

Measurement of tumor necrosis factor- α , interleukin-6, Fas ligand, interleukin-1 α , and interleukin-1 β in the aqueous humor of patients with open angle glaucoma using multiplex bead analysis

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Purpose: Various cytokines, including tumor necrosis factor- α (TNF- α), Fas ligand (FasL), interleukin-1 α (IL-1 α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6), contribute to the pathogenesis of primary open angle glaucoma (POAG). The present study was set to measure these cytokines in the aqueous humor of patients with POAG and in control subjects using multiplex bead analysis.

Methods: Twenty-five patients with POAG and 29 control subjects were enrolled in this case-control study. Aqueous humor concentrations of the cytokines (IL-1 α , IL-1 β , IL-6, FasL, and TNF- α) were measured using multiplex bead analysis.

Results: Mean aqueous humor levels of IL-6 were significantly lower in patients with POAG compared to the control subjects (9.3 \pm 23.7 versus 55.3 \pm 94.4 pg/ml; $p=0.002$). No significant difference in the aqueous humor concentration of IL-1 β was found between patients with POAG and control subjects (0.5 \pm 0.8 versus 0.4 \pm 0.8 pg/ml; $p=0.85$). Concentrations of IL-1 α , TNF- α , and FasL were below limits of detection. No significant correlation was found between IL-6 concentration and age, duration of disease, cup/disc ratio, or mean deviation.

Conclusions: In the present study, we found significantly lower concentrations of IL-6 in the aqueous humor of patients with POAG.

Primary open angle glaucoma (POAG) is characterized by the slow and progressive degeneration of retinal ganglion cells (RGCs) and their axons, which eventually leads to blindness [1]. Affecting about 33 million people worldwide, POAG is the second leading cause of blindness globally [2,3]. The precise pathomechanism resulting in glaucomatous damage is still elusive. However, high intraocular pressure (IOP) is the most important and still the only therapeutically modifiable risk factor. Nonetheless, immunologic and neurotoxic factors have also been shown to contribute to the pathogenesis of POAG [4-12]. IOP is regulated by the balance between secretion of the aqueous humor by the ciliary body and drainage into the venous circulation mainly via the trabecular meshwork [1]. In POAG, elevated levels of IOP are exclusively generated through altered trabecular meshwork (i.e., decreased cellularity, increased amounts of extracellular matrix) [13,14].

Histologically, glaucomatous RGC death occurs by apoptosis, a distinct morphological form of cell death [15,16]. In

contrast to necrosis, apoptosis is a subacute process provoked by cellular degradation rather than disruption. Apoptotic stimuli lead to initiation of biochemical cascades and ultimately activate a family of proteases called caspases, which are the major executioners of apoptosis [17,18]. Activation of caspases is induced mainly via two mechanisms: The extrinsic pathway is initiated by binding of certain proteins (tumor necrosis factor- α [TNF- α], Fas ligand [FasL]) to their specific receptors, while the intrinsic pathway is initiated by the release of cytochrome c from the intermembrane space of mitochondria. As outlined by Almasieh and coworkers in their review, extrinsic and intrinsic activation of apoptosis is implicated in glaucomatous damage of RGCs and components of both pathways including TNF- α , FasL, interleukin-1 α (IL-1 α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) have been linked to the pathogenesis of glaucomatous damage of RGCs as well as reduced cellularity of the trabecular meshwork in glaucomatous eyes [19-27].

Multiplex bead analysis has the advantage of measuring numerous analytes in parallel in relatively small volumes, as they are typically provided in ophthalmologic samples from the aqueous humor, vitreous, or tear fluid [28-30]. Thus, the present study was set to investigate concentrations of specific cytokines (TNF- α , FasL, IL-1 α , IL-1 β , and IL-6) in

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the aqueous humor of patients with POAG using a multiplex bead analysis.

METHODS

Twenty-five patients with POAG and 29 control subjects were enrolled in this retrospective case-control study. All participants were of Caucasian origin, lived in the same geographic area (southern part of Austria), and were seen at the local Department of Ophthalmology, Medical University of Graz, between April and November 2010. The study was approved by the Institutional Review Board of the Medical University of Graz and followed the principles of the Declaration of Helsinki. Before the participants were enrolled, written informed consent was obtained.

All patients underwent slit-lamp biomicroscopy, testing for best-corrected visual acuity, Goldmann applanation tonometry, gonioscopy, and standard automated perimetry (Interzeag Octopus 101, program G2) or, in cases of profoundly decreased visual acuity, Goldmann perimetry. In all patients, photographs of the optic disc were taken.

