Special Issue

Iris Damage Is Associated With Elevated Cytokine Levels in Aqueous Humor

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Purpose. To evaluate the association between iris damage and cytokine levels in the aqueous humor (AqH).

Methods. A total of 201 AqH samples from 201 consecutive patients (mean age 73.7 ± 10.6) were collected at the beginning of corneal transplantation or cataract surgery. Iris damage of each case was assessed from preoperative slit-lamp findings based on its severity. The subjects were classified into three groups: eyes without iris damage (126 eyes), eyes with mild iris damage (51 eyes), and eyes with severe iris damage (24 eyes). The levels of cytokines (IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17a, interferon gamma-induced protein [IP]-10, monocyte chemotactic protein [MCP]-1, IFN-α, IFN-γ, macrophage inflammatory protein [MIP]-1α, MIP-1β, P-selectin, E-selectin, soluble intercellular adhesion molecule [sICAM]-1, TNF-α, and granulocyte-macrophage colony-stimulating factor [GM-CSF]) in AqH were measured by multiplex beads immunoassay.

Results. The levels of aqueous protein, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17A, MCP-1, TNF-α, E-selectin, P-selectin, and sICAM-1 in eyes with mild and severe iris damage were higher than in those without iris damage ($P < 0.033$). Multivariate analyses of clinical factors revealed that iris damage was associated with the history of complicated glaucoma, and the number of previous intraocular surgeries. The levels of AqH IL-6, IL-8, IL-13, MIP-1α, TNF-α, and sICAM-1 were significantly elevated in eyes with mild and severe iris damage in phakic eyes, and the levels of AqH IL-8 and sICAM-1 were significantly elevated in eyes with severe iris damage in pseudophakic eyes, compared with the eyes without iris damage ($P < 0.045$).

Conclusions. Iris damage was associated with the elevation in the levels of aqueous protein and cytokines.

Keywords: iris, aqueous humor, cytokine, immune privilege, glaucoma

Aqueous humor (AqH) maintains a normal homeostatic environment in the eye and is essential for the proper functioning of tissues in its anterior chamber.[1] Immunomodulatory properties of AqH protect the delicate internal structures of the visual axis from the blinding conditions of innate and adaptive immune inflammations.[2–4] Aqueous humor contains a variety of important immunomodulatory factors, that inhibit T cells and complement activation in AqH.[5–8] The concentrations of protein and ion content in AqH are different from those in plasma; some of the proteins and ions are secreted from tissues in the anterior segment.[1] Several laboratory studies have demonstrated that AqH as well as cultured explants of whole iris and ciliary body (I/CB), or single-cell suspensions derived from these tissue-secreted supernatants, suppress T cell activation in vitro.[9,10] The changes in AqH proteome can correlate with the prognosis of eye diseases.[1,11] However, in clinical setting, the association between I/CB factors and anti-inflammatory effects has been poorly understood.

Graft rejection and chronic loss of endothelial cell density (ECD) are the major concerns in improving the prognosis of corneal transplantation. Recently, using Cox hazard analysis, we identified that preexistence of severe iris damage was one of the clinical factors for graft failure and rapid ECD loss after Descemet's stripping automated endothelial keratoplasty (DSAEK)[12]; in eyes with severe iris damage, ECD decreases rapidly, leading to graft failure. Moreover, we recently showed the elevation of inflammatory cytokine levels in AqH in eyes with bullous keratopathy and reduced ECD.[13] A question that arises is why ECD decreases in eyes with severe iris damage. Anatomically, the AqH is present between the corneal endothelium and the iris, and it has been reported that inflammatory cytokines in AqH are elevated during various pathological processes.[14–16] In a previous study,[17] a combination of proinflammatory cytokines were observed to synergistically induce the apoptosis of corneal endothelial cells in vitro. In addition, ECDs are lower in eyes with a history of uveitis, and are correlated with flare in the anterior chamber,[18] suggesting that inflammatory factors in AqH directly influence ECD. Thus, we hypothesized that the iris damage might be associated with increased cytokine levels in AqH. In the present study, we examined the association between the severity of iris damage and elevated cytokine levels in AqH. Moreover, we evaluated the clinical factors that predispose iris to damage, and analyzed their correlation with the levels of inflammatory cytokines in AqH.

Table 1. Demographics of the Subjects

Clinical Factors	Total	Iris Damage		
		No	Mild	Severe
Number of subjects	201	126	51	24
Age	73.7 ± 10.6	73.6 ± 10.5	75.3 ± 10.0	70.8 ± 11.7
Sex (male/female)	80/121	52/74	17/34	11/13

Methods

This prospective consecutive study was performed in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Review Board of Tokyo Dental College, Ichikawa General Hospital (I-15-42). Written informed consent was obtained from all the participants.

Patients

A total of 201 consecutive patients who underwent corneal transplantation and cataract surgery at Tokyo Dental College in the period from October, 2015 to September, 2016 were included. The demographics of the participants are shown in Table 1. The diseases for which surgeries were performed included bullous keratopathy (72 eyes), cataract (61 eyes), corneal scar (28 eyes), Fuchs' endothelial corneal dystrophy (FECD; 19 eyes), hereditary epithelial/stromal dystrophies (11 eyes; lattice corneal dystrophy, 5 eyes; macular corneal dystrophy, 3 eyes; granular corneal dystrophy, 3 eyes), and keratoconus (10 eyes). We did not perform corneal transplantation or cataract surgery in eyes with active inflammation of the cornea or anterior chamber, and such eyes were not included in the study. We confirmed that the anterior chamber did not contain cells, ciliary injections, or keratoprecipitates using slit-lamp microscopy before surgery. As for flare in the anterior chamber, we measured aqueous protein levels directly, because it was often difficult to assess the flare accurately using slit-lamp microscopy in the eyes with corneal opacity. We excluded 18 eyes with ocular comorbidities (8 eyes with a history of exfoliation syndrome, 3 eyes with chemical burn, 2 eyes with ocular cicatricial pemphigoid, 1 eye with aniridia, 1 eye with endotheliitis, 1 eye with Stevens-Johnson syndrome, 1 with exposure keratitis, and 1 eye with iritis), because previous studies showed that aqueous cytokine levels are elevated in eyes with exfoliation syndrome due to breakdown of the blood–aqueous barrier (BAB),[19] and that tear cytokine levels are elevated in inflammatory ocular surface diseases,[20,21] which can affect aqueous cytokine levels due to breakdown of the corneal epithelial barrier.[22] In the eyes with cataract and bullous keratopathy, when we perform two-step surgery, we always perform cataract surgery first, followed by DSAEK, with an interval of more than 3 months. In the eyes with cataract after penetrating keratoplasty (PKP), we make it a rule to wait for an interval of more than 6 months after PKP to perform the cataract surgery.

