Aqueous Humor in Primary Open-Angle Glaucoma Contains an Increased Level of CD44S

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PURPOSE. To determine whether the cell adhesion molecule CD44, the principal receptor of hyaluronan, is altered in the aqueous humor and the anterior segment of patients with primary open-angle glaucoma (POAG).

METHODS. The trabecular meshwork (TM), iris, ciliary body, and sclera of POAG and age-matched control eyes preserved in ethanol were microdissected and subjected to 1% Triton X-100 solubilization at 4°C. Western blot analysis was performed using monoclonal antibodies that recognize either CD44H (hematopoietic; extracellular domain) or CD44S (soluble ectodomain). The concentration of soluble CD44S in aqueous and microdissected tissues was measured by enzyme-linked immunosorbent assay (ELISA).

RESULTS. ELISA of soluble CD44S of aqueous from eyes of patients with POAG indicated that the concentration of soluble CD44S is increased in comparison with that of aqueous from normal eyes (P < 0.0003). Western blot analysis and densitometry of POAG iris and ciliary body revealed a statistically significant increase in the Triton X-100 extraction of CD44H. The predominant increases were in the 180-kDa (P < 0.001) and the 85-kDa (P < 0.001) forms. ELISA of soluble CD44S indicated that the concentration is statistically decreased in iris (P < 0.05), ciliary body (P < 0.001), and TM (P < 0.005) of POAG eyes.

CONCLUSIONS. Increased amounts of soluble CD44S in POAG aqueous and Triton X-100–solubilized CD44H characterized POAG in the iris and ciliary body. These soluble CD44 isoforms may influence the activity of the transmembrane CD44H by acting as inhibitors of CD44H and, thereby, adversely influence the cell survival of TM and retinal ganglion cells in POAG. (Invest Ophthalmol Vis Sci. 2002;43:153–159)

Primary open-angle glaucoma (POAG) is a major blinding disease affecting approximately 67 million persons worldwide.1 It is likely that several biochemical and cellular factors influence the glaucoma process. A variety of cellular insults or molecular defects2–5 may intersect, leading individually or collectively to cell death in the trabecular meshwork (TM)6 or retinal ganglion cells.5 Moreover, evidence suggests that activated immunity6 and alterations in the extracellular matrix7–10 may be etiologic factors in POAG. The extracellular matrix is a diverse group of macromolecules that assemble to form a functional network. The majority of aqueous outflow resistance in both normal and POAG eyes is within the TM, especially within the extracellular matrix of the juxta-acanalicular connective tissue, which is a glycosaminoglycan-enriched area.10–12 Biochemical studies10 and computer-aided image analysis11 indicate that POAG is associated with a marked decrease in hyaluronan (HA) and an increase in chondroitin sulfate in the TM. HA is a key factor in promoting cell motility, adhesion, and proliferation.13,14 These cell events are orchestrated by three HA15 cell receptors—CD44, receptor for HA-mediated motility (RHAMM), and intercellular adhesion molecule (ICAM)-1—all of which have been identified in the human TM.16

One receptor for HA, CD44, is a multifunctional receptor and cell-adhesion molecule that increases with the aging of T lymphocytes.17,18 CD44H is an integral cell membrane glycoprotein with postulated roles in a wide variety of biological processes, including cell adhesion,19 inflammation,20 autoimmunity,21 and apoptosis.22 CD44 exists as both an 80- to 90-kDa type 1 transmembrane glycoprotein, CD44 hematopoietic (CD44H), and a 30- to 50-kDa soluble form, soluble CD44S, generated by the release of the extracellular domain by hydrolytic cleavage.23 Hydrolysis of CD44H involves either the cleavage of glycosylphosphatidylinositol (GPI)-anchor and/or the limited proteolysis of the extracellular domain. These soluble CD44 isoforms may regulate the effects of cognate ligands of CD44H by acting as soluble inhibitors of CD44H. In addition, the proteolytically cleaved soluble CD44S and the loss of the GPI-anchor of CD44H probably constitute, at least in part, a turnover mechanism for downregulating the CD44 receptor.24

