes and 16 trabeculectomy specimens of primary open angle glaucoma (POAG, 11 eyes from 8 cases), congenital glaucoma (2 eyes from 1 case), and juvenile glaucoma (3 eyes from 2 cases) were embedded in Lowicryl K4M at low temperature. The distribution of elastin was studied by the protein A-gold technique.

Results. In normals, the gold particles indicating the antigenic sites for elastin existed mainly in the central amorphous element of the elastic-like fibers, and a few gold particles were observed within the area containing fine granular-like material and fine fibrillar-like material. No labeling was observed in cellular materials or other ECMs. In congenital and juvenile glaucoma, labeling was similar to that observed in normals. In POAG specimens compared to normals, there was an increased amount of elastin-bound immunogold particles along the inner canal endothelium. The increased gold particles, which did not have a fibrillar arrangement and were not enclosed by electron-dense microfibrils, were found within the area containing fine fibrillar-like material. However, labeling within the elastic-like fibers was similar to that observed in normals.

Conclusions. Under electron microscopy, elastin could be localized in the normal and glaucomatous human TM. The results of this investigation suggest that elastin may play an important role in the etiology of POAG. Invest Ophthalmol Vis Sci. 1994;35:486-494.

It is generally accepted that the trabecular meshwork (TM) plays an important role in the normal bulk flow of the aqueous humor and is presumed to be the major site of aqueous outflow resistance in glaucomatous human eyes. Histologically, the increase of extracellular materials in the TM has been demonstrated in cases of open angle glaucoma.1-5 Thus, the extracellular materials in the TM are believed to play a crucial role in aqueous outflow resistance. Although histochemical and biochemical analysis techniques have been developed to investigate these extracellular materials, it is difficult to study the TM directly because of the limited amount of tissue available. To avoid this difficulty, ultrastructural studies were performed using immunohistochemical technique from recent investigations.6-9

Elastic fibers, which are important extracellular constituents of many organs and tissues, are composed predominantly of elastin. In human eyes, it is known that elastin is a major component of Bruch's membrane, lamina cribrosa, blood vessels, and conjunctival stroma.10,11 In these tissues, the ultrastructural appearance of the elastic fibers consists of the central amorphous element and the peripheral microfibrillar component. Generally, the elastin is embedded in amorphous element that is less electron-dense than the surrounding small layer of osmiophilic microfibrils.11-13 However, in the human TM, the ultrastructural appearance of the elastic fibers with a large amount of microfibrillar component and a small amount of amorphous element differs from that of normal elastic tissues.2,6,14-16 They are, therefore, termed elastic-like fibers.2

From the Departments of *Ophthalmology and †Cell Biology, Shinshu University School of Medicine, Matsumoto, Japan.

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Reprint requests: Junichi Umihira, MD, Department of Ophthalmology, Shinshu University School of Medicine, 3-1-1, Asahi, Matsumoto 390, Japan.


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Elastic-like fibers may serve as a mechanical link between the meshwork and the adjacent corneal and scleral tissues\(^{16,17}\) and may stain positively with stain presumed to be specific to elastin.\(^{6,14,15}\) The amorphous element of these elastic-like fibers can be digested by pancreatic elastase.\(^{26,15}\) These findings suggest that the amorphous element may contain elastin. This suggestion, however, has been controversial because the elastin could not be observed under customary transmission electron microscopy and had not been visualized at the ultrastructural level until a recent study by Gong et al, who successfully demonstrated the presence of elastin in the trabecular tissue using immunogold technique.\(^6\)

The present study is designed to localize elastin by ultrastructural immunohistochemistry and to compare findings in the normal and glaucomatous human TM. For this purpose, antibodies to \(\alpha\) elastin were labeled with colloidal gold and reacted in ultrathin sections of TM embedded in Lowicryl K4M. The results of this investigation show that in cases of primary open angle glaucoma (POAG), there are marked changes in the distribution and the quantity of elastin in the human TM.

**MATERIALS AND METHODS**

Eight eye bank eyes that had undergone keratoplasty and had never been diseased were used as normal human trabecular tissue. All eye bank eyes were fixed within 24 hours of the donors’ deaths. Donor ages and cup-to-disc ratios of the eyes are shown in Table 1. Sixteen trabeculectomy specimens, which included eight cases (eleven eyes) of POAG, one case (two eyes) of congenital glaucoma, and two cases (three eyes) of juvenile glaucoma were used as glaucomatous human trabecular tissue. All trabeculectomy specimens were removed by the same operative procedure and were fixed as soon as possible. The ages of the cases of glaucoma and the status of diseases are shown in Table 2.

