Multiplex Cytokine Analysis of Aqueous Humor in Eyes with Primary Open-Angle Glaucoma, Exfoliation Glaucoma, and Cataract

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Purpose. To measure levels of various inflammatory cytokines in the aqueous humor of patients with primary open-angle glaucoma (POAG), exfoliation glaucoma (EXG), and senile cataract.

Methods. Aqueous humor samples were obtained from 64 eyes of 64 Japanese subjects (POAG, 20 eyes; EXG, 23 eyes; and cataract, 21 control eyes). The levels of eight cytokines including interleukin (IL)1-β, IL-6, IL-8, transforming growth factor (TGF)-β1, tumor necrosis factor (TNF)-α, serum amyloid A (SAA), migration inhibitory factor (MIF), and vascular endothelial growth factor (VEGF)-A were estimated using the multiplex bead immunoassay technique.

Results. Compared with the cataract group, the levels of TGF-β1, IL-8, and SAA were significantly higher in aqueous humor samples from the POAG (5.0-fold, 2.3-fold, and 11.9-fold, respectively) and EXG (12.5-fold, 4.0-fold, and 18.3-fold, respectively) groups. Except for a significant decrease in the IL-6 level in the POAG (0.23-fold) group, no other cytokine levels differed in the POAG and EXG groups compared with the cataract group. The levels of TGF-β1, IL-8, and SAA were positively correlated with each other ($\rho = 0.723–0.786$; $P < 0.0001$), the intraocular pressure (IOP) ($\rho = 0.392–0.662$; $P < 0.0001–0.0019$), and the number of glaucoma medications ($\rho = 0.478–0.659$; $P < 0.0001–0.0001$).

Conclusions. Cytokine networks including TGF-β1, IL-8, and SAA in aqueous humor may have critical roles in IOP elevations in patients with open-angle glaucoma. (*Invest Ophthalmol Vis Sci.* 2012;53:241–247) DOI:10.1167/iovs.11-8434

Glaucoma is characterized by a progressive "glaucomatous" optic neuropathy and corresponding visual field loss, in which elevated intraocular pressure (IOP) is the main risk factor. In open-angle glaucoma (OAG) including primary open-angle glaucoma (POAG) and exfoliation glaucoma (EXG), the IOP increases because of reduced aqueous humor outflow at the trabecular meshwork (TM).[1] This results from increased aqueous humor outflow resistance due to changes in the amount and quality of the extracellular matrix (ECM) in the TM.[2] In EXG, an age-related, complex, generalized disorder of the ECM, the progressive accumulation of intraocular production of abnormal fibrillar materials in the TM is considered the primary cause of chronic IOP elevation.[3,4] Although the involvement of various genetic and internal/external stress factors such as immune reactions,[5,6] inflammation,[7] ischemia,[8] hypoxia,[9] and oxidative stress,[10] have been proposed, the exact mechanism of the ECM changes in POAG and EXG is still not well understood.

If an immune reaction and/or chronic inflammation at the TM or anterior segment is associated with elevated IOP in glaucoma, changes in ocular inflammatory cytokine expression might be detectable as changes in cytokine concentrations in the aqueous humor, because of the cytokine autocrine and paracrine mechanisms of action. Until now, several studies that measured cytokine concentrations in aqueous humor samples have detected elevated cytokine levels including transforming growth factor (TGF)-β, interleukin (IL)-6, and IL-8.[11–15] In human donor eyes, perfusion of TGF-β2 increased both fibronection concentration in anterior segments and IOP.[16,17] Therefore, evaluation of the aqueous humor composition of various cytokines may expand the understanding of glaucoma pathophysiology.

A major limitation of testing aqueous humor is that only small sample volumes (typically 50 to 150 μL of fluid) can be obtained from human eyes; these amounts are barely sufficient to test a few cytokines using traditional enzyme-linked immunosorbent assay (ELISA) techniques. Multiplex bead immunoassays, which allow for simultaneous detection of multiple cytokines in small volume clinical samples,[18] have been used to determine cytokine expression profiles in the aqueous humor of patients with uveitis[19–21] and branch retinal vein occlusion.[22] A recent study used this technique to identify cytokine levels in aqueous humor obtained primarily from eyes with POAG.[14] In the present study, to investigate the possible roles of the cytokine network in the pathologic mechanism of glaucoma, we measured multiple cytokines related to inflammation in the aqueous humor of eyes with POAG, EXG, and cataract using a multiplex bead immunoassay technique.

Methods

Subjects

All subjects provided written informed consent for aqueous humor collection in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of Shimane University Hospital. Aqueous humor samples were obtained from 64 eyes of 64 Japanese subjects (20 eyes with POAG, 23 eyes with EXG, and 21 eyes with a cataract as controls). All subjects underwent ophthalmologic examinations including measurements of best-corrected visual acuity (VA), IOP measured by Goldmann applanation tonometry, and slit-lamp, gonioscopic, and funduscopic examinations under pupillary dilation. POAG was defined by the clinical findings including an open iridocorneal angle, the characteristic appearance of glaucomatous optic neuropathy such as enlargement of the optic disc cup or focal thinning of the neuroretinal rim, and corresponding visual field defects tested (Humphrey Visual Field Analyzer; Carl Zeiss Meditec, Dublin, CA), and no evidence of secondary glaucoma. EXG was defined by the presence of

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an open iridocorneal angle, characteristic appearance of glaucomatous optic neuropathy and its corresponding visual field defects, and the presence of characteristic pseudoexfoliation material deposits on the anterior capsule and/or pupillary margin. Cataract was defined by the absence of glaucomatous optic neuropathy and no history of IOP exceeding 21 mm Hg. Except for a cataract and/or glaucoma, no subjects had ocular pathologies such as clinically detectable ocular inflammation, infection, neuropathies, retinopathies, or maculopathies. The demographic data of the subjects, including age, sex, IOP measured at the day before the surgery, number, and details of glaucoma medication use are summarized in Table 1.

