CD44H Localization in Primary Open-Angle Glaucoma

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PURPOSE. Primary open-angle glaucoma (POAG) is associated with a decreased content of hyaluronan in the trabecular meshwork and in the juxtasclanicular connective tissue. In this study, the authors examined selected regions of the anterior segment to localize and determine the content of CD44H, a transmembrane multifunctional glycoprotein and the principal receptor of hyaluronan.

METHODS. Sections of ethanol-fixed anterior segments of six POAG and six normal postmortem eyes were analyzed by immunostaining with and without the nonionic detergent Triton X-100, using the CD44H monoclonal antibody, and the avidin/biotin complex. They were visualized by Vector VIP substrate and were quantitated by computer-aided color image analysis.

RESULTS. CD44H was expressed in all regions. Statistically significant decreased content of CD44H was observed in the POAG regions compared with normal regions—ciliary muscle (P < 0.001), ciliary stroma (P < 0.001), anterior iris (P < 0.05), iris root (P < 0.05), and trabecular meshwork (P < 0.05)—and in a subgroup of nonlaser POAG juxtasclanicular connective tissue (P < 0.05) and trabecular meshwork (P < 0.01). In sections treated with Triton X-100 a further increase in immunostaining was observed in normal eyes. As evidenced by scattergram plots of the ciliary body stroma region of the change in the optical density of CD44H between pretreatment with Triton X-100 and without Triton X-100 (y axis) versus the optical density of CD44H without Triton X-100 (x axis), individual cases of POAG were separated from normals.

CONCLUSIONS. These results indicate that CD44H may represent a marker of POAG and an etiologic factor in the POAG disease process. (Invest Ophthalmol Vis Sci. 1998;39:673–680)

CD44 is an 80- to 90-kDa, type 1, transmembrane multifunctional glycoprotein, and it is the principal receptor of the glycosaminoglycan, hyaluronan.1,2 CD44 is expressed in a wide variety of cell types, including mature T-cells, B-cells, medullary thymocytes, granulocytes, macrophages and fibroblasts, and the corneal epithelium3 and retina.4,5 CD44 binds to the actin cytoskeleton, mediates cell attachment to the extracellular matrix,6 and participates in fibroblast migration in provisional wound healing7 and in immunologic activation.8 CD44 participates in the uptake and degradation of hyaluronan.9 In addition to hyaluronan, CD44 has multiple extracellular matrix-unrelated ligands of CD44.8,9 Each ligand interaction is influenced by the CD44 exon and glycosylation patterns.2

Primary open-angle glaucoma (POAG) has no recognized cause.14,15 By the year 2000, the number of persons in the United States with POAG is estimated to be 2.47 million.16 It is likely that several biochemical and cellular factors influence the glaucoma process. A variety of cellular insults or molecular defects17,18 may intersect, leading individually or collectively to cell loss in the trabecular meshwork19 or in retinal ganglion cells.20 Alterations in the extracellular matrix, namely, the level and expression of matrix metalloproteinases21 or the type and amount of glycosaminoglycans22,23 influence the POAG disease process. Laser trabeculoplasty induces stromelysin in the juxtasclanicular connective tissue.24 Recent evidence from our laboratory indicates that the content of hyaluronan22,23 in the trabecular meshwork and in the juxtasclanicular connective tissue of POAG eyes is decreased statistically. Biochemical studies of microdissected anterior segments of POAG eyes and age-matched normal eyes using Triton X-100 characterized CD44H in the iris and ciliary body CD44.24

In the present study, to clarify the mechanism(s) for the decrease in hyaluronan in POAG and to explore the possibility that CD44H may be altered in POAG, we compared the regional variations of CD44H in the anterior segment of normal eyes and in POAG eyes by immunostaining and by computer-assisted densitometry. The results of this study indicated that CD44H is altered in POAG and may serve as a marker of POAG.

