IgG antibody patterns in aqueous humor of patients with primary open angle glaucoma and pseudoexfoliation glaucoma

Stephanie C. Joachim, Diana Wuenschig, Norbert Pfeiffer, Franz H. Grus

Experimental Ophthalmology, Department of Ophthalmology, Johannes Gutenberg University, Mainz, Germany

Purpose: There has been growing body of evidence indicating an immunological component in the pathogenesis of glaucoma. Several studies found specific antibodies in sera of glaucoma patients. Recently, we detected antibody profiles in aqueous humor of patients with normal tension glaucoma that showed significant differences when compared to control subjects. The aim of this study was to compare the IgG antibody patterns against retinal antigens in aqueous humor of patients with primary open angle glaucoma, pseudoexfoliation glaucoma, and control subjects then to identify some of the important biomarkers of this study.

Methods: Aqueous humor of 44 patients was analyzed: 15 patients with primary open angle glaucoma (POAG), 14 patients with pseudoexfoliation glaucoma (PEX), and 15 control subjects (CO). The aqueous was tested against western blots of bovine retinal antigens. The IgG antibody patterns were analyzed by multivariate statistical techniques. Some of the important biomarkers were identified via mass spectrometry (Maldi-TOFTOF).

Results: All patients showed complex patterns of IgG antibodies against retinal antigens. The discriminant analysis revealed a statistically significant difference between the antibody profiles of the POAG and the CO group (p=0.00018). There was also a statistically significant difference between the antibody profiles of the PEX and the CO group (p=0.0013). Not only were up-regulations in the glaucoma groups observed, compared to controls, but also down-regulations. There was no significant difference between the antibody patterns of the POAG and PEX group. The identified biomarkers included heat shock protein 27, α-enolase, actin, and GAPDH.

Conclusions: We could show significant differences between the IgG antibody profiles of the glaucoma groups (PEX and POAG) and controls. The analysis of intraocular antibodies could provide further hints for autoimmune involvement in glaucoma.

Glaucoma, the second most common cause of blindness worldwide [1,2], is a group of ocular disorders where the progressive loss of retinal ganglion cells and atrophy of the optic nerve occurs. Besides the primary forms of glaucoma, e.g. primary open angle glaucoma, where patients have elevated intraocular pressure [3], there are also secondary glauomas. The most common type of secondary glaucoma is called pseudoexfoliation glaucoma. Forty to sixty-nine percent of patients with pseudoexfoliation syndrome develop glaucoma and it accounts for about 25% of all forms of glaucoma [4]. Pseudoexfoliation syndrome is characterized by accumulation of abnormal fibrillar extracellular material in intraocular tissues such as non-pigmented ciliary epithelium, iris pigment epithelium, and trabecular endothelium and by accumulation of extraocular tissues like skin or connective tissue of visceral organs [5,6]. Pseudoexfoliation (PEX) syndrome is currently the most identifiable risk factor for open angle glaucoma [7].

There has been growing evidence of an immunological component in the pathogenesis of glaucoma. Several studies analyzed antibodies in sera of patients with primary open angle glaucoma and normal tension glaucoma (NTG) [8-14]. Our group already analyzed the antibody patterns in aqueous humor of NTG patients [15].

Dooremal first observed that the eye is one of the immune-privileged regions of the body [16], which was later described by Streilein et al. [16,17]. Multiple factors contribute to the immune privilege of the eye [18] such as the blood-aqueous-barrier and the fact that eyes do not have a lymph drainage system. Aqueous humor is, for example, capable of suppressing cytokine production by activated T-lymphocytes [19]. The anterior chamber of the eye has an active immunomodulation. This so called “anterior chamber associated immune deviation” (ACAID) could be shown in several studies [17,20-22]. The reaction to intraocular antigens is established by the eye itself.

Several studies could show that the analysis of the local antibody production can give more information than serum analysis, for example, an aqueous humor analysis in patients with uveitis [23,24]. Torun et al. [25] showed that the analysis of aqueous humor is superior to the analysis of serum in ocular toxoplasmosis.

Recently we analyzed the antibody pattern in aqueous humor of normal tension glaucoma patients [15]. We not only found significant differences (p>0.5) between the NTG and the control group but also identified some of the important antigens like heat shock protein 70 and vimentin.
The aim of this study was to analyze if POAG and PEX, the most common form of secondary glaucoma, reveal different antibody profiles compared to control subjects.