Aqueous Humor Collection

Aqueous humor samples (100 to 200 μL) were aspirated at the beginning of glaucoma or cataract surgery through a limbal paracentesis using a 0.5-mL syringe with a 30-gauge needle (BD Japan, Tokyo, Japan), with care taken to prevent blood and intraocular tissue contamination. After obtaining the aqueous humor samples, the anterior chamber was reformed with balanced salt solution and the planned surgeries were performed. All samples were immediately frozen and stored at −80°C until the analyses were performed. As the preoperative medications, all patients used topical 0.5% levofloxacin eye drops three times a day for 3 days before surgery. In the patients who underwent cataract surgery alone or combined cataract and glaucoma surgeries, topical mydriatics (1% tropicamide and 5% phenylephrine; Santen, Osaka, Japan) and anesthetics (0.4% oxybuprocaine, Santen) were instilled into the surgical eye every 30 minutes for 2 hours before the surgery. In the patients who underwent glaucoma surgery alone, topical miotics (2% pilocarpine; Santen) and anesthetics (0.4% oxybuprocaine; Santen) were instilled into the surgical eye every 30 minutes for 2 hours before the surgery.

TABLE 1. Demographic Data

	Groups		
	Cataract (n = 21)	**POAG (n = 20)**	**EXG (n = 23)**
Age, y			
Mean ± SD	75.2 ± 5.3	73.4 ± 11.5	79.4 ± 4.5
Range	67–84	58–89	70–87
P value, vs. cataract*	—	0.6956	0.0133†
P value, vs. POAG*	—	—	0.1534
Sex			
Men	11	11	13
Women	10	9	10
P value, vs. cataract‡	—	>0.9999	>0.9999
P value, vs. POAG‡	—	—	>0.9999
IOP, mm Hg			
Mean ± SD	13.6 ± 2.4	19.1 ± 4.7	26.9 ± 11.4
Range	10–18	14–32	11–52
P value, vs. cataract*	—	<0.0001§	<0.0001§
P value, vs. POAG*	—	—	0.0115†
Glaucoma medications, *n*			
Mean ± SD	0	2.3 ± 1.1	2.3 ± 1.0
Range	0	0–3	0–4
P value, vs. cataract*	—	<0.0001§	<0.0001§
P value, vs. POAG*	—	—	0.6312
Glaucoma medications, *n* (%)			
Prostaglandin analogs	0 (0)	17 (85)	22 (96)
β-blockers	0 (0)	15 (75)	16 (70)
Carbonic anhydrase inhibitors	0 (0)	13 (65)	14 (61)

* *P* values are calculated between each pair of groups using the Mann-Whitney *U* test.

† Significance level at 5% ($P < 0.0167$) by Bonferroni correction for multiple comparisons.

‡ *P* values are calculated between each pair of groups using the Fisher's exact probability test.

§ Significance level at 1% ($P < 0.0033$), by Bonferroni correction for multiple comparisons.

Multiplex Bead Immunoassay

Cytokine concentrations were analyzed using a multiplex bead immunoassay system (Procarta Cytokine Assay Kit; Affymetrix, Inc., Santa Clara, CA), based on multiplexing technology (xMAP; Luminex, Austin, TX), according to the manufacturer's instructions. The data were acquired using a Luminex-compatible workstation and its manager software (Bio-Plex workstation and version 6.0 software; Bio-Rad, Tokyo, Japan), according to the manufacturer's instructions. To analyze seven cytokines related to the inflammatory process (IL 1-β, IL-6, IL-8, tumor necrosis factor [TNF]-α, serum amyloid A [SAA], migration inhibitory factor [MIF], and vascular endothelial growth factor [VEGF]-A), 25 μL of undiluted aqueous humor samples was analyzed simultaneously. To analyze TGF-β1, 40 μL of undiluted aqueous humor sample was activated by adding 10 μL of 1 N HCl and then neutralized by adding 8 μL of 1.2 n NaOH/0.5 M HEPES; 25 μL of preactivated samples were proceeded to the cytokine assay for TGF-β1. Thus, we measured total (both active and inactive forms) amount of TGF-β1 in the aqueous humor. Based on the information provided by the manufacturer, the multiplex assay kit can quantitatively measure multiple cytokines from as little as 25 μL of bodily fluids, with a lower limit of detection of 1 pg/mL per cytokine. Each sample was run as a single measurement for limited quantity of collected aqueous humor.

Statistical Analysis

Data are presented as the mean ± SD and were analyzed using statistical software (StatView version 5.01; SAS Institute, Inc., Cary, NC). For comparisons between each pair of cataracts and the POAG and EXG groups, the differences in continuous data including age, IOP, number of glaucoma medications, cytokine concentrations, and in categorical data including sex were calculated using the Mann-Whitney *U* test and Fisher's exact probability test, respectively. Correlations between cytokine concentrations and subjects' demographic data including age, IOP, and number of glaucoma medications were calculated using Spearman's correlation test. For the correction of multigroup comparisons, *P* values of 0.0167 and 0.0033 for the Mann-Whitney *U* test or Fisher's exact probability test and 0.0071 and 0.0014 for Spearman's correlation test were considered statistically significant with significance levels of 5% and 1%, respectively, based on Bonferroni's methods.

