
References

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Protein Quantification and Electrophoresis in Aqueous Humor of Pseudoexfoliation Eyes

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Purpose. Pseudoexfoliation (PSX) eyes frequently show clinical signs of blood–aqueous barrier impairment. To analyze these alterations, the authors examined aqueous humor of human eyes with and without PSX.

Methods. After aqueous humor samples had been obtained during cataract or filtering glaucoma surgery, a modified Pierce–bicinchoninic acid assay was used to quantify total aqueous protein concentration in 27 PSX eyes and 37 eyes without clinical signs of PSX (12 cataract eyes and 25 eyes with primary open-angle glaucoma). In addition, aqueous protein composition was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis, silver staining, and laser densitometry in 27 PSX eyes and 59 eyes without PSX.

Results. Aqueous protein concentration was significantly higher in PSX (mean 0.42 ± 0.16 mg/ml) than in normal cataract eyes (0.22 ± 0.08 mg/ml, P < 0.0001) and in eyes with open-angle glaucoma (0.26 ± 0.09 mg/ml, P < 0.0001, Wilcoxon-Mann-Whitney test). Electrophoresis revealed a characteristic increase of a 12.5-kDa band in 15 of 27 PSX eyes but in only 1 of 59 eyes without PSX (P < 0.00001, chi-square test).

Conclusions. These results substantiate increased aqueous protein concentration and aqueous barrier impairment in PSX. The additional finding of an increased 12.5-kDa band in 56% of PSX eyes may be related to the pathogenesis of PSX in the anterior ocular segment. Invest Ophthalmol Vis Sci. 1994;35:748–752.

Biomicroscopic examination of pseudoexfoliation (PSX) eyes frequently reveals signs of discrete intraocular inflammation such as aqueous flare and postoperative fibrin formation. These findings appear to be related to alterations of the blood–aqueous barrier in PSX that have been observed using various methods such as iris fluorescein angiography, and fluorophotometry. In addition, quantitative assessment of aqueous flare with the laser flare-cell meter revealed significantly increased flare values in PSX eyes indicating increased aqueous protein concentration.

It was the aim of this study to search for alterations of the aqueous humor in PSX eyes. Therefore, we performed both quantitative determination of total aqueous protein and qualitative assessment of the protein composition in eyes with and without PSX.

METHODS. Materials. Aqueous humor was obtained during intraocular surgery from 27 eyes of 27 patients (age 72.6 ± 9.1 years; 16 men and 11 women) with the characteristic clinical signs of PSX and from 72 eyes of 72 patients (age 53.9 ± 21.9 years; 33 men and 26 women) without any clinical signs of PSX with various clinical diagnoses. All patients underwent a complete ophthalmologic examination, and the type and severity of cataract and the presence and morphol-
ogy of maculopathies were determined. Additional medical data (systemic hypertension, cardiac problems, or chronic obstructive airway disease) were recorded. Ten of the PSX eyes showed signs of secondary open-angle glaucoma (OAG), including increased intraocular pressure and glaucomatous optic nerve cupping.

Aqueous humor was obtained by a total of three experienced surgeons. During extracapsular cataract extraction (17 PSX eyes and 23 eyes without PSX), during trabeculectomy (10 PSX eyes and 48 eyes without PSX), or during penetrating keratoplasty (1 eye without PSX), after preparation of a fornix-based conjunctival flap and before entering the anterior chamber, a self-sealing 0.3-mm stab incision was made with a sharp lancet through the peripheral cornea. Immediately thereafter, the anterior chamber was entered through the stab incision with a blunt Sautter cannula on a disposable tuberculin syringe, and 50 \( \mu l \) of aqueous humor was withdrawn quickly (within 2 seconds) from the central anterior chamber. The anterior chamber then was refilled with hyaluronic acid, and the aqueous sample was immediately frozen. During stab incision and aqueous aspiration, great care was taken to avoid touching any intraocular structure and contaminating the sample.

