Distribution and Characterization of Sulfated Proteoglycans in the Human Trabecular Tissue

Akihiko Tawara, Hugh H. Varner, and Joe G. Hollyfield

The distribution of proteoglycans in the trabecular tissue of human eyes was evaluated histochemically, using Cupromeronic Blue in combination with a series of enzyme digestions and nitrous acid treatment. Within the extracellular matrix of the trabecular meshwork, many Cupromeronic Blue-positive filaments were observed in association with collagen fibrils, basal lamina, a basal lamina-like material and a fine fibrillar-like material. Pretreatment with chondroitinase AC reduced the staining associated with the collagen fibrils, whereas filament staining in this location was completely eliminated by pretreatment with chondroitinase ABC. Nitrous acid treatment eliminated almost all the filament staining associated with the basal lamina and basal lamina-like material. When the tissue was treated with chondroitinase AC, chondroitinase ABC or nitrous acid, filament staining associated with the fine fibrillar-like material was reduced. After a combined treatment with nitrous acid followed by incubation with chondroitinase ABC, all filament staining in the trabecular meshwork was eliminated. These results are consistent with an interpretation that human trabecular tissue contains three distinct types of sulfated proteoglycans, namely chondroitin sulfate, dermatan sulfate and heparan sulfate-type proteoglycans. Proteoglycans containing chondroitin sulfate and dermatan sulfate are present in association with collagen fibrils. Proteoglycans of heparan sulfate-type are associated with the basal lamina and the basal lamina-like material. Chondroitin sulfate, dermatan sulfate and heparan sulfate-type proteoglycans are present in association with the fine fibrillar-like component. The basal lamina, basal lamina-like material and fine fibrillar-like components associated with these negatively charged proteoglycans may be important contributors to aqueous outflow resistance in the juxtacanalicular connective tissue. Invest Ophthalmol Vis Sci 30:2215–2231, 1989

It is generally accepted that a major portion of the aqueous humor leaves the anterior chamber by a process of bulk flow through the trabecular meshwork into the canal of Schlemm. Histologically, the trabecular meshwork can be divided into three parts: the uveal and corneoscleral meshworks, and the juxtacanalicular connective tissue. The corneoscleral meshwork is formed by flat interlacing sheets of loose, fibrous connective tissue components, in contrast to the uveal meshwork, which has a cord-like structure. Although their gross morphologies are different, their cellular and extracellular components are quite similar. The central core of the trabecular lamellae (trabecular beam), which consists of extracellular matrix, is covered by a single layer of endothelial cells. The inter trabecular spaces in the uveal and corneoscleral meshworks are extremely expansive and offer virtually no resistance to aqueous outflow in these portions of the meshwork. The juxtacanalicular connective tissue, located just beneath the inner wall of Schlemm’s canal, has an architecture consisting of several layers of endothelial cells that are embedded in an extracellular matrix of fibrous connective tissue elements and ground substance. In the juxtacanalicular connective tissue, aqueous humor flows through the spaces in the extracellular matrix and reaches the lumen of Schlemm’s canal by way of the giant vacuoles that traverse the endothelial cells covering the inner wall of the canal. The main resistance to aqueous humor outflow via Schlemm’s canal is thought to reside at the level of the juxtacanalicular connective tissue. Although the identity of the components in the trabecular meshwork responsible for resistance to aqueous outflow are not known, glycosaminoglycans (GAGs) have been implicated by the reports that outflow resistance was reduced by testicular hyaluronidase or Streptomyces hyaluronidase in the perfusion fluid. Histochemical attempts

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to demonstrate GAGs in the aqueous outflow pathway in the normal mammalian eyes and glaucomatous human eyes have been made. However, since the identity and distribution of GAGs in the trabecular meshwork have been inconsistent among the previous reports and because of the potentially important role of these connective tissue elements in aqueous outflow resistance, additional investigations in this area are needed.

GAGs, with the exception of hyaluronic acid and heparin, exist as components of proteoglycan molecules, covalently linked as side chains to a core protein. Histochemical procedures for staining proteoglycans in tissues employing a cationic copper phtha-

Lysis at the ultrastructural level. We have studied the distribution of proteoglycans in nonglaucomatous human trabecular meshwork using Cupromeronic Blue in combination with a series of specific enzyme digestions and nitrous acid treatment.

Materials and Methods

Materials

Cupromeronic Blue was a gift from Dr. Tom Wickersham of Aldrich Chemical Company (Milwaukee, WI). Chondroitinase AC, chondroitinase ABC and keratanase were purchased from Sigma Chemical Co. (St. Louis, MO).