RESULTS

The concentrations of eight cytokines analyzed are shown in Table 2. All cytokines were detected in > 90% of the samples analyzed in each group, except for SAA in the cataract group in which five (24%) of the 21 samples were below the detection limit of the assay and TNF-α in the EXG group in which three (13%) of the 23 samples were below the detection limit. Compared with the cataract group, the concentrations of IL-8 ($P = 0.0004$), TGF-β1 ($P < 0.0001$), and SAA ($P = 0.0102$) were significantly higher and IL-6 ($P = 0.0123$) was significantly lower in the POAG group, and the concentrations of IL-8 ($P < 0.0001$), TGF-β ($P < 0.0001$), and SAA ($P < 0.0001$) were significantly higher in the EXG group. Compared with the POAG group, IL-6 ($P < 0.0001$), IL-8 ($P = 0.0113$), TGF-β ($P = 0.0010$), and SAA ($P = 0.0064$) were significantly higher in the EXG group.

The correlations among the cytokine concentrations are shown in Table 3. Positive correlations were found between IL1-β and TNF-α ($\rho = 0.911$; $P < 0.0001$), IL1-β and VEGF-A ($\rho = 0.793$; $P < 0.0001$); IL-6 and IL-8 ($\rho = 0.334$; $P = 0.0080$); IL-8 and TGF-β1 ($\rho = 0.786$; $P < 0.0001$), IL-8 and SAA ($\rho = 0.723$; $P < 0.0001$); TGF-β1 and SAA ($\rho = 0.727$; $P < 0.0001$); TNF-α and MIF ($\rho = 0.330$; $P = 0.0089$), and TNF-α and

TABLE 2. Detection of Aqueous Humor Cytokines by Multiplex Bead Immunoassay

	Groups		
	Cataract	**POAG**	**EXG**
IL1-β			
Detectable samples, *n*	21/21	18/20	23/23
Mean ± SD (range)	0.3 ± 0.2 (0.02–1.0)	0.3 ± 0.3 (0.02–1.0)	0.6 ± 0.6 (0.02–2.2)
P value, vs. cataract	—	0.7114	0.0722
P value, vs. POAG	—	—	0.0530
IL-6			
Detectable samples, *n*	21/21	20/20	23/23
Mean ± SD (range)	64.3 ± 112.2 (2.2–401.9)	15.1 ± 19.1 (1.5–84.1)	105.1 ± 124.2 (3.1–441.8)
P value, vs. cataract	—	0.0123*	0.0842
P value, vs. POAG	—	—	<0.0001†
IL-8			
Detectable samples, *n*	21/21	20/20	23/23
Mean ± SD (range)	1.4 ± 1.2 (0.2–5.2)	3.2 ± 1.9 (0.9–6.9)	5.6 ± 3.6 (1.5–16.2)
P value, vs. cataract	—	0.0004†	<0.0001†
P value, vs. POAG	—	—	0.0113*
TGF-β1			
Detectable samples, *n*	21/21	20/20	23/23
Mean ± SD (range)	5.4 ± 4.6 (0.8–19.9)	26.9 ± 44.2 (1.1–208.5)	67.7 ± 58.7 (6.9–232.3)
P value, vs. cataract	—	<0.0001†	<0.0001†
P value, vs. POAG	—	—	0.0010†
TNF-α			
Detectable samples, *n*	21/21	19/20	20/23
Mean ± SD (range)	1.5 ± 0.7 (0.8–3.4)	1.6 ± 1.2 (0.4–4.4)	2.3 ± 1.7 (0.4–5.7)
P value, vs. cataract	—	0.6513	0.0800
P value, vs. POAG	—	—	0.1063
SAA			
Detectable samples, *n*	16/21	18/20	23/23
Mean ± SD (range)	7.2 ± 11.8 (0.7–37.2)	85.8 ± 224.1 (0.7–878.5)	132.1 ± 226.2 (0.7–859.3)
P value, vs. cataract	—	0.0102*	<0.0001†
P value, vs. POAG	—	—	0.0064*
MIF			
Detectable samples, *n*	21/21	20/20	23/23
Mean ± SD (range)	2927.4 ± 3013.7 (559.9–13634.0)	9358.7 ± 33497.8 (291.0–151557.7)	4220.4 ± 7903.0 (380.3–35443.8)
P value, vs. cataract	—	0.1792	0.1767
P value, vs. POAG	—	—	0.7888
VEGF-A			
Detectable samples, *n*	21/21	20/20	23/23
Mean ± SD (range)	533.1 ± 242.1 (220.3–1105.9)	431.8 ± 197.2 (77.6–833.0)	722.9 ± 534.8 (50.4–2379.2)
P value, vs. cataract	—	0.2010	0.2448
P value, vs. POAG	—	—	0.0385

Cytokine concentrations are expressed as the mean ± SD (pg/mL). *P* values are calculated between each pair of groups using the Mann-Whitney *U* test.

* Significance level at 5% ($P < 0.0167$), by Bonferroni correction for multiple comparisons.

† Significance level at 1% ($P < 0.0033$), by Bonferroni correction for multiple comparisons.

VEGF-A ($\rho = 0.828$; $P < 0.0001$). A negative correlation was found between TGF-β1 and MIF ($\rho = -0.342$; $P = 0.0067$).

