

Preoperative Aqueous Cytokine Levels are Associated With Endothelial Cell Loss After Descemet's Stripping Automated Endothelial Keratoplasty

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PURPOSE. To evaluate the association between endothelial cell density (ECD) after Descemet's stripping automated endothelial keratoplasty (DSAEK) and preoperative cytokine levels in the aqueous humor (AqH).

METHODS. This prospective consecutive case series included 97 consecutive patients who underwent DSAEK (64 eyes) or cataract surgery (33 eyes). AqH samples were collected at the beginning of each surgery. The levels of cytokines (IL-1 α , IL-1 β , IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IFN- α , IFN- γ , monocyte chemoattractant protein [MCP]-1, E-selectin, P-selectin, and soluble intercellular adhesion molecule [sICAM]-1) in the AqH were measured by multiplex beads immunoassay. The correlations between preoperative aqueous cytokine levels and the ECD at 12 months after DSAEK were analyzed.

RESULTS. The ECD decreased from 2747 ± 259 cells/mm² in the donor graft to 1235 ± 607 cells/mm² at 12 months after DSAEK. In all subjects undergoing DSAEK, the postoperative ECD at 12 months was significantly correlated with the preoperative levels of MCP-1 ($r = -0.467$, 95% confidence interval [CI]: -0.650 to -0.222 , $P = 0.0003$). In an analysis excluding Fuchs endothelial corneal dystrophy (11 eyes), the ECD at 12 months after DSAEK was significantly correlated with preoperative levels of IL-17A ($r = -0.635$, 95% CI: -0.819 to -0.319 , $P = 0.0004$), MCP-1 ($r = -0.605$, 95% CI: -0.779 to -0.345 , $P < 0.0001$), IFN- γ ($r = -0.633$, 95% CI: -0.796 to -0.385 , $P < 0.0001$), E-selectin ($r = -0.516$, 95% CI: -0.756 to -0.276 , $P = 0.0004$), and sICAM-1 ($r = -0.537$, 95% CI: -0.735 to -0.253 , $P = 0.0005$).

CONCLUSIONS. Higher preoperative levels of IL-17A, MCP-1, IFN- γ , E-selectin, and sICAM-1 in the AqH were associated with lower ECD after DSAEK for bullous keratopathy.

Keywords: aqueous humor, cytokine, endothelial cell density, Descemet's stripping automated endothelial keratoplasty

Descemet's stripping automated endothelial keratoplasty (DSAEK) for the treatment of endothelial dysfunction has several advantages over standard penetrating keratoplasty (PKP).¹⁻³ By removing only the Descemet's membrane and dysfunctional endothelium, and retaining healthy portions of the patient's cornea, DSAEK offers rapid visual recovery,^{2,3} resulting in less graft rejections than PKP and leading to a favorable long-term graft survival rate up to 80% to 87% at 5 years.^{4,5} The primary cause of graft failure after DSAEK is endothelial decompensation, even in eyes without evidence of immunologic rejection.⁴⁻⁶ The endothelial cell density (ECD) decreases with age, and in various conditions including uveitis and postintraocular surgeries.⁷⁻¹⁰ The risk factors for endothelial cell loss after DSAEK include a history of glaucoma surgery and graft rejection.^{5,11-13} In contrast, the ECD prognosis is reported to be favorable in eyes with Fuchs' endothelial corneal dystrophy (FECD) compared with pseudophakic bullous

keratopathy.¹⁴ However, the exact mechanism of chronic endothelial cell loss is still poorly understood.

Recently, we reported that severe pre-existing iris damage was one of the clinical factors for graft failure and rapid endothelial cell loss after DSAEK.⁶ However, the reasons behind decreased ECD in eyes with severe iris damage remain elusive. Anatomically, the aqueous humor (AqH) is present between the corneal endothelium and the iris, and it has been reported that inflammatory cytokines in the AqH increase during various pathological processes.¹⁵⁻¹⁸ In an in vitro study, a combination of proinflammatory cytokines synergistically induced the apoptosis of corneal endothelial cells.¹⁹ We recently showed that inflammatory cytokine levels were elevated in the AqH of eyes with bullous keratopathy and reduced ECDs,²⁰ and that iris damage was associated with an elevation in aqueous cytokine levels.²¹ Collectively, these results suggest that inflammatory factors in the AqH directly influence endothelial



cell loss. However, an elevation in aqueous cytokine levels can just be the result, not the direct cause, following endothelial cell loss. Thus, we hypothesized that the AqH conditions, such as elevated cytokine levels, can cause rapid loss of ECD after corneal transplantation. In our latest study, we showed that higher preoperative aqueous levels of specific cytokines were associated with rapid ECD loss after PKP.²² The purpose of this study is to evaluate the association between preoperative cytokine levels in the AqH and reduction of ECD following DSAEK.