METHODS

Patients: Forty-four patients were included in this study: 15 patients with primary open angle glaucoma (POAG; mean age 75.6, SE±7), 14 patients with pseudoxfoliation glaucoma (PEX; mean age 79.9, SE±6), and 15 control subjects (CO; mean age 68.9, SE±13). The patient classification was done in accordance with the guidelines of the European Glaucoma Society and the groups were matched for age and gender.

The diagnostic criteria for POAG were the following factors: patients had glaucomatous optic disc damage with corresponding glaucomatous changes in the visual field (examined by Goldmann perimeter, Haag-Streit, Schlieren, Switzerland); the patients’ eyes had open angles; there was an absence of alternative causes of optic neuropathy (e.g. infection, inflammation, meningeal disease, ischemic disease, and compressive lesions); and intraocular pressure (IOP) was greater than 21 mmHg (Goldmann applation tonometer, Haag-Streit, Schlieren, Switzerland).

The diagnostic criteria for PEX were the following: IOP was greater than 21 mmHg without treatment, which was determined by applation tonometer; there was visual field loss as in POAG; all had open angles; and during the lit lamp examination, dandruff-like exfoliation material could be seen on the pupil border and white deposits on the anterior lens surface.

The CO group was composed of patients with no history of glaucoma, no pathologic fundus, and no elevated IOP. All CO subjects were undergoing cataract surgery and had no history of other eye diseases.

Exclusion criteria for all groups were: acute attack of glaucoma, diabetes mellitus and retinopathy, and other eye diseases.

Aqueous humor samples: Aqueous humor was collected as described previously [15]. Informed consent was obtained from all patients before surgery. Aqueous humor from control subjects was collected during cataract surgery. Aqueous humor from POAG and PEX patients was collected during trabeculectomy. Fifty to one-hundred microliters of aqueous humor were taken at the beginning of eye surgery. Care was taken to ensure that the aqueous humor did not become contaminated with blood and that the corneal epithelium, iris, and lens were not damaged. All samples were stored at -80 °C for later analysis.

Western blot analysis: The preparation of the retinal antigens was done as described earlier [8,15].

Retinae of bovine eyes were dissected and homogenized. The samples were first boiled then centrifuged at 15,000 rpm for one h. The pellet was discarded and the supernatant was stored at -80 °C for later analysis.

The bovine retina extracts were used for 13.5% SDS-PAGE using a MultiGel-Long (Biometra, Goettingen, Germany).

After electrophoresis, the gels were transferred onto Protran BA 83 nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) by using a Semi-Dry Blotter (Biometra, Goettingen, Germany). The blots were blocked with blocking buffer which contains 5% non-fat dry milk.

The nitrocellulose membranes were cut into strips and one strip was used per aqueous humor sample. The strips were incubated overnight with aqueous humor (1:40). After washing the strips with wash buffer (0.5% non-fat dry milk with Tween20 in PBS) several times, they were incubated with a secondary antibody (1:1000) for one h (peroxidase-conjugated Immuno Pure® Goat Anti-Human IgG (H+L); Pierce, Rockford, IL). After washing the strips with wash buffer the bands were visualized by staining with chemiluminescence (LumiLight Western Blotting Substrate, Roche, Mannheim, Germany), which was prepared according to the manufacturer’s instructions. Molecular weights were estimated for each band based on the distance migrated for 10 known molecular weight standards (BenchMark, Invitrogen, Karlsruhe, Germany).

Data analysis: The data was acquired through a video camera (Sony XC-75CE; Sony, Koeln, Germany) and a digital image analysis system. The evaluation of western blots was performed by BioDocAnalyze (Biometra, Goettingen, Germany), which created densitometry data of the blots showing the grey-intensity values versus relative mobility (RF-values). BioDocAnalyze evaluated the height, area, and molecular weight of all peaks of the densitographic data file. The maximum extinction (the band intensity of an individual blot) was set to 100%. The other extinction values of this blot were transformed into relative percentages, thus reducing the influence of different absolute staining intensities of individual blots. Only those peaks on the western blots were included in the calculation that exceeded a specific cut-off value to assure that only “real” peaks and not artifacts were taken into account.

