Hyaluronan in the Human Trabecular Meshwork

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Purpose. Hyaluronan (HA) is a high-molecular weight glycosaminoglycan that can affect water and solute fluxes in the extracellular matrix. The distribution of HA in the human trabecular meshwork of nonglaucomatous eyes was examined to help understand the potential role of HA in the regulation of aqueous outflow resistance.

Methods. Histolocalization of HA was established in situ in the trabecular meshwork of human eyes with no known diseases of the anterior segment. A specific biotinylated HA-binding peptide was used as a probe for this study, with enhanced sensitivity of HA detection achieved by modifications of the fixation and staining procedures.

Results. Evaluation of HA staining in the aqueous outflow pathway in comparison to that in other ocular structures (e.g., the vitreous) showed pronounced staining in the trabecular meshwork. The staining intensity was similar between various layers of the meshwork. Both the filtering and the anterior nonfiltering portions of the trabecular meshwork showed pronounced HA staining. The staining was localized primarily to the trabecular meshwork endothelial cells.

Conclusions. Pronounced HA staining observed in the various layers of the trabecular meshwork suggests that substantial amount of HA is present in the nonglaucomatous outflow pathway. The staining pattern suggests that HA is associated with the endothelial cells lining the trabecular beams. This finding supports potential roles for this glycosaminoglycan in the regulation of the physiological aqueous outflow resistance or in the maintenance of the outflow channels or both. Histochemical localization of HA in the various layers of the nonglaucomatous meshwork provides a useful basis for future comparative studies of HA distribution and relative amounts in the trabecular meshworks of eyes affected by various types of glaucoma. Invest Ophthalmol Vis Sci. 1997;38:1222-1228.
not rule out protease contamination of the hyaluronidase preparations used in these studies. However, hyaluronidase perfusion of primate eyes has produced variable effects on the outflow facility. Such variations have been attributed to interspecies differences, the type and source of hyaluronidase, and the conditions used for the enzymatic digestion that may have contributed to a variable and incomplete degradation of HA.

A variety of approaches have been used to study HA in the trabecular meshwork. However, direct visualization of HA has been difficult because of the lack of sufficiently sensitive detection techniques specific for HA. Studies using polyacrylonitrile observed low levels of HA or only low levels of HA staining, primarily in the anterior nonfiltering segment and in the deepest aspect of the meshwork. However, other investigators who recently used a b-HABP probe to study animal eyes reported none or only low levels of HA staining, primarily in the anterior nonfiltering segment and in the deepest aspect of the filtering portion of the TM, adjacent to the aqueous plexus. Such localization of HA in the TM would argue against a direct role for this molecule in the regulation of aqueous outflow facility, although interspecies differences are possible between human and animal eyes. In addition, the technique of tissue processing and staining, as well as the storage and handling of the b-HABP probe, is critical to obtaining optimal and reproducible results.

In the current investigation, HA distribution was examined in the TM of 11 nonglaucomatous eyes using modifications of the tissue fixation and the b-HABP staining procedure. These modifications allowed highly sensitive and reproducible HA staining. The staining was observed in all layers of the human TM in both the filtering and the anterior nonfiltering portions of the meshwork. The distribution of HA in various layers of the human TM is important for understanding the roles of HA in the regulation of the aqueous outflow resistance.

MATERIALS AND METHODS
Materials
In the current study, we evaluated trabecular meshworks of 11 human eyes obtained from donors of 50 to 80 years of age. Research procedures were in accordance with institutional guidelines and the Declaration of Helsinki. Ten normal eyes from five persons were transported from the eyebank to the laboratory on ice and fixed within 24 to 48 hours after death. One eye was enucleated for choroidal melanoma of the posterior segment and fixed immediately after surgery. None of the donors of the eyebank eyes had a known history of any ocular disease (e.g., glaucoma or uveitis). The eye with melanoma did not have any prior radiation treatment. Each globe was bisected by a sagittal incision before it was submerged in fixative.

Fixation
Tissue specimens were fixed overnight at room temperature in 10% formaldehyde in 0.22 M phosphate-buffered saline, pH 7.2, followed by immersion in 70% ethanol for 5 days. After fixation, the tissues were embedded in paraffin according to a routine protocol. The tissue blocks were cut in 4- to 8-μm sections. The slides used had a positively charged coating (ProbeOn Plus; Fisher Scientific, Pittsburgh, PA), ensuring proper tissue adherence during the multiple steps of the immunohistochemical staining procedure.