**TABLE 1. Data of Normal Eyes**

<table>
<thead>
<tr>
<th>Specimen no.</th>
<th>Age</th>
<th>Cup-to-Disc Ratio*</th>
<th>Particle Density† Within FFM</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>51</td>
<td>0</td>
<td>0.4 ± 1.2</td>
</tr>
<tr>
<td>N2</td>
<td>63</td>
<td>0.5</td>
<td>0.3 ± 0.4</td>
</tr>
<tr>
<td>N3</td>
<td>68</td>
<td>0</td>
<td>0.6 ± 0.9</td>
</tr>
<tr>
<td>N4</td>
<td>74</td>
<td>0.3</td>
<td>0.8 ± 0.7</td>
</tr>
<tr>
<td>N5</td>
<td>74</td>
<td>0</td>
<td>0.6 ± 0.8</td>
</tr>
<tr>
<td>N6</td>
<td>75</td>
<td>0</td>
<td>0.3 ± 0.4</td>
</tr>
<tr>
<td>N7</td>
<td>85</td>
<td>0.3</td>
<td>1.1 ± 1.1</td>
</tr>
<tr>
<td>N8</td>
<td>88</td>
<td>0.3</td>
<td>1.5 ± 1.0</td>
</tr>
</tbody>
</table>

* Cup-to-disc ratio was measured using the biomicroscope.
† Density of gold particles within the area containing fine fibrilar-like material (no. of gold particles/\(\mu m^2\) ± SD).

Under light microscopy, in all examined specimens it was confirmed that the laminated structure of the TM and the Schlemm’s canal were maintained. In the eye bank eyes, there were no significant postmortem changes at the electron microscopic level.

**Antibody**

Rabbit antiserum to human aortic \(\alpha\) elastin was obtained from Elastin Products (St. Louis, MO). The specificity of this antibody was reported in previous papers\(^6,11,12\) and was tested by light microscopic analysis using the avidin-biotin peroxidase complex technique.\(^18\) Western blot analysis was performed to establish immunoreactivity in the TM by SDS-PAGE according to the method of Laemmli\(^19\) on 10% polyacrylamide gels followed by electrophoretical transfer to a nitrocellulose membrane.\(^20\)

**Tissue Preparation for Electron Microscopic Immunohistochemistry**

The normal trabecular tissues cut into small (4 × 4 mm) blocks and trabeculectomy specimens were fixed for 24 to 48 hours in a fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.1 M sodium phosphate (pH 7.4). Tissues were given three 20-minute washes with sodium phosphate buffer containing 0.1 M lysine and 0.15 M NaCl, pH 7.4. Tissues were dehydrated in a graded series of ethanol and were then embedded in Lowicryl K4M at −20°C for 50 hours. Ultrathin sections were cut on an ultramicrotome and were mounted on membrane-coated nickel grids.

**Immunohistochemical Staining for Electron Microscopy**

Protein A-gold technique was applied for immunoelectron microscopy according to Roth\(^21\) with some modifications. The sections were then: (1) incubated on drops of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.05% (vol/vol) Triton X-100 and 5 mg/ml bovine serum albumin for 1 hour; (2) transferred onto drops of antibody solution (1:100 dilution in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.05% Triton X-100 and 5 mg/ml of bovine serum albumin) and incubated for 4 hours at room temperature; (3) washed rigorously several times with drops of 0.05 M Tris-HCl buffer with 0.05% Triton X-100; (4) floated on a 1:30 diluted solution of 15 nm protein A-gold particle complex (EY Laboratories; San Mateo, CA) for 2 hours at room temperature; (5) washed rigorously several times with drops of 0.05 M Tris-HCl buffer with 0.05% Triton X-100; (6) counterstained with uranyl acetate and lead citrate; (7) examined in a Hitachi HS-9 transmission electron microscope at 75 kV.
TABLE 2. Data of Glaucomatous Eyes