AqH Samples

Aqueous humor was obtained under sterile conditions at the beginning of surgery after retrobulbar anesthesia in corneal transplantation or topical anesthesia in cataract surgery. First, paracentesis was placed at the clear cornea. The AqH samples (70–300 μL) were obtained using a 27-G needle, taking care not to touch the iris, lens, or corneal endothelium. The samples were centrifuged at 3000*g* for 5 minutes. The soluble fractions were collected and stored at −80 °C until cytokine levels were measured.

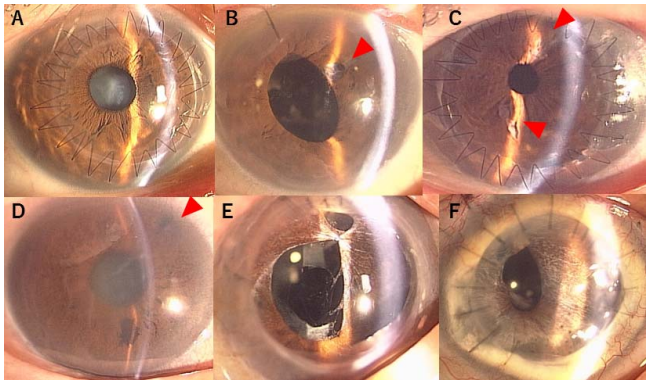

Figure 1. Representative cases with iris damage. (**A**) No iris damage with a healthy normal iris. (**B**) Mild iris damage limited to one quadrant. (**C**) Mild iris damage limited to two quadrants. (**D**) After laser iridotomy. (**E**) Severe iris damage in three quadrants. (**F**) Devastating severe iris damage and atrophy in whole iris.

Measurement of Protein Concentration

The concentrations of protein in the AqH samples were determined using the DC protein assay kit (Bio-Rad, Hercules, CA, USA). The reactions were based on the Lowry assay, and were performed according to the manufacturer's instructions. In brief, BSA in the concentration range of 0.23 to 1.37 mg/mL was used as a standard. Samples (5 μL) of BSA and AqH were added to 96-well microplates; this was followed by immediate addition of a mixture containing 25 μL of reagent A+S and 200 μL of reagent C. After 15-minutes incubation at room temperature in the dark, the microplates were read at 690 and 405 nm using a microplate reader (Model550; Bio-Rad). The concentrations were calculated by the subtraction method using a microplate manager system (Bio-Rad).

Measurement of Cytokine Levels

The levels of cytokines (IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IFN-α, IFN-γ, monocyte chemotactic protein [MCP]-1, TNF-α, E-selectin, P-selectin, soluble intercellular adhesion molecule [sICAM]-1, granulocyte-macrophage colony-stimulating factor [GM-CSF], macrophage inflammatory protein [MIP]-1α, MIP-1β, and interferon gamma-induced protein [IP]-10) in the AqH samples were measured using Luminex (ProcaPlex kit; Luminex, San Antonio, TX, USA) beads-based multiplex immunoassay according to previous reports.[13,23] Briefly, 50 μL of AqH samples were incubated with antibody-coated capture beads in an incubation buffer at room temperature. After 2-hours incubation, the beads were washed thrice using washing buffer and phycoerythrin-labeled streptavidin was added and allowed to bind with in the dark at room temperature for 30 minutes. After three washes with the washing buffer, 150 μL of the reading buffer was added to the plates, and the assays were performed using Luminex 200 system.

Definition of the Severity of Iris Damage

Iris damage was defined as iris depigmentation, laser iridotomy, or iris defect due to intraocular surgeries. The severity grade of iris damage for each case was determined based on its severity from slit-lamp findings. Briefly, healthy eyes are regarded as "no iris damage" (Fig. 1A), eyes with "mild iris damage" are

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defined as iris damage limited to only one (Fig. 1B) to two quadrants (Fig. 1C), or with laser iridotomy (Fig. 1D), and eyes with "severe iris damage" are defined as iris damage from three (Fig. 1E) to four quadrants (Fig. 1F). The causes for iris damage in the samples used in the current study, included laser iridotomy (LI; 29 eyes), previous corneal transplantation (22 eyes), complicated cataract surgery (14 eyes), complicated vitreoretinal surgery (5 eyes), glaucoma surgery (trabeculectomy in 8 eyes, goniosynechialysis in 3 eyes and trabeculotomy in 3 eyes), and trauma (5 eyes).

Data Analysis

To identify clinical parameters associated with iris damage, we selected the following variables, based on the previous studies and on our knowledge of iris damage: the presence of intraocular lens (IOL), a history of corneal transplantation, complicated glaucoma, diabetes mellitus (DM), axial length, patient age, and number of previous intraocular surgeries. Complicated glaucoma was defined as glaucoma with a history of single or multiple glaucoma surgeries or secondary glaucoma after complicated intraocular surgeries. Clinical factors, such as a history of IOL, corneal transplantation, complicated glaucoma, and DM, were dichotomized for univariate and multivariate analyses. To assess the association between the clinical factors and iris damage, univariate analyses were conducted for each variable using Spearman's rank correlations. Multiple linear regression analysis was conducted using stepwise analysis (Model 1) and backward elimination analysis (Model 2).

Statistical Analysis

Stata statistical software for Windows, version 14.1 (StataCorp LP, College Station, TX, USA) was used for all the statistical analyses, and a P value less than 0.05 was considered statistically significant. The data are expressed as averages with SE and median for cytokine levels. The Shapiro-Wilk test was used to assess whether the data showed a normal distribution. The Mann-Whitney U-test was used to compare cytokine levels between each iris damage subgroup.