METHODS

Specimens

Six normal eyes from three donors were obtained from the Illinois Eye Bank and the Lions Eye Bank of Washington and Northern Idaho. Six POAG eyes from three donors were ob-
TABLE 1. Clinical History, Ocular Findings,* and Specimen Code of Donor Eyes

<table>
<thead>
<tr>
<th>Eye Number</th>
<th>Specimen Number</th>
<th>Patient Age (years/sex)</th>
<th>Cause of Death</th>
<th>Ocular Medications</th>
<th>Laser Trabeculoplasty</th>
<th>Duration of POAG</th>
<th>Treated IOP</th>
<th>Cap/ Disc Ratio</th>
<th>Visual Field Condition†</th>
<th>Condition</th>
</tr>
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<tbody>
<tr>
<td>POAG eyes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1R</td>
<td>2026</td>
<td>57/F</td>
<td>Pulmonary fibrosis</td>
<td>Iopidine 0.5% tid OU</td>
<td>None</td>
<td>2 years</td>
<td>21</td>
<td>0.4</td>
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<tr>
<td>L</td>
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<td></td>
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<td></td>
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<td>OS, 1 year before death</td>
</tr>
<tr>
<td>2R</td>
<td>1907</td>
<td>70/M</td>
<td>Prostate carcinoma</td>
<td>Betoptic 0.5% bid OU</td>
<td>None</td>
<td>7 years</td>
<td>22</td>
<td>0.8</td>
<td>3</td>
<td>OS, 3 years before death</td>
</tr>
<tr>
<td>L</td>
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<td>3R</td>
<td>1786</td>
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<td>Lung carcinoma</td>
<td>Timoptic 0.5% bid OU</td>
<td>None</td>
<td>17 years</td>
<td>22</td>
<td>0.6</td>
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<td>OS, 6 years before death</td>
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<tr>
<td>Normal eyes</td>
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<tr>
<td>1R</td>
<td>2119</td>
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<td>Congestive heart failure</td>
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<td>OS, 8 years before death</td>
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<td></td>
<td>OS, 9 years before death</td>
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<tr>
<td>2R</td>
<td>2065</td>
<td>74/M</td>
<td>Myocardial infarction</td>
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<td>OS, 11 years before death</td>
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<tr>
<td>3R</td>
<td>2066</td>
<td>74/M</td>
<td>Colon carcinoma</td>
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<td>OS, 12 years before death</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>OS, 13 years before death</td>
</tr>
</tbody>
</table>

*IOP, intraocular pressure; POAG, primary open-angle glaucoma; OU, both eyes; OD, right eye; OS, left eye; F, female; M, male; bid, two times daily; tid, three times daily; qid, four times daily.

*The ocular findings were from the last available examination.

†1, normal; 2, scotoma; 3, significant defect; 4, loss of central field.

Tained from the Rochester Eye and Human Parts Bank, the Medical Eye Bank of Florida, and the Michigan Eye-Bank and Transplantation Center through the efforts of the Foundation for Glaucoma Research (San Francisco, CA). After enucleation, the eyes were sent to the laboratory and were placed in 100% ethanol within 24 hours. A 6-mm wedge of the anterior segment including the cornea, anterior sclera, trabecular meshwork, iris, and ciliary body was resected and was placed in fresh 100% ethanol. Each wedge resection was assigned a specimen number, which was used in a subsequent analysis. The remainder of the anterior segment was microdissected for biochemical analysis. The mean age of the normal donor eyes was 72.9 years, and the mean age of the donor POAG eyes was 67.0 years. None of the eyes had evidence of uveitis or cataract at the time of enucleation.

Immunocytochemistry

Each specimen was processed for paraffin embedding, and serial 8-μm paraffin sections were cut and placed on polylysine–coated slides. The sections were deparaffinized and rehydrated. In sections pretreated with Triton X-100 to expose the CD44H epitope, sections were treated with 5% Triton X-100 at 4°C for 1 hour, were washed with cold Triton X-100, and were treated with fresh 5% Triton X-100 at 4°C for an additional 1 hour. Untreated and Triton X-100–treated sections then were treated with normal horse serum as a blocking agent for 20 minutes and were incubated overnight at 4°C with a mouse antihuman monoclonal antibody (BBA 10) against CD44H (R&D Systems, Minneapolis, MN). The sections were washed with buffer and were incubated with biotinylated secondary antibody in normal serum (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). Finally, the sections were incubated with avidin/biotin complex (ABC reagent, Vector Laboratories) and were visualized by Vector VIP substrate (Vector Laboratories) to reveal a purple reaction product. The sections were not counterstained. The slides were coverslipped with Pro-Texx (Baxter Diagnostics, Deerfield, IL). Control sections treated with H2O2 to eliminate endogenous peroxidase and sections treated to block endogenous avidin or biotin before reaction with the antibody appeared the same as the experimental sections. Sections treated only with secondary antibody demonstrated no staining.