<table>
<thead>
<tr>
<th>Specimen No.</th>
<th>Eye</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Cup-to-Disc Ratio</th>
<th>Visual Field*</th>
<th>IOP (mm Hg)</th>
<th>Particle Density† Within FFM</th>
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</thead>
<tbody>
<tr>
<td>P1L L 42 POAG</td>
<td>0.8</td>
<td>2</td>
<td>38</td>
<td>4.9 ± 1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2R R 52 POAG</td>
<td>0.9</td>
<td>4</td>
<td>32</td>
<td>6.3 ± 3.2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>P3R L 52 POAG</td>
<td>0.9</td>
<td>3</td>
<td>26</td>
<td>4.5 ± 4.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P3L R 52 POAG</td>
<td>0.9</td>
<td>3</td>
<td>23</td>
<td>3.7 ± 1.7</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>P4R L 60 POAG</td>
<td>0.9</td>
<td>4</td>
<td>42</td>
<td>6.3 ± 3.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4L R 60 POAG</td>
<td>0.6</td>
<td>3</td>
<td>36</td>
<td>5.0 ± 2.7</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>P5L L 67 POAG</td>
<td>0.8</td>
<td>2</td>
<td>18</td>
<td>6.4 ± 3.1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>P6R R 67 POAG</td>
<td>1.0</td>
<td>5</td>
<td>27</td>
<td>6.6 ± 2.0</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>P6L L 67 POAG</td>
<td>1.0</td>
<td>4</td>
<td>18</td>
<td>6.3 ± 2.1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>P7R R 72 POAG</td>
<td>1.0</td>
<td>5</td>
<td>50</td>
<td>12.4 ± 5.2</td>
<td></td>
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<tr>
<td>P8R R 81 POAG</td>
<td>0.9</td>
<td>4</td>
<td>18</td>
<td>7.4 ± 3.0</td>
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<tr>
<td>C1L R 4 Congenital</td>
<td>1.0</td>
<td>Unknown</td>
<td>40</td>
<td>0.8 ± 0.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J1L L 4 Congenital</td>
<td>1.0</td>
<td>Unknown</td>
<td>40</td>
<td>0.6 ± 0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J1R R 19 Juvenile</td>
<td>0.5</td>
<td>1</td>
<td>19</td>
<td>0.7 ± 0.7</td>
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<tr>
<td>J2R R 41 Juvenile</td>
<td>1.0</td>
<td>3</td>
<td>24</td>
<td>2.0 ± 1.7qe</td>
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<tr>
<td>J2L L 41 Juvenile</td>
<td>1.0</td>
<td>4</td>
<td>25</td>
<td>1.8 ± 1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IOP = preoperative intraocular pressure with medication.
* Visual field: 1, no defect; 2, scotoma; 3, mild defect; 4, significant defect; 5, only central visual field.
† Density of gold particles within the area containing fine fibrillar-like material (no. of gold particles/μm² ± SD).

Controls
Control staining to demonstrate the immunohistochemical specificity included replacement of antiserum by Tris-HCl buffer or nonimmune rabbit serum and elastase digestion (0.1 mg/ml in 0.05 M Tris-HCl buffer, pH 7.5) for 2 hours at room temperature before immunostaining.

Quantitative Analysis of Elastin-Bound Immunogold Particles
Quantitation of the number of elastin-bound immunogold particles performed to establish the quantitative difference of elastin between normal and glaucomatous specimens. Ten electron micrographs were taken at >30 μm intervals along the subendothelial layer of Schlemm’s canal of each specimen. Random electron micrographs were taken at a low power so that we could not directly observe the immunogold with the electron microscope. These electron micrographs were enlarged to a final magnification of ×15,000, and then the number of gold particles within the area containing some extracellular materials per square micrometer was calculated.

RESULTS
Observation under light microscopy of 6 μ frozen sections immunohistochemically stained for elastin showed that immunoreactivity was similar in all parts of the TM examined and in neighboring tissues.

Immunoblots of homogenates of the normal human TM obtained from the fellow eye of the normal specimens demonstrated two immunoreactive polypeptides at approximately 61 kD and 50 kD. There was no significant staining of the background with the antibody we used.

Elastin in the Normal Trabecular Meshwork
Under electron microscopy using the protein A-gold technique, elastin could be directly visualized and localized. The gold particles indicating the antigenic sites for elastin in the normal trabecular tissues existed predominantly in the elastic-like fibers. No regional differences in either the intensity or the pattern of immunogold labeling for elastin within the area of elastic-like fibers were noted among the uveal, corneoscleral, or endothelial meshworks. At higher magnifications, the gold particles were observed mainly in the amorphous element of the elastic-like fibers (Figs. 1A and 1C).

In the endothelial meshwork, a few gold particles were occasionally observed within the area containing fine granular-like material that surrounded the elastic-like fiber and was restricted to the subendothelial layer of Schlemm’s canal. Besides, a few gold particles were occasionally observed within the area containing fine fibrillar-like material that was present principally in the subendothelial layer of Schlemm’s canal (Fig. 2A). However, the number of gold particles was less than that detected within the elastic-like fibers.