Results

Aqueous Protein Levels in Eyes With and Without Iris Damage

The aqueous protein levels in eyes without iris damage were 0.51 ± 0.46 mg/mL, which significantly increased to 098 ± 0.14 mg/mL in eyes with mild iris damage ($P = 0.0033$) and 1.70 ± 0.29 mg/mL in eyes with severe iris damage ($P < 0.0001$). The aqueous protein level in eyes with severe iris damage was significantly higher than in eyes with mild iris damage (Fig. 2, $P = 0.016$).

Increased Aqueous Cytokine Levels in Eyes With Iris Damage

The mean cytokine levels in AqH, in terms of iris damage severity in all the subjects are shown in Table 2. The levels of IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17A, MCP-1, TNF-α, E-selectin, P-selectin, and sICAM-1 in eyes with mild and severe iris damage were higher than those without iris damage ($P < 0.033$). The levels of IL-13, MIP-1α, IFN-α and IFN-γ in eyes with mild iris damage were higher than those without iris damage ($P < 0.0036$), and IP-10 in eyes with severe iris damage was higher than that of no iris damage ($P = 0.0013$). None of the cytokines in eyes with severe iris damage was lower than that in eyes without iris damage ($P > 0.05$), and only IP-10 was lower in eyes with mild iris damage, compared with that in eyes without iris damage ($P < 0.05$). Interferon-α was higher in eyes with mild iris damage than in eyes with severe iris damage. There were no other significant differences in the cytokine levels between eyes with mild and severe iris damage.

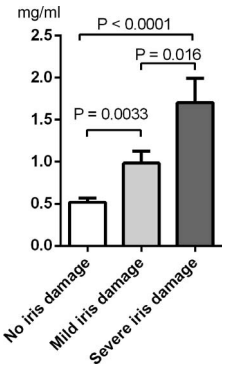

Figure 2. Protein concentration in the AqH. The protein concentration in the AqH significantly increased to 0.98 ± 0.14 mg/mL in eyes with mild iris damage ($P = 0.0033$) and to 1.70 ± 0.29 mg/mL in eyes with severe iris damage, compared with eyes without iris damage (0.51 ± 0.46 mg/mL). The protein concentration in the AqH significantly correlated with the severity of iris damage ($r = 0.434$, $P < 0.001$).

Associations Between Clinical Factors and the Severity of Iris Damage

Table 3 shows the univariate and multivariate regression analyses of the association between iris damage severity and clinical factors. Univariate correlation analysis showed iris damage severity was correlated with the number of previous intraocular surgeries ($\rho = 0.754$, $P = 0.0001$), presence of IOL ($\rho = 0.516$, $P = 0.0001$), complicated glaucoma ($\rho = 0.491$, $P = 0.0001$), history of corneal transplantation ($\rho = 0.371$, $P = 0.0001$), and axial length ($\rho = -0.170$, $P = 0.018$). Multiple regression analyses showed that iris damage severity had significant correlations with the number of previous intraocular surgeries ($\beta = 0.336$, $P = 0.0001$) and history of complicated glaucoma ($\beta = 0.355$, $P = 0.013$).

Aqueous Cytokine Levels Stratified With the Severity of Iris Damage in Phakic and Pseudophakic Eyes

Having demonstrated that the levels of aqueous protein and cytokines were increased in eyes with iris damage, we assessed the levels of aqueous protein and cytokines by dividing the samples into two categories—phakic and pseudophakic eyes—because previous report has shown that specific cytokine levels alter after the cataract surgery.[15] In phakic eyes (Table 4, total 142 eyes), the levels of IL-6, IL-8, IL-13, MIP-1α, TNF-α, and sICAM-1 in the eyes with mild and severe iris damage were significantly higher than those in the eyes without iris damage ($P < 0.045$). The levels of protein, IL-1β, IL-17A, GM-CSF, IFN-α, IFN-γ, and P-selectin in the eyes with mild iris damage were significantly higher than that in the eyes without iris damage ($P < 0.04$). The levels of IL-6 and MCP-1 in eyes with severe iris damage were significantly higher than that in the eyes without iris damage ($P = 0.0007$ and $P = 0.0027$, respectively).

In pseudophakic eyes (Table 5, total 59 eyes), the IL-8 levels in the eyes with mild and severe iris damage were significantly

Table 2. Aqueous Cytokine Levels in All Eyes

Aqueous Cytokine	No Iris Damage, $N = 126$	Mild Iris Damage, $N = 51$	Severe Iris Damage, $N = 24$	*P* Value*	*P* Value†	*P* Value‡
IL-1α	62.9 ± 7.2 (46.9)	102 ± 19.5 (71.6)	97.6 ± 17.7 (68.6)	0.012	0.55	0.0026
IL-1β	2.7 ± 0.6 (1.1)	11.3 ± 3.1 (3.1)	24.3 ± 18.1 (1.6)	0.0001	0.46	0.0064
IL-4	26.3 ± 2.6 (20.9)	48.1 ± 5.9 (29.2)	40.3± 5.4 (30.8)	<0.0001	0.95	0.0001
IL-6	380 ± 110 (9.4)	1497 ± 466 (335)	1185 ± 701 (210)	<0.0001	0.50	<0.0001
IL-8	41.0 ± 9.4 (16.6)	70.7 ± 11.4 (39.5)	78.5 ± 19.9 (51.9)	<0.0001	0.77	<0.0001
IL-10	2.6 ± 0.2 (1.8)	8.5 ± 3.0 (3.7)	12.5 ± 5.5 (4.1)	<0.0001	0.57	<0.0001
IL–12p70	7.9 ± 0.6 (6.5)	14.0 ± 1.7 (8.5)	11.8 ± 1.4 (9.0)	0.0001	0.95	0.001
IL-13	8.4 ± 0.8 (7.2)	10.6 ± 1.0 (9.6)	9.6 ± 1.3 (9.2)	<0.0001	0.41	0.11
IL-17A	5.6 ± 0.4 (4.6)	10.6 ± 2.0 (7.2)	8.3 ± 1.0 (7.2)	0.0089	0.84	0.0073
MIP-1α	11–2 ± 0.9 (9.2)	20.1 ± 3.2 (12.3)	14.5 ± 2.0 (11.8)	0.0081	0.67	0.07
MIP-1β	338 ± 42.9 (303.7)	384 ± 70.4 (323)	365 ± 75.7 (294)	0.661	0.99	0.75
MCP-1	627 ± 70.6 (466.7)	860 ± 146 (615)	756 ± 86.5 (694)	0.0036	0.57	0.0005
TNF-α	110 ± 8.1 (80.9)	326 ± 157 (102)	139 ± 14.9 (122)	0.033	0.68	0.011
GM-CSF	5.4 ± 1.1 (3.6)	8.0 ± 1.9 (5.1)	8.8 ± 2.8 (8.5)	0.16	0.52	0.068
IFN-α	4.5 ± 0.4 (3.9)	6.5 ± 0.8 (4.9)	4.1 ± 0.4 (4.0)	0.0003	0.035	0.95
IFN-γ	62.7 ± 5.0 (51.9)	102.1 ± 13.0 (66.6)	68.9 ± 4.9 (62.6)	<0.0001	0.26	0.95
E-Selectin	2739 ± 236 (2295)	4326 ± 732 (2812)	3863 ± 512 (2783)	0.0004	0.83	0.002
P-Selectin	5477 ± 432 (3895)	14147 ± 2625 (7540)	10026 ± 3409 (6850)	<0.0001	0.21	0.004
sICAM-1	2093 ± 235 (1313)	4721 ± 737 (3100)	5279 ± 1054 (3242)	<0.0001	0.30	<0.0001
IP-10	377 ± 160 (95.8)	302.9 ± 56.8 (151)	400 ± 115 (181.9)	<0.0001	0.56	0.0013