The human eyes, with no history of glaucoma, used in these studies were obtained through the Lions Eye Bank, Houston. The clinical data related to these donations are as follows; Donor A: a 34-year-old male who died from a closed head injury. Fifteen minutes after death, his eyes were enucleated and immersed in fixative. Both eyes were used in this analysis. Donor B: a 59-year-old male who died from pneumonia. His eyes were enucleated and fixed 35 min after death. Only the right eye was used. Donor C: a 60-year-old female who died from renal cancer. Her left eye, which was fixed 45 min after death, was used in this analysis. Donor D: a 76-year-old female who died from complications following coronary bypass surgery. Her eyes were enucleated 25 min after death and fixed immediately. Only the left eye was used in this analysis. All eyes were fixed in 1% formaldehyde, 2% glutaraldehyde in 0.1 M Sorensen’s phosphate buffer, pH 7.4 and remained in fixative for several days to 1 month before being used in these studies. The duration of fixation had no effect on the pattern or intensity of the staining with Cupromeronic Blue. The eyes were transected by an equatorial incision while in the fixative. After removing the lens, the anterior chamber angle tissues, including the trabecular tissue, the periphery of the cornea, iris and ciliary body were cut out as a 2–3 mm³ block.

Morphometric Analysis of Proteoglycans in the Trabecular Meshwork

The anterior chamber angle tissues from all eyes were stained with Cupromeronic Blue according to the procedure described below. Several anterior chamber angle tissues from each eye served as control tissues which were treated with the same solutions as for Cupromeronic Blue staining except that the dye was omitted from the staining mixture.

Enzyme and Nitrous Acid Treatments

The anterior chamber angle tissues from right eye of the 34-year-old man (Donor A) and left eye of the 60-year-old woman (Donor C) were used for this analysis. After removal of the iris, cornea, sclera and ciliary body as much as possible, the anterior chamber angle tissues were cut into 200 μm wide tissue strips using a LKB tissue chopper. They were kept in 0.1 M phosphate buffer overnight, then divided into ten groups that were processed with one of the following treatments.

1. Chondroitinase AC: After storing in cold, enriched Tris buffer (0.25 M Tris–HCl, 0.18 M NaCl, 0.05% BSA, 5 mM benzamidine-HCl and 0.1 M 6-amino-caproic-acid, pH 8.0) for 24 hr, the tissues were incubated in 0.5 ml enriched Tris buffer with 1 U/ml chondroitinase AC for 24 hr at 37°C.

2. Chondroitinase ABC: Anterior chamber angle tissues were stored in cold, enriched Tris buffer for 24 hr, then replaced in 0.5 ml enriched Tris buffer with 1 U/ml chondroitinase ABC for 24 hr at 37°C.

3. Control for 1 and 2: The tissues were stored in cold, enriched Tris buffer for 24 hr, then incubated in 0.5 ml enriched Tris buffer with no enzyme for 24 hr at 37°C.

4. Keratanase: After the tissues were stored in cold Tris buffer (0.1 M Tris-HCl, 5 mM benzamidine-HCl, 0.1 M 6-amino-caproic-acid, pH 8.0) for 24 hr, they were replaced in 0.5 ml of Tris buffer with 1 U/ml keratanase for 24 hr at 37°C.

5. Control for 4: After storing in cold Tris buffer, the tissues were incubated in 0.5 ml Tris buffer without the enzyme for 24 hr at 37°C.

6. Nitrous acid: Equal volumes of 5% sodium nitrite and 33% acetic acid (v/v) were mixed and al-
lowed to stand for 90 min at room temperature, after which 5 mM benzamidine–HCl and 0.1 M 6-aminocaproic acid (protease inhibitors) were added. After rinsing twice in 0.2 M acetic acid containing protease inhibitors, anterior chamber angle tissues were incubated in the solution for 90 min at room temperature. Then, the tissues were washed in 0.2 M acetic acid containing protease inhibitors.

7. Control for 6: The tissues were treated according to the same procedure with the same solution as in treatment 6 except that 0.2 M acetic acid containing protease inhibitors was substituted for the nitrous acid solution.

8. Nitrous acid+chondroitinase AC: After incubation in nitrous acid containing protease inhibitors for 90 min at room temperature, the anterior chamber angle tissues were washed in 0.2 M acetic acid containing protease inhibitors. They were stored in 0.1 M phosphate buffer for 30 min and replaced in enriched Tris buffer for 60 min, then, incubated in 0.5 ml enriched Tris buffer with 1 U/ml chondroitinase AC at 37°C for 24 hr.

9. Nitrous acid+chondroitinase ABC: Incubation of the tissues in nitrous acid containing protease inhibitors for 90 min at room temperature was followed by two washes in 0.2 M acetic acid containing protease inhibitors. After storing in 0.1 M phosphate buffer for 30 min and in enriched Tris buffer for 60 min, they were incubated in 0.5 ml enriched Tris buffer with 1 U/ml chondroitinase ABC at 37°C for 24 hr.