Image Analysis

Image analysis was performed using a Zeiss SEM-IPS image processing system (Carl Zeiss, Thornwood, NY). Real-color digital images were obtained by sequentially averaging 10 inputs from red, green, and blue color of a video camera (3-CCD, 7000E; Hitachi, Tokyo, Japan) attached to a Zeiss Universal light microscope with a ×25 Planapo objective (Carl Zeiss). The resultant images were corrected for illumination inconsistencies by a shading correction using a blank background image a short distance from the original image. A color editor was used to determine interactively the red, green, and blue parameters that were used in the segmentation of desired features. Practically, each of the three signals—red, green, and blue—has a 256 gray-level resolution, or 2563 possibilities that could be used for feature extraction. By choosing interactively the parameters of a local 5 × 5-pixel matrix repeatedly (up to...
Figure 1. Low magnification of CD44 immunostaining visualized by Vector VIP substrate of a normal anterior segment. The boxed-in areas are representative areas that were analyzed: trabecular meshwork (TM); juxtacanalicular connective tissue (JCT); anterior iris (AI); iris root (IR); ciliary muscle (CM); and ciliary stroma (CS). * signifies Schlemm's canal. Magnification, ×80.

10 times), the color hue and intensity of a desired feature to be extracted were defined and displayed; that is, VIP-stained CD44H.

The parameters determined by the color editor then were used to perform color discrimination, resulting in a binary image. This binary image then was used to mask the previously

Figure 2. Composite computer image acquisition and image analysis of the intensity of CD44H staining in an 8-μm section of the ciliary body stroma. Computer images of normal ciliary body stroma without (a) and with Triton X-100 (c), and corrected computer images, using real-color discrimination to eliminate pigment and background, of normal ciliary body stroma without Triton X-100 (b), as in a, and with Triton X-100 (d), as in c, are shown. Computer images of primary open-angle glaucoma (POAG) ciliary body stroma without (e) and with (h) Triton X-100, and corrected computer images, using real-color discrimination to eliminate pigment and background, without Triton X-100 (f), as in e, and treated with Triton X-100 (g), as in g, are shown. The optical density of CD44H immunostaining was measured in the colored areas. Magnification, ×440.
We used an immunostaining approach and computer-aided densitometry to identify and quantitate CD44H in selected regions of the anterior segment of normal eyes and POAG eyes (Fig. 1). CD44H immunostaining was observed in all regions of the anterior segment. The ciliary body stroma appeared moderately positive and the trabecular meshwork exhibited a substantial increase in CD44H staining (Table 2).

### Statistical Analysis

Unpaired Student’s t-tests were used to analyze the differences between normal (n = 6) and POAG (n = 6) regions, whereas Student’s t-tests for paired data were used to analyze differences in CD44H patterns within normal and POAG regions and subgroups.

### RESULTS

We used an immunostaining approach and computer-aided densitometry to identify and quantitate CD44H in selected regions of the anterior segment of normal eyes and POAG eyes (Fig. 1). CD44H immunostaining was observed in all regions of the anterior segment. The ciliary body stroma appeared strongly positive for CD44H, whereas the anterior iris, ciliary body muscle, iris root, and juxtacanalicular connective tissue were moderately positive and the trabecular meshwork appeared slightly positive.

To avoid subjective impressions of the staining intensity and pigment in the various regions, we used computer-aided image analysis to quantitate the CD44H immunostaining. Images of CD44H immunostaining without pretreatment with Triton X-100 (Fig. 2a, normal; Fig. 2e, POAG) were corrected to exclude pigment and background by real-color discrimination (Fig. 2b, normal; Fig. 2f, POAG). Similarly, images of CD44 immunostaining pretreated with Triton X-100 (Fig. 2c, normal; Fig. 2g, POAG) were corrected to exclude pigment and background by real-color discrimination (Fig. 2d, normal; Fig. 2h, POAG).