For total aqueous protein determination, 27 PSX eyes, 25 eyes with primary chronic OAG (age 63.3 ± 15.5 years), and 12 normal cataract eyes (age 57.6 ± 20.4 years) were included. Ten of the 27 PSX eyes had clinical signs of secondary OAG. Eighteen of the 25 eyes with OAG and 8 of the 10 PSX eyes with secondary OAG were from patients receiving antiglaucoma medication. Patients with a history of uveitis, diabetic retinopathy, previous ocular trauma, or previous ocular surgery were excluded from total aqueous protein determination.

For sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE), 27 PSX eyes and 59 eyes without clinical signs of PSX were included. The PSX eyes were identical to the PSX eyes in which total aqueous protein was determined, but there were no exclusion criteria in non-PSX eyes undergoing SDS-PAGE. The clinical diagnoses of the eyes without PEX are listed in Table 1.

The tenets of the World Medical Association Declaration of Helsinki were followed, and local Ethical Committee approval was granted. Prior informed consent was obtained from all patients.

Aqueous samples were stored at —70°C.

**Determination of Protein Concentration by Bicin Choninic Acid Assay.** Protein concentrations were determined by a modified Pierce—bicin choninic acid method with bovine albumin as standard. Aqueous humor (10 \( \mu l \) containing up to 5 \( \mu g \) of protein) was incubated for 15 min at room temperature with sodium deoxycholate (150 \( \mu l \), 0.17 mg/ml). Trichloroacetic acid (50 \( \mu l \), 25%) was added and incubated for 10 min. The solution was centrifuged at 14,500g for 10 min. The supernatant solution containing interfering reducing substances was removed. Pierce reagent (300 \( \mu l \)) was added. After incubation at 37°C for 2 hours, the absorbency at 562 nm was determined and compared to a bovine serum albumin standard curve. The observed absorbency was linear up to 0.6 corresponding to 5 \( \mu g \) of serum albumin.

The spindown assay used here was originally introduced by Bensadoun and Weinstein for removing interfering substances before protein determination by the Lowry assay. We were able to demonstrate that adding up to 5 mM ascorbate or glutathione did not interfere with the assay described here (Hannappel 1993, unpublished data).

**TABLE 1. Clinical Diagnoses of 59 Eyes Without Signs of PSX that were Examined by SDS-PAGE Electrophoresis**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of Eyes</th>
</tr>
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<tbody>
<tr>
<td>Primary open angle glaucoma</td>
<td>30</td>
</tr>
<tr>
<td>Cataract</td>
<td>11</td>
</tr>
<tr>
<td>Secondary open angle glaucoma</td>
<td>8</td>
</tr>
<tr>
<td>&quot;Pigmentary glaucoma&quot;</td>
<td>5</td>
</tr>
<tr>
<td>Status post iridocyclitis</td>
<td>1</td>
</tr>
<tr>
<td>Status post ocular trauma</td>
<td>2</td>
</tr>
<tr>
<td>Fuchs’ cyclitis</td>
<td>1</td>
</tr>
<tr>
<td>Congenital hereditary corneal</td>
<td>1</td>
</tr>
<tr>
<td>endothelial dystrophy</td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 1.** Total aqueous protein in normal cataract eyes, eyes with primary chronic OAG, and eyes with pseudoexfoliation (PSX) with and without OAG. The bars represent the mean aqueous protein concentration for each group.
PCOAG
n = 25

Number

PSX
n = 27

Normal cataract
n = 12

Protein concentration (mg/ml)

FIGURE 2. Distribution of frequency of total protein concentration of the different groups (normal cataract eyes, primary chronic OAG eyes, and pseudoexfoliation (PSX) eyes).

SDS-ExcelGel Electrophoresis. Aqueous humor (10 μl) was separated by SDS-PAGE. Precasted polyacrylamide gradient gels (ExcelGel 8% to 18%, Pharmacia Biotech GmbH [Freiburg, Germany]) and buffer strips were used. Before electrophoresis the samples were brought to final concentrations of 0.05 M Tris-HCl, 10 g/l SDS, 5 mM dithiothreitol, and 0.1 g/l bromophenol blue, pH 7.5. The samples were heated to 95°C. The following proteins were used as molecular weight standards: phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa), and prealbumin (13.7 kDa).

Silver Staining. The gels were fixed overnight in 40% methanol–10% acetic acid. They were silver stained according the procedure of Heukeshoven and Dernick. The relative intensities of the stained bands were determined by means of laser scanning densitometry (Ultrosan XL, Pharmacia Biotech GmbH).