CD44H has diverse functional properties owing to sequence differences arising from alternate splicing of mRNA, as well as extensive posttranslational glycosylation.25 CD44H is expressed on a variety of ocular cells, including retinal ganglion cells26 and axons.27 CD44S is present in serum26 and aqueous humor.28 In vitro, axon growth of retinal ganglion cells is inhibited by the presence of CD44H.29 Aging leads to replacement of virgin T cells by memory T cells and to the accumulation of cells with signal transduction defects.30 Aged CD44H-positive cells are less responsive to antigenic stimuli.31 Yonemura et al.32 demonstrated that CD44H is the membrane-binding partner for ezrin-radixin-moesin (ERM) proteins. The CD44-ERM complex acts as a regulatable protein scaffold that anchors actin filaments to the plasma membrane. Consequently, increased turnover of CD44H in POAG may disrupt several normal cell functions and adversely affect TM cell function33 and survival. Therefore, we determined the concentration of soluble CD44S in the aqueous humor of normal subjects and patients with POAG in comparison with CD44H and soluble CD44S in donor eyes, both of which appear to

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characterize POAG. Our data support the hypothesis that CD44H may represent a protein marker of the disease.34

**METHODS**

**Aqueous Humor Samples**

Samples of aqueous humor were obtained from normal subjects (n = 41; age range, 46–85 years; mean, 72.5 ± 8.6) who were undergoing elective cataract surgery, patients with glaucoma (n = 29; age range, 46–85 years; mean, 71.5 ± 9.8) who were undergoing elective cataract surgery (n = 9) or filtration surgery (n = 20), and one patient with ocular hypertension who was undergoing elective cataract surgery. None of the subjects had had incisional ocular surgery. The available clinical histories of uveitis or incisional ocular surgery. The available clinical histories of the normal and POAG donor specimens are shown in Table 1.

The microdissected anterior segment tissues were incubated for 2 hours at 4°C with end-over-end mixing in 1 mL 20 mM Tris (pH 7.5); 0.15 M NaCl-1% Triton X-100 containing the inhibitors 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine HCl, 10 mM N-ethyl maleimide, 100 mM 6-amino hexanoic acid, 50 mM iodoacetamide, and 1 μg/mL leupeptin; and 10 μg/mL soybean trypsin inhibitor. The resultant homogenate was clarified by centrifugation at 10,000 g for 10 minutes, and the supernatant was stored at −80°C. The pellet was resuspended in 1 mL of the above-mentioned buffer with the addition of 0.5% sodium deoxycholate (Doc) and 0.1% sodium dodecyl sulfate (SDS) to further extract CD44H.35 The solubilization was performed for 2 hours at 4°C with end-over-end mixing and clarified by centrifugation at 10,000g for 10 minutes, and the supernatant was stored at −80°C.

**Human Donor Eyes**

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 obtained from the Rochester Eye and Human Parts Bank, Inc., 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 either immersed in alcohol or refrigerated and sent to the laboratory and placed in 100% ethanol within 12 hours of death. The anterior segment was microdissected.10,11 The mean age of the normal donor eyes was 72.9 years, and the mean age of the POAG donor eyes was 67.0 years. None of the eyes had evidence of

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Patient</th>
<th>Cause of Death*</th>
<th>Ocular Medications</th>
<th>Laser Trabeculoplasty</th>
<th>Duration of POAG</th>
<th>Treated IOP (mm Hg)</th>
<th>Cup-to-Disc Ratio</th>
<th>Visual Field Condition†</th>
</tr>
</thead>
<tbody>
<tr>
<td>L 2067 — — — — —</td>
<td>None — — — — —</td>
<td>Pulmonary fibrosis/3R 2066 74/M Colon</td>
<td>Iopidine 0.5% RID</td>
<td>Both eyes</td>
<td>None</td>
<td>2 years</td>
<td>21</td>
<td>0.4</td>
</tr>
<tr>
<td>3R 2065 74/M Prostate cancer/2R 2065 74/M Myocardial failure/</td>
<td>Iopidine 0.5% RID</td>
<td>5h</td>
<td>Betoptic 0.5% BID both eyes</td>
<td>Left eye, 2 years before death</td>
<td>None</td>
<td>7 years</td>
<td>22</td>
<td>0.8</td>
</tr>
<tr>
<td>L 2064 74/M Lung carcinoma/</td>
<td>Pilocarpine 2.0% QID both eyes</td>
<td>Both eyes</td>
<td>Both eyes, 3 years before death</td>
<td>Left eye, 1 year before death</td>
<td>None</td>
<td>17 years</td>
<td>22</td>
<td>0.6</td>
</tr>
<tr>
<td>L 2067 — — — — —</td>
<td>None — — — — —</td>
<td>Lung carcinoma/</td>
<td>Both eyes</td>
<td>Both eyes, 3 years before death</td>
<td>Left eye, 1 year before death</td>
<td>None</td>
<td>17 years</td>
<td>22</td>
</tr>
</tbody>
</table>

The ocular findings were from the last available examination.