METHODS

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

Study Participants

A total of 97 consecutive patients who underwent DSAEK (DSAEK group, 64 eyes) and cataract surgery (control group, 33 eyes) at Ichikawa General Hospital, Tokyo Dental College, from October 26, 2015 to August 10, 2016 were included (Table 1). We excluded eyes with active inflammation of the cornea or the anterior chamber and patients systemically administered steroids from the study. The etiologies of DSAEK in the studied eyes included pseudophakic bullous keratopathy (25 eyes), postlaser iridotomy bullous keratopathy (11 eyes), FECD (11 eyes), posttrabeculectomy bullous keratopathy (10 eyes), uveitis (4 eyes), birth injury (2 eyes), and unknown cause (1 eye). We performed solitary DSAEK in 43 eyes and DSAEK combined with simultaneous cataract surgery in 21 eyes. Control participants were defined as patients who underwent cataract surgery without uveitis or systemic inflammatory diseases, such as ulcerative colitis or rheumatoid arthritis, and had not undergone corneal or intraocular surgeries previously. All participants in the control group had an ECD exceeding 2000 cells/mm².

Surgical Technique

DSAEK surgery was performed using double-glide technique.^{23,24} All DSAEK surgeries were performed by one of three experienced surgeons (TY, YS, or JS). After sub-Tenon anesthesia with injection of 2% lidocaine, a 5.0-mm temporal corneoscleral incision was made. An anterior chamber maintenance cannula was inserted through the 2 or 10 o'clock paracentesis, and Descemet stripping was performed with a reverse-bent Sinsky hook (Asico, Westmont, IL, USA). The recipient's endothelium and Descemet's membrane were carefully removed using forceps. Precut donor grafts were trephined and the endothelial surface of the donor lenticle was coated with a small amount of viscoelastic material. Donor tissue was gently inserted into the anterior chamber using a Busin glide (Asico) and Shimazaki DSAEK forceps (Inami, Tokyo, Japan). Air was carefully injected into the anterior chamber to unfold the graft. At 10 minutes after air injection, half of the air was replaced by balanced salt solution (BSS; Alcon, Fort Worth, TX, USA). At the end of the surgery, 2-mg subconjunctival betamethasone was administered. In patients with significant lens opacity (21 eyes), standard phacoemulsification, and aspiration were performed with implantation of an IOL, followed by the DSAEK procedure. All DSAEK procedures were successful and uneventful, without any excessive intraoperative manipulation. In 5 eyes, early postop-

TABLE 1. Preoperative Demographics of Patients

	DSAEK Group	Control	P Value
Eyes, <i>n</i>	64	33	
Sex, <i>n</i> (%)			
Male	23 (36.0)	16 (48.5)	0.23*
Female	41 (64.0)	17 (51.5)	
Age, y	74.3 ± 9.3	75.9 ± 7.6	0.47
BCVA, logMAR	1.29 ± 0.74	0.38 ± 0.36	<0.001
IOP, mm Hg	12.4 ± 4.1	14.3 ± 2.6	0.04
Axial length, mm	23.63 ± 2.08	23.84 ± 1.58	0.58
ECD, cells/mm ²	422 ± 54	2760 ± 343	<0.001
CCT, μm	741 ± 111	534 ± 19	<0.001
DM, <i>n</i> (%)	10 (16)	1 (3)	0.06*
Presence of glaucoma, <i>n</i> (%)	17 (27)	0 (0)	0.001*
IOL, <i>n</i> (%)	43 (67)	0 (0)	<0.001*
Previous intraocular surgeries, <i>n</i>	1.6 ± 1.5	0.0 ± 0.0	<0.001

Mean ± SD. BCVA, best-corrected visual acuity; AL, axial length; CCT, central corneal thickness; DM, diabetes mellitus; NA, not available; SD, standard deviation.