The correlations between each cytokine and age, IOP, and the number of glaucoma medications are shown in Table 4. Positive correlations were found between age and IL-6 ($\rho = 0.353$; $P = 0.0050$), IOP and IL-8 ($\rho = 0.523$; $P < 0.0001$), IOP and TGF-β1 ($\rho = 0.662$; $P < 0.0001$), IOP and SAA ($\rho = 0.392$; $P = 0.0019$), and the number of glaucoma medications and IL-8

TABLE 3. Correlations among Cytokines

***ρ*/*P* value**	**IL1-β**	**IL-6**	**IL-8**	**TGF-β1**	**TNF-α**	**SAA**	**MIF**	**VEGF-A**
IL1-β		0.192	0.208	0.159	0.911	0.234	0.260	0.793
IL-6	0.2848		0.334	0.153	0.178	0.318	0.100	0.319
IL-8	0.0981	0.0080		0.786	0.153	0.723	−0.187	0.187
TGF-β1	0.2060	0.2251	<0.0001*		0.107	0.727	−0.342	0.067
TNF-α	<0.0001*	0.1578	0.2246	0.3950		0.152	0.330	0.828
SAA	0.0630	0.0117	<0.0001*	<0.0001*	0.2281		−0.290	0.229
MIF	0.0392	0.4289	0.1388	0.0067†	0.0089	0.0212		0.272
VEGF-A	<0.0001*	0.0113	0.1377	0.5957	<0.0001*	0.0688	0.0306	

Correlation coefficient (ρ) and *P* values for each pair of cytokines are calculated by Spearman's correlation test.

* Significance level at 1% ($P < 0.0014$), by Bonferroni correction for multiple comparisons.

† Significance level at 5% ($P < 0.0071$), by Bonferroni correction for multiple comparisons.

Table 4. Correlations between Cytokines and Age, IOP, and Number of Glaucoma Medications

	Age (y)		IOP (mm Hg)		Glaucoma Medications (*n*)	
	ρ	*P* Value	ρ	*P* Value	ρ	*P* Value
IL1-β	0.275	0.0289	0.042	0.7362	−0.022	0.8627
IL-6	0.353	0.0050*	−0.085	0.4974	−0.267	0.0340
IL-8	0.123	0.3287	0.523	<0.0001†	0.581	<0.0001†
TGF-β1	0.058	0.6438	0.662	<0.0001†	0.659	<0.0001†
TNF-α	0.273	0.0300	0.020	0.8741	−0.049	0.6945
SAA	0.142	0.2583	0.392	0.0019*	0.478	0.0001†
MIF	0.298	0.0182	−0.222	0.0776	−0.379	0.0026*
VEGF-A	0.269	0.0324	−0.023	0.8559	−0.088	0.4864

The correlation coefficient (ρ) and *P* values for each pair of cytokines are calculated by Spearman's correlation test.

* Significance level at 5% ($P < 0.0071$), by Bonferroni correction for multiple comparisons.

† Significance level at 1% ($P < 0.0014$), by Bonferroni correction for multiple comparisons.

($\rho = 0.581$; $P < 0.0001$), the number of glaucoma medications and TGF-β1 ($\rho = 0.659$; $P < 0.0001$), and the number of glaucoma medications and SAA ($\rho = 0.478$; $P = 0.0001$). A negative correlation was found between the number of glaucoma medications and MIF ($\rho = -0.379$; $P = 0.0026$).

Discussion

Using the bead immunoassay, we found clear elevations of cytokines related to an immune reaction or inflammation compared with control eyes, that is, IL-8, TGF-β1, and SAA in the aqueous humor samples from the POAG and EXG groups. Previously, an elevated concentration of TGF-β1 was detected using a conventional ELISA technique in aqueous humor samples from patients with pseudoexfoliation syndrome (PXS) and EXG,[23,24] and IL-8 by bead immunoassay in aqueous humor from OAG,[14] suggesting good reproducibility of the multiplex bead immunoassay in the present study. This is one of the earliest studies to report successful detection of multiple cytokines using the multiplex bead immunoassay in aqueous humor from POAG and EXG.

TGF-β signaling acts in various biologic processes throughout the body and the eye, including ECM turnover, proliferation, fibrotic responses, apoptosis, and modulation of the immune system.[25–27] Considerable amount of TGF-β, mainly in the form of TGF-β2, have been detected in normal human aqueous humor.[28] Perfusion of TGF-β2 in human[16,17] and porcine[29] anterior segments resulted in decreased outflow, increased IOP, and increased ECM gene expression. Increased level of TGF-β2 in aqueous humor of POAG eyes[11–13] and production of TGF-β2 in cultured human ciliary body epithelium[30] have been reported, thus previous reports had almost exclusively focused on TGF-β2 isoform in the pathogenesis of POAG. On the other hand, to the best of our knowledge, no study reported the elevated TGF-β1 level in POAG. Increased TGF-β1 expression was found in the ciliary body epithelium of donor eyes with PXS and EXG,[23] suggesting that the ciliary epithelium is a local source of TGF-β1 secretion in glaucomatous eyes. TGF-β1 increases ECM production in human eye[31] and cause IOP elevation in rat eye.[32] We found a positive correlation between levels of TGF-β1 and IOP, suggesting that TGF-β1 has a role in remodeling of the ECM in both EXG and POAG.

IL-8, also called CXCL8, has a role in chemokine functions.[33] Although IL-8 was not detected by immunohistochemistry in the TM of human cadaver eyes without glaucoma,[14] cultured TM cells can secrete IL-8 into the aqueous humor after treatment with TNF-α and IL1-β and chronic oxidative stress[34,35]; thus, TM cells could be a local source of IL-8 secretion. Although the molecular mechanisms of action of IL-8 on the TM are unclear, the positive correlation between IL-8 and IOP found in the present study suggested the role of IL-8 in the positive regulation of outflow resistance. In addition, positive correlations were found between TGF-β1 and IL-8 in the present study; thus, the levels of TGF-β1 and IL-8 may commonly reflect the status of ECM remodeling and severity of the reduction in aqueous humor outflow at the TM in eyes with POAG and EXG.