* Time interval between death and immersion of the globe into 100% alcohol.

† 1, normal; 2, scotoma; 3, significant defect; 4, loss of central field.
propriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:2500 dilution), and was quantitated according to a densitometry scale of 0, no detectable product; 1+, trace; 2+, positive; 3+, strongly positive; and 4+, intensely positive.36-37

**Enzyme Immunoassay of CD44S**

Soluble CD44S was measured in the aqueous humor from patients, the Triton X-100 extract, and the Doc-SDS extract, using the standard commercially available CD44 soluble ELISA kit (Bender Med Systems, Vienna, Austria) as described by Asplund and Heldin.35 Briefly, 1 μg protein equivalent of each sample of aqueous, Triton X-100 extract, and Doc-SDS extract protein was subjected to 1:60 dilution in the sample dilution buffer; 20 μL of the diluted sample and 80 μL of the sample diluent buffer were added to each well. The samples were treated with 50 μL of diluted HRP-CD44S antibody conjugate and incubated for 3 hours at room temperature. Each well was washed three times with buffer, and 100 μL freshly prepared tetramethylbenzidine substrate buffer was added and incubated for 15 minutes at room temperature. Color development was stopped by adding 100 μL of 4 N H₂SO₄; the optical density was read in a ELISA reader at 450 nm. The concentration of soluble CD44S was determined by the standard curve, using the manufacturer’s standard CD44S soluble protein.

**Statistical Analysis**

CD44H and CD44S immunoblots of normal and POAG tissues were performed in duplicate, and CD44S ELISA assays were performed in triplicate. For validation of the standard curve, the slope, intercept, and correlation coefficient were within the manufacturer’s suggested ranges. Data are presented as mean ± SEM. All statistical analyses were conducted with Student’s t-test. *P* < 0.05 was considered the level of significance.

**RESULTS**

**Aqueous Humor and CD44S**

In 41 normal aqueous samples the soluble CD44S concentration was 9.58 ± 0.79 ng/mL (SEM), whereas in 26 POAG aqueous samples the soluble CD44S concentration was 15.96 ± 1.37 ng/mL (an approximately twofold increase, *P* < 0.0003; Table 2). Western blot analysis of aqueous humor showed three immunoreactive bands: a predominant 31-kDa band, the soluble form of CD44, which was markedly increased in the POAG aqueous, a minor 55-kDa band, another soluble form of CD44, and an 85-kDa band (Fig. 1). The aqueous humor profile of immunoreactive bands was distinct from that of the positive control, serum, which has 60- to 80-kDa and 100- to 150-kDa bands (data not shown).

**Donor Eyes and Triton X-100 Extraction: CD44H in the Iris and in the Ciliary Body**

In the iris and ciliary body microdissected tissues, Triton X-100 extracted proteins were identified on immunoblots with an anti-CD44H monoclonal antibody that recognizes the extracellular domain of CD44. A CD44H-positive protein of an apparent molecular weight of 85 kDa was identified in almost all cases (Fig. 2A, 2B). In addition, heterogenous high-molecular-weight proteins were observed, typical of glycosylated CD44H.32 Immunoblots of the iris and ciliary body revealed a distinctive pattern in POAG eyes compared with normal eyes. All six cases of POAG but only one of the normal eyes, 3L, demonstrated a marked increase in 180-kDa and 85-kDa CD44H proteins in the iris and in the ciliary body (Figs. 2A, 2B). Analysis of the intensity of CD44H immunostaining between the POAG and normal eyes was highly significant for the 180-kDa protein (*P* < 0.001) and for the 85-kDa protein (*P* < 0.001). The CD44H immunoblot pattern of iris and ciliary body was correlated with the available clinical histories (Table 1). In eyes from donor 2, the most advanced glaucoma, was the most positive for CD44H; eyes from donor 3, with moderate glaucoma, were moderately positive; those from donor 1, early glaucoma, were slightly positive.