#### CD44 Profile of Primary Open-Angle Glaucoma and Normal Anterior Segment Regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Pretreatment with Triton X-100 (Optical Density)</th>
<th>No Pretreatment with Triton X-100 (Optical Density)</th>
<th>Δ in Optical Density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>POAG</td>
<td>Normal</td>
</tr>
<tr>
<td>Ciliary body stroma</td>
<td>0.218 ± 0.030</td>
<td>0.164 ± 0.030**</td>
<td>0.140 ± 0.018</td>
</tr>
<tr>
<td>Ciliary body muscle</td>
<td>0.192 ± 0.025</td>
<td>0.126 ± 0.030**</td>
<td>0.133 ± 0.044</td>
</tr>
<tr>
<td>Anterior iris</td>
<td>0.183 ± 0.096</td>
<td>0.106 ± 0.017*</td>
<td>0.116 ± 0.032</td>
</tr>
<tr>
<td>Iris root</td>
<td>0.164 ± 0.064</td>
<td>0.099 ± 0.010*</td>
<td>0.098 ± 0.028</td>
</tr>
<tr>
<td>TM</td>
<td>0.178 ± 0.051</td>
<td>0.113 ± 0.035*</td>
<td>0.129 ± 0.055</td>
</tr>
<tr>
<td>Laser-treated</td>
<td>0.166 ± 0.043</td>
<td>0.132 ± 0.035*</td>
<td>0.094 ± 0.016</td>
</tr>
<tr>
<td>No laser</td>
<td>0.140 ± 0.042</td>
<td>0.101 ± 0.002*</td>
<td>0.088 ± 0.032</td>
</tr>
</tbody>
</table>

POAG, primary open-angle glaucoma; n, number of eyes; TM, trabecular meshwork; JCT, juxtacanalicular connective tissue.

*P < 0.05; **P < 0.01; ***P < 0.001 are Student’s t-test significance values of normal compared with POAG eyes.

<table>
<thead>
<tr>
<th>Pretreatment with Triton X-100 (Optical Density)</th>
<th>No Pretreatment with Triton X-100 (Optical Density)</th>
<th>Δ in Optical Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>POAG</td>
<td>Normal</td>
</tr>
<tr>
<td>Iris root</td>
<td>0.082 ± 0.021*</td>
<td>0.046 ± 0.040</td>
</tr>
<tr>
<td>Ciliary body stroma</td>
<td>0.101 ± 0.002*</td>
<td>0.064 ± 0.028</td>
</tr>
<tr>
<td>Ciliary body muscle</td>
<td>0.080 ± 0.046</td>
<td>0.052 ± 0.030</td>
</tr>
<tr>
<td>Anterior iris</td>
<td>0.072 ± 0.041</td>
<td>0.052 ± 0.030</td>
</tr>
<tr>
<td>Iris root</td>
<td>0.072 ± 0.029</td>
<td>0.052 ± 0.030</td>
</tr>
<tr>
<td>TM</td>
<td>0.072 ± 0.030</td>
<td>0.052 ± 0.030</td>
</tr>
<tr>
<td>Laser-treated</td>
<td>0.072 ± 0.041</td>
<td>0.052 ± 0.030</td>
</tr>
<tr>
<td>No laser</td>
<td>0.072 ± 0.029</td>
<td>0.052 ± 0.030</td>
</tr>
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</table>

**P < 0.05; ***P < 0.001 are Student’s t-test significance values of normal compared with POAG eyes.

Δ in optical density is the difference between sections pretreated with Triton X-100 and sections without Triton X-100.

Δ in optical density is the difference between sections pretreated with Triton X-100 and sections without Triton X-100.