POAG was defined by an open anterior chamber angle, optic disc changes characteristic of glaucoma (notching, thinning of the neuroretinal rim, increased cup/disc ratio in relation to the optic disc size), visual field defects characteristic of glaucoma (inferior or superior arcuate scotoma, nasal step, paracentral scotoma), and absence of conditions leading to secondary glaucoma. Patients with POAG were admitted to our department for glaucoma (trabeculectomy) or cataract surgery.

The control group consisted of 29 patients with no morphological or functional damage indicative for primary or secondary open angle or angle closure glaucoma. Control subjects were admitted to our department for cataract surgery.

Exclusion criteria for both groups were signs of ocular inflammation or infection, neuropathy, retinopathy or maculopathy, and previous ocular surgery less than 1 year and ocular laser surgery less than 6 months before enrollment. Moreover, individuals with systemic inflammatory or immunomodulating diseases were excluded.

Aqueous humor collection: Each participant received routinely povidone-iodine 5% (prepared at the Department of Ophthalmology, Medical University of Graz, Graz, Austria) preoperatively, while patients scheduled for cataract surgery also received standard mydriatic drops (phenylephrine 1% and cyclopentolate 0.5%, prepared at the Department of Ophthalmology, Medical University of Graz). Anesthesia was achieved using a retroperibulbar block with 2.5 ml ropivacaine (Naropin 5mg/ml, AstraZeneca, Norway), 2.5

ml lidocainhydrochloride (Xylanaest 2%, Gebro Pharma, Austria), and 150IE hyaluronidase (Hylase Dessau 300 I.E., Riemser, Germany) in all participants. Aqueous humor was collected via limbal paracentesis using a blunt 30 gauge cannula at the beginning of the surgery and was placed immediately on ice. Each sample was centrifuged at $1036 \times g$ for 10 min, aliquoted, and stored at -70°C until assayed.

Cytometric bead analysis: For determination of the five cytokines, the BD CBA Flex Set System and the BD Human Soluble Protein Master Buffer Kit (Cat. No. 558264, BD Bioscience-PharMingen, San Diego, CA) and the BD CBA Flex Sets for the measurement of IL-1 α (Cat. No. 560153, BD Bioscience-PharMingen), IL-1 β (Cat. No. 558279, BD Bioscience-PharMingen), IL-6 (Cat. No. 558276, BD Bioscience-PharMingen), FasL (Cat. No. 558330, BD Bioscience-PharMingen), and TNF- α (Cat. No. 558273, BD Bioscience-PharMingen) were used. Each BD CBA Flex Set contained one bead population with distinct fluorescence intensity as well as the appropriate phycoerythrin (PE) detection reagent and standard. Five bead populations coated with capture antibodies specific to IL-1 α , IL-1 β , IL-6, FasL, and TNF- α were mixed with each other. The tests were performed according to the manufacturer's instructions, and the samples were run in duplicate. As previously described [31], the mixed bead populations were incubated with recombinant standards or test samples to form sandwich complexes. After the PE-conjugated detection antibodies were added, the samples were incubated again and then resolved in the FL-3 channel of a BD FACSCalibur flow cytometer (BD Bioscience). The results were generated in graphic and tabular format by using the CBA analysis software (BD Bioscience-PharMingen). The assay sensitivities were as follows: IL-1 α 6.54 pg/ml, IL-1 β 1.74 pg/ml, IL-6 1.87 pg/ml, FasL 2.05 pg/ml, and TNF- α 1.95 pg/ml.

To detect IL-1 α , IL-1 β , IL-6, FasL, and TNF- α , the aqueous humor samples were run without predilution. The tests were performed and analyzed according to the manufacturer's instructions. Briefly, 50 μl of the five mixed and single capture beads were mixed with 50 μl of the provided standards or samples and incubated in the dark for 1 h at room temperature. Subsequently, 50 μl of the mixed and the single PE detection reagent(s) were added, and the samples were incubated in the dark for 2 h at room temperature. After the second incubation step, the samples were washed, centrifuged (at $200 \times g$ for 5 min), and resuspended in 300 μl of wash buffer. The BD FACSCalibur flow cytometer (BD Bioscience) was calibrated with setup beads, and 300 events were acquired for each factor and each sample. Individual analyte concentrations were indicated by their fluorescence

intensities and were computed by their respective standard reference curve and BD CBA software.

Descriptive statistics were used to calculate the frequencies and percentages of the discrete variables. Continuous data are given as mean±standard deviation (SD). Means were compared using the Mann–Whitney test. Proportions of groups were compared with a chi-square test. Correlation coefficients were calculated using Pearson's correlation test. The criterion for statistical significance was $p \leq 0.05$. Statistical analysis was performed using the SPSS statistical package (SPSS, version 18.0, Chicago, IL).