Based on the densitographs of each western blot, multivariate statistical techniques were used to detect differences in the distribution of antibodies against retinal antigens in aqueous humor of all three groups. The densitographic data such as peak height, localization, and area under the curve were exported to Statistica® (Statsoft, Tulsa, AR) then the statistical calculations were performed by Statistica®. In the present study, the profiles were compared by an analysis of discriminance.

Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF TOF) mass spectrometry: We cut the corresponding immunoreactive bands out of the SDS-gel, transferred the pieces into reaction tubes, and incubated them with 50 µl acetonitrile (100%). The tubes were centrifuged and the acetonitrile was removed. The gel pieces were dried in a concentrator (Eppendorf, Hamburg, Germany) at 30 °C for 10 min.

Ammonium bicarbonate (50 mM; pH 8.0) and trypsin (15 µg/µl; Roche, Grenzach-Wyhlen, Germany) were placed in the tubes and they were incubated overnight at 37 °C. The next day the supernatant was placed in another tube and the
gel pieces were treated with 20 μl of 25 mM ammonium bicarbonate at room temperature for 30 min. Then, they were incubated for another 30 min with 5% formic acid in 50% acetonitrile and 45% water. The supernatant was transferred into another tube and the latter step was repeated once. The collected supernatants were dried in a concentrator.

Cinnamic acid matrix (0.5 μl; 0.02 g cinnamic acid, 5 ml water, 5 ml acetonitrile, and 10 μl trifluoroacetic acid) were spotted on an anchor chip target (Bruker, Bremen, Germany) then 1 μl samples were placed on top of each spot followed by another 0.5 μl matrix. All identifications were performed with an Ultraflex Maldi-TOFTOF (Bruker, Bremen, Germany).

The MS/MS (tandem mass spectrometry) spectra were used for data base searches with MASCOT using NCBI and SwissProt databases. Only the identifications with significant scores were used.

RESULTS

All aqueous humor samples showed complex IgG antibody patterns against retinal antigens in all three groups (Figure 1). The multivariate discrimination analysis showed a significant difference between the pattern of the POAG and the CO group (p=0.00018). There was also a significant difference between the PEX and the CO group (p=0.0013). We not only found up-regulations of the antibody reactivities of the glaucoma groups in comparison to controls but also down-regulations. There was no significant difference between the antibody patterns of the POAG and the PEX group (p=0.18).

DISCUSSION

Recent studies detected antibodies in sera of patients with normal tension and primary open angle glaucoma [8,10-13,26].
We could also show significant differences between the antibody profiles of normal tension glaucoma patients and control subjects in aqueous humor [15]. These findings could be hints for an immunological component in the pathogenesis of glaucoma.

This study illustrates that there are significant differences between the antibody patterns of the POAG and of the CO group (p=0.00018) and between the PEX and the CO group (p=0.0013) but no significant differences between the antibody patterns of the POAG and of the PEX group. Interestingly, similar to our previous studies, we not only found increased antibody reactivities in the glaucoma groups in comparison to controls but also decreases for example, at 21 kDa or 46 kDa.

Several of the important biomarkers in this study were identified through mass spectrometry. The antigen corresponding with a decreased antibody reactivity in the glaucoma groups at around 46-48 kDa was identified as α-enolase, a member of the heat shock protein family, via Maldi-TOF/TOF (Figure 5). Anti-α-enolase antibodies have been found in sera of patients with autoimmune retinopathy [27], membranous nephropathy [28], and rheumatoid arthritis [29]. The question of whether this decrease in antibody reactivity in the glaucoma groups plays a role in the pathogenesis needs to be further studied.

The identified antigen around 36 kDa is called glyceraldehyde-3-phosphate dehydrogenase (Figure 3), a key enzyme in glycolysis. GAPDH is involved in nuclear pathways [30]
and in apoptosis [31]. Mazzola et al. [32] reported an impairment of GAPDH glycolytic function in Alzheimer’s and Huntington’s disease subcellular fractions. Antibodies against GAPDH have been found in sera of patients with systemic lupus erythematosus [33]. The loss of some of these naturally-occurring anti-GAPDH and possible protective autoantibodies may lead to a loss of immune protection or to an increased risk to develop glaucoma.