10. Control for 8 and 9: The anterior chamber angle tissues were treated according to the same procedure for treatment 8 or 9. However, acetic acid containing protease inhibitors was used instead of nitrous acid solution, and 0.5 ml enriched Tris buffer was used instead of the same buffer with the chondroitinase enzymes.

After incubation, all tissues were stored in cold 0.1 M phosphate buffer overnight and were then stained with Cupromeronic Blue as described below.

Histochemical Procedures

Cupromeronic Blue staining followed the method of Scott.16,17 The fixed anterior chamber angle tissues were first equilibrated for 1 hr in several changes of 25 mM sodium acetate, 0.2 M MgCl₂ and 2.5% (w/v) glutaraldehyde, pH 5.7. The tissues were then placed in 0.05% Cupromeronic Blue in the sodium acetate fixative, where they remained overnight. Tissues were rinsed three times in the fixative without the dye, followed by three washes in aqueous 0.5% sodium tungstate, and three washes in 0.5% sodium tungstate in 50% (v/v) ethanol. All tissues were dehydrated in graded concentrations of ethanol and embedded in Epon. Ultrathin sections were analyzed by electron microscopy, with uranyl acetate staining. Some sections of the control tissue for Cupromeronic Blue staining were observed after staining with uranyl acetate and lead citrate.

Results

The origin and identity of the constituents present in the extracellular matrix of the trabecular meshwork have not been well defined nor is there a unified terminology for designation of the various matrix components. In the subsequent description of our results and discussion, we will use the following terminology in referring to the morphologically different components that can be resolved with electron microscopy:

The extracellular matrix in the trabecular tissue can be divided into two broad categories, the fibrous elements and the ground substance. The fibrous elements include collagen fibrils and elastin-like fibers. The latter represent the electron-dense clumps of fibrils which are scattered in the trabecular beams and in the juxtacanalicular connective tissue (Fig. 1A, B). Since the properties of these fibers are reported to be different from elastic fibers present in other connective tissues, the term elastin-like fibers18-20 has been used. The ground substance includes the basal lamina, basal lamina-like material, fine granular-like material and fine fibrillar-like material. The basal lamina is represented by the homogenous linear profile aligned with the basal surface of the endothelial cells covering the trabecular beams, bordering the inner wall of Schlemm’s canal and located in the juxtacanalicular connective tissue. The basal lamina is not always continuous along the basal border of endothelial cells, and frequently gaps occur where, what appears to be the basal side of an endothelial cell, is free of the basal lamina material. Basal lamina-like material also appears in the form of a homogeneous linear profile. However, this material is present away from the endothelial cells and is frequently observed in stack-like arrays forming a lamellar pattern in the juxtacanalicular connective tissue (Fig. 1A). The fine granular-like material is comprised of a homogeneous component with electron-lucent profiles that have a granular particulate appearance (Fig. 1B). This component is frequently present surrounding bundles of elastin-like fibers. The fine fibrillar-like material is present principally in the juxtacanalicular connective tissue, especially near Schlemm’s canal, and shows a mixture of short fibrils (10–20 nm in diameter) that appear to be embedded in an electron-lucent, homogeneous matrix (Fig. 1C).
Fig. 1. Electron micrographs of the extracellular matrix in the juxtacanalicular connective tissue of the control tissue incubated in absence of Cupromeronic Blue. (A) The displaced basal lamina-like material (small arrows) and the bundles of elastin-like fibers (EL) (×37,000). (B) The fine granular-like material (asterisks) (×29,000). (C) The fine fibrilar-like material consisting of a mixture of fine fibrils (large arrows) and a fine homogeneous component (small arrows) (×50,000). No other electron-dense staining in the extracellular matrix except for the large comma-like deposits (open arrows) present in (A). This section was stained with uranyl acetate and lead citrate. EN: endothelial cell. Bars indicate 0.5 μm. Left eye from Donor A (34 years old).
Cupromeronic Blue Staining in the Normal Trabecular Tissue

The relative proportion of components in the extracellular matrix of the trabecular meshwork varied somewhat among the different tissue samples studied. In some of the older eyes, age-related changes such as increased appearance of long-spacing (lattice) collagen and thickening of basal lamina and basal lamina-like material were observed. However, the composition and localization of the components of the extracellular matrix in the trabecular tissue were basically identical and distribution of filaments following Cupromeronic Blue staining was virtually identical in the tissues from all five eyes. There was no noticeable difference in the staining associated with each component among uveal and corneoscleral meshworks or the juxtacanalicular connective tissue, although the fine fibrillar-like material was observed principally in the juxtacanalicular region. Three types of Cupromeronic Blue-positive filaments could be distinguished based on differences in size, electron density and location in the trabecular meshwork (Fig. 2). The largest Cupromeronic Blue-positive filaments, which will be referred to as CB-1 filaments, were 80–85 nm long and 10–13 nm wide (the sizes of the filament type were estimated on the prints by measuring the 30 largest typical filaments in each class). Intermediate size filaments, which we refer to as CB-2 filaments, were 50–55 nm long and 7–10 nm wide. Both CB-1 and CB-2 filaments show similar electron densities following Cupromeronic Blue staining. CB-3 filaments, which stained less intensely than CB-1 or CB-2 filaments, were only slightly smaller than CB-2 filaments, measuring 45–50 nm in length and 6–8 nm in width.