RESULTS

The present study included 25 patients with POAG (15 women and ten men) and 29 controls (18 women and 11 men) with a mean age of 77.3±9.4 years, and 76.4±8.9 years, respectively (Table 1). Six patients each had diabetes mellitus ($p=0.63$). The mean hemoglobin A1c (HbA1c) levels of the probands with diabetes mellitus were 7.5±0.5%, and none had diabetic retinopathy. Nine patients with POAG were admitted for glaucoma surgery, while 16 were admitted for cataract surgery.

Mean duration of POAG disease was 8.4±8.1 years. The visual fields of patients with POAG had a mean mean deviation (MD) of 11.8±7.3 dB, and a mean cup/disc ratio of 0.78±0.15. IL-6 was detected in ten patients and 24 controls. The mean concentration was 9.3 pg/ml in the patients and 55.3 pg/ml in the controls, respectively ($p=0.002$). IL-1 β was detected in six patients and six controls with mean

concentrations of 0.5 and 0.4 pg/ml, respectively ($p=0.85$). The IL-1 α , TNF- α , and FasL concentrations were below limits of detection (Table 2).

The mean IOP of the nine patients who donated aqueous humor during glaucoma surgery was 22.9±5.0 mmHg, while the mean IOP of the 16 patients who donated aqueous humor during cataract surgery was 16.9±5.8 mmHg. The mean concentration of IL-6 of the former group was 3.6±10.1 pg/ml versus 12.4±28.6 pg/ml in the cataract surgery group. The difference was statistically not significant ($p=0.16$).

Three patients with POAG received no IOP-lowering therapy at the time of surgery. The mean IL-6 concentration in these patients was 14.2 pg/ml, while the mean IL-6 concentration in the patients with recent IOP-lowering therapy was 8.6 pg/ml ($p=0.71$). No significant correlation was found between IL-6 concentrations and age, duration of disease, cup/disc ratio, or MD.

DISCUSSION

In the present study using multiplex bead analysis, we found significantly decreased concentrations of IL-6 in the aqueous humor of patients with POAG, while the IL-1 β concentrations did not differ significantly between patients and the control subjects. Patients admitted for glaucoma surgery had the highest mean IOP but interestingly, the lowest mean concentration of IL-6. However, the difference was statistically not significant.

TABLE 1. CLINICAL CHARACTERISTICS OF PATIENTS WITH PRIMARY OPEN ANGLE GLAUCOMA (POAG) AND PATIENTS WITH CATARACTS.

Variables	POAG (n=25)	Cataract (n=29)	Significance P value
Female	15 (60)	18 (62)	0.88
Mean years ± SD	77.3±9.4	76.4±8.9	0.59
Diabetes mellitus	6 (24)	6 (20.7)	0.63
Arterial hypertension	17 (68)	18 (62.1)	0.62
IOP mmHg ± SD	19.1±6.1	14.7±2.9	0.005
Mean duration of disease (years ± SD)	8.4±8.1		
Glaucoma medication			
PGA	19 (76)		
BB	11 (44)		
AA	7 (28)		
CAI	13 (52)		
No medication	3 (12)		
Single medication	6 (24)		
Double medication	6 (24)		
Triple medication	10 (40)		

Numbers are given as n (%); PGA: prostaglandin analogon; BB: beta blocker; AA: alpha agonist; CAI: carbonic anhydrase inhibitor

TABLE 2. MEAN LEVELS OF IL-6 AND INTERLEUKIN-1 β IN PATIENTS WITH PRIMARY OPEN ANGLE GLAUCOMA (POAG) AND PATIENTS WITH CATARACT IN THE AQUEOUS HUMOR USING MULTIPLEX BEAD ANALYSIS.

Variables	POAG (n=25)	Cataract Subjects (n=29)	Significance P value
IL-6			
Detectable*	10 of 25 (40)	22 of 29 (75.9)	0.01
Mean \pm SD pg/ml	9.3 \pm 23.7	55.3 \pm 94.4	0.002
Range pg/ml	0 - 113.2	0 - 344.2	
IL-1 β			
Detectable*	6 of 25 (24)	5 of 29 (17.2)	0.54
Mean \pm SD pg/ml	0.5 \pm 0.8	0.4 \pm 0.8	0.72
Range pg/ml	0-2.0	0-2.0	

* Numbers are given as n (%)

Interleukin-6 acts as a proinflammatory and neuroprotective cytokine. It is secreted by T-cells and macrophages to stimulate immune response to trauma or other tissue damage leading to inflammation. As an antiapoptotic factor, IL-6 activates the Janus kinase pathway leading to the expression of various stress-related factors, including heat shock protein 70 and heat shock protein 90.