**TABLE 2. Aqueous CD44 Concentration in POAG**

<table>
<thead>
<tr>
<th>Type of Glaucoma</th>
<th>Number</th>
<th>CD44 Concentration</th>
<th>Comparison</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>41</td>
<td>9.58 ± 0.79</td>
<td>Normal vs. POAG</td>
<td><em>P</em> &lt; 0.0003</td>
</tr>
<tr>
<td>POAG</td>
<td>26</td>
<td>15.96 ± 1.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>17</td>
<td>15.80 ± 1.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>8</td>
<td>14.90 ± 1.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>1</td>
<td>27.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filtration surgery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Successful</td>
<td>17</td>
<td>13.91 ± 1.61</td>
<td>Success vs. failed surgery</td>
<td><em>P</em> &lt; 0.004</td>
</tr>
<tr>
<td>Failed</td>
<td>3</td>
<td>24.42 ± 1.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ocular Hypertension</td>
<td>1</td>
<td>14.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JOAG</td>
<td>3</td>
<td>7.74 ± 2.78</td>
<td>Normal vs. JOAG</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>JOAG vs. POAG</td>
<td><em>P</em> &lt; 0.04</td>
</tr>
</tbody>
</table>

CD44 concentration of aqueous humor was determined by ELISA and is expressed as nanograms per milliliter (± SEM). JOAG, juvenile open-angle glaucoma.
CD44H in the TM and Sclera

A comparison of normal and POAG immunoblots of the TM and sclera was unrevealing (Figs. 2C, 2D); the intensity of the CD44H profiles was statistically insignificant. There was a large amount of CD44H in the TM in the most advanced POAG (in donor eye 2R but less in 2L, which recently had undergone laser trabeculoplasty), a moderate amount of CD44H in the eyes with a less-advanced form (3R and 3L), and a minimal amount in an early diagnosed POAG (eyes 1R and 1L).

0.5% Doc-0.1% SDS Extraction and CD44H

To test whether the Triton X-100 extracted all the CD44H, we subjected the Triton X-100-insoluble material to extraction with 0.5% Doc-0.1% SDS (Fig. 3). In both the normal and POAG tissues, Doc-SDS extracted additional amounts of CD44; however, results of Doc-SDS extraction and Western blot analysis were similar for normal and POAG and statistically insignificant in all tissues.

Soluble CD44S

To explore whether a CD44S monoclonal antibody, which preferentially recognizes soluble CD44S, was useful in distinguishing normal and POAG tissues, we used Triton X-100 and Doc-SDS extracts of all tissues on immunoblots. The CD44S-positive proteins were predominantly 55, 67, and 85 kDa, which was distinctly different from the CD44H immunoblots (compare Figs. 2 and 4). There were no differences between normal and POAG tissues when CD44S was used on immunoblots (Fig. 4).

CD44S ELISA

The concentrations of tissue CD44S, as determined by ELISA, are shown in Figure 5 for iris, TM, ciliary body, and sclera. The POAG ciliary body was significantly decreased (range, 150–200 pg/mg) in comparison with the normal ciliary body (range, 300–400 pg/mg; \( P < 0.001 \)). The POAG iris also contained lower concentrations of CD44S than did the normal iris (\( P < 0.05 \)). The POAG TM was statistically different from the normal TM. The range of normal TM was 80 to 120 pg/mg whereas the range in POAG was 10 to 80 pg/mg (\( P < 0.005 \)). There was no significant difference in laser- versus non–laser-treated TM. There was no statistically significant difference between normal and POAG in the concentration of CD44S in sclera.

0.5% Doc-0.1% SDS Extraction and CD44H

To test whether the Triton X-100 extracted all the CD44H, we subjected the Triton X-100-insoluble material to extraction with 0.5% Doc-0.1% SDS (Fig. 3). In both the normal and POAG tissues, Doc-SDS extracted additional amounts of CD44; however, results of Doc-SDS extraction and Western blot analysis were similar for normal and POAG and statistically insignificant in all tissues.
The source of soluble CD44S is presumably the ciliary body–iris epithelium, because these tissues have the highest concentration of CD44. CD44S monoclonal antibody preferentially recognizes the soluble form of CD44. Western blot analysis in POAG demonstrated a marked increase of the Triton X-100–extracted CD44H in the iris and ciliary body. Western blot analysis of the iris and ciliary body in all six cases of POAG demonstrated a statistically significant increase in CD44H—the 85- to 180-kDa proteins. Western blot analyses using the CD44S antibody were insignificant but confirmed the specificity of the antibody, which preferentially recognized the lower molecular weight CD44S. Triton X-100, a nonionic detergent, is useful in solubilizing a number of integral membrane proteins, such as CD44H. 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 CD44H is an absolute requirement for detergent solubility. Because the transmembrane portion of CD44H associates with phospholipid microdomains, the activity of the cell determines the Triton X-100 solubility of CD44H. In POAG, the predominant increase in the Triton X-100-solubilized CD44H was in the 180- and 85-kDa forms of CD44, the membrane CD44.