CB-1 and CB-3 filaments were observed associated with collagen fibrils present in all regions of the trabecular meshwork (Figs. 2, 3, 4). When collagen was sectioned across the long axis of the fibril, both CB-1 and CB-3 filaments appeared to span the space between adjacent collagen fibrils. Since CB-1 filaments...
Fig. 3. Longitudinal section of collagen fibrils in a trabecular beam treated with Cupromeronic Blue. CB-1 filaments (large arrowheads) appear irregularly arrayed in contrast to CB-3 (small arrowheads), which are present at regular intervals along the collagen fibrils ($\times 110,000$). Bar indicates 0.1 $\mu$m. Right eye from Donor B (59 years old).

Fig. 4. Basal lamina-like material in the juxtacanalicular connective tissue stained with Cupromeronic Blue. Many CB-2 filaments (large arrowheads) are associated with basal lamina-like material. CB-3 filaments (small arrowheads) are present around the collagen fibrils. Few filaments are observed in the elastin-like fiber bundles (EL). EN: endothelial cell ($\times 62,000$). Bar indicates 0.5 $\mu$m. Left eye from Donor D (76 years old).
were longer than CB-3 filaments, the distance between adjacent collagen fibrils bridged by CB-1 filaments was greater than the space bridged by CB-3 filaments (Fig. 2). When collagen fibrils were cut along their long axis, CB-3 filaments radiated at right angles to the long axis and were spaced at regular intervals, appearing to be attached to the collagen fibril at or near the "d" or "e" band (Fig. 3). CB-1 filaments were associated with the collagen fibrils in random orientations (Fig. 3).

Elastin-like fibers in the trabecular tissue were virtually free of Cupromeronic Blue-positive filaments (Figs. 2, 4).

CB-2 filaments were associated with the basal lamina below the endothelial cells covering the trabecular beams and the inner wall of Schlemm's canal, and in the juxtacanalicular connective tissue (Fig. 2). When sections were obtained at near right angle to the orientation of the basal lamina, CB-2 filaments were present on both sides of the basal lamina. When thicker profiles of basal lamina were encountered, CB-2 filaments were arranged in a single layer on the endothelial side of the basal lamina (Fig. 2). In the juxtacanalicular connective tissue and the trabecular beams, several layers of basal lamina-like material that were not closely associated with endothelial cells were also present. CB-2 filaments were also associated with the surface of this displaced basal lamina-like material (Figs. 2, 4).

Fine fibrillar-like material was usually present in the juxtacanalicular connective tissue near Schlemm's canal. In places where the basal lamina was interrupted, this fine fibrillar-like material was sometimes observed in close proximity to the basal surface of the endothelial cells (Fig. 5). CB-1 and smaller filaments were always observed in this fine fibrillar-like material (Fig. 5). It was not always apparent whether the smaller filaments consisted of both CB-2 and CB-3 filaments or only one of these filament types. However, judging from differences in electron density and width, the smaller filaments seemed to be comprised of both CB-2 and CB-3 filaments (Fig. 5).

Few electron-dense filaments were observed in the fine granular-like ground substance in the trabecular beams or juxtacanalicular connective tissue (Figs. 2,

![Fig. 5. Fine fibrillar-like material in the juxtacanalicular connective tissue below the inner wall of Schlemm's canal in the tissue stained with Cupromeronic Blue. Many CB-1 (large arrowheads), CB-2 (medium arrowheads) and CB-3 (small arrowheads) filaments are present. A few electron-dense profiles are present associated with the apical surface of the endothelial cells (EN) lining the canal. The fine granular-like material (asterisks) rarely contains any Cupromeronic Blue-positive filaments (×63,000). Bar indicates 0.5 μm. Left eye from Donor D (76 years old).](https://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933375/)
In some areas, several filaments of all three types were observed surrounding the material. However, it was not clear whether they were associated with the fine granular-like material or with other matrix components located just adjacent to the material (Fig. 5).

Virtually no Cupromeronic Blue-positive filaments were present on the apical surface of the endothelial cells that covered the trabecular beams or the inner wall of Schlemm’s canal (Figs. 2, 5).

In the control tissues treated with the same solutions as for Cupromeronic Blue staining except that the dye was omitted from the staining mixture, no electron-dense filaments were present in the trabecular tissue (Fig. 1A–C). However, electron-dense comma- to dot-like profiles, which may be deposits of tungsten, were occasionally observed (Fig. 1A).