* χ^2 test.

erative double chamber necessitated air injection and resolved without any serious complications. We excluded these eyes from the correlation analyses, because air injection is associated with ECD loss after DSAEK.^{5,6,25} One patient had mild IOP elevation up to 22 mm Hg, which resolved with a topical antiglaucoma agent. There was no case with graft rejection up to the 12-month follow-up. Patients were prescribed topical eyedrops levofloxacin (Cravit; Santen, Osaka, Japan) and betamethasone 0.1% eyedrops (Sanbetazon; Santen) five times a day. Topical betamethasone eyedrops were tapered over the following 6 months. Starting from 6 months after DSAEK, we prescribed fluorometholone 0.1% eyedrops (Flumetholone 0.1; Santen) three times a day for up to 12 months after surgery.

Aqueous Humor Samples

The AqH samples containing 70 to 300 μL were obtained under sterile conditions at the beginning of surgery after topical anesthesia in DSAEK and cataract surgery. First, paracentesis was placed at the clear cornea. AqH sample was obtained using a 27-G needle taking care not to touch the iris, the lens, or corneal endothelium. The samples were centrifuged at 3000g for 5 minutes. The soluble fractions were collected and stored at -80°C until measurements.

Protein and Cytokine Level Measurements

The protein concentrations of AqH samples were determined using the DC protein assay (Bio-Rad, Hercules, CA, USA).²⁰ In brief, bovine serum albumin (BSA) was used as a standard in the range of 0.23 to 1.37 mg/mL. Samples (5 μL) of BSA and AqH were added to 96-well microplates, followed by immediate addition of a mixture containing 25 μL reagent A and 200 μL reagent C. After 15 minutes of incubation at room temperature in the dark, the microplates were read at 690 and 405 nm using a microplate reader (Model 550; Bio-Rad).

The cytokine levels of IL-1 α , IL-1 β , IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IFN- α , IFN- γ , monocyte chemoattractant protein [MCP]-1, TNF- α , E-selectin, P-selectin, soluble intercellular adhesion molecule [sICAM]-1, macrophage inflammatory protein [MIP]-1 α , MIP-1 β , and interferon gamma-induced protein [IP]-10 in AqH samples were measured using Luminex

(ProcaPlex kit; Luminex, San Antonio, TX, USA) beads-based multiplex immunoassay according to previous reports.²⁶ Briefly, 50 μ L of AqH samples were incubated with antibody-coated capture beads in an incubation buffer at room temperature. After 2-hour incubation, the beads were washed three times using washing buffer, and phycoerythrin-labeled streptavidin was added for 30 minutes in the dark at room temperature. After being washed three times with washing buffer, plates were resuspended in 150 μ L of reading buffer, and the assays were performed using a Luminex 200.

Data Analysis

Routine examinations, including slit-lamp evaluation, best spectacle-corrected distance visual acuity, IOP, and ECD, were performed preoperatively and at 1, 3, 6, and 12 months after surgery. The ECD was measured by masked orthoptists using a specular microscopy system (EM-4000; TOMEY, Nagoya, Japan). Approximately, 50 cells were analyzed for mean cell density. ECD was determined by the automated software of EM-4000. In eyes in which the automated cell counts failed or misidentified endothelial cells, ECD was determined using the center method for manual counting. We analyzed ECD as absolute ECD and percentage ECD loss (%ECD loss) to assess the correlation with preoperative cytokine levels. Percent ECD loss was defined as follows: %ECD loss = [(graft ECD – postoperative ECD) / graft ECD] \times 100. There were eight patients with graft failure within 1 year after DSAEK (primary graft failure in four eyes, graft failure at 3 months in 2 eyes and at 6 months in 2 eyes). We defined the ECD as 300 cells/mm² as previously reported,⁶ because the ECD could not be directly measured due to corneal edema. At 1 and 3 months, ECD measurement was difficult in some patients due to residual corneal edema or interface irregularity. As a result, the number of eyes with successful ECD measurements at 1, 3, 6, and 12 months following DSAEK were 49, 58, 58, and 56, respectively. We classified patients into two groups based on the ECD at 12 months after DSAEK: one group included eyes where the ECD was more than 1200 cells/mm² and the other group included eyes where the ECD was less than 1200 cells/mm² at 12 months. The cut-off value of 1200 was set following our previous study.²²