At about 42-43 kDa, we found decreased antibody reactivity in the POAG and the PEX group in comparison to the control group. The corresponding antigen was identified as actin (Figure 4). In a study by Czaja et al. [34,35], anti-actin antibodies were present in the majority of patients with autoimmune hepatitis and they assumed the expression of these antibodies has a prognostic implication. Antibodies to actin have also been proposed as markers for autoimmune hepatitis by other groups [36-38]. Anti-actin antibodies have also been found in sera of patients with atherosclerosis [39] or with autoimmune inner ear disease [40].

In the 26 kDa molecular weight region, the statistical analysis revealed increased antibody reactivity in both glaucoma groups. This antigen was identified as heat shock pro-

<table>
<thead>
<tr>
<th>protein</th>
<th>mass</th>
<th>score</th>
<th>peptides</th>
<th>MSMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Q9XSJ4) Alpha-enolase (2-phospho-D-glycerate hydro-lyase) ENOA_BOVIN</td>
<td>47507</td>
<td>96</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>

Figure 5. Antigen at about 47 kDa. A Mascot search identified this protein as α-enolase with a highly significant score of 96 (A) and several peptides could be matched (C). The box plot (B) shows a down-regulation of the antibody against this biomarker in the glaucoma groups (POAG and PEX) with comparison to controls (CO). There was a significant difference between all 3 groups (p=0.0041).

<table>
<thead>
<tr>
<th>protein</th>
<th>mass</th>
<th>score</th>
<th>peptides</th>
<th>MSMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Q3TI49) Heat-shock protein beta-1 (Heat shock 27 kDa protein) HSPB1_BOVIN</td>
<td>22436</td>
<td>80</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 6. The antigen at 27 kDa was identified as HSP27. The antibody reactivity in the PEX group was upregulated in comparison to the other groups (C, p=0.0072).
tein 27 (Figure 6). Studies by Tezel and Wax [12,13] found an increased anti-HSP27 antibody level in sera of patients with normal tension glaucoma and POAG in comparison to control subjects. These anti-HSP antibodies are thought to induce neuronal apoptosis through the attenuation of the ability of native HSP27 to stabilize retinal actin cytoskeleton [41].

We found increased antibody reactivity around 27 kDa against optic nerve antigens [10] and human retinal antigens [42] in sera of normal tension glaucoma patients. To our knowledge, antibodies against HSP27 have not been analyzed in aqueous humor of PEX patients.

Increased levels of anti-HSP27 antibodies were also found in cerebrospinal fluid of patients with Guillain-Barré syndrome [43]. In this study, bovine retinal antigens were used. To exclude the possibility that the detected antibodies are only cross reactivities to bovine material, further studies with human antigens will be necessary. This would prove the existence of autoantibodies.

To gain more information about an immune component in glaucoma, we must learn if these antibodies play a role in the pathogenesis of glaucoma or if they are an epiphenomenon of the disease.

The specific pathogenesis of PEX is still not known. Studies could show that the blood aqueous barrier is impaired in patients with pseudoexfoliation syndrome [44,45]. Further information is also needed about the influence of antiglaucoma drugs on the aqueous humor composition since animals treated with timolol showed a significantly different aqueous humor protein composition than non-treated animals [46].

Another possible existence for antibodies against retinal antigens could be molecular mimicry [47]. Similar phenomena have been observed in other diseases like arthritis [48].

Shoenfeld et al. [49] discuss the existence of protective autoantibodies among the regular repertoire of autoantibodies in the disease process. We could identify antigens in primary open angle and pseudoexfoliation glaucoma that are found in other neurodegenerative diseases, e.g. HSP27. But the role of these antibodies in the pathogenesis of glaucoma is not clear.

Comparison of aqueous humor samples and serum samples from the same patient could provide further information about the differences in antibody profiles. In summary, we showed significant differences between the IgG antibody profiles in aqueous humor of POAG patients in comparison to that of controls as well as between the profiles of PEX patients in comparison to controls and we identified some of the important antigens like α-enolase, GAPDH, actin, and HSP27.

ACKNOWLEDGEMENTS

Supported by: DFG (Deutsche Forschungsgemeinschaft) Gr1463/4-1, MAIFOR, and Schwerpunkt Praeventive Medizin, University of Mainz

REFERENCES


