Ultrastructural Immunocytochemical Analysis of Elastin in the Human Lamina Cribrosa

Changes in Elastic Fibers in Primary Open-Angle Glaucoma

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The elastic fiber consists of several components: a central core of \( \alpha \)-elastin and a microfibrillar sheath containing three components: fibrillin, microfibril-associated glycoprotein, and a 35-kD protein with amine oxidase activity. Elastin is a major component of the elastic fibers of the extracellular matrix (ECM) of the lamina cribrosa, and elastic fibers undergo marked changes in primary open-angle glaucoma (POAG). These changes, as demonstrated previously, include loss and fragmentation of elastic fibers at the bottom of the glaucomatous cup and disorganization in the peripheral walls of the cup. The author characterized the changes in elastic fibers with age and in POAG at the ultrastructural level, using colloidal gold immunostaining and anti-human \( \alpha \)-elastin antibody. In fetal eyes, there was no detectable elastin in the ECM of the lamina cribrosa. In infant eyes, elastin was present in microfibrillar aggregates in the core of the plates. In young adults, thin elastic fibers were present that ran longitudinally in the core of the plates. With age, elastic fibers become thicker, tubular, and surrounded by densely packed collagen fibers. In mild POAG, tubular elastic fibers no longer were identifiable. Fragments of elastic fibers and microfibrillar aggregates stained positively for elastin suggested new synthesis of elastin that was not organized into tubular elastic fibers. In advanced POAG, masses of nonfibrillar elastin-positive material had a spotted appearance. Throughout the cribiform plates, there was a loss of collagen fibers, proliferation of basement membranes, and bundles of elastin-negative microfibrils not associated with collagen or elastic fibers. The progression of marked changes in elastic fibers and the disorganization of the ECM of the lamina cribrosa was associated with the loss of function and continuous remodeling of the optic nerve head in POAG. Invest Ophthalmol Vis Sci 33:2891-2903, 1992

Our previous studies described the extracellular macromolecules that compose the connective tissue plates of the lamina cribrosa in normal human eyes and in those with primary open-angle glaucoma (POAG). The lamina cribrosa from normal young eyes contains the extracellular matrix (ECM) of a compliant tissue that may be resilient to acute mechanical changes after increases in intraocular pressure. In the young adult, the core of the cribiform plates contains abundant fibers of elastin, a network of filamentous basement membranes, and small amounts of fibrillar collagens. As the lamina cribrosa ages, there is a gradual increase in fibrillar collagens in the core of the plates and increases in the fibers of elastin and basement membranes. These changes suggest that, in normal humans, the connective tissue of the lamina cribrosa may retain flexibility and resiliency with age.

In POAG, there are marked changes in the composition and organization of the ECM of the lamina cribrosa. We observed an increase in density and the area occupied by basement membranes in the prelaminar region and lamina cribrosa of human glaucomatous eyes. A similar observation recently was reported in experimental glaucoma in primates. Histo-pathologic examination of glaucomatous human eyes in early or moderate stages of damage demonstrated glial hyperplasia in both the laminar and prelaminar regions. These proliferating glial cells are the most likely source of newly synthesized basement membranes and probably represent a response to stressful conditions associated with the loss of axons during the glaucomatous process.

In the cribiform plates of the glaucomatous lamina cribrosa, granular masses of elastin appear, and the fibers of elastin increasingly are disorganized during disease progression. In severe POAG, there is appar-

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ently marked loss of elastin from the cribiform plates immediately bordering the disc surface.3

Mature elastic fiber consists of several components: a central electron-lucent core of \( \alpha \)-elastin, responsible for the elastic properties, and a microfibrillar sheath containing at least three components: a 31,000-D microfibril-associated glycoprotein; a 35-kD protein with amine oxidase activity; and fibrillin, a 350,000-D glycoprotein.6 Identification of elastic fibers, at the ultrastructural level, is difficult because of the lack of contrast of amorphous material. Enhancement of staining affinities has been obtained using combinations of heavy metals, such as uranyl acetate with tannic acid or palladium chloride.7 However, these methods cause electron-dense staining of the fibers that obscures the fine structural detail. Thus, characteristic changes with age and diseases of the elastic fibers are difficult to determine.

Immunoelectron microscopic examination of tissues has been used successfully to study elastic fibers in various normal, developing, and diseased tissues, allowing us to localize \( \alpha \)-elastin while preserving the structure of the other components of the elastic fiber.8–13 Using colloidal gold probes and specific antibodies that recognize \( \alpha \)-elastin, we found age-related and glaucomatous changes in the elastic fibers of the human lamina cribrosa.