Statistical Analysis

Data were analyzed using Prism for Windows software (version 6.04; Graphpad Software, Inc., La Jolla, CA, USA). The D'Agostino & Pearson omnibus normality test was used to assess whether the data showed a normal distribution. Spearman's correlation analyses were used to evaluate the correlations among AqH cytokine levels and ECD. From the correlation analyses, we excluded five eyes in which air injection was performed for the treatment of postoperative double chamber, because air injection is associated with the ECD loss after DSAEK.^{5,6,25} To assess the differences in the time courses of decreases in postoperative ECD between the ECD \geq 1200 and the ECD <1200 groups, a one-way ANOVA was used. To compare the differences in protein and cytokine levels across the groups, the Mann-Whitney *U* test was used. For multivariate analyses, we used STATA/IC 14.0 for Windows (StataCorp LP, College Station, TX, USA). To assess the clinical factors that can be correlated with the postoperative ECD, we selected five clinical factors (graft size, the presence of glaucoma, preoperative steroid use, history of laser iridotomy [LI]/trabeculectomy, and the lens status) and conducted multiple linear regression analyses. The presence of glaucoma, preoperative steroid use, history of LI/trabeculectomy, and the lens status were dichotomized as independent variables

TABLE 2. Preoperative Aqueous Cytokine Levels

	DSAEK (N = 64)	Control (N = 33)	P Value*
Protein	1.25 \pm 0.13 (1.18)	0.31 \pm 0.40 (0.25)	<0.0001
IL-1 α	69.3 \pm 14.8 (55.9)	49.3 \pm 4.4 (44.2)	0.046
IL-1 β	5.2 \pm 1.7 (1.8)	3.1 \pm 1.7 (1.1)	0.128
IL-4	45.0 \pm 6.5 (29.8)	20.5 \pm 1.0 (20.8)	0.005
IL-6	1668 \pm 450 (426)	85 \pm 69 (5.1)	<0.0001
IL-8	93.7 \pm 18.2 (49.8)	46.4 \pm 25.3 (21.4)	<0.0001
IL-10	10.6 \pm 4.2 (3.3)	1.9 \pm 0.1 (1.7)	<0.0001
IL-12p70	12.9 \pm 1.7 (8.6)	6.4 \pm 0.2 (6.4)	<0.0001
IL-13	9.5 \pm 0.9 (9.2)	7.1 \pm 0.2 (7.1)	0.0047
IL-17A	10.3 \pm 1.6 (7.2)	4.0 \pm 0.4 (3.5)	<0.0001
MIP-1 α	14.7 \pm 3.2 (11.2)	9.4 \pm 0.4 (8.7)	0.335
MIP-1 β	345 \pm 79.1 (226)	345 \pm 20.3 (321)	0.100
MCP-1	1022 \pm 111 (720)	592 \pm 84.4 (470)	0.0005
TNF- α	162 \pm 178 (97.2)	76.4 \pm 5.4 (70.2)	0.0116
IFN- α	4.8 \pm 0.6 (4.2)	4.1 \pm 0.1 (3.9)	0.520
IFN- γ	106.3 \pm 15.8 (70.3)	55.6 \pm 2.1 (54.6)	0.0018
E-selectin	4170 \pm 640 (3104)	2149 \pm 46.0 (2145)	<0.0001
P-selectin	11323 \pm 2242 (7431)	3724 \pm 131 (3581)	<0.0001
sICAM-1	5971 \pm 882 (3966)	2027 \pm 450 (1304)	<0.0001
IP10	295 \pm 319 (157)	257 \pm 89.4 (98.3)	0.0251

Mean \pm SE (Median); Protein:(mg/mL); Cytokines:(pg/mL). Statistically significant values are in bold.

* Mann-Whitney *U* test. SE, standard error.

(variance inflation factors [VIF] = 1.14–1.16). The data are expressed as means \pm standard deviation (SD). *P* values < 0.05 were considered to be statistically significant except cytokine data. Cytokine data were also controlled with Bonferroni correction. Because there were 20 different comparisons (1 protein and 19 cytokines), *P* values < 0.0025 (i.e., *P* = 0.05/20) were considered to be statistically significant after Bonferroni correction.