The concentration of soluble CD44S increased with age. Linear regression analysis showed that soluble CD44S increased approximately 0.075 ng/mL per year in normal aqueous and approximately 0.12 ng/mL per year in POAG aqueous. In eyes at age 50, the normal projected soluble CD44S was approximately 7.0 ng/mL; by age 80, it was 9.25 ng/mL. The data for patients with POAG indicate that at age 50 the projected soluble CD44S was 12.5 ng/mL and by age 80 was 16.2 ng/mL in this study. It is quite possible that within the 41 normal aqueous specimens a few individuals may have undiagnosed POAG or will manifest POAG in the future. It appears that the soluble CD44S concentration in POAG is higher than that in juvenile open-angle glaucoma. Notably, the soluble CD44S concentration in patients in whom filtration surgery was unsuccessful was higher than in patients with successful filtration surgery.

Soluble CD44S is shed as the result of ligand binding, alternate splicing, and enzymatic cleavage from the cell surface. The source of soluble CD44S is presumably the ciliary body–iris epithelium, because these tissues have the highest concentrations of CD44H and are known to release proteins. ELISA analysis of the soluble CD44S of POAG iris, ciliary body, and TM showed a statistically significant decrease; however, ELISA of CD44S in aqueous showed a statistically significant increase in soluble CD44S, which may indicate that soluble CD44S is shed into the aqueous.

The ectodomain shedding of proteins from the cell surface is common. Many proteins lead a dual existence as both integral membrane proteins and soluble proteins; the classic examples are immunoglobulin and acetylcholinesterase. Alternate processing of each produces membrane-bound or soluble forms, each form having distinct physiologic roles. CD44H also has a dual existence. Soluble CD44S is shed into aqueous and may act as a signaling molecule that may influence target cells, both in the anterior and the posterior segments. It is interesting that increased concentrations of soluble CD44S have been found in the sera of patients with autoimmune diseases. If CD44S acts as a decoy receptor, it would interfere with normal ligand-induced signal transduction by membrane CD44H. Notably, the shedding of CD44S participates in turnover and remodeling of the extracellular matrix and cell surface, and in binding studies of CD44S, the shed soluble CD44S retains its biologic activity.

Western blot analysis of CD44S in aqueous humor from normal subjects and patients with POAG demonstrated a statistically significant increase in soluble CD44S, which may indicate that soluble CD44S concentration in POAG is higher than that in normal. Computer-aided color image analysis of normal and POAG sections analyzed by immunostaining with CD44H antibody clearly separated individual cases of POAG from the normal. Computer-aided color image analysis of normal and POAG sections analyzed by immunostaining with CD44H antibody clearly separated individual cases of POAG from the normal. Computer-aided color image analysis of normal and POAG sections analyzed by immunostaining with CD44H antibody clearly separated individual cases of POAG from the normal. Computer-aided color image analysis of normal and POAG sections analyzed by immunostaining with CD44H antibody clearly separated individual cases of POAG from the normal. Computer-aided color image analysis of normal and POAG sections analyzed by immunostaining with CD44H antibody clearly separated individual cases of POAG from the normal.
influence over migration. CD44H influences cell survival.51 Previous studies have shown that fibroblasts undergo apoptosis after anti-CD44 antibody treatment.52 In addition to anti-CD44 antibodies’ inducing fibroblast apoptosis, soluble CD44 has been shown to impair tumor metastasis by inhibiting the function of CD44H as a cell-adhesion molecule and causing mammary carcinoma cells to undergo apoptosis.53 The ability of soluble CD44 to induce apoptosis by binding to membrane-bound CD44H elicits the hypothesis that elevated levels of soluble CD44S will be accompanied by programmed cell death. Shed soluble CD44S binds to membrane-bound CD44H and abrogates the CD44 receptor.

Thus, CD44H is a multifunctional receptor involved in cell-cell54,55 and cell–matrix interactions,56 cell trafficking, presentation of chemokines and growth factors, and transmission of growth signals.57 An increased concentration of soluble CD44S in POAG may block the binding of CD44H to HA.58 Also CD44H is required for certain high-affinity receptors, e.g., erbB2 phosphorylation and erbB2-erbB3 heterodimerization, for cell survival.51 If soluble CD44S interferes with CD44H activity, then it is downregulated and erbB2 is less active, and this causes programmed cell death. Thus, our working hypothesis is that POAG is characterized biochemically by decreased concentration of HA and increased turnover and downregulation of the HA receptor CD44, which, in turn, may influence cell survival of TM and retinal ganglion cells.

References