Δ in optical density is the difference between sections pretreated with Triton X-100 and sections without Triton X-100.
FIGURE 3. A summary of the results of each anterior segment component by scattergram plots of the optical density of CD44H immunostaining without Triton X-100 pretreatment (x axis) versus the change in optical density of CD44 between pretreatment with Triton X-100 and without Triton X-100 (y axis). Individual cases of primary open-angle glaucoma (POAG) eyes are indicated by solid, numbered circles, and individual cases of normal eyes are indicated by open, numbered circles. In the trabecular meshwork and juxtacanalicular connective tissue plots, the POAG group is indicated by an open box, the POAG laser-treated subgroup is indicated by a shaded box, and the POAG nonlaser-treated subgroup is indicated by a crosshatched box.
CD44H Profile of Primary Open-Angle Glaucoma Juxtacanalicular Connective Tissue and Trabecular Meshwork: Effect of Laser Trabeculoplasty

The CD44H profile of the trabecular meshwork and juxtacanalicular connective tissue of the POAG eyes treated only with topical medications (specimens 1R and 2R) or treated by laser trabeculoplasty (specimens 1L, 2L, 3R, and 3L) was compared with normal eyes (Table 2). The subclass of nonlaser-treated POAG trabecular meshwork and juxtacanalicular connective tissue revealed statistically significant differences when compared with the normal trabecular meshwork and juxtacanalicular connective tissue. Pretreatment of POAG sections with Triton X-100 resulted in less change in optical density in the trabecular meshwork (P < 0.02) and an increase in optical density in the normal trabecular meshwork (P < 0.01) and in the normal juxtacanalicular connective tissue (P < 0.05). There were no significant differences when the subclass of laser-treated POAG trabecular meshwork and juxtacanalicular connective tissue were compared with normal trabecular meshwork and juxtacanalicular connective tissue. In sections of POAG eyes, no statistically significant differences were noted in laser- versus nonlaser-treated trabecular meshwork or juxtacanalicular connective tissue optical density with or without Triton X-100.

 Scattergram Plots of CD44H and Primary Open-Angle Glaucoma Compared with Normal Plots

As evidenced by scattergram plots of regional change in the optical density of CD44H between pretreatment with Triton X-100 and without Triton X-100 (y axis) versus the optical density of CD44H without Triton X-100 (x axis), individual cases of normal eyes and POAG eyes clustered into distinctive patterns in the ciliary body stroma, the ciliary body muscle, and the iris root (Fig. 3). However, the anterior iris, trabecular meshwork, and juxtacanalicular connective tissue had indeterminate patterns. Separating POAG cases into laser-treated and nonlaser-treated groups of the trabecular meshwork and juxtacanalicular connective tissue regions delineated the two subgroups on the scattergram plots (Fig. 3). The nonlaser POAG subgroup was statistically significant and distinct from the normal group, whereas the laser-treated subgroup was more similar to the normal group (Table 2).

Discussion

This study provides the first immunostaining marker of individual cases of POAG. To identify the CD44H marker, we pretreated sections with Triton X-100, a nonionic detergent that is useful in solubilizing numerous integral membrane proteins,27 such as CD44.28 Triton X-100 binds to the hydrophobic domains of proteins without disrupting protein–protein interactions. A 20-amino acid hydrophobic region in the transmembrane portion of the CD44 is an absolute requirement for detergent solubility.29 At higher concentrations, as used in this study, Triton X-100 also separates lipids from membrane bilayers to facilitate dyes,30 histologic stains,31,32 and immunostaining,33,34 as observed in the present study of normal eyes and POAG eyes. The optimal concentration of Triton X-100 is dependent on the composition of the cell membranes and varies with the cell type.

Scattergram plots of the change in optical density of CD44H between pretreatment with Triton X-100 and without Triton X-100 (y axis) versus the optical density of CD44H without Triton X-100 (x axis) were sufficiently distinctive in identifying individual cases of POAG in the ciliary body stroma, the ciliary body muscle, and the iris root. The prominent CD44H immunostaining in the POAG ciliary body stroma was statistically significant and striking compared with normal eyes. The ciliary body stroma is composed of cells, collagen, muscle, and nerves,35 and it is thickest over the pars plicata.