No labeling for elastin was observed in cellular materials or other extracellular materials, such as collagen fibrils, long spacing fibers, basal lamina, and basal lamina-like material. The pattern of immunogold labeling did not vary among all examined normal specimens.
FIGURE 1. Ultrathin section of Lowicryl K4M embedded human trabecular meshwork stained for elastin by the protein A-gold technique. Elastin is labeled by 15 nm gold particles. (A) High-power view of the subendothelial layer of Schlemm's canal in a 88-year-old normal eye (specimen No. N8, ×20,000). Immunogold labeling for elastin is seen within the elastic-like fibers (arrowheads). SC = Schlemm's canal; g = fine granular-like material; f = fine fibrillar-like material. (B) Low-power view of corneoscleral meshwork in a 52-year-old POAG eye (specimen No. P2R, ×15,000). In POAG eyes, immunogold labeling for elastin within the elastic-like fibers is similar to that observed in normal eyes. (C) Cross-sectional view of elastic-like fiber in a 68-year-old normal eye (specimen No. N3, ×30,000). Immunogold labeling for elastin is observed mainly in the amorphous element of the elastic-like fibers that is less electron-dense than the surrounding microfibrillar component. (D) Oblique-sectional view of elastic-like fiber in a 52-year-old POAG eye (specimen No. P2R, ×18,000). Immunogold labeling within elastic-like fibers in POAG eyes is similar to that observed in normal eyes.
Changes in the Glaucomatous Trabecular Meshwork

The most significant finding was that in all examined cases of POAG, the amount of positively labeled gold particles for elastin within the area containing fine fibrillar-like material that was present in the subendothelial layer of Schlemm's canal was distinctly larger than in the age-matched normal eyes (Fig. 3). However, labeling for elastin within the elastic-like fibers was similar to that observed in normal eyes (Fig. 1B and 1D).

The elastin-bound immunogold particles within the area containing fine fibrillar-like material was not related to that observed within the elastic-like fibers because the increased gold particles did not have a fibrillar arrangement and were not enclosed by electron-dense microfibrils. Labeling for elastin increased throughout the area containing fine fibrillar-like material, and aggregations of gold particles were evident within the several regions involving fine fibrillar-like material (Fig. 3B). In some regions, the electron-dense microfibrils that had a nonfibrillar arrangement were observed near or around a few aggregations of the...
FIGURE 3. Electron micrographs of the area containing fine fibrillar-like material in the subendothelial layer of Schlemm's canal. (A) Severe POAG, 67-year-old. In POAG eyes, there is an increased amount of elastin-bound immunogold particles along the inner Schlemm's canal endothelium compared to normals (specimen No. P6R, ×20,000). SC = Schlemm's canal. (B) Mild POAG, 60-year-old. In POAG eyes, aggregations of gold particles are evident within the several regions involving fine fibrillar-like material (specimen No. P4R, ×20,000). (C) Severe POAG, 72-year-old (specimen No. P7R, ×7,500). (D) High-power view of the area outlined in (C) demonstrates the area containing fine fibrillar-like material (×30,000). The increased gold particles do not have a fibrillar arrangement and are not enclosed by electron-dense microfibrils.

gold particles, indicating the antigenic sites for elastin (Fig. 4).

In the most severe case of POAG (specimen No. P7R) which had significant visual field defect and large cup-to-disc ratio, the labeling for elastin within the area containing fine fibrillar-like material was more outstanding.

In cases of congenital and juvenile glaucoma, the labeling pattern for elastin in the TM was similar to that observed in normal specimens. Particularly, the
FIGURE 4. An electron micrograph of the area containing fine fibrillar-like material in a 72-year-old POAG eye. The electron-dense microfibrils that have a nonfibrillar arrangement are observed around the aggregations of the gold particles indicating the antigenic sites for elastin (specimen No. NRT, x27,000).

amount of positively labeled gold particles within the area containing fine fibrillar-like material and elastic-like fibers was similar to that observed in normal eyes (Figs. 2B, 2C, 2D).

The labeling density of elastin-bound immunogold particles within the area containing fine fibrillar-like material in each specimen is described in Tables 1 and 2. Statistical analysis using Student's t-test indicated that the labeling density in POAG specimens (6.3 ± 2.1/μm²) was significantly more than that in normal (0.7 ± 0.5/μm²), congenital glaucomatous (0.7 ± 0.5/μm²), and juvenile glaucomatous specimens (1.5 ± 0.5/μm²).

Controls

In control sections, no specific labeling for elastin was found when the primary antiserum was replaced by either Tris-HCl buffer or nonimmune rabbit serum, nor when the sections were digested with elastase.

DISCUSSION

Generally, in various normal organs and tissues, elastin has a similar arrangement of amino acids. Thus, in this study, we used an antibody to human aortic α elastin for the immunohistochemical detection of elastin in the human TM. Again, the immunoreactivity of the antibody in the TM was established by Western blot analysis.