RESULTS

Pre- and Postoperative ECD

In the 64 eyes that underwent DSAEK, the ECD was 2747 \pm 259 cells/mm² in the donor graft, 1815 \pm 592 cells/mm² at 1 month, 1470 \pm 623 cells/mm² at 3 months, 1294 \pm 600 cells/mm² at 6 months, and 1235 \pm 607 cells/mm² at 12 months after DSAEK. There were significant correlations among the postoperative ECD at 3, 6, and 12 months (Supplementary Table S1). Between the two groups (ECD \geq 1200 group and ECD <1200 group), there were no significant differences in age (72.4 \pm 10.8 and 74.5 \pm 8.5 years, respectively; *P* = 0.90) and graft ECD (2774 \pm 179 and 2700 \pm 327 cells/mm², respectively; *P* = 0.49). There were significant differences in ECD at 1, 3, 6, and 12 months after DSAEK between the two groups (Supplementary Fig. S1, *P* = 0.02, *P* = 0.01, *P* < 0.001, and *P* < 0.001, respectively).

Preoperative Protein and Cytokine Levels in Aqueous Humor

The preoperative levels of AqH protein, IL-6, IL-8, IL-10, IL-12p70, IL-17A, MCP-1, IFN- γ , E-selectin, P-selectin, and sICAM-1 were significantly higher in eyes undergoing DSAEK compared with the control group (Table 2, all *P* \leq 0.0018). In the ECD \geq 1200 group, the levels of IL-6, IL-10, E-selectin, and P-selectin were significantly higher compared with those of the control group (Supplementary Table S2, all *P* \leq 0.0025). In the ECD <1200 group, the levels of protein, IL-4, IL-6, IL-

TABLE 3. Correlations Between Preoperative Cytokine Levels and Endothelial Cell Density After Descemet's Automated Stripping Endothelial Keratoplasty ($N = 64$)

	ECD at 6 mo			ECD at 12 mo		
	<i>r</i>	<i>P</i> Value	95% CI	<i>r</i>	<i>P</i> Value	95% CI
Protein	-0.275	0.068	-0.532 to 0.029	-0.256	0.086	-0.515 to 0.046
IL-1 α	0.248	0.092	-0.051 to 0.506	0.159	0.292	-0.146 to 0.437
IL-1 β	-0.128	0.396	-0.411 to 0.177	-0.037	0.810	-0.335 to 0.268
IL-4	-0.109	0.408	-0.360 to 0.157	-0.287	0.032	-0.517 to -0.018
IL-6	0.008	0.995	-0.258 to 0.260	-0.039	0.769	-0.304 to 0.231
IL-8	-0.008	0.995	-0.266 to 0.252	-0.090	0.505	-0.350 to 0.182
IL-10	0.095	0.470	-0.170 to 0.348	0.016	0.907	-0.255 to 0.285
IL-12p70	-0.125	0.349	-0.378 to 0.145	-0.297	0.031	-0.527 to 0.020
IL-13	-0.051	0.763	-0.373 to 0.283	-0.106	0.539	-0.428 to 0.240
IL-17A	-0.399	0.0088	-0.633 to -0.100	-0.389	0.011	-0.625 to -0.087
MIP-1 α	0.248	0.118	-0.074 to 0.523	0.226	0.154	-0.098 to 0.512
MIP-1 β	0.237	0.075	-0.033 to 0.475	0.145	0.302	-0.139 to 0.406
MCP-1	-0.320	0.018	-0.545 to -0.052	-0.467	0.0003	-0.650 to -0.222
TNF- α	0.177	0.261	-0.143 to 0.464	0.240	0.131	-0.082 to 0.517
IFN- α	0.245	0.128	-0.082 to 0.524	0.144	0.381	-0.189 to 0.447
IFN- γ	-0.317	0.018	-0.543 to -0.100	-0.387	0.0029	-0.593 to -0.133
E-selectin	-0.336	0.017	-0.567 to -0.055	-0.336	0.045	-0.519 to 0.001
P-selectin	-0.275	0.042	-0.510 to -0.003	-0.275	0.042	-0.510 to -0.003
sICAM-1	-0.351	0.0086	-0.569 to -0.009	-0.355	0.0067	-0.569 to -0.0096
IP10	0.110	0.497	-0.153 to 0.359	0.067	0.618	-0.204 to 0.329

Statistically significant values are in bold.

12p70, IL-17A, IFN- γ , E-selectin, P-selectin, and sICAM-1 were significantly higher compared with those of the control group (all, $P \leq 0.0025$). Although there were no statistically significant differences, the levels of MCP-1 and IFN- γ were higher in the ECD <1200 group compared with the ECD ≥ 1200 group ($P = 0.01$ and $P = 0.02$, respectively).