We found surprising elevations of SAA in the aqueous humor samples from the glaucomatous eyes (an 11.9-fold increase in POAG eyes and an 18.3-fold increase in EXG eyes). Previously, increased SAA mRNA levels were detected in TM tissues derived from donor eyes with POAG compared with controls[36]; thus, to the best of our knowledge, the present study was the first to detect an elevated SAA concentration in aqueous humor samples from living eyes with POAG and EXG. SAA plays important roles in infection,[37] inflammation,[37] tissue repair,[38] and amyloid deposition.[39] An acute-phase response in inflammation is mediated primarily by a proinflammatory cytokine-induced upregulation of SAA biosynthesis in the liver,[40] macrophages, smooth muscle cells, and endothelial cells,[41] although the exact source of SAA detected in the aqueous humor is unclear. SAA activates many cellular signal transduction pathways, such as the extracellular signal-regulated kinase, P38 mitogen-activated protein kinase,[42] and nuclear factor-kappa B (NF-κB)-dependent pathways[43]; increases the production of matrix metalloproteinases,[44] cytokines, and cytokine receptors[45]; and stimulates release of TNF-α, IL-1β, and IL-8 in human blood neutrophils.[46,47] With perfusion of exogenous SAA through the anterior chambers of donor eyes, the IOP increased; however, the IOP did not increase in response to other proteins such as bovine serum albumin, suggesting that the IOP did not simply increase as a result of physical blockade of the outflow pathway by the protein.[36] Published evidence has suggested that amyloid deposits are associated with glaucoma[48,49]; however, ocular amyloidosis occurs primarily in EXG and certain secondary glaucomas but not in POAG.[50–52] Amyloid deposits were not found in the TM of SAA-induced ocular hypertension in donor eyes.[36] In the present study, increased SAA in the aqueous humor was found in eyes with EXG and POAG. Accordingly, SAA is likely involved in elevated outflow resistance as the modulator of cytokine expressions rather than a factor in amyloid deposition.

Proinflammatory cytokine IL-6 decreased in POAG compared with the control and EXG groups; the level was higher in the EXG group. Previously, expression of IL-6 mRNA in normal

donor TM[15] and secretion of IL-6 in cultured human TM cells after TGF-β1 treatment[53] have been detected; thus, TM cells could be a local source of IL-6. Loss of TM cells associated with aging can be responsible for functional abnormality of TM tissue in POAG.[54–56] Because IL-6 was correlated positively with age in the present study, the difference in age between groups may explain the discrepancy. Increased levels of IL-6 were reported in the aqueous humor of a murine model of uveitis[57] and early-stage PXS.[15] Because IL-6 is an important mediator of increased vascular permeability and endothelial barrier dysfunction in vitro and in vivo,[58,59] an elevated IL-6 level may be involved in the iris vasculopathy and chronic breakdown of the blood-aqueous barrier that are characteristic of EXG.[4,60,61]

The present study did not detect significant differences in the concentrations of TNF-α, IL1-β, MIF, and VEGF-A among the groups. TNF-α was elevated in the optic nerve[62] and retina[63] of the human glaucomatous eyes, although a recent study reported no elevation of TNF-α in the aqueous humor of eyes with POAG.[64] Thus, TNF-α may play a role in damaging the retinal ganglion cells rather than the increased aqueous outflow resistance in OAG. Previous studies have detected increased levels of IL-1β mRNA in TM cells derived from glaucomatous cadaver eyes[65] and VEGF protein in aqueous humor from eyes with POAG, angle closure glaucoma, and EXG.[66] Although they did not reach significance, the mean levels of these two cytokines were higher in the EXG group in the present study; thus, a larger sample size may yield statistical significance but needs clarification. MIF, lymphokine recruiting macrophages at inflammatory loci,[67] increases in the aqueous humor of eyes with uveitis.[68] MIF is expressed in normal human TM, secreted from cultured TM cells,[69] and induced by IL-1, TNF-α, and TGF-β1 in conjunctival fibroblasts from ocular cicatricial pemphigoid.[70] The present study, however, did not support MIF involvement in the pathogenesis of POAG and EXG.

In the present study, the number of glaucoma medications was correlated positively with the levels of IL-8, TGF-β1, and SAA in the anterior chamber; thus, use of glaucoma medications might affect cytokine production. Previously, increased proinflammatory cytokines such as IL1-β, IL-6, IL-8, IL-10, IL-12, and TNF-α in the tears or conjunctival epithelium of glaucomatous eyes treated with topical antiglaucoma drugs have been reported.[71–73] However, the levels of IL1-β and TNF-α did not differ between the groups, IL-6 was lower in the POAG group, and the correlations between the number of glaucoma medications and IL1-β, TNF-α, or IL-6 did not reach significance in the present study. It was reported that topical latanoprost decreased aqueous humor TGF-β1 to normal level in EXG,[74] while most POAG and EXG patients used latanoprost in our study. Because we found a positive correlation between IOP and the number of glaucoma medications, the increased levels of cytokines in the aqueous humor are more likely related to the increased IOP rather than direct induction by the use of glaucoma medication. For the correlation analyses, we used preoperative IOP value measured in the afternoon at the day before the aqueous humor sampling for all studied eyes. We believe that this minimizes the influences of diurnal variation of IOP and closely reflects the IOP at the time of aqueous humor sampling.