Mean ± SE (median) (pg/mL).
* Compared between eyes without iris damage and eyes with mild iris damage, Mann-Whitney *U*-test.
† Compared between eyes with mild iris damage and eyes with severe iris damage, Mann-Whitney *U*-test.
‡ Compared between eyes without iris damage and eyes with severe iris damage, Mann-Whitney *U*-test.

higher than that in the eyes without iris damage ($P = 0.0001$ and $P = 0.0031$, respectively). The levels of protein and sICAM-1 in the eyes with severe iris damage were significantly higher than that in the eyes without iris damage ($P = 0.03$ and $P = 0.034$, respectively).

Aqueous Protein/Cytokine Levels and Iris Damage Score (IDS)

Having demonstrated that the aqueous protein level was correlated with the severity of iris damage, we sought to evaluate the association of iris damage and aqueous levels of protein and cytokines in detail. We assessed the aqueous protein and cytokine levels after classifying the iris damage into four grades as reported previously.[12] In brief, IDS 0 indicates no iris damage (Fig. 1A); IDS 1 is defined as iris damage limited only to one quadrant (Fig. 1B); or no iris damage with laser iridotomy (Fig. 1D); IDS 2 is defined as iris damage in two quadrants (Fig. 1C); IDS 3 is defined as iris damage in three quadrants (Fig. 1E); and IDS 4 is defined as iris damage in four quadrants (Fig. 1F). The aqueous protein levels in the eyes with IDS 0 increased from 0.52 ± 0.05 mg/mL (Fig. 3) to 0.86 ± 0.18 mg/mL in IDS1 ($P > 0.99$), 1.22 ± 0.24 mg/mL in IDS2 ($P = 0.024$), 1.62 ± 0.30 mg/mL in IDS3 ($P < 0.001$), and 1.80 ± 0.52 mg/mL in IDS4 ($P < 0.001$). The protein concentration in the AqH significantly correlated with iris damage score ($r = 0.469$, $P < 0.001$). On the contrary, the aqueous cytokine levels were more complex (Table 6; Fig. 4). Although the levels of some aqueous cytokines were significantly elevated in eyes with IDS 1 to 4 compared with those in eyes with IDS 0, there were no statistically significant differences in aqueous cytokine levels among eyes with IDS 1, 2, 3, and 4 (Fig. 4). However, IDS was significantly positively correlated with the levels of IL-1α, IL-1β, IL-4, IL-6, IL -8, IL -10, IL-12p70, IL-13, IL-17A, IFN-α, IFN-γ, MCP-1, TNF-α, E-selectin, P-selectin, sICAM-1, and IP-10 (Table 6; Spearman's correlation, $P < 0.028$).

Discussion

In the current study, we demonstrated that the iris damage was associated with elevated cytokine levels in AqH. Furthermore,

Table 3. Association Between Iris Damage Severity and Clinical Factors

	Univariate Models*		Multifactorial Model			
			Model 1		Model 2	
Clinical Factors	ρ	*P* Value	*B*	*P* Value	*B*	*P* Value
Number of previous intraocular surgery	0.754	0.0001	0.336	0.0001	0.336	0.0001
IOL (+=1)	0.516	0.0001				
Complicated Glaucoma (+=1)	0.491	0.0001	0.355	0.013	0.355	0.013
History of corneal transplantation (+=1)	0.371	0.0001				
Axial length	−0.170	0.018				
DM (+=1)	−0.032	0.66				
Age	−0.013	0.86				

* Spearman's correlation analysis.