Correlations Between Preoperative Aqueous Cytokine Levels and Postoperative ECD

Table 3 shows the correlations between preoperative aqueous cytokine levels and absolute ECDs at 6 and 12 months after DSAEK in all subjects. The ECD at 12 months was inversely correlated with the levels of MCP-1 ($r = -0.467$, 95% confidence interval [CI]: -0.650 to -0.222, $P = 0.0003$). The %ECD loss at 12 months was inversely correlated with the levels of MCP-1 (Supplementary Table S4, $r = 0.470$, 95% CI: 0.232-0.656, $P = 0.0002$). In contrast, all of the preoperative

cytokine levels were not correlated with ECD and %ECD loss at 6 months after cataract surgery (Supplementary Table S5).

Associations Between Endothelial Cell Density and Presence of Glaucoma

Lens status, history of LI and presence of glaucoma have been reported to be factors related to ECD reduction.^{5,8,9,27} To evaluate the association between ECD and these factors, we conducted multivariate regression analyses in which the presence of glaucoma, history of LI, graft size, preoperative steroid use, and lens status were included as independent variables (Table 4). The presence of glaucoma was significantly associated with lower ECD ($\beta = -0.321$, $P = 0.024$ at 3 months, $\beta = -0.339$, $P = 0.009$ at 6 months and $\beta = -0.317$, $P = 0.010$ at 12 months). History of LI was associated with lower ECD ($\beta = -0.257$, $P = 0.046$ at 6 months, and $\beta = -0.333$, $P = 0.008$ at 12 months). History of trabeculectomy was associated with lower ECD after DSAEK ($\beta = -0.352$, $P = 0.011$ at 3 months, $\beta = -0.287$, $P = 0.028$ at 6 months, and $\beta = -0.278$, $P = 0.026$ at 12 months; Supplementary Table S6).

TABLE 4. Multivariate Analysis: Presence of Glaucoma Was Associated With Postoperative Endothelial Cell Density

	ECD at 3 mo		ECD at 6 mo		ECD at 12 mo	
	β	<i>P</i> Value	β	<i>P</i> Value	β	<i>P</i> Value
Glaucoma	-0.321	0.024	-0.339	0.009	-0.317	0.010
Laser iridotomy	0.074	0.588	-0.257	0.046	-0.333	0.008
Lens status	-0.089	0.531	-0.068	0.597	-0.197	0.113
Graft size	0.086	0.516	0.135	0.270	0.185	0.112
Prerogative steroid use	-0.044	0.752	0.064	0.626	-0.032	0.801
Adjusted R^2	0.075		0.169		0.361	

Presence of glaucoma, laser iridotomy and lens status were dichotomized as categorical variables for multivariate regression analysis as follows: presence of glaucoma: absence = 0, presence = 1, laser iridotomy: absence = 0, presence = 1, lens status: phakia = 0, pseudophakia = 1. VIF = 1.16. Statistically significant values are in bold.

Correlations Between Preoperative Aqueous Cytokine Levels and Postoperative ECD in Subjects Excluding Fuchs Endothelial Corneal Dystrophy

A limitation of this study is the heterogeneity in the causative diseases. Then, we conducted correlation analyses between preoperative aqueous cytokine levels and postoperative ECD excluding the eyes with FECD (Table 5; Supplementary Table S7). The ECDs were inversely correlated with the levels of IL-17A ($r = -0.652$, $P = 0.0002$ at 6 months and $r = -0.631$, $P = 0.0004$ at 12 months), MCP-1 ($r = -0.605$, $P < 0.0001$ at 12 months), IFN- γ ($r = -0.528$, $P = 0.0008$ at 6 months and $r = -0.633$, $P < 0.0001$ at 12 months), E-selectin ($r = -0.588$, $P < 0.0003$ at 6 months and $r = -0.516$, $P = 0.0004$ at 12 months), and sICAM-1 ($r = -0.537$, $P = 0.0005$ at 12 months). The %ECD loss was correlated with the levels of IL-17A, MCP-1, IFN- γ , E-

TABLE 5. Correlation Between Preoperative Cytokine Levels and Endothelial Cell Density After DSAEK in Eyes Excluding Fuchs' Endothelial Corneal Dystrophy