RESULTS. Total aqueous protein concentrations were significantly higher in PSX eyes (0.42 ± 0.16 mg/ml, range 0.18 to 0.99 mg/ml) than in both the cataract group (0.22 ± 0.08 mg/ml, range 0.04 to 0.34 mg/ml) and the OAG group (0.26 ± 0.09 mg/ml, range 0.12 to 0.50 mg/ml) (P < 0.0001, Wilcoxon-Mann-Whitney test) (Figs. 1, 2 and Table 2). The difference of total aqueous protein concentration in PSX eyes with secondary OAG (0.46 ± 0.20 mg/ml, range 0.30 to 0.99) and in PSX eyes without secondary OAG (0.40 ± 0.13 mg/ml, range 0.18 to 0.70) was statistically not significant (P > 0.3). There also was no significant difference between cataract eyes and OAG eyes (P > 0.1) or between OAG eyes and PSX eyes with secondary OAG with and without antiglaucoma medication (P > 0.3). Type and severity of cataract did not seem to affect aqueous protein concentration in either patient group.

SDS-PAGE revealed a very striking increase of a 12.5-kDa band (defined as more than 3% of total aqueous protein) in 15 of 27 PSX eyes but only in 1 of 59 eyes without PSX (Fig. 3). The molecular weight of the observed increased band was approximately 1 kDa smaller than the four subunits (13.7 kDa) of prealbumin (Fig. 3). The presence of the increased 12.5-kDa band in PSX eyes did not appear to be correlated with other clinical characteristics. Apart from this band, no striking differences in the aqueous protein composition between PSX and non-PSX eyes were observed with albumin showing the highest concentration (Fig. 3).

TABLE 2. Total Aqueous Protein Concentration (mg/ml) and P Values (Wilcoxon-Mann-Whitney Test)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean ± SD (range)</th>
<th>Significance</th>
<th>(P Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cataract eyes</td>
<td>12</td>
<td>0.22 ± 0.08 (0.04–0.34)</td>
<td>&gt;0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PCOAG eyes</td>
<td>25</td>
<td>0.26 ± 0.09 (0.12–0.50)</td>
<td>&gt;0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>All PSX eyes</td>
<td>27</td>
<td>0.42 ± 0.16 (0.18–0.99)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>PSX glaucoma eyes</td>
<td>10</td>
<td>0.46 ± 0.20 (0.30–0.99)</td>
<td>&gt;0.3</td>
<td></td>
</tr>
<tr>
<td>PSX eyes without glaucoma</td>
<td>17</td>
<td>0.40 ± 0.13 (0.18–0.70)</td>
<td>&gt;0.3</td>
<td></td>
</tr>
</tbody>
</table>

PCOAG = primary chronic open angle glaucoma; PSX = pseudoexfoliation.
FIGURE 3. SDS-PAGE electrophoresis. Lanes 1, 2: Aqueous humor of two eyes without pseudoexfoliation (PSX). Lanes 3, 4: Aqueous humor of two PSX eyes. Localization and molecular weights of protein standards are indicated (right). Note the 12.5-kDa band (arrows) that is barely visible in non-PSX eyes but very prominent in PSX eyes and that lies just inferior to the prealbumin band (arrowheads).

DISCUSSION. There are a number of clinical signs that suggest impairment of the blood-aqueous barrier in PSX: aqueous flare; aqueous cells that mostly are melanin granules from the iridal pigment epithelium; and marked inflammatory reaction with frequent fibrin formation after intraocular surgery or laser trabeculoplasty. Impairment of the blood-aqueous barrier in PSX has been further objectivated using iris fluorescein angiography, fluorophotometry, and the laser flare-cell meter. Thus, our finding of increased total aqueous protein concentration in PSX eyes is well in concordance with previous clinical observations and was somewhat expected. The total aqueous protein concentration of our group of normal cataract eyes was higher than previously reported values, a difference that may be accounted for by different methods of protein determination. The origin of the increased aqueous proteins in PSX appears to be mostly the iris vessels. Alterations of iridal vessels in PSX have been noted using iris fluorescein angiography that demonstrated fluorescein leakage and microaneurysmalization. Electron microscopic studies of iris vessels in PSX revealed deposition of PSX material in the vessel walls with consecutive disorganization of the normal vessel structure, loss of endothelial and adventitial cells, and thinning and fenestration of the endothelial lining. Schlötzer-Schrehardt et al were able to further trace the defect of the blood-aqueous barrier in PSX. By using immunoelectron microscopic staining for endogenous albumin and immunoglobulin G, they localized the primary sites of protein leakage in the iris root and the anterior ciliary body.