Table 4. Aqueous Cytokine Levels in Phakic Eyes

Aqueous Cytokine	No Iris Damage, $N = 110$	Mild Iris Damage, $N = 27$	Severe Iris Damage, $N = 5$	*P* Value*	*P* Value†	*P* Value‡
Protein	0.44 ± 0.04 (0.33)	0.82 ± 0.17 (0.61)	0.52 ± 0.11 (0.31)	0.025	0.68	0.43
IL-1α	59.4 ± 7.7 (45.9)	76.7 ± 13.3 (63.9)	103 ± 23.6 (82.6)	0.083	0.24	0.0007
IL-1β	2.7 ± 0.6 (1.1)	9.7 ± 4.3 (3.1)	56.3 ± 54.1 (2.3)	0.0003	0.77	0.20
IL-4	25.4 ± 2.8 (20.3)	42.1 ± 5.8 (27.9)	42.9 ± 14.9 (27.9)	0.0001	0.89	0.07
IL-6	223 ± 86.2 (8.0)	969 ± 291 (335)	3525 ± 3310 (228)	<0.0001	0.97	0.0024
IL-8	33.0 ± 8.8 (16.2)	51.1 ± 11.8 (34.8)	49.6 ± 12.0 (38.8)	0.0001	0.58	0.0031
IL-10	2.4 ± 0.2 (1.8)	4.5 ± 0.6 (3.3)	4.1 ± 0.7 (3.9)	<0.0001	0.66	0.0019
IL–12p70	7.6 ± 0.6 (6.4)	12.6 ± 1.7 (8.5)	10.0 ± 1.6 (10.8)	0.0013	0.96	0.046
IL-13	8.1 ± 0.9 (6.9)	12.1 ± 1.7 (9.5)	14.5 ± 3.8 (13.6)	<0.0001	0.52	0.012
IL-17A	5.6 ± 0.5 (3.9)	12.9 ± 3.6 (7.4)	9.9 ± 3.8 (7.4)	0.0041	0.99	0.082
MIP-1α	11.1 ± 1.0 (9.1)	18.0 ± 2.7 (12.8)	16.6 ± 3.9 (13.6)	0.0045	0.83	0.019
MIP-1β	332 ± 47.5 (304)	345 ± 65.9 (279)	424 ± 154 (392)	0.82	0.56	0.30
MCP-1	590 ± 77.3 (458)	634 ± 64.3 (595)	662 ± 72.5 (690)	0.058	0.84	0.0027
TNF-α	104 ± 8.2 (61.4)	214 ± 54.2 (74.1)	164 ± 26.6 (154)	0.012	0.63	0.0073
GM-CSF	5.1 ± 1.1 (3.6)	9.9 ± 3.0 (5.1)	13.7§	0.036		
IFN-α	4.5 ± 0.4 (3.9)	5.8 ± 0.9 (5.0)	4.6 ± 1.1 (6.5)	0.04	0.49	0.86
IFN-γ	62.5 ± 5.7 (52.0)	93.8 ± 12.1 (68.8)	71.6 ± 17.4 (56.8)	<0.0001	0.31	0.27
E-Selectin	2728 ± 264 (2268)	3424 ± 475 (2677)	2783 ± 771 (2404)	0.043	0.66	0.51
P-Selectin	5256 ± 472 (3723)	10518 ± 1786 (7540)	6907 ± 1132 (8154)	<0.0001	0.72	0.062
sICAM-1	1974 ± 248 (1301)	3721 ± 501 (2845)	3147 ± 1000 (2278)	<0.0001	0.68	0.045
IP-10	406 ± 183 (94.1)	254 ± 82 (129)	422 ±166 (447)	0.015	0.39	0.16

Mean ± SE (median), protein (mg/mL), cytokines (pg/mL).
* Compared between eyes without iris damage and eyes with mild iris damage, Mann-Whitney *U*-test.
† Compared between eyes with mild iris damage and eyes with severe iris damage, Mann-Whitney *U*-test.
‡ Compared between eyes without iris damage and eyes with severe iris damage, Mann-Whitney *U*-test.
§ Only one sample could be measured using multiplex beads assay among the samples in each group.

multivariate analysis showed that iris damage was correlated with complicated glaucoma surgery and number of previous intraocular surgeries. These results suggest that iris damage lead to elevation in the levels of aqueous inflammatory cytokines, which would be a useful clinical finding for increased cytokine levels in AqH; however, there was no significant difference in the aqueous cytokine levels between mild and severe iris damage.

Corneal transplants are among the most successful solid tissue transplants. The primary cause of graft failure is endothelial decompensation, even in eyes without any evidence of immunologic rejection.[24] After corneal transplan-

Table 5. Aqueous Cytokine Levels in Pseudophakic Eyes

Aqueous Cytokine	No Iris Damage, $N = 16$	Mild Iris Damage, $N = 24$	Severe Iris Damage, $N = 19$	*P* Value*	*P* Value†	*P* Value‡
Protein	1.03 ± 0.20 (1.0)	1.17 ± 0.23 (1.1)	1.95 ± 0.3 (1.7)	0.89	0.06	0.031
IL-1α	93.6 ± 18.5 (71.4)	126 ± 35.6 (72.4)	95.4 ± 23.2 (64.4)	0.73	0.83	0.63
IL-1β	3.2 ± 1.3 (1.3)	13.3 ± 4.7 (3.3)	8.3 ± 5.4 (1.6)	0.11	0.61	0.39
IL-4	33.7 ± 4.5 (35.1)	54.9 ± 10.7 (33.3)	39.6 ± 5.9 (31.8)	0.39	0.78	0.78
IL-6	1461 ± 613 (87.4)	2092 ± 931 (280)	570 ± 227 (191)	0.47	0.41	0.98
IL-8	33.0 ± 8.8 (16.2)	51.1 ± 11.8 (34.8)	49.6 ± 12.0 (38.8)	0.0001	0.58	0.0031
IL-10	3.5 ± 0.5 (3.3)	13.3 ± 6.4 (3.8)	14.7 ± 6.97 (4.4)	0.52	0.95	0.16
IL–12p70	10.5 ± 1.5 (7.9)	15.6 ± 3.2 (8.5)	12.3 ± 1.8 (8.9)	0.52	0.95	0.55
IL-13	10.7 ± 1.3 (9.2)	9.2 ± 1–2 (9.9)	8.1 ± 1.1 (8.1)	0.94	0.34	0.16
IL-17A	7.8 ± 1.1 (7.1)	8.6 ± 2.0 (7.2)	7.9 ± 1.0 (7.2)	0.74	0.71	0.94
MIP-1α	12.5 ± 1.4 (12.9)	22.4 ± 6.1 (11–2)	13.8 ± 2.4 (11.3)	0.98	0.68	0.90
MIP-1β	414 ± 79.4 (362)	426 ± 129 (348)	349 ± 88.3 (261)	0.50	0.90	0.31
MCP-1	902 ± 162 (670)	1115 ± 296 (617)	805 ± 107 (697)	0.98	0.94	0.88
TNF-α	158 ± 28.6 (122)	130.9 ± 25.6 (99.7)	128 ± 17.7 (109)	0.24	0.52	0.49
GM-CSF	13.2 ± 3.9 (13.2)	5.9 ± 2.1 (3.6)	6.3 ± 2.2 (6.3)	0.16	0.56	0.88
IFN-α	4.4 ± 0.7 (3.6)	7.3 ± 1.3 (4.8)	3.9 ± 0.4 (4.0)	0.021	0.02	0.99
IFN-γ	65 ± 7.0 (50.9)	112 ± 24.3.1 (64.3)	68.2 ± 4.6 (68.3)	0.12	0.44	0.53
E-Selectin	2821 ± 395 (2404)	5304 ± 1425 (3018)	4147 ± 606 (2840)	0.12	0.99	0.11
P-Selectin	6959 ± 1038 (5105)	18230 ± 5139 (8395)	10847 ± 4301 (6161)	0.13	0.18	0.86
sICAM-1	3081 ± 726 (2201)	5846 ± 1445 (3151)	5840 ± 1285 (3390)	0.039	0.32	0.034
IP-10	179 ± 52 (117)	358 ± 77 (191)	394 ± 141 (169)	0.02	0.57	0.14