### Materials and Methods

Six pairs of normal human eyes with no history of eye disease or diabetes (age range, newborn to 83 yr) and two pairs of fetal eyes (ages, 20 and 21 weeks' gestation) were used for age-related studies. Eleven eyes with diagnoses of POAG were obtained through the Foundation for Glaucoma Research, the New England Eye Bank, and several other eyebanks throughout the United States. Information on the type of glaucoma and status of the disease (visual field and/or cup-to-disc ratio) was obtained by contacting the treating ophthalmologists. The eyes then were classified as having mild, moderate, or advanced glaucoma (Table 1), using this information.

### Tissue Preparation

The posterior poles were dissected and fixed either by the eyebank technician or by us. The interval between time of death and time of fixation was 1–10 hr. The tissues were fixed in either 10% buffered formaldehyde in 0.1 mol/l phosphate buffer for 2 hr. The optic nerve heads were dissected free of adjacent tissues (leaving a small rim of sclera) and then divided into four blocks of tissue. The fetal and newborn optic nerves were processed in whole. The specimens were washed several times in the same buffer of the fixative, postfixed in 2% osmium tetroxide for 2 hr, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Semithin sections were cut for light microscopy for cross or sagittal orientation of the lamina cribrosa and insertion region. Thin sections were cut of selected areas and mounted on uncoated nickel grids for electron microscopic observation.

### Postembedding Immunolabeling

Postembedding immunocytochemical analysis was done, using the protein A–gold technique.8,9 The grids were floated, specimen-side down, onto drops of the following reagents at room temperature. To block nonspecific binding, the sections were incubated in 4% nonfat dry milk for 20 min and then transferred without washing (but after removing excess solution) onto drops of primary antibody. We used antiserum against human aorta \( \alpha \)-elastin (working dilution, 1:100; Elastin Products, Owenville, MD). The specificity of this antibody was reported in the literature and was tested by immunocytochemical analysis, enzyme-linked immunosorbent assay, and western blot techniques.14,15 This antibody reacts strongly with human \( \alpha \)-elastin and tropoelastin and less strongly with elastin from other species. No reactivity with other components of the elastic fiber has been reported.10,11 The grids were incubated in the primary antibody diluted with 0.05 mol/l Tris containing 1% bovine serum albumin (BSA) for 2 hr. After rinsing with 0.05 mol/l Tris, pH 7.4, and 0.05 Tris with 0.2% BSA, pH 7.4, the grids were incubated for 1 hr in a solution

| Table 1. Data of eyes with the diagnosis of primary open-angle glaucoma |
|---|---|---|
| **Age of subject (yr)** | **Eye** | **Visual field** | **Cup/disc ratio** |
| 83 | RE | 1 | 0.5 |
| 83 | LE | 1 | 0.6 |
| 74 | RE | 1 | 0.2 |
| 74 | LE | 1 | 0.3 |
| 83 | RE | 2 | 0.8 |
| 83 | LE | 2 | 0.8 |
| 78 | RE | 3 | NA |
| 78 | LE | 3 | NA |
| 74 | RE | 4 | >0.8 |
| 83 | RE | 4 | >0.8 |
| 83 | LE | 4 | >0.8 |

* 1, no defect; 2, increasing scotoma; 3, significant defect; 4, loss of central field; NA, not available.
containing colloidal gold-labeled immunoglobulin G (Jansen Biotech, Olen, Belgium) diluted 1:12 with 0.05 mol/l Tris and 1.5% BSA, pH 8.3. The sections then were rinsed sequentially in 0.05 mol/l Tris and 0.2% BSA, 0.05 mol/l Tris, and distilled water. All specimens were counterstained in uranyl acetate and lead citrate and examined in a Phillips 410 electron microscope (Eindhoven, The Netherlands).

Light microscopic observation was used to select areas of the lamina cribrosa to be examined. At the electron microscopic level, we examined cribiform plates from the center and the periphery of the lamina and the insertion region in normal eyes and those with mild POAG. In eyes with moderate and advanced POAG, we examined the cribiform plates in the periphery of the glaucomatous cup, the deeper and superficial aspects of the lamina in regions bordering the surface of the disc, and the insertion region.