The significant correlations among the TGF-β1, IL-8, and SAA levels and the significant correlations of these cytokines and the IOP levels suggested that the cytokine networks play important roles in IOP elevations in OAG, although the exact mechanism of the interactions among these cytokines are unclear. A previous study showed that adding recombinant SAA to cultured TM cells potently stimulated IL-8 secretion[36]; thus, cytokine-like properties of SAA may induce an immune reaction specifically related to IL-8. Based on their known activities, IL-8 and TGF-β1 may act by mechanisms as diverse as stimulation of ECM synthesis and remodeling. Cross-talk pathways among TGF-β1, IL-8, and SAA and the exact source of these cytokines could be targets of future investigation of the pathologic mechanisms of IOP elevation in glaucoma.

References

1. Alvarado JA, Murphy CG. Outflow obstruction in pigmentary and primary open angle glaucoma. *Arch Ophthalmol.* 1992;110:1769–1778.
2. Lutjen-Drecoll E, Shimizu T, Rohrbach M, Rohen JW. Quantitative analysis of 'plaque material' in the inner- and outer wall of Schlemm's canal in normal- and glaucomatous eyes. *Exp Eye Res.* 1986;42:443–455.
3. Ritch R, Schlotzer-Schrehardt U. Exfoliation syndrome. *Surv Ophthalmol.* 2001;45:265–315.
4. Schlotzer-Schrehardt U, Naumann GO. Ocular and systemic pseudoexfoliation syndrome. *Am J Ophthalmol.* 2006;141:921–937.
5. Grus FH, Joachim SC, Wuenschig D, Rieck J, Pfeiffer N. Autoimmunity and glaucoma. *J Glaucoma.* 2008;17:79–84.
6. Tezel G, Wax MB. The immune system and glaucoma. *Curr Opin Ophthalmol.* 2004;15:80–84.
7. Li G, Luna C, Liton PB, Navarro I, Epstein DL, Gonzalez P. Sustained stress response after oxidative stress in trabecular meshwork cells. *Mol Vis.* 2007;13:2282–2288.
8. Nakabayashi M. Review of the ischemia hypothesis for ocular hypertension other than congenital glaucoma and closed-angle glaucoma. *Ophthalmologica.* 2004;218:344–349.
9. Helbig H, Schlotzer-Schrehardt U, Noske W, Kellner U, Foerster MH, Naumann GO. Anterior-chamber hypoxia and iris vasculopathy in pseudoexfoliation syndrome. *Ger J Ophthalmol.* 1994;3:148–153.
10. Yu AL, Fuchshofer R, Kampik A, Welge-Lussen U. Effects of oxidative stress in trabecular meshwork cells are reduced by prostaglandin analogues. *Invest Ophthalmol Vis Sci.* 2008;49:4872–4880.
11. Tripathi RC, Li J, Chan WF, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. *Exp Eye Res.* 1994;59:723–727.
12. Inatani M, Tanihara H, Katsuta H, Honjo M, Kido N, Honda Y. Transforming growth factor-beta 2 levels in aqueous humor of glaucomatous eyes. *Graefes Arch Clin Exp Ophthalmol.* 2001;239:109–113.
13. Picht G, Welge-Luessen U, Grehn F, Lutjen-Drecoll E. Transforming growth factor beta 2 levels in the aqueous humor in different types of glaucoma and the relation to filtering bleb development. *Graefes Arch Clin Exp Ophthalmol.* 2001;239:199–207.
14. Kuchtey J, Rezaei KA, Jaru-Ampornpan P, Sternberg P Jr, Kuchtey RW. Multiplex cytokine analysis reveals elevated concentration of interleukin-8 in glaucomatous aqueous humor. *Invest Ophthalmol Vis Sci.* 2010;51:6441–6447.
15. Zenkel M, Lewczuk P, Junemann A, Kruse FE, Naumann GO, Schlotzer-Schrehardt U. Proinflammatory cytokines are involved in the initiation of the abnormal matrix process in pseudoexfoliation syndrome/glaucoma. *Am J Pathol.* 2010;176:2868–2879.
16. Gottanka J, Chan D, Eichhorn M, Lutjen-Drecoll E, Ethier CR. Effects of TGF-beta2 in perfused human eyes. *Invest Ophthalmol Vis Sci.* 2004;45:153–158.
17. Fleenor DL, Shepard AR, Hellberg PE, Jacobson N, Pang IH, Clark AF. TGFbeta2-induced changes in human trabecular meshwork: implications for intraocular pressure. *Invest Ophthalmol Vis Sci.* 2006;47:226–234.
18. Vignali DA. Multiplexed particle-based flow cytometric assays. *J Immunol Methods.* 2000;243:243–255.
19. Curnow SJ, Falciani F, Durrani OM, et al. Multiplex bead immunoassay analysis of aqueous humor reveals distinct cytokine profiles in uveitis. *Invest Ophthalmol Vis Sci.* 2005;46:4251–4259.
20. Ooi KG, Galatowicz G, Towler HM, Lightman SL, Calder VL. Multiplex cytokine detection versus ELISA for aqueous humor: IL-5, IL-10, and IFNgamma profiles in uveitis. *Invest Ophthalmol Vis Sci.* 2006;47:272–277.