Mean ± SE (median), protein (mg/mL), cytokines (pg/mL).
* Compared between eyes without iris damage and eyes with mild iris damage, Mann-Whitney *U*-test.
† Compared between eyes with mild iris damage and eyes with severe iris damage, Mann-Whitney *U*-test.
‡ Compared between eyes without iris damage and eyes with severe iris damage, Mann-Whitney *U*-test.

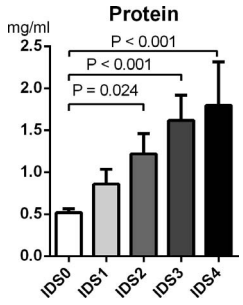

Figure 3. Protein level in AqH stratified based on iris damage score. Aqueous protein level increased with iris damage score.

tation, the ECD reduces in a chronic fashion over years,[25] and rates of ECD reduction are exacerbated from 2.6% to 9.7% annually.[26–29] The risk factors for postoperative endothelial cell loss after corneal transplantation include donor age, recipient age, graft diameter, lens status, glaucoma, graft rejection, bullous keratopathy (compared with FECD), and peripheral corneal diseases.[25,30–34] However, the exact mechanism of chronic ECD loss is still poorly understood. Previous laboratory studies have shown that inflammatory cytokines induce apoptosis in corneal endothelial cells.[17,35,36] We recently reported that severe preexisting iris damage was a risk factor for graft failure and rapid ECD loss after DSAEK.[12] Furthermore, we found that the inflammatory cytokine levels in AqH increased in eyes with BK and reduced ECD.[13] We postulate that the results of the elevated proinflammatory cytokine levels may represent "chronic pathological inflammation," which might induce the elevation of IL-10, an anti-inflammatory cytokine, due to the negative feedback mechanism.[37,38] Chronic inflammation may induce endothelial cell loss over time after intraocular surgeries or affect the graft survival after corneal transplantation, especially in eyes with iris damage. It would be valuable to study the correlation analyses between preoperative aqueous cytokines and the decrease in ECD over time after corneal transplantation, to assess the influence of elevated levels of cytokines on the endothelial cell loss and for identifying the cytokines that determine the fate of endothelial cell survival after corneal transplantation.

Multifactorial regression analyses identified that the iris damage severity significantly correlated with the presence of complicated glaucoma and number of previous intraocular surgeries. Regarding the impact of complicated glaucoma on the aqueous cytokine levels, the current study included 23 eyes with complicated glaucoma (12 eyes after trabeculectomy for the treatment of primary open angle glaucoma [POAG], 8 eyes with secondary glaucoma after complicated intraocular surgeries, and 3 eyes with congenital glaucoma after multiple glaucoma surgeries). We expected higher levels of AqH cytokines in the eyes with histories of complicated glaucoma, because glaucoma surgery has been reported as a factor for reduced ECD after corneal transplantation.[25,31,33] Among the cytokines, the levels of the following cytokines were high especially in the eyes with severe iris damage ($N = 12$): IL-1α (108 ± 29.6 pg/mL), IL-6 (799 ± 344 pg/mL), MCP-1 (942 ± 154 pg/mL), IFN-γ (72.4 ± 6.5 pg/mL), and TNF-α (166 ± 27.1 pg/mL). In contrast, all the four eyes with POAG, which had normal IOPs at the time of AqH collection, had no iris damage and had normal AqH cytokine levels (IL-1α: 48.9 ± 4.3 pg/mL, IL-6: 16.0 ± 6.8 pg/mL, MCP-1: 648 ± 80 pg/mL, IFN-γ: 56.9 ± 2.4 pg/mL, and TNF-α: 77.1 ± 5.3 pg/mL).[13] Although we had to increase the number of eyes with complicated glaucoma and uncomplicated POAG for statistical analyses, the results suggest that the presence of complicated glaucoma leads to elevated levels of aqueous cytokines as reported previously.[13,39]

Table 6. Correlation Between Aqueous Cytokine Levels and Iris Damage Score

Aqueous Cytokine	Correlation Coefficient	*P* Value
IL-1α	0.276	0.0002
IL-1β	0.272	0.0004
IL-4	0.392	<0.0001
IL-6	0.490	<0.0001
IL-8	0.459	<0.0001
IL-10	0.457	<0.0001
IL-12p70	0.316	<0.0001
IL-13	0.278	0.0006
IL-17A	0.294	0.0002
MIP-1α	0.161	0.055
MIP-1β	0.028	0.695
MCP-1	0.293	<0.0001
TNF-α	0.279	0.0007
GM-CSF	0.206	0.08
IFN-α	0.174	0.028
IFN-γ	0.305	<0.0001
E-Selectin	0.289	<0.0001
P-Selectin	0.352	<0.0001
sICAM-1	0.454	<0.0001
IP-10	0.343	<0.0001

Spearman's correlation analysis.