Histochemistry
Biotinylated HA-binding peptide was prepared from bovine nasal cartilage using affinity chromatography on immobilized HA. The specificity of this probe was documented by two separate procedures and gave similar results: (1) predigestion of the specimens with 50 U/ml Streptomyces hyaluronidase (Calbiochem–Behring, La Jolla, CA), 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM ethylenediaminetetraacetic acid and 1 mg/ml Prefablock (Boehringer Mannheim, Indianapolis, IN) overnight at 37°C and (2) overnight absorption of the b-HABP with excess HA at 4°C (ICN Biochem, Costa Mesa, CA). The latter control specimen was designed to block the binding sites for HA on the probe before its use for the staining procedure. Secondary control specimens were performed by incubating some slides with the buffer used for hyaluronidase digestion under conditions similar to those used for hyaluronidase digestion to avoid potential loss of HA during this additional step. To control for possible nonspecific decrease of the sensitivity of the b-HABP probe during incubation with exogenous HA solution, we used an additional incubation step with HA solvent (ddH2O). Certain specimens were predigested with 1 mg/ml Protease XIV (Sigma, St. Louis, MO) in 0.05 M Tris–HCl, pH 7.4, at 37°C for 1 hour. No enhancement of staining caused by increased availability of the b-HABP binding sites was observed. This step also was used as an additional control for possible protease contamination of the Streptomyces hyaluronidase preparation. As a posi-
FIGURE 1. Hyaluronan staining (brown) localized immunohistochemically in the aqueous outflow pathway of a nonglaucomatous human eye (Table 1, #1-OD). The anterior chamber is designated as AC; the Schlemm’s canal is designated as SC. Counterstained with light green (A, top left). Hyaluronan staining is observed in both the anterior nonfiltering portion and the posterior filtering region of the human trabecular meshwork (TM). The TM beams show staining in the uveal, corneoscleral, and the juxtacanalicular layers of the TM (original magnification, ×50) (B, top right). HA staining is localized to the TM endothelial lining (arrows). The staining intensity is similar in all areas of the TM (original magnification, ×300). The TM beams stain with light green (C, bottom right). No HA staining is observed after pretreatment of slides with Streptomyces hyaluronidase. Scattered granules of pigment are seen as brown dots. Original magnification, ×50.

As a positive control, we used the staining of vitreous and subconjunctival connective tissue. Finally, three different batches of b-HABP were used. Sensitivity of the probe varied between batches, and dilutions of the stock solution were prepared (ranging from 1:60 to 1:120) to yield comparable staining intensity.

The staining for HA was performed according to a modification of a protocol described previously. Briefly, after deparaffinization and dehydration in graded ethanol, the slides were incubated with 2% hydrogen peroxide followed by incubation with 3% bovine serum albumin solution at 37°C for 1 hour. The b-HABP stock solution, appropriately diluted with HA-binding reaction buffer (25 mM disodium acid phosphate, 1.5 M sodium chloride, 0.3 M GuHCl, 0.08% bovine serum albumin, 0.02% NaN₃, pH 7) was applied and sections incubated overnight at 4°C in a humidifying box. After washing, avidin–biotin–horseradish peroxidase complex (ABC) was added (Vectastain ABC Standard Kit, Peroxidase PK 4000, or the ABC Elite Kit; Vector Laboratories, Burlingame, CA). The slides were incubated with a chromogenic horseradish peroxidase substrate. Several alternative substrates initially were compared, including DAB, VIP, and AEC (horseradish peroxidase substrate kits, Vector Laboratories); all of the subsequent experiments were performed using the DAB Kit (3,3′-diaminobenzidine, Peroxidase Substrate Kit, DAB SK-4100; Vector Laboratories, Burlingame, CA). The sections were counterstained alternatively with either methyl green or light green. The slides were examined by light microscopy and microphotographs were taken using an Olympus Vanox AHBT3 Microscope (Olympus, Woodbury, NY) with an integrated Olympus C-35AD-4 camera using Kodak Gold Plus 200 ASA, 35-mm film (Eastman Kodak, Rochester, NY).

To facilitate comparison of staining intensities between different layers of the trabecular meshwork, a progressive scale from 1 (absent staining as observed in Descemet’s membrane) to 6 (staining intensity visually comparable to that observed in the vitreous) was chosen. This approach was applied to each specimen and permitted semiquantitative analysis of HA staining in different regions of the outflow pathway using staining intensity in other ocular structures as internal control. Both low and high magnifications (×40 and ×400, respectively) were used. The staining intensity was evaluated separately by two observers. They agreed on 9 of 11 slides, and the remaining 2 specimens were reevaluated and the data averaged.
TABLE 1. Distribution of Hyaluronan in Human Trabecular Meshwork*

<table>
<thead>
<tr>
<th>Donor/Source</th>
<th>Eye</th>
<th>Age (years)</th>
<th>Hyaluronan in HTM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/Eyebank</td>
<td>OD</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>2/Eyebank</td>
<td>OD</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>3/Eyebank</td>
<td>OS</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>4/Eyebank</td>
<td>OS</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>5/Eyebank</td>
<td>OS</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>6/Choroidal melanoma</td>
<td>OD</td>
<td>80</td>
</tr>
</tbody>
</table>

OD = right eye; OS = left eye; HTM = human trabecular meshwork; JXT = juxtacanalicular tissue.