Control Specimens

The specificity of the immunolabeling was tested with the following control specimens: (1) replacement of the antiserum by nonimmune rabbit serum or the antiserum adsorbed overnight at 4°C with the corresponding antigen, and (2) before immunolabeling, elastase digestion. Briefly, grids were immersed in elastase type III (Sigma, St. Louis, MO) diluted in 0.5 mol/l Tris, pH 8.8, for 1 hr at room temperature.9,10

Results

Fetal and Postnatal Development of Elastic Fibers in the Lamina Cribrosa

There was no labeling for α-elastin in the ECM between layers of cells in the fetal lamina cribrosa (Fig. 1A). Occasional bundles of collagen fibers, microfibrils, and basement membranes were present in the scanty ECM of the core of the cribiform plates. Larger accumulations of collagen fibers were observed around blood vessels (Fig. 1B). By contrast, abundant staining for α-elastin in the form of fibers was evident in the sclera adjacent to the optic nerve head (Fig. 1C).

In the newborn eye, α-elastin was present in irregularly shaped aggregates of microfibrils in the ECM of the plates (Fig. 2A). These microfibrillar aggregates usually were associated with the surface of cells and basement membranes (Fig. 2B). Not all microfibrillar aggregates were labeled with colloidal gold particles. As in the fetal eye, α-elastin staining was prominent in the sclera adjacent to the optic nerve head (Figs. 3A–B).

Elastic Fibers in the Adult Lamina Cribrosa

In the young adult, α-elastin was localized in thin long elastic fibers running longitudinally in the ECM of the core of the plates (Fig. 4A). Electron-lucent areas labeled for α-elastin appeared interspersed in the microfibrillar framework of the fiber. In old normal eyes, α-elastin was present in the form of long tubular fibers. Gold particles were distributed homogeneously in the thickness of the fibers (as seen in longitudinal views, Fig. 4B). Elastic fibers appeared thicker than in young eyes and were embedded in a dense collagenous matrix in the core of the cribiform plates. The elastic fibers in close contact with this matrix were parallel to the collagen fibers; these were oriented longitudinally. Occasionally, bundles of microfibrils also were present in the collagenous matrix in older eyes. In cross sections, the central area of the elastic fibers appeared electron lucent. The α-elastin was localized to the electron-lucent areas of the core. A microfibrillar sheath surrounded the central core (Fig. 5A).

Changes in POAG

At the ultrastructural level, in mild POAG, there were marked changes in the ECM throughout the entire lamina cribrosa in all eyes examined compared with age-matched control eyes. There was thickening of the basement membranes lining the plates. In the core of the plates, there was an abundance of basement membrane-like material not associated with cellular processes. The collagen matrix appeared loose; there was a marked loss of collagen fibers. The characteristic thick tubular appearance of the elastic fibers of normal tissues was no longer visible in eyes with POAG (Figs. 6A–B). At high magnifications (Figs. 7A–B), α-elastin was localized to fragments of elastic fibers interspersed throughout the core of the cribiform plates. In POAG, the electron-lucent central area of the fragments was not clearly visible, and the fragments were surrounded by an irregular sheath of microfibrils. When long elastic fibers were present, they were labeled poorly with the antibody and were associated with basement membrane-like material. Numerous bundles of microfibrils, not labeled with α-elastin, were present throughout the core of the cribiform plates. Occasionally, long-spacing collagen also was present.

In moderate POAG, changes in the ECM were similar to those described for mild POAG, but they were more pronounced throughout the lamina cribrosa and insertion region. Loss of collagen fibers, abundance of bundles of microfibrils, and proliferation of basement membranes were evident in the core of the cribiform plates. Elastic fibers of normal appearance...
Fig. 1. (A) Sagittal view of the lamina cribrosa in a 21-week-old (gestational age) fetal eye. Notice the absence of immunogold labeling in the spaces between cells in the cribriform plates. Asterisk indicates a basement membrane (×17,680). (B) Small blood vessel (BV) in the lamina cribrosa. Notice collagen fibers and microfibrils around the vessel. A well developed basement membrane and glial cells (G) separate the perivascular extracellular matrix from the nerve axons (Ax) (×24,000). (C) Immunogold labeling of elastin in the sclera adjacent to the optic nerve head in the same eye shown in (A) and (B) (×26,000).

were less frequent; instead, elastin-staining aggregates of irregular and varied shapes were abundant in the core of the cribriform plates and the insertion region (Figs. 8A–B). We found α-elastin was localized either to the electron-lucent and/or electron-dense areas inside the aggregates. The elastic fibers and the elastin-staining aggregates were surrounded by a granular matrix unlike the dense collagen fiber matrix observed in normal eyes.