	ECD at 6 mo			ECD at 12 mo		
	<i>r</i>	<i>P</i> Value	95% CI	<i>r</i>	<i>P</i> Value	95% CI
Protein	-0.262	0.170	-0.581 to +0.127	-0.099	0.624	-0.471 to 0.303
IL-1 α	0.292	0.124	-0.094 to 0.602	0.290	0.135	-0.105 to 0.605
IL-1 β	-0.207	0.320	-0.565 to 0.216	-0.043	0.844	-0.449 to 0.378
IL-4	-0.346	0.039	-0.612 to -0.010	-0.439	0.0065	-0.674 to -0.125
IL-6	0.080	0.648	-0.270 to 0.569	0.070	0.699	-0.290 to 0.412
IL-8	0.020	0.909	-0.324 to 0.360	0.081	0.654	-0.280 to 0.422
IL-10	0.078	0.662	-0.277 to 0.414	0.121	0.507	-0.248 to 0.459
IL-12p70	-0.368	0.032	-0.635 to -0.024	-0.430	0.010	-0.673 to -0.102
IL-13	-0.243	0.276	-0.611 to 0.212	-0.220	0.351	-0.612 to 0.260
IL-17A	-0.652	0.0002	-0.831 to -0.352	-0.631	0.0004	-0.819 to -0.319
MIP-1 α	0.356	0.081	-0.058 to +0.665	0.482	0.017	0.085 to 0.747
MIP-1 β	0.365	0.034	0.021 to 0.632	0.266	0.141	-0.102 to 0.570
MCP-1	-0.452	0.0050	-0.682 to -0.140	-0.605	<0.0001	-0.779 to -0.345
TNF- α	0.194	0.341	-0.220 to 0.550	0.335	0.102	-0.082 to 0.652
IFN- α	0.360	0.091	-0.074 to 0.680	0.314	0.152	-0.137 to 0.657
IFN- γ	-0.528	0.0008	-0.732 to -0.236	-0.633	<0.0001	-0.796 to -0.385
E-selectin	-0.588	<0.0001	-0.802 to -0.372	-0.516	0.0004	-0.756 to -0.276
P-selectin	-0.263	0.127	-0.555 to 0.083	-0.163	0.366	-0.487 to 0.202
sICAM-1	-0.481	0.0026	-0.702 to -0.177	-0.537	0.0005	-0.735 to -0.253
IP10	0.281	0.101	-0.067 to +0.569	0.306	0.083	-0.052 to 0.595

Statistically significant values are in bold.

selectin, and sICAM-1 (Supplementary Table S7, $P < 0.0025$), which is similar to the results we obtained for the absolute ECD values.

Influence of Simultaneous Cataract Surgery on Postoperative Endothelial Cell Density

Regarding the influence of simultaneous cataract surgery, we compared postoperative ECD between solitary DSAEK (43 eyes) and DSAEK combined with simultaneous cataract surgery (21 eyes; Supplementary Table S8), which showed no significant difference between them at any point (Supplementary Fig. S2; Mann-Whitney U test, all $P > 0.05$).

Correlations Among the Preoperative Aqueous Protein and Cytokine Levels and Endothelial Cell Density

The Figure shows the correlations among aqueous protein and cytokine levels in eyes that underwent DSAEK. All correlation coefficients were positive. The red lines represent strong positive correlations ($P < 0.0001$) and the blue lines represent moderate positive correlations ($P < 0.0025$). The color gradations of the circles represent the differences in cytokine levels among healthy eyes, eyes with low postoperative ECDs (<1200 cells/mm²) and eyes with high postoperative ECDs (≥ 1200 cells/mm²). The levels of IL-17A, MCP-1, IFN- γ , E-selectin, and sICAM-1 (shown in green circles), were associated with ECD at 12 months after DSAEK.

DISCUSSION

Late endothelial dysfunction is the major cause of visual loss after DSAEK.^{5,28} The average annual reduction rate of ECD has been reported to be 0.6% in healthy eyes,²⁹ 2.5% after cataract surgery,⁹ and 2.6% to 7.8% after PKP.³⁰ Recent studies reported that the 10-year ECD correlated with the 6-month ECD after DSAEK and PKP, whereas it did not correlate with the baseline

donor ECD.^{14,31,32} Regarding the eyes after PKP, the risk factors for endothelial cell loss after corneal transplantation include donor and recipient ages, graft diameter, lens status, glaucoma, and graft rejection.³²⁻³⁴ In contrast, regarding DSAEK, the risk factors for the late endothelial cell loss include history of glaucoma surgery, small diameter of graft, and severe iris damage.^{5,6,11-13} However, the exact mechanism for the reduction of ECD is still poorly understood.