* A progressive scale was chosen from 1 (absent staining as observed in Descemet’s membrane) to 6 (staining intensity visually comparable to that in the vitreous) to facilitate comparison of hyaluronan staining in different layers of the trabecular meshwork. This approach controls for the interexperimental fluctuation in staining intensity.

RESULTS

Hyaluronan staining was observed in all layers of the nonglaucomatous human TM and occurred in both the filtering and the anterior nonfiltering regions of the meshwork (Fig. 1A). Evaluation of staining intensity was performed in a semiquantitative manner as described in the Methods section. Comparison of the staining intensities in the different layers of the meshwork, presented in Table 1, suggested that comparable amounts of HA were present in the uveal and corneoscleral meshworks as well as in the area closest to the Schlemm’s canal, the juxtacanalicular tissue (JXT). No difference in the pattern or intensity of HA staining was apparent in the TM of the specimen with choroidal melanoma of the posterior segment compared with that of the eyebank specimens (Table 1, #6-OD).

In all areas of the TM, pronounced staining was localized to the endothelial cells lining the trabecular beams (Fig. 1B). The resolution of the light microscopy technique used in this study does not distinguish whether HA was located inside the cells or associated with the cell surface. The connective tissue of the beams showed none-to-mild staining. Hyaluronan was visualized in both anterior and posterior walls of the Schlemm’s canal, in the walls of the collector channels, and in the ciliary muscle (Fig. 1A). Intense staining was observed consistently in the vitreous and in conjunctival connective tissue. Descemet’s membrane showed no staining.

After serial dilution of the b-HABP probe, the staining intensity decreased proportionally in all ocular tissues, including vitreous, conjunctival connective tissue, and the TM. The staining was eliminated in all layers of the TM at the same dilution of the probe in the majority of sections, further indicating that comparable amounts of HA were located in different layers of the meshwork. However, at the dilution of the b-HABP probe that produced no staining in the TM, staining still was present in the vitreous, known to have a rather high HA content.

Staining was completely absent from sections pretreated with Streptomyces hyaluronidase (Fig. 1C). The b-HABP probe preincubated with an excessive amount of exogenous HA before the staining procedure produced negative staining as well (data not shown). To avoid nonspecific reduction of staining intensity caused by additional incubation steps in the control experiments, secondary controls were performed. Some sections were either pretreated with hyaluronidase buffer alone or stained using the b-HABP probe that had been preincubated only with HA solvent. These sections showed no changes in staining intensity when compared with those of the experimental slides.

DISCUSSION

In the current investigation, HA was visualized in the human TM that supports its proposed role in the regulation of physiological outflow resistance in the human eye. In fact, all layers of both the filtering and nonfiltering portions of the TM (i.e., the JXT, corneoscleral, and uveal meshwork) showed HA staining, with similar staining intensity in different layers of the TM in most specimens. Some specimens did show slightly more pronounced staining in the JXT compared with staining intensity of the inner TM. This may reflect internal variations of HA deposition in the TM of different subjects. In addition, it is possible that the staining intensity is affected by variations in connective tissue density in various ocular structures (e.g., there is more connective tissue in the JXT than in the uveal meshwork), which could cause the appearance of increased HA staining. This visual impression is accentuated when sections are examined under low magnification. Examination under high magnification is necessary to achieve a more objective evaluation of staining.

Although many studies implicate a role for HA in the aqueous outflow resistance in various animal models, some investigators have raised questions whether a similar role is possible in the human outflow pathway. In normal eyes, HA may provide the necessary coating for the beams, preventing the fusion of trabecular lamellae as observed in glaucoma. Decreased amounts of HA in the human outflow pathway...

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The physiochemical properties of HA may contribute to the elasticity and resilience of the nonglaucomatous trabecular meshwork and help the recovery of the meshwork structure after contraction and relaxation cycles of the ciliary muscle.