Biochemical investigation of the aqueous in various forms of OAG has so far received relatively little attention. Most studies concentrated on aqueous proteins with high molecular weights, whereas small proteins with molecular weights below 20 kDa received even less attention. In our study, eyes with primary chronic OAG did not show significantly increased total aqueous protein concentrations in comparison with the normal cataract group.

The exact composition of PSX material and the pathogenesis of PSX syndrome are still unclear. Our findings in SDS-PAGE indicates that not only total aqueous protein concentrations is increased in PSX, but also that—at least in part of the PSX eyes—aqueous protein composition differs from non-PSX eyes with a prominent 12.5-kDa band. Ringvold et al studied lens capsules and pooled aqueous humor from four PSX eyes using SDS-PAGE. In the lens capsule, they observed two polypeptides of 14.4 and 16.3 kDa that were not present in the control material. They noted no marked differences between the PSX positive and negative aqueous, although they reported a barely visible band at 14.4 kDa in the PSX specimen. The corresponding findings of atypical aqueous and lens capsule proteins in the range of 12 to 15 kDa may be the consequence of aberrant biochemical processes in PSX syndrome leading to accumulation of this atypical aqueous protein. However, the exact nature and
structure of this 12.5-kDa protein reported here has yet to be clarified.

In our study, elevated amounts of a 12.5-kDa protein were observed in 56% of PSX eyes. It is unclear why this elevation was not observed in 44% of the PSX eyes but was found in 1 of 59 eyes without clinical signs of PSX. Possible explanations include that PSX might be a heterogeneous disease process with expression of characteristic signs in only a disease subgroup, or that the accumulation of the 12.5-kDa protein is not directly related to the PSX disease process. Interestingly, neither the accumulation of the 12.5-kDa protein nor the increase of total aqueous protein appeared to be correlated with presence or absence of secondary glaucoma capsulare. Therefore, neither the impairment of the blood–aqueous barrier nor the presence of the 12.5-kDa band appears to be directly related to the development of secondary OAG in PSX.

Recently, typical PSX material have been identified at several extraocular and systemic locations. Therefore, further elucidation of protein alterations and of the pathogenesis of PSX syndrome may have implications transcending ocular aspects of this disease.

Key Words
pseudoexfoliation syndrome, aqueous humor, aqueous protein, sodium dodecylsulfate polyacrylamide gel electrophoresis, blood–aqueous barrier

References

Influence of HLA-DRB1 Gene Variation on the Clinical Course of Vogt-Koyanagi-Harada Disease

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Purpose. To investigate the difference, if any, in the immunogenetic backgrounds between two clinical subtypes of Vogt-Koyanagi-Harada disease (VKH).

Methods. HLA-DR4 gene variations were investigated in 46 Japanese patients, 28 with the prolonged type and 18 with the nonprolonged type of VKH. HLA-DR4 genes were amplified with polymerase chain reaction (PCR) and then analyzed for its variation with single-strand conformation polymorphism (SSCP) and restriction fragment length polymorphism (RFLP) methods.

Results. Significant differences were found in the DR4 gene variation in the two clinical subtypes. All the patients with the prolonged type had either the DRB1*0405 or DRB1*0410 variant, whereas 39% of the patients with the nonprolonged type had neither of them. This difference in frequency was statistically highly significant (P = 0.00059, Pc = 0.0041). DRB1*0405 was also more frequent in the prolonged type (93%) than in the nonprolonged type (56%) (P = 0.0044, Pc = 0.030). In the prolonged type, relative risk was highest for DRB1*0405/0410 (128), whereas in the nonprolonged type it was highest for DR4 (8.6).

Conclusion. This preliminary study showed that DR4 gene variants differed significantly between the two...