Enzyme or Nitrous Acid Treatment

Enzyme or nitrous acid treatment differentially altered the staining of the three filament types. Following incubation with chondroitinase AC, CB-1 filament staining was eliminated (Fig. 6). Digestion with chondroitinase ABC eliminated the staining of CB-1 and CB-2 filaments (Fig. 7A, B). Following treatment with nitrous acid, CB-2 filament staining was eliminated (Fig. 8A, B). In contrast, incubation with keratanase caused no notable changes in staining of the three filament types (micrograph not shown).

Within the collagen bundles, following chondroitinase AC treatment CB-1 filament staining was eliminated whereas CB-3 filament staining persisted. The latter was closely associated with the “d” or “e” band of individual collagen fibrils (Fig. 6A, B). Digestion with chondroitinase ABC eliminated both CB-1 and CB-3 filament staining associated with the collagen fibrils (Fig. 7A, B), whereas treatment with nitrous acid did not alter filament staining in this compartment (Fig. 8A, B).

In the basal lamina of the endothelial cells as well as in the displaced basal lamina-like material, CB-2 filament staining was eliminated following incubation with nitrous acid (Fig. 8A, B). Digestion with chondroitinase AC or chondroitinase ABC did not alter staining of CB-2 filaments (Fig. 7A, B). In some areas, after digestion with chondroitinase ABC, border layers of CB-2 filaments present along opposite surfaces of the basal lamina and basal lamina-like material were much more apparent because of the absence of staining of CB-1 and CB-3 filaments associated with collagen fibrils (Fig. 7B).

In the fine fibrillar-like material, after digestion

Fig. 6. Collagen fibrils in a trabecular beam from tissue incubated with chondroitinase AC before Cupromeronic Blue staining. (A) Only CB-3 filaments, occurring at regular intervals along the collagen fibril are stained. Compare with Figure 3 (×110,000). (B) Enlargement showing close association of CB-3 filaments with the “d” or “e” band of collagen fibrils (arrowheads) (×150,000). Bars indicate 0.1 µm. Right eye from Donor A (34 years old).
Fig. 7. Trabecular beam from tissue incubated with chondroitinase ABC prior to Cupromeronic Blue staining. Almost all the Cupromeronic Blue staining is eliminated from the collagen fibrils. (A) Along the surface of basal lamina (BL) associated with an endothelial cell (EN), a layer of CB-2 filaments are present (arrowheads) (×63,000). (B) Along both surface of basal lamina adjacent to the endothelial cells (EN), two layers of electron-dense CB-2 filaments (arrowheads) are evident. The displaced basal lamina-like material (arrows) located away from the endothelial cells also contains CB-2 filaments (arrowheads). EL: elastin-like fiber (×62,000). Bars indicate 0.5 µm. Right eye from Donor A (34 years old).
Fig. 8. Trabecular tissue treated with nitrous acid before Cupromeronic Blue staining. Almost all the CB-2 filament staining associated with basal lamina (BL) in the trabecular beam (A) and with basal lamina-like material (arrows) in the juxtacanalicular connective tissue (B) has been eliminated. Many filaments of CB-1 (large arrowheads) and CB-3 (small arrowheads) persist around the collagen fibrils. Asterisk indicates a profile of fine granular-like material. EL: elastin-like fibers, EN: endothelial cell (A, B: X62,000). Bars indicate 0.5 μm. Right eye from Donor A (34 years old).
with chondroitinase AC, CB-1 filament staining was lost, but there were still many filaments remaining, which appeared to be composed of a mixture of CB-2 and CB-3 (Fig. 9A). Incubation with chondroitinase ABC failed to remove all the filaments, leaving small filaments which appeared to be CB-2 type (Fig. 9B). When treated with nitrous acid, although the number of the filaments were reduced, CB-1 filaments, and smaller and less electron-dense filaments of the CB-3 type remained (Fig. 9C). The combined treatments with nitrous acid and chondroitinase AC resulted in the loss of CB-1 and CB-2 filaments with only CB-3 filaments remaining. The latter appeared to be associated with fibrils rather than the homogeneous components which make up this fine fibrillar-like material, (Fig. 9D). When the tissues were treated with nitrous acid followed by incubation in chondroitinase ABC, all Cupromeronic Blue staining was eliminated from the fine fibrillar-like material (Fig. 10).

Therefore, after the combined treatment with nitrous acid and chondroitinase ABC, virtually all Cupromeronic Blue staining was eliminated from the extracellular matrix in the trabecular meshwork (Fig. 10). The combined results of filament staining following these enzyme and nitrous acid treatments are summarized in the Table 1.