In advanced POAG, the boundaries and internal structure of cribriform plates disappeared at the border of the surface of the disc at the base of the cup. The ECM was composed of loose basement membranes, isolated bundles of collagen fibers, and abnormal elastin-staining aggregates occupying the space between the surface of the disc and the beginning of the myelinated nerve. Aggregates of nonfibrillar elastin-staining material were abundant and had a "honeycomb" or "spotted" appearance (Fig. 9A). The immunogold particles were localized preferentially to the electron-dense areas; the microfibrils present within and around the aggregates were not labeled. The density of collagen fibers was reduced greatly and replaced by spaces filled with granular matrix. As in

Fig. 2. (A) Sagittal view of cribriform plates in a newborn eye. Gold-labeled antibody localization of elastin (arrows) on a microfibrillar aggregate in close association to a lamina cribrosa cell (LCC) (×26,000). (B) Cross-sectional view of cribriform plates in a 1-month-old infant eye. As in (A), note microfibrillar aggregates adjacent to the surface of lamina cribrosa cells. Asterisks indicates basement membrane (×26,000).
mild and moderate POAG, basement membrane-like structures and bundles of microfibrils were present in the ECM. Cross-sectional views of the bottom of the glaucomatous cup, at the level of myelinated nerves, revealed a thick dense collagogenous matrix separating the remnant nerve bundles. Interspersed throughout collagen matrix, disorganized electron-dense ribbon-like fibers (labeled for α-elastin) were observed (Fig. 9B).

Control Studies

Specific label was abolished when the primary antibody was replaced by either normal rabbit serum or primary antibody preadsorbed with human α-elastin (Figs. 3B, 5B). Digestion with elastase partially reduced the density of colloidal gold particles from elastic fibers in the lamina cribrosa and sclera (Fig. 5C).

Discussion

The elastic properties of tissues are a result of the presence of elastic fibers in the extracellular matrix. Ultrastructural examination demonstrated that elastic fibers are composed of two morphologically distinct components: a central amorphous core and a microfibrillar component that surrounds the amorphous core and also is scattered and enmeshed throughout the core. Elastin is the protein that comprises the amorphous core of the elastic fiber and is responsible for the elastic properties. This substance reacts poorly with conventional ultrastructural stain-
Fig. 5. Cross-sectional views of elastic fibers in the cribriform plates of an 81-year-old normal donor eye. (A) Immunogold labeling of the electronlucent areas of the core of the fibers. The electron-dense areas in the core of the fibers are not labeled. A thin sheath of microfibrils surrounds the core of the fibers. Elastic fibers are surrounded by a dense collagen fiber matrix in adult eyes (×26,000). (B) No staining for elastin is evident when the primary antibody is replaced by nonimmune serum (×26,000). (C) Elastase digestion diminishes the density of gold particles (×26,000).

In fetal eyes, there was no staining for α-elastin in the lamina cribrosa, indicating the absence of this macromolecule. The developing cribiform plates are formed by layers of glial cells separated by basement membranes interspersed by small spaces containing a few collagen fibers and microfibrils. At this stage of fetal development, the sclera adjacent to the optic nerve head contains abundant elastic fibers stained positively for α-elastin.

At birth, abundant aggregates of microfibrils appear in the extracellular matrix of the cribiform plates; positive staining for α-elastin is detectable first in these aggregates. As reported previously in other tissues undergoing elastogenesis, microfibrils are in close proximity with the surface of the predominant cell, in this case, the lamina cribrosa cells. The appearance of microfibrils precedes that of elastin; thus, microfibrils hypothetically determine the pattern of the mature elastic fiber. During the morphogenesis of the elastic fiber, elastin molecules first appear on preexisting microfibrillar structures. By forming loci on the immature elastic fiber, elastin deposits coalesce to form the electron-lucent core characteristic of the mature fiber. Subsequently displacing the microfibrils to the periphery of the fiber during maturation. Fibrillin, a component of the microfibrils associated with elastic fibers, has been described previously in the lamina cribrosa of newborn eyes.

In the young adult, elastic fibers are thin, long, and run longitudinally in the core of the cribiform plates. Elastin localizes to electron-lucent areas interspersed throughout the thickness of the elastic fiber. With age, elastic fibers increase in thickness to form long tubular structures. Elastin localizes to the electron-lucent areas of the core of the fiber. Little is known about the aging of elastin. The age-related increase in the thickness of the elastic fibers in the lamina cribrosa may be caused by continuous synthesis of the macromolecule with age and/or to increased cross linking. In tissues rich in elastin, such as aorta, elastin is synthesized postnaturally for a short period. Cross linking occurs continuously with age, leading to stiffer tissues; no measurable turnover of elastin occurs with age.