Furthermore, the study showed that the levels of aqueous protein and cytokine were elevated in the eyes with iris damage both in phakic and pseudophakic conditions. In phakic eyes, the causes of iris damage were LI and surgical iris damage during corneal transplantation and trabeculectomy, whereas the phakic eyes without iris damage included cataract eyes and eyes with FECD. We previously reported the elevation of aqueous cytokines in eyes with a history of LI and past intraocular surgeries.[13] To assess the association between iris damage and the cytokine levels in eyes after corneal transplantation, we compared the levels of aqueous cytokines among the different iris damage severity after selecting the eyes after corneal transplantation (Supplementary Table S1, 19 eyes after PKP, 6 eyes after DSAEK, and 2 eyes after anterior lamellar keratoplasty). The intraocular surgeries that caused the iris damage were performed 6 months to 10 years before the AqH collection. Although we have to evaluate the association among the kind of surgeries and the interval between surgeries and AqH collection after increasing the number of subjects, we postulated that iris damage during complicated intraocular surgery might induce the elevated levels of aqueous cytokines over the years in a chronic fashion.

The previous laboratory studies showed that the interaction among iris pigment epithelial cells (I-CB cells in the literature), and the components of the AqH might maintain the homeostasis of the immune system in the anterior chamber. The previous reports on the immunosuppressive effects of the iris pigment epithelium are shown in Table 7.[8,9,12,40–48] Streilein et al.[7] reported that the iris pigment epithelial cells have immunomodulatory properties. The elevated cytokine levels might be attributed to the breakdown of the BAB because the aqueous protein level correlated significantly with the iris damage severity ($r = 0.434$, $P < 0.001$). It is tempting to speculate that the results of this study suggest that the lack of immunomodulatory factors from the I-CB cells can change the microenvironment in the AqH, such as elevation in the levels of aqueous inflammatory cytokines, considering the results of previous laboratory reports. In general, cytokines are produced

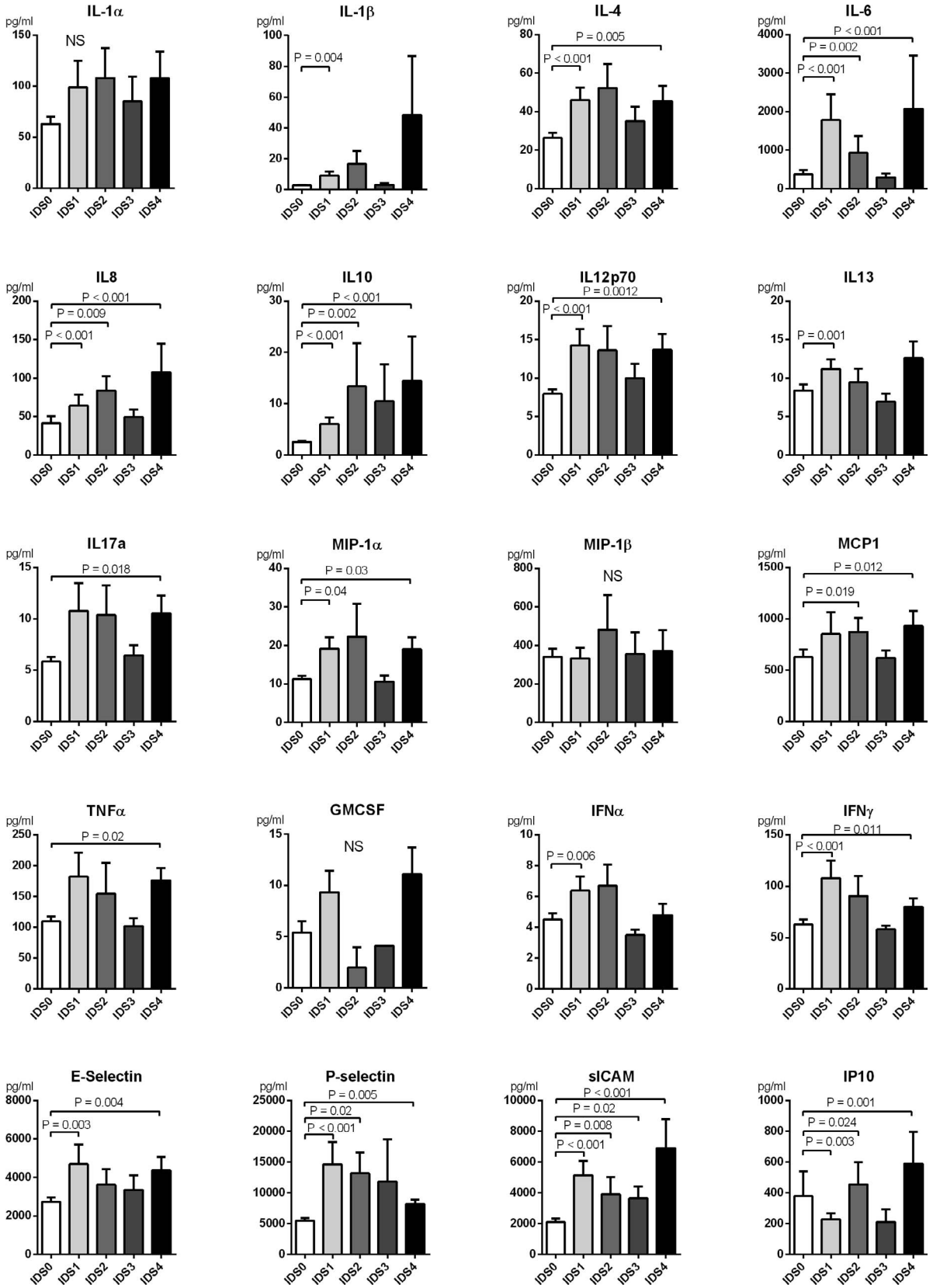

Figure 4. Cytokine levels in aqueous humor stratified based on iris damage score. Levels of some cytokines were higher in the eyes with IDS 1-4 compared with the eyes with IDS 0.