Discussion

In the extracellular matrix of the trabecular tissue stained with Cupromeronic Blue, three types of filaments were distinguished on the basis of their length, width, electron density, location and sensitivity to enzyme degradation. When Cupromeronic Blue is applied at a critical electrolyte concentration, this dye selectively stains sulfated proteoglycans. Extracellular matrix filaments that stain with Cupromeronic Blue or the closely related dye, Cuprolinic Blue, are thought to represent proteoglycan monomers. Any differences in filaments profile appearance is thought to represent differences in chemical composition and size of the different types of proteoglycans. Thus, our morphological identification of three filament types within the trabecular meshwork suggests that there are at least three different types of sulfated proteoglycan present within this tissue. The differential sensitivity of the Cupromeronic Blue-stained filaments to the enzyme and nitrous acid treatments strengthens the suggestion that these three filament types represent distinctly different sulfated proteoglycans.

CB-1 and CB-3 filaments were present within the bundles of collagen fibrils in the trabecular tissue. Treatment with chondroitinase AC, which degrades chondroitin sulfate and hyaluronic acid, eliminated the staining of CB-1 filaments. Treatment with chondroitinase ABC, which degrades chondroitin sulfate, dermatan sulfate and hyaluronic acid, virtually eliminated CB-1 and CB-3 filament staining. Hyaluronic acid, because of the absence of sulfate groups, is not stained with Cupromeronic Blue at the critical electrolyte concentration used in this study. In fact, incubation with Streptomyces hyaluronidase did not remove these filament staining from the tissue (data not shown). Therefore, a reasonable interpretation of these results is that the large CB-1 filaments randomly arrayed around the collagen fibrils represent chondroitin sulfate-type proteoglycans, whereas the CB-3 filaments usually associated with the "d" or "e" band of collagen fibrils represent dermatan sulfate-type proteoglycans. Similar findings that these two types of proteoglycans are associated with collagen fibrils have been reported for mouse lung alveoli and bovine arterial wall after staining with Cuprolinic Blue or Cupromeronic Blue. In the trabecular meshwork of human, monkey, rabbit and cat eyes, and in trabeculectomized specimens from eyes with primary open-angle glaucoma, GAGs have been demonstrated around collagen histochemically, although their classes were not identified. Richardson stained the aqueous outflow system of the cat with ruthenium red and observed a coating of stained material around the collagen fibers. Because testicular hyaluronidase partially degraded the stained material, he suggested that the collagen fibers contained testicular hyaluronidase-resistant GAGs such as dermatan sulfate, keratan sulfate or heparan sulfate, acid glycoprotein, or acid glycolipids in addition to hyaluronic acid, chondroitin or chondroitin sulfate.

CB-2 filaments were associated with the basa lamina below the endothelial cells as well as the displaced basal lamina-like material in the trabecular tissue. CB-2 filament staining was eliminated following treatment with nitrous acid, which degrades heparan sulfate-type GAGs. Therefore, a reasonable interpretation of these results is that CB-2 filaments represent heparan sulfate-type proteoglycans. Several histochemical studies have identified GAGs in the trabecular meshwork associated with basal lamina. However, the class of the GAGs was not consistent among these reports, with chondroitin sulfate, dermatan sulfate, as well as hyaluronic acid among those suggested. Murphy and coworkers studied the human trabecular tissue...
Fig. 9. Fine fibrillar-like material in the juxtacanalicular area of the tissue treated with enzyme and/or nitrous acid prior to Cupromeronic Blue staining. (A) (chondroitinase AC) CB-1 filaments are absent but many CB-2 (medium arrowheads) and CB-3 (small arrowheads) filament staining persists (×53,000). (B) (chondroitinase ABC) CB-2 filament (medium arrowheads) staining persists (×53,000). (C) (nitrous acid) Although overall filament staining is reduced, CB-1 (large arrowheads) and CB-3 (small arrowheads) filament staining still persists (×62,000). (D) (combination of nitrous acid and chondroitinase AC) Many of the CB-3 filaments appear to be associated with the fine fibrillar component rather than homogeneous component (×63,000). Asterisk indicates a profile of fine granular-like material. EN: endothelial cell lining the inner wall of Schlemm's canal. Bars indicate 0.5 μm. Right eye from Donor A (34 years old).
using indirect immunofluorescence technique and demonstrated heparan sulfate-type proteoglycan along the subendothelial border of the trabecular beams, which corresponds well with our results on the distribution and identity of CB-2 filaments.