We selected the anterior portion, at the beginning of the first ciliary process, as a representative region. The other regions were selected on the basis of staining patterns, namely, the external longitudinal portion of the ciliary body muscle, the anterior portion of the iris, the iris root, and, of course, the mid-portion of the trabecular meshwork and juxtacanalicular connective tissue, to determine whether laser trabeculoplasty may influence CD44H immunostaining. Scattergram plots of the trabecular meshwork and juxtacanalicular connective tissue nonlaser-treated group of POAG were useful in classifying this POAG subgroup.

CD44 is the principal receptor for hyaluronan. CD44 is a cell adhesion molecule, and, notably, it increases in the aging process.36,37 CD44 is a type 1 transmembrane glycoprotein homologous to the cartilage link protein38 and is known to have diverse functional properties caused by sequence differences arising from the alternate splicing of mRNA and post-translational modifications.39 CD44 is encoded on a single gene on the short arm of chromosome 11. CD44 has 20 exons, of which 10 exons are absent or present in various combinations by RNA splicing.8 In addition to the inclusion or exclusion of whole exons, more diversity of CD44 is generated through the use of internal splice donor and acceptor sites. The genomic structure of CD44 is remarkable for its structural and functional complexity, based on the concept of alternate splicing.

Because of the alternate splicing of the CD44 receptor, CD44 expression is correlated with malignant transformation,40-45 interaction with its ligands,44-47 and cell locomotion.48 Ezrin, ardid, and moesin family members are located just beneath the plasma membrane and act as molecular linkers between a cytoplasmic domain of CD44 and the actin-based cytoskeleton.50 Camp et al.49 have shown two pools of CD44, one containing nonphosphorylated, cytoskeleton-associated CD44 and the other containing phosphorylated, unassociated CD44.

Although CD44 has been shown to participate in lymphocyte adhesion to high endothelial venules, lymphocyte maturation is closely associated with increased levels of CD44. In vitro cultures of embryonic retinal ganglion cells are inhibited by the presence of CD44.50 In multiple sclerosis, increased CD44 expression has been associated with fibrous astrocytes in inflamed areas of white matter.51 Aging leads to the replacement of virgin T-cells by memory T-cells and to the accumulation of cells with signal transduction defects.52 Aged CD44-positive cells are less responsive to antigenic stimuli. The studies of Alvarado et al.19 have demonstrated that the trabecular meshwork endothelial cells are frequently absent in POAG, creating naked trabecular meshwork beams. The inability of trabecular meshwork endothelial cells to migrate may...
CD44H Localization in Primary Open-Angle Glaucoma

indicating a lack of cell locomotion and a lack of regenerative capacity of a trabecular meshwork. Recent studies suggest CD44 may represent a trigger molecule for cell lysis.33-35 The ligation of CD44 by selected monoclonal antibodies results in the rapid enhancement of natural killer cytotoxic activity36 and in the stimulation of the release of macrophage colony-stimulating factor, interleukin-1, and tumor necrosis factor from monocytes.37 The differences in CD44H in the laser-treated subgroup compared with the topically treated glaucoma subgroup support the notion that laser trabeculectomy influences the trabecular meshwork cell population and that laser treatment stimulates naive, naive CD44H in comparison with older trabecular meshwork cells.

The difference in the Triton X-100 effect on CD44 immunostaining patterns of POAG eyes compared with normal eyes may indicate changes in the associated lipid in the cell membrane or translational or post-translational modifications of the CD44 protein. Alternatively, Triton X-100 may unmask epitopes in normal eyes. In support of the enhanced extraction in POAG eyes, we have identified a distinctive pattern of CD44H in POAG by biochemical methods. Western blot analysis of all six cases described in this present study also demonstrated a marked increase in a Triton X-100-soluble CD44H in the iris and the ciliary body.26 In summary, the results of this immunostaining study agree with the western blot analysis of CD44H,26 and they suggest that CD44H may prove to be a useful marker of POAG. Additional studies are required to further characterize the functionality and structural aspects of the CD44 receptor in normal eyes compared with POAG eyes.

Acknowledgments

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References


38. Brissett NC, Perkins SJ. The protein fold of the hyaluronate-binding proteoglycan tandem repeat domain of link protein, aggrecan and CD44 is similar to that of the C-type lectin superfamily. FEBS Lett. 1996;388:211–216.