Future studies in our laboratory will address the biosynthesis, cross linking, and degradation of elastin with age in the human lamina cribrosa.

In POAG, there are marked changes in the ECM of the lamina cribrosa. Decreases in collagen content and proliferation of basement membranes are evident in the core of the plates; these were noticed in earlier studies in human and in monkey glaucomatous optic nerves. Changes in the elastic fibers were apparent; there was loss of the tubular structure of the fibers and irregular poor staining pattern in the fibers that remained intact. In mild POAG, abundant bundles of microfibrils, unlike those in age-matched normal eyes, were present throughout the entire lamina cri-
Elastic fibres in normal and glaucomatous lamina cribrosa. Some of these microfibrillar bundles stained positively for elastin, showing a fibrillar or granular ultrastructure, as in diseases characterized by abnormal or increased elastogenesis. Thus, in mild POAG, elastin molecules might be newly synthesized.

As the disease advances, the changes in the elastic component of the lamina cribrosa are more obvious. Masses of aggregated material stained positively for elastin were present in the periphery of the cup and the insertion region. The dense collagenous matrix that normally surrounds elastic fibers in the cribiform plates was replaced by a granular matrix. Spotted patterns of elastin-staining aggregates with a honeycomb appearance were seen in advanced POAG.

The localization of elastin in these aggregates was different from that in normal elastic fibers. The dark electron-dense areas were labeled with the antibody to α-elastin; in normal elastic fibers, elastin is localized to electron-lucent areas of the fiber. Similar pathologic changes occur in various human diseases affecting either the production or degradation of elastic fibers. For example, honeycomb-like elastic fibers are present in Marfan syndrome and in certain forms of Ehlers-Danlos syndrome; presumably, these are caused by a lack of polymerization and/or abnormal maturation of elastic fibers. Alterations in elastic fiber maturation observed in Marfan syndrome are consistent with the recent discovery of a mutation in the gene that encodes for fibrillin in these patients. Moth-eaten or spotted appearance of elastic material is characteristic of elastotic degeneration of elastic fibers observed in actinic-damaged skin. This may be the consequence of excessive production of abnormal elastic fibers and progressive elastolysis.

Based on our findings and by analogy with other disease states, in early stages of POAG, there appears to be new formation of elastin, proliferation of basement membranes, and decreases in collagen density in the cribiform plates. This listing is similar to the events that characterize the early phase of atherosclerosis. Proliferating smooth muscle cells in the intima of the aorta synthesize basement membranes and elastic material, maintaining both ECM macromolecules in close association during this process. In later stages of POAG, the abnormal elastic fibers ("elastotic fibers") suggest abnormal enzymatic activity, either enhanced degradation or impaired maturation of newly formed material.

Furthermore, these glaucomatous changes in the elastic fibers are accompanied by a decrease in collagen fiber density in the cribiform plates. Function-
Fig. 7. (A) Mild POAG, 74-year-old. Elastic fibers (E) poorly labeled with elastin antibody are in close association with basement membrane-like material (asterisk). Note the abundance of aggregates of microfibrils (Mf) and the sparsity of collagen fibers surrounding the elastic fibers. Cross-sectional view (x26,000). (B) Mild POAG, 74-year-old. Irregular aggregates of microfibrils labeled with elastin antibody (E) and long spacing collagen bundles (LSC) are present in a sagittal view of the cribiform plates (x26,000). (C) Normal, 83-year-old. Immunogold staining for elastin in an elastic fiber with normal ultrastructure. Notice densely packed collagen fibers surrounding the fiber (x26,000).

Fig. 8. Moderate POAG, 78-year-old. (A) Elastin-staining aggregates in the core of cribiform plates. Notice bundles of microfibrils (Mf) and sparsity of collagen fibers around the aggregates (x26,000). (B) Insertion region. Cross-section of abnormal elastic fibers surrounded by granular matrix. Arrow points to small group of remnant collagen fibers (x26,000).
ally, elastic fibers and collagen act as parallel mechanical elements to applied stress or strain. At low levels of strain, collagen fibers can be extended easily with most of the stress borne by elastic fibers. At high levels of strain, collagen fibers limit further distension, providing strength and support to the tissue. The decrease in density of collagen and the progression of changes in the elastic fibers in the core of the plates observed in mild POAG must alter the mechanical properties of the tissue early in the disease significantly and may lead to the collapse, compression, and remodeling of the cribriform plates in advanced stages of this disease.

**Key words:** elastin, lamina cribrosa, immunocytochemistry, human, glaucoma

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**References**

12. Li Z-Y, Streten BW, and Wallace RN: Association of elastin