The basal lamina in the trabecular tissue, the homogeneous linear profile aligned with the basal surface of the endothelial cells, shows an atypical appearance with frequent breaks and uneven thickness. It is, however, considered that the material has the same basic composition as that in other tissues,²⁷ because the presence of type IV collagen, proteoglycans, and glycoproteins such as laminin and fibronectin, which are thought to be the characteristic components of the basal lamina in a variety of tissue,²⁷,²⁸ has been demonstrated in the trabecular tissue.²⁷,²⁹,³⁰ Van Kuppevelt et al stained mouse²³,²⁴ and human³¹ lung alveoli with Cuprolinic Blue, and demonstrated nitrous acid-sensitive, electron-dense filaments in the basal lamina of epithelial cells, which appeared to lie closely together in one plane, although sometimes a second layer was apparent. In this study, CB-2 filaments associated with the basal lamina showed the identical pattern of distribution of heparan sulfate-type proteoglycan to that in the basal lamina of other tissues,²³,²⁴,³¹ which strengthens the possibility that the basal lamina in the trabecular tissue has basically the same composition as the basal lamina in other connective tissues.

The basal lamina-like material located away from the endothelial cells showed the same histological appearance as the basal lamina located adjacent to the endothelial cells in the trabecular tissue. The CB-2 filaments, identified as heparan sulfate-type proteoglycans, were also associated with the displaced basal lamina-like material in a pattern identical to the distribution of heparan sulfate type proteoglycan in the basal lamina below endothelial cells. From these similarities in histological appearance and in the histochemical characteristics, these two components, though located in different positions, are thought to be of similar origin and composition. Rohen²⁰,³² Rohen et al,¹⁹ and Lütjen-Drecoll et al¹⁸ have previously suggested that homogeneous ground substance located immediately underneath the inner wall of Schlemm’s canal, which they call type I plaque, was derived from the basal lamina.

Fine fibrillar-like material in the juxtacanalicular...
Table 1. Results of enzyme digestion

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</tbody>
</table>

Components of extracellular matrix stained with Cupromeronic Blue are listed at the top of the table. Enzymes and nitrous acid used for proteoglycan degradation are shown in the left column with their substrate specificity. * Cupromeronic Blue-positive filament types. † Chondroitin sulfate. ‡ Hyaluronic acid. § Dermatan sulfate. ¶ Keratan sulfate. ¶¶ Heparan sulfate.

Connective tissue contained CB-1 filaments that were easily identified because of their distinct morphology. It was not completely clear based on morphology alone whether the smaller filaments consisted of CB-2 or CB-3, or both, or contained other filament types as well. The treatment with nitrous acid followed by chondroitinase ABC digestion eliminated all Cupromeronic Blue-positive filament staining associated with the fine fibrillar-like material, while the treatment with chondroitinase AC, chondroitinase ABC, nitrous acid, or the combined treatment with nitrous acid and chondroitinase AC reduced, but not removed, the filament staining. These results are consistent with the interpretation that fine fibrillar-like material in the juxtacanalicular connective tissue contains CB-1, CB-2 and CB-3, which represent chondroitin sulfate, heparan sulfate and dermatan sulfate-type proteoglycans, respectively.

The fine fibrillar-like material appeared to be composed of both fine fibrils as well as a more electronlucent homogeneous substance, somewhat similar to basal lamina-like material. After combination treatment with nitrous acid and chondroitinase AC, CB-3 filaments, which represent dermatan sulfate-type proteoglycan, were observed closely associated with the fine fibrils in the material. On the basis of its histological appearance and histochemical characteristics, it is likely that the fine fibrillar-like material is a mixture of fine collagen fibrils and basal lamina-like material. The presence of fine collagen fibrils and basal lamina-like material has been reported to occur adjacent to the inner wall of Schlemm's canal. The suggestion that these components occur together in combination to form these fine fibrillar-like components is novel. Immunohistochemical study that demonstrated the basal lamina-related antigens as well as type III collagen antigen adjacent to the endothelial cells lining Schlemm's canal in the human trabecular tissue may support this possibility.

No significant filament staining was associated with the elastin-like fibers, the fine granular-like material or the apical surface of the endothelial cells. In early histochemical studies, GAGs associated with elastin-like fibers and the surface of endothelial cells lining the trabecular beams and Schlemm's canal were reported in mammalian trabecular tissues. Furthermore, staining of GAGs was observed on the amorphous ground substance or so-called sheath of the elastin-like fibers, which we refer to as the fine granular-like material. However, the dyes, ruthenium red, colloidal iron and colloidal thorium employed in the early studies, stain not only GAGs but also tissue polyanions other than GAGs, such as glycoproteins and acid glycolipids, while Cupromeronic Blue reacts selectively with sulfated proteoglycans. Therefore, it is considered that the elastin-like fibers, the fine granular-like material and the apical surface of the endothelial cells in the human trabecular tissue do not contain sulfated proteoglycans, although they may include hyaluronic acid.