Our results show that HA staining was most apparent in association with TM cells, which suggests that HA may be important in maintaining an appropriate environment for the cells. It is still possible that in vivo HA partially or completely fills the intertrabecular spaces, which may be difficult to visualize using a histochemical approach because of the inevitable artifacts of any fixation method as it collapses the HA molecule onto the cell surface. In addition, this GAG is known to be lost from tissue specimens during preparation and staining procedures, perhaps because of its weak anchoring to tissues. In addition, the b-HABP probe labels the large molecules of HA at the site of the probe’s attachment, but the entire molecule may not be visualized. The number of binding sites for the b-HABP per molecule of HA is unknown and probably varies depending on several factors, including relative molecular mass of HA, the number of other HABPs that interact with the molecule, the number of intact binding sites, and the conformational changes in the three-dimensional structure of the molecule that may facilitate or weaken the interactions. Staining also was observed in the Schlemm’s canal, collector channels, and ciliary muscle.

The results of the current study are supported by a number of prior biochemical and histochemical studies of HA in the TM. There are several possible factors that may explain the discrepancy between our findings and those reported by other investigators who also used the b-HABP technique but were unable to show prominent staining in the TM. The staining for HA may be affected by the method of tissue fixation. In addition, the fact that the anchoring of HA to the tissues is generally weak may result in extensive loss of this water-soluble polysaccharide during paraffin embedding and the multistep staining procedure. In fact, during tissue processing, HA may be lost to a greater extent from a loose connective tissue (e.g., TM) than from more dense tissues (e.g., sclera or conjunctival stroma). Prolonged washing of the samples as described in the original staining protocol also may contribute to loss of HA. The sensitivity of the b-HABP probe may vary between different batches depending on the concentration of the peptide, percent of biotinylation, storage time and conditions, and functional activity of the peptide. The buffer used for the b-HABP staining reaction, described in the Methods section, is particularly important for the sensitivity and specificity of the staining.

Inclusion of ethanol in tissue fixation appeared to enhance the detectability of HA in various ocular tissues, including trabecular meshwork. The exact mechanism of the ethanol effect on tissues is unknown. Ethanol may either stabilize HA in situ, which would reduce its loss from the tissue samples (e.g., by precipitating HA) during the preparation procedure and the multistep immunohistochemical staining, or may unmask the potential b-HABP binding sites on the HA molecule. Careful control of the washing steps also may reduce the loss of HA during the staining procedure. Because of the possible variations related to the staining conditions and reagents, it appears crucial that each laboratory perform similar optimizing steps in their own settings to maximize the detectability of HA. It also is possible that the evaluation of HA in glaucomatous eyes may require different conditions for optimal staining resulting from possible variations in HA attachment to diseased tissue structures.

In the TM, HA may provide an important regulatory mechanism of the outflow resistance; the hydraulic conductivity (flow per unit pressure) of HA has been shown in vitro to be concentration- and molecular weight-dependent. Hyaluronan also may form complexes with its binding proteins or proteoglycans as has been documented in other tissues (e.g., a corticosteroid-induced glycoprotein appears to bind to HA in cultured HTM cells [JR Polansky, personal communication, 1996]). Such complexes may affect the aqueous humor outflow, and their effects can be modulated by hyaluronidase or protease degradation. In fact, high levels of hyaluronidase activity have been documented in cultured HTM cells. In addition, certain biologic effects of HA in the TM may depend on the presence of HA-binding proteins. The size of HA also may be important for its biologic effects in the TM.

Several receptors for HA have been identified that mediate biologic effects of HA. The family of HA receptors include CD44, RHAMM, CD38, and ICAM-1. The interactions of HA with its receptors may prove to be of particular relevance for the TM. In preliminary experiments, CD44 was immunohisto-localized in all layers of the human TM. Its expression suggests a possible mechanism for HA attachment to the meshwork. Local metabolism of HA also may provide a mechanism for the aqueous outflow control. Other factors that can potentially modulate HA metabolism include hyaluronidase inhibitors and exoglycosidase activities.

Further investigations of HA in the TM should focus on local mechanisms of autoregulation, including investigation of HA-binding proteins and receptors, as well as further characterization of hyaluronidase activity produced by HTM cells. This will help define the proposed role of HA in the regulation of...
outflow resistance, with potential implications for the mechanisms of the homeostatic response of the TM to environmental stresses and for the pathogenesis of various glaucoma syndromes. However, the role of HA in the TM may differ depending on each particular syndrome. The intense HA staining throughout the filtering region of the TM, reported here, suggests the presence of substantial amounts of HA in the outflow pathway. This finding supports a role for HA in the regulation of physiological aqueous humor outflow, with important implications for the pathology of various types of glaucoma.

**Key Words**

aqueous outflow, glycosaminoglycan, histochemistry, hyaluronic acid, trabecular meshwork

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