It has been suggested that IL-6 protects neurons from N-methyl-d-aspartate-induced excitotoxicity via Janus kinase pathways [32]. In an in vitro study, Sappington and coworkers investigated the impact of glia-derived IL-6 on the pressure-induced death of retinal ganglion cells. They found that increased IL-6 concentrations counteract not only proapoptotic signals from the microglia but also the pressure-induced apoptotic cascade in ganglion cells. Furthermore, in glaucomatous optic nerves, TNF- α expression of astrocytes and microglia increases with disease severity, whereas IL-6 increases retinal ganglion cell survival [33]. In an animal glaucoma model, Johnson and coworkers provided evidence that in early optic nerve head injury expression of IL-6 is increased [34]. Noma and coworkers provided evidence that the aqueous level of IL-6 is strongly correlated with the vitreous level of IL-6 ($\rho=0.81$, $p=0.0012$), so that the aqueous level of IL-6 can be assumed to be surrogate for IL-6 levels in the back of the eye [35].

Several studies looked at the concentration of IL-6 in the aqueous humor of patients with glaucoma. In a multiplex cytokine analysis study including 29 patients with POAG and 30 controls, Kuchtey and coworkers reported significantly elevated concentrations of IL-8 in the aqueous humor of patients with POAG, suggesting that immune activation may be associated with glaucoma. In that study, the concentration of IL-6 was lower in patients with glaucoma, although the difference was not statistically significant (1.6 pg/ml versus 2.7 pg/ml) [36]. In another study investigating hepcidin

prohormone levels and IL-6 in the aqueous humor of 20 patients with POAG and 25 controls using enzyme-linked immunosorbent assay (ELISA), Sorkhabi and coworkers found no significant difference between aqueous humor IL-6 concentrations of POAG and the control group (4.4 pg/ml versus 5.8 pg/ml) [37]. Using multiplex analysis, Takai and coworkers found significantly decreased aqueous humor concentrations of IL-6 in 20 patients with POAG compared to 21 patients with cataract (15.1 pg/ml versus 64.3 pg/ml), while Chua and coworkers reported no significant differences between patients with POAG and controls (2.85 pg/ml versus 2.23 pg/ml) [38,39]. According to differing mean ages, ethnicities, and methods (ELISA versus multiplex analysis), a comparison between these studies and the present study is not valid. The most obvious obstacle remains the limited number of probands in these studies. Likewise, Takai and coworkers reported significantly increased levels of IL-1 β in the aqueous humor of patients with POAG as well as exfoliation glaucoma (5.4 pg/ml versus 26.9 pg/ml versus 67.7 pg/ml), while Chua and coworkers found no significant difference after correction for multiple testing (0.6 pg/ml versus 0.72 pg/ml) [38,39].

Upregulation of TNF- α in the optic nerve heads and retina sections of glaucomatous eyes has been shown in ex vivo studies, while an in vitro study provided evidence that glial cells exposed to elevated hydrostatic pressure or stimulated ischemia secreted increased amounts of TNF- α , subsequently leading to apoptotic death of cocultured RGCs [20-22]. A recent review summarizing four studies investigating levels of TNF- α in the aqueous humor of patients with OAG found higher TNF- α levels in patients with OAG (standardized mean difference=0.517 [95% confidence interval: 0.207-0.826; $p=0.0001$]), but different methods were used, and three of these studies were from Asia [40].

Endothelial leukocyte adhesion molecule-1 is a cell-adhesion molecule consistently present in the outflow region

of glaucomatous eyes, but absent in the outflow region of normal eyes. Expression of endothelial leukocyte adhesion molecule-1 has been suggested to be under the control of IL-1 α , IL-1 β , and IL-6 [23]. Decreased cellularity of the trabecular meshwork has been reported in glaucomatous eyes, and this cell loss has been suggested to be due to apoptotic death via the Fas/FasL pathway [13,25,26]. In a recent study using ELISA, no significant difference in FasL concentration was found in the aqueous humor of patients with POAG and controls [27]. In our study, these cytokines (TNF- α , IL-1 α , FasL) were below the limits of detection, which is in contrast to recently published studies and may be due to the different methods used. In conclusion, we found significantly lower concentrations of IL-6 in the aqueous humor of patients with POAG. However, since results regarding IL-6 up to now have been inconclusive, more studies are needed to clarify the pathogenic role of IL-6 in POAG.

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