In this study, almost all Cupromeronic Blue-positive filaments in the human trabecular tissue were eliminated by the combination treatment with nitrous acid and chondroitinase ABC. The keratanase treatment did not alter the staining of any of the fila-
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tement types in the trabecular meshwork. These findings indicate that the human trabecular tissue contains chondroitin sulfate, dermatan sulfate and heparan sulfate-type of proteoglycans, and that the tissue includes little or no keratan sulfate-type proteoglycan. There may be some concern that keratan sulfate-type proteoglycan in the trabecular tissue is not detected by the histochemical method applied in this study. However, since keratan sulfate-type proteoglycan has been demonstrated in rabbit cornea using Cupromeronic Blue in combination with chondroitinase ABC and keratanase digestions following methods identical to those used in this study, it is unlikely that keratan sulfate-type proteoglycan, if present in the trabecular meshwork, would not have been demonstrated in this study. These results also fit well with biochemical data obtained from studies using cultured human and calf trabecular meshwork cells. However, other reports indicate that human, primate and rabbit trabecular meshwork cells synthesized keratan sulfate as well as the other GAG types we have identified in this analysis.

Since corneal cells are known to produce keratan sulfate, one possibility for this apparent discrepancy could be due to the inclusion of nontrabecular cells from the adjacent cornea in the cultures used for these biochemical studies. Another possibility is that the biosynthetic capabilities of the trabecular cells change in vitro and that in that environment become capable of keratan sulfate synthesis. The types of proteoglycan and their distribution in the human trabecular tissue are summarized in Table 2.

Table 2. Distribution of proteoglycans in the human trabecular tissue

<table>
<thead>
<tr>
<th>Component</th>
<th>Chondroitin sulfate</th>
<th>Heparan sulfate</th>
<th>Dermatan sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrous elements</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen fibrils</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Elastin-like fibers</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ground substances</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal lamina</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Basal lamina-like material</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Fine granular-like material</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fine fibrillar-like material</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Types of glycosaminoglycan associated with proteoglycans are listed at the top of the table. Components of the extracellular matrix in the trabecular tissue are shown in the left column. +: present, -: absent.

A filtering function of the heparan sulfate-type proteoglycan in basal lamina has been suggested in the lung alveoli and in the renal glomeruli. In renal tissue, removal of the heparan sulfate-type of GAG led to increased permeability of the glomerular basal lamina to serum albumin. In the human trabecular tissue, heparan sulfate-type proteoglycan was demonstrated with close relation to basal lamina, basal lamina-like material and fine fibrillar-like material that also contained chondroitin sulfate and dermatan sulfate proteoglycans. In the juxtacanalicular connective tissue, the trabecular surface of the inner wall of the canal is lining by basal lamina and/or fine fibrillar-like material with negatively charged proteoglycans, although there were some breaks. Furthermore, there are many layers of basal lamina-like material containing highly charged heparan sulfate-type proteoglycan in the tissue. There is a distinct possibility, based on these findings, that these basal lamina, basal lamina-like material and fine fibrillar-like material, which are rich in heparan sulfate-type proteoglycans, may play an important role in establishing aqueous outflow resistance via Schlemm’s canal in the juxtacanalicular connective tissue.

Studies of trabecular tissues obtained by trabeculectomy from patients with congenital or so-called juvenile glaucoma report extensive accumulations of basal lamina-like material in the trabecular meshwork. A similar deposition of basal lamina-like material in the trabecular tissue has been observed in cases of steroid glaucoma. The proteoglycan distribution in the basal lamina-like material present in these types of glaucoma is not known. However, heavy accumulation of basal lamina-like and/or fine fibrillar-like material in the trabecular meshwork seems to have a close relationship to the manifestation of glaucoma, although there may be other primary cause or causes which result in the deposition of this material. In vitro age-related studies of GAG synthesis in cultured trabecular meshwork cells show a relative increase in the synthesis of heparan sulfate, suggesting that under certain conditions, the proportions of proteoglycans synthesized by trabecular cells can be altered. Additional
studies of the trabecular meshwork from glaucomatous eyes using the methods employed in this study should provide important new information on the distribution and identity of sulfated proteoglycans in diseased tissue.

From this study, we conclude that the sulfated proteoglycans present in human trabecular meshwork tissue consist principally of heparan sulfate, dermatan sulfate and chondroitin sulfate-type proteoglycans. In the extracellular matrix of the trabecular tissue, chondroitin sulfate and dermatan sulfate-type proteoglycans are associated with collagen fibrils. Heparan sulfate-type proteoglycan is present in association with the basal lamina and displaced basal lamina-like material. Chondroitin sulfate, dermatan sulfate and heparan sulfate-type proteoglycans all are present in the fine fibrillar-like material. Basal lamina, basal lamina-like material and fine fibrillar-like material may play an important role in establishing aqueous outflow resistance in the juxtacanalicular connective tissue.

**Key words:** trabecular meshwork, Cupromeronic Blue, proteoglycans, glycosaminoglycans, electron microscopy

**References**