21. Takase H, Futagami Y, Yoshida T, et al. Cytokine profile in aqueous humor and sera of patients with infectious or noninfectious uveitis. *Invest Ophthalmol Vis Sci.* 2006;47:1557–1561.
22. Kaneda S, Miyazaki D, Sasaki S, et al. Multivariate analyses of inflammatory cytokines in eyes with branch retinal vein occlusion: relationships to bevacizumab treatment. *Invest Ophthalmol Vis Sci.* 2011;52:2982–2988.
23. Schlotzer-Schrehardt U, Zenkel M, Kuchle M, Sakai LY, Naumann GO. Role of transforming growth factor-beta1 and its latent form binding protein in pseudoexfoliation syndrome. *Exp Eye Res.* 2001;73:765–780.
24. Koliakos GG, Schlotzer-Schrehardt U, Konstas AG, Bufidis T, Georgiadis N, Dimitriadou A. Transforming and insulin-like growth factors in the aqueous humour of patients with exfoliation syndrome. *Graefes Arch Clin Exp Ophthalmol.* 2001;239:482–487.
25. Imanishi J, Kamiyama K, Iguchi I, Kita M, Sotozono C, Kinoshita S. Growth factors: importance in wound healing and maintenance of transparency of the cornea. *Prog Retin Eye Res.* 2000;19:113–129.
26. Lutjen-Drecoll E. Morphological changes in glaucomatous eyes and the role of TGFbeta2 for the pathogenesis of the disease. *Exp Eye Res.* 2005;81:1–4.
27. Streilein JW, Ksander BR, Taylor AW. Immune deviation in relation to ocular immune privilege. *J Immunol.* 1997;158:3557–3560.
28. Jampel HD, Roche N, Stark WJ, Roberts AB. Transforming growth factor-beta in human aqueous humor. *Curr Eye Res.* 1990;9:963–969.
29. Bachmann B, Birke M, Kook D, Eichhorn M, Lutjen-Drecoll E. Ultrastructural and biochemical evaluation of the porcine anterior chamber perfusion model. *Invest Ophthalmol Vis Sci.* 2006;47:2011–2020.
30. Helbig H, Kittredge KL, Coca-Prados M, Davis J, Palestine AG, Nussenblatt RB. Mammalian ciliary-body epithelial cells in culture produce transforming growth factor-beta. *Graefes Arch Clin Exp Ophthalmol.* 1991;229:84–87.
31. Kottler UB, Junemann AG, Aigner T, Zenkel M, Rummelt C, Schlotzer-Schrehardt U. Comparative effects of TGF-beta 1 and TGF-beta 2 on extracellular matrix production, proliferation, migration, and collagen contraction of human Tenon's capsule fibroblasts in pseudoexfoliation and primary open-angle glaucoma. *Exp Eye Res.* 2005;80:121–134.
32. Robertson JV, Golesic E, Gauldie J, West-Mays JA. Ocular gene transfer of active TGF-beta induces changes in anterior segment morphology and elevated IOP in rats. *Invest Ophthalmol Vis Sci.* 2010;51:308–318.
33. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med.* 2006;354:610–621.
34. Luna C, Li G, Liton PB, et al. Resveratrol prevents the expression of glaucoma markers induced by chronic oxidative stress in trabecular meshwork cells. *Food Chem Toxicol.* 2009;47:198–204.
35. Shifera AS, Trivedi S, Chau P, Bonnemaison LH, Iguchi R, Alvarado JA. Constitutive secretion of chemokines by cultured human trabecular meshwork cells. *Exp Eye Res.* 2010;91:42–47.
36. Wang WH, McNatt LG, Pang IH, et al. Increased expression of serum amyloid A in glaucoma and its effect on intraocular pressure. *Invest Ophthalmol Vis Sci.* 2008;49:1916–1923.
37. Eckersall PD, Bell R. Acute phase proteins: biomarkers of infection and inflammation in veterinary medicine. *Vet J.* 2010;185:23–27.
38. Migita K, Kawabe Y, Tominaga M, Origuchi T, Aoyagi T, Eguchi K. Serum amyloid A protein induces production of matrix metalloproteinases by human synovial fibroblasts. *Lab Invest.* 1998;78:535–539.
39. Husebekk A, Skogen B, Husby G, Marhaug G. Transformation of amyloid precursor SAA to protein AA and incorporation in amyloid fibrils in vivo. *Scand J Immunol.* 1985;21:283–287.
40. Ramadori G, Van Damme J, Rieder H, Meyer zum Buschenfelde KH. Interleukin 6, the third mediator of acute-phase reaction, modulates hepatic protein synthesis in human and mouse. Comparison with interleukin 1 beta and tumor necrosis factor-alpha. *Eur J Immunol.* 1988;18:1259–1264.
41. Upragarin N, Landman WJ, Gaastra W, Gruys E. Extrahepatic production of acute phase serum amyloid A. *Histol Histopathol.* 2005;20:1295–1307.
42. Baranova IN, Vishnyakova TG, Bocharov AV, et al. Serum amyloid A binding to CLA-1 (CD36 and LIMPII analogous-1) mediates serum amyloid A protein-induced activation of ERK1/2 and p38 mitogen-activated protein kinases. *J Biol Chem.* 2005;280:8031–8040.
43. Jijon HB, Madsen KL, Walker JW, Allard B, Jobin C. Serum amyloid A activates NF-kappaB and proinflammatory gene expression in human and murine intestinal epithelial cells. *Eur J Immunol.* 2005;35:718–726.
44. Vallon R, Freuler F, Desta-Tsedu N, et al. Serum amyloid A (apoSAA) expression is up-regulated in rheumatoid arthritis and induces transcription of matrix metalloproteinases. *J Immunol.* 2001;166:2801–2807.
45. Patel H, Fellowes R, Coade S, Woo P. Human serum amyloid A has cytokine-like properties. *Scand J Immunol.* 1998;48:410–418.
46. Furlaneto CJ, Campa A. A novel function of serum amyloid A: a potent stimulus for the release of tumor necrosis factor-alpha, interleukin-1beta, and interleukin-8 by human blood neutrophil. *Biochem Biophys Res Commun.* 2000;268:405–408.
47. Ribeiro FP, Furlaneto CJ, Hatanaka E, et al. mRNA expression and release of interleukin-8 induced by serum amyloid A in neutrophils and monocytes. *Mediators Inflamm.* 2003;12:173–178.
48. McKinnon SJ. Glaucoma: ocular Alzheimer's disease? *Front Biosci.* 2003;8:s1140–s1156.
49. Vickers JC. The cellular mechanism underlying neuronal degeneration in glaucoma: parallels with Alzheimer's disease. *Aust N Z J Ophthalmol.* 1997;25:105–109.
50. Berlau J, Lorenz P, Beck R, et al. Analysis of aqueous humour proteins of eyes with and without pseudoexfoliation syndrome. *Graefes Arch Clin Exp Ophthalmol.* 2001;239:743–746.
51. Janciauskiene S, Krakau T. Alzheimer's peptide: a possible link between glaucoma, exfoliation syndrome and Alzheimer's disease. *Acta Ophthalmol Scand.* 2001;79:328–329.
52. Nelson GA, Edward DP, Wilensky JT. Ocular amyloidosis and secondary glaucoma. *Ophthalmology.* 1999;106:1363–1366.
53. Liton PB, Li G, Luna C, Gonzalez P, Epstein DL. Cross-talk between TGF-beta1 and IL-6 in human trabecular meshwork cells. *Mol Vis.* 2009;15:326–334.
54. Caballero M, Liton PB, Challa P, Epstein DL, Gonzalez P. Effects of donor age on proteasome activity and senescence in trabecular meshwork cells. *Biochem Biophys Res Commun.* 2004;323:1048–1054.
55. Caballero M, Liton PB, Epstein DL, Gonzalez P. Proteasome inhibition by chronic oxidative stress in human trabecular meshwork cells. *Biochem Biophys Res Commun.* 2003;308:346–352.
56. De La Paz MA, Epstein DL. Effect of age on superoxide dismutase activity of human trabecular meshwork. *Invest Ophthalmol Vis Sci.* 1996;37:1849–1853.
57. Xu Y, Chen W, Lu H, et al. The expression of cytokines in the aqueous humor and serum during endotoxin-induced uveitis in C3H/HeN mice. *Mol Vis.* 2010;16:1689–1695.
58. Campbell IL, Abraham CR, Masliah E, et al. Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. *Proc Natl Acad Sci U S A.* 1993;90:10061–10065.
59. Maruo N, Morita I, Shirao M, Murota S. IL-6 increases endothelial permeability in vitro. *Endocrinology.* 1992;131:710–714.
60. Kuchle M, Nguyen NX, Hannappel E, Naumann GO. The blood-aqueous barrier in eyes with pseudoexfoliation syndrome. *Ophthalmic Res.* 1995;27(suppl 1):136–142.
61. Ritch R. Exfoliation syndrome. *Curr Opin Ophthalmol.* 2001;12:124–130.
62. Yuan L, Neufeld AH. Tumor necrosis factor-alpha: a potentially neurodestructive cytokine produced by glia in the human glaucomatous optic nerve head. *Glia.* 2000;32:42–50.
63. Tezel G, Li LY, Patil RV, Wax MB. TNF-alpha and TNF-alpha receptor-1 in the retina of normal and glaucomatous eyes. *Invest Ophthalmol Vis Sci.* 2001;42:1787–1794.
64. Sawada H, Fukuchi T, Tanaka T, Abe H. Tumor necrosis factor-alpha concentrations in the aqueous humor of patients with glaucoma. *Invest Ophthalmol Vis Sci.* 2010;51:903–906.
65. Wang N, Chintala SK, Fini ME, Schuman JS. Activation of a tissue-specific stress response in the aqueous outflow pathway of the eye