AqH has a unique composition that includes proteins, ascorbate, glutathione, and other biologically active substances. In recent years, elevated levels of cytokines in the AqH have been reported to be associated with pathogenesis in various ocular diseases, such as FECD,³⁵ glaucoma,^{17,18,36} ocular surface diseases,³⁷ and graft rejection.^{38,39} We reported an elevation of inflammatory cytokines in eyes with bullous keratopathy.²⁰ Moreover, the iris damage was associated with the elevation of aqueous cytokine levels.²¹ However, these recent reports were cross-sectional studies and the elevated cytokine levels might just be the results, not the cause, of endothelial cell loss. Thus, we conducted this prospective study to assess the association of preoperative cytokine levels and postoperative ECD after corneal transplantation.

Regarding the association between preoperative cytokine levels and ECD after PKP, we showed that the preoperative levels of specific aqueous cytokines, such as IL-10, MCP-1, and IFN- γ , were inversely correlated with ECD at 3 and 6 months after PKP.²² After DSAEK, the preoperative levels of MCP-1, IFN- γ , IL-17A, E-selectin, and sICAM-1 were correlated with postoperative ECD in eyes with pseudophakic bullous keratopathy. MCP-1 is the main chemotactic factor for the migration of monocytes/macrophages and the pathogenesis of chronic inflammation.⁴⁰ MCP-1 directly enhances the production of inflammatory cytokines⁴¹ and causes cell apoptosis via MCP-induced protein.⁴² IFN- γ activates the immune cells and upregulates major histocompatibility complex (MHC) class I and II molecules.¹⁹ IFN- γ induces apoptosis of endothelial cells in vitro.¹⁹ Recently, Chen et al.⁴³ reported that IL-17A⁺ Th17 cells produce IFN- γ and mediate ocular surface autoimmunity. ICAM-1 mediates the recruitment of immune cells to sites of

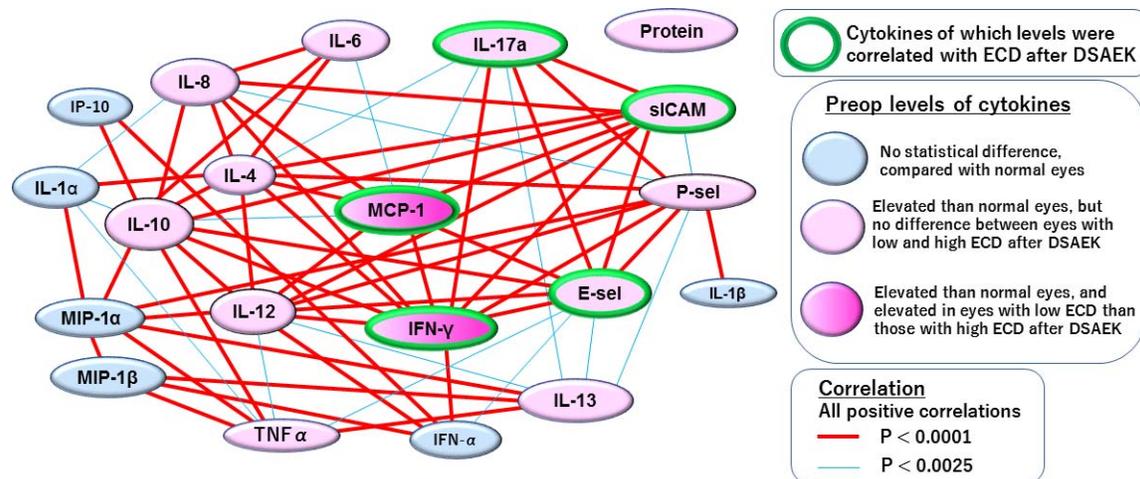


FIGURE. Correlation among aqueous protein and cytokine levels in eyes undergoing Descemet stripping automated endothelial keratoplasty. Preoperative cytokine levels were correlated with each other in eyes that underwent Descemet stripping automated endothelial keratoplasty (DSAEK). The red thick lines and blue thin lines represent strong ($P < 0.0001$) and moderate ($P < 0.0025$) correlations, respectively. All correlations were positive. The color of the circles represents the protein/cytokine levels compared with those in healthy eyes. The color gradation of the circles represents the differences in cytokine levels among healthy eyes, eyes with low ECD (< 1200 cells/mm² at 12 months after DSAEK) and high ECD (≥ 1200 cells/mm²). The cytokines associated with post-DSAEK ECD are denoted by green circles. It is noteworthy