In a previous study of Murphy et al., which they used 1 μm sections for immunofluorescence staining of elastin, they demonstrated labeling for elastin in the uveal and corneoscleral meshwork, but the endothelial meshwork was negative for elastin. Similarly, by light microscopic immunohistochemical analysis using avidin-biotin peroxidase complex technique, we could not determine the distribution and characterization of the elastin in the TM because the sections were too thick to investigate the minute structure of elastin in the TM.

The elastic tissue in the aorta has a large amount of elastin. On the other hand, the elastic tissue in immature tissues, such as tendons and TM, has a large amount of microfibrillar strands and remaining small amounts of elastin. Thus, it is difficult to extract elastin from these immature tissues. By electron microscopic analysis using the highly sensitive and specific immunogold localization technique with tissues embedded in Lowicryl K4M, elastin could be localized and directly visualized in greater detail. In the preliminary study to determine the method for tissue preparation and immunohistochemical staining, we tested various fixation conditions, serial dilutions of antibody, and staining conditions. The procedure described here was optimal to show the strong immunoreactivity of elastin. Thus, we applied this procedure to the immunohistochemical study of the TM that was necessary to maintain their laminated structures.

The elastic fibers consist of central amorphous elements and peripheral microfibrillar components. Gong et al., using the immunogold staining method to stain elastin in normal trabecular tissues embedded in Epon-Araldite, found elastin at the center of elastic-like fibers in the TM. Our high magnification photographs of the elastic-like fibers demonstrated that the amorphous elements are the most immunoreactive for elastin. These findings reveal that elastic-like fibers existing throughout the TM are undoubtedly truly elastic fibers.

An ultrastructural study based on a morphologic classification demonstrated that the sheath materials surrounding the elastic-like fibers were increased in the TM of glaucomatous eyes. However, the constituents of the sheath materials have not yet been identified. The increase of the sheath materials might be related to the synthesis of some extracellular materials associated with changes of the elastic tissues. Elastin-
synthesizing cells respond to hormones and other stimuli. Yun et al. have demonstrated an increase of elastin synthesis by cultured human trabecular cells in the presence of dexamethasone using an immunofluorescence staining method. These findings could suggest a possible mechanism for aqueous outflow resistance in steroid glaucoma, and maybe even in POAG. If changes of some extracellular materials in the TM play a crucial role in aqueous outflow resistance, our finding that elastin within the area containing fine fibrillar-like material increased in the cases of POAG suggests that elastin might be responsible for increased aqueous outflow resistance.

In this study, the glaucomatous changes observed in trabeculectomy specimens should be compared with normal tissues obtained from intact eyes by the same procedure. Because we could not obtain normal specimens from absolutely intact eyes, we used eye bank eyes instead—they were relatively fresh and had suffered no significant postmortem changes. Congenital and juvenile glaucomatous specimens were actually important controls here because their findings were essentially similar to normals regarding immunoreactivity for elastin.

In the cases of congenital and juvenile glaucoma, the amount of positively labelled gold particles for elastin within the area containing fine fibrillar-like material was less than in the cases of POAG. This result suggests that the pathogenesis of POAG is different from that of congenital and juvenile glaucoma, and the changes in elastin distribution in cases of POAG are not responsible for the chronic elevation of the intraocular pressure.

Alvarado et al. described that the morphometric difference in extracellular materials between nonglaucomatous and POAG specimens was probably too small to account for the decrease in outflow facility characteristic of POAG. Under conventional electron microscopy, elastin could be observed only as an electron-lucent amorphous element among electron-dense microfibrils or could not be observed directly in the TM. Also, the quantitative changes of elastin in the cases of POAG could not be observed by previous morphometric ultrastructural analysis. Our analysis using immunogold staining method is the first investigation in which the changes of elastin in the primary open angle glaucomatous TM could be visualized.

Most recently, Hernandez reported marked changes of elastic fibers in the lamina cribrosa in POAG by using an ultrastructural immunocytochemical technique. She noticed that these changes were more pronounced in severe cases of POAG, and she found masses of nonfibrillar elastin-positive material in advanced POAG. Her observations are similar to ours concerning the presence of the elastin in the TM. It remains to be elucidated, however, whether the changes of elastin in the POAG eyes are the cause of POAG or the result of POAG. The immunohistochemical technique we have developed could be employed to elucidate the pathogenesis of POAG.

Key Words

trabecular meshwork, immunogold technique, elastin, primary open angle glaucoma, glaucomatous change

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