defines the glaucoma disease phenotype. *Nat Med.* 2001;7:304–309.

66. Hu DN, Ritch R, Liebmann J, Liu Y, Cheng B, Hu MS. Vascular endothelial growth factor is increased in aqueous humor of glaucomatous eyes. *J Glaucoma.* 2002;11:406–410.
67. Bloom BR, Bennett B. Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. *Science.* 1966;153:80–82.
68. Taguchi C, Sugita S, Tagawa Y, Nishihira J, Mochizuki M. Macrophage migration inhibitory factor in ocular fluids of patients with uveitis. *Br J Ophthalmol.* 2001;85:1367–1371.
69. Takase H, Sugita S, Rhee DJ, et al. The presence of macrophage migration inhibitory factor in human trabecular meshwork and its upregulatory effects on the T helper 1 cytokine. *Invest Ophthalmol Vis Sci.* 2002;43:2691–2696.
70. Razzaque MS, Foster CS, Ahmed AR. Role of macrophage migration inhibitory factor in conjunctival pathology in ocular cicatricial pemphigoid. *Invest Ophthalmol Vis Sci.* 2004;45:1174–1181.
71. Malvitte L, Montange T, Vejux A, et al. Measurement of inflammatory cytokines by multicytokine assay in tears of patients with glaucoma topically treated with chronic drugs. *Br J Ophthalmol.* 2007;91:29–32.
72. Manni G, Centofanti M, Oddone F, Parravano M, Bucci MG. Interleukin-1beta tear concentration in glaucomatous and ocular hypertensive patients treated with preservative-free nonselective beta-blockers. *Am J Ophthalmol.* 2005;139:72–77.
73. Baudouin C, Hamard P, Liang H, Creuzot-Garcher C, Bensoussan L, Brignole F. Conjunctival epithelial cell expression of interleukins and inflammatory markers in glaucoma patients treated over the long term. *Ophthalmology.* 2004;111:2186–2192.
74. Konstas AG, Koliakos GG, Karabatsas CH, et al. Latanoprost therapy reduces the levels of TGF beta 1 and gelatinases in the aqueous humour of patients with exfoliative glaucoma. *Exp Eye Res.* 2006;82:319–322.