Table 7. Literature Review on the Association Between Iris Pigment Epithelium and Anterior Chamber Inflammation

Author (y)	Cell Line/ Animal/ Human	Model/ Subjects	Molecules	Principal Source	Principal Cellular Targets and Biologic Effects
Streilein, et al. (1990)[8]	Cell line	MLR	TGF-β	AqH	T cell; suppress proliferation
Streilein, et al. (1991)[9]	Cell line	MLR	NA	I-CB	T cell; suppress proliferation
Suzuma, et al. (1997)[40]	Rat	EIU	P-selectin	I-CB	Immune cells; increased cell infiltration in the iris and aqueous protein levels
Suzuma, et al. (1998)[41]	Rat	EIU	E-selectin	I-CB, retina	Immune cells; increased cell infiltration in the iris and aqueous protein levels
Marie, et al. (1999)[42]	Rat	EIU	TNF-α, IL-6, IL-13	I-CB	Immune cells; IL-13 suppresses cell infiltrates in the iris by downregulating TNF-α and IL-6
Yoshida, et al. (2000)[43]	Cell line/ mouse	MLR/ACAID	TGF-β	I-CB/AqH	T cell; suppress proliferation Downregulate IFN-γ, IL-2, IL-4 and IL-10
Ohta, et al. (2000)[44]	Mouse	EIU/MLR	TGF-β		T cell; suppress proliferation
Yoshida, et al. (2000)[45]	Cell line/ mouse	MLR	CD95	I-CB	T cell; suppress proliferation Downregulate IFN-γ, IL-2, IL-4.
Lemaitre, et al. (2001)[46]	Mouse	EIU	IL-13	Exogenous IL-13	Exogenous IL-13 suppresses cell infiltrates in the iris by downregulating MCP-1 and MIP
Mo, et al. (2003)[47]	Mouse	PDS/ACAID	CD95	I-CB	I-CB contribute to ACAID and suppress T cell proliferation
Sugita, et al. (2007)[48]	Cell line	Treg assay	B7	I-CB	I-CB induce Treg
Ishii, et al. (2016)[12]	Human	EK	NE	NE	Iris damage was associated with endothelial cell loss, leading to graft failure
The current study	Human	Cataracts/corneal disease	Cytokine	NE	Iris damage was associated with elevated cytokine levels in AqH

MLR, mixed lymphocyte reactio; NA, not available; EIU, endotoxin-induced uveitis; ACAID, anterior chamber associated immune deviation; PDS, pigment dispersion syndrome; Treg, regulatory T cell; EK, endothelial keratoplasty; NE, not evaluated; BK, bullous keratopathy.

in immune cells in response to specific stimuli, such as infection, trauma, or autoimmune diseases. Hence, further evaluation of the causes of chronic inflammation in the anterior chamber is needed in future studies.

Because cytokines are very sensitive, some systemic condition can affect the aqueous cytokine levels, as reported for the elevation of tear cytokine levels with deterioration of rheumatoid arthritis.[49] Systemic inflammatory conditions can also affect AqH cytokine levels via elevation of serum cytokines or breakdown of the BAB. We thoroughly checked the clinical records of all patients regarding steroid use and systemic diseases. There were nine patients with systemic inflammatory diseases: three patients with rheumatoid arthritis, two patients with asthma, one patient with atopic dermatitis, one patient with polymyositis, one patient with polyneuritis, and one patient with Sjogren syndrome (2 patients without iris damage, 3 patients with mild iris damage, and 4 patients with severe iris damage). We compared the aqueous protein and cytokine levels between these nine patients and 192 patients without systemic diseases and found that there were no statistically significant differences in the aqueous protein and cytokine levels, except IL-8 (39.7 ± 32.0 pg/mL in the 9 patients with systemic inflammatory diseases and 54.0 ± 102pg/mL in the 192 patients without systemic inflammatory diseases). Topical steroids can also potentially be used as a treatment modality to suppress AqH cytokine levels. Regarding steroid use, topical steroids were administered in the eyes after corneal transplantation (10 eyes), pseudophakic bullous keratopathy (PBK; eight eyes), and trabeculectomy (three eyes), when the AqH samples were collected. No patients received systemically administered steroids. We analyzed the differences in protein and cytokine levels between the eyes administered with topical steroids and the eyes that did not receive such treatment. However, we did not find any clinically relevant difference.

This study has some limitations. Firstly, this study included only patients who had undergone corneal transplantation and cataract surgery, and did not include patients with retinal or glaucoma diseases with normal ECD. All the eyes that underwent multiple complicated intraocular surgeries had severe iris damage and this study did not include eyes with a healthy iris, which underwent several intraocular surgeries. Such heterogenous selection of subjects can induce selection bias. In such eyes, the elevation in the levels of aqueous cytokines has been reported and we have to consider other reasons for the elevated levels of aqueous cytokines.[15,16,50,51] We will have to conduct comprehensive studies to evaluate the effect of different kinds of surgeries on aqueous cytokines in the future. Second, whereas the aqueous proteins level was directly correlated with the severity of iris damage (Figs. 3), there were no statistically significant differences in aqueous cytokine levels between the eyes with mild and severe iris damage (Supplementary Fig. S1). Iris damage was associated with various clinical factors and the measurement of cytokines is very sensitive, which might make the results of cytokine levels more complex in the context of iris damage. Thus, we will conduct proteomics analysis of AqH to find the essential molecular mechanism for the results in a comprehensive way. Thirdly, we included only Japanese eyes with brown pigmented iris. In Asian eyes, iris damage is easy to identify, compared with in eyes with less pigmentation as in the iris of Caucasian eyes. We need further studies to evaluate the effect of iris damage on the levels of aqueous cytokines in other races. Forth, the interval between the previous glaucoma surgery and AqH collection can affect the aqueous levels of cytokines. The mean interval was 14.4 ± 14.8 years, ranging from 2 to 54 years (total 15 eyes: 12 eyes after trabeculectomy and 3 eyes with multiple glaucoma surgeries). The aqueous levels of protein and cytokines were not significantly correlated with the interval between the glaucoma surgery and AqH collection, although we have to increase the number of subjects after glaucoma surgery. The other limitation is that we could not

grade flare in the AqH owing to the presence of corneal opacities in some of the subjects.

In conclusion, we have shown that the iris damage is associated with elevated levels of aqueous protein as well as inflammatory cytokines, such as IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17A, MCP-1, TNF-α, E-selectin, P-selectin, MIP-1α and sICAM-1. The multivariate analyses revealed that the iris damage severity is associated with the number of previous intraocular surgeries and with the presence of complicated glaucoma. Thus, the preexisting iris damage can be one of the useful clinical parameters for chronic breakdown of BAB and elevated inflammatory cytokines, although there was no direct correlation between the extent of iris damage and the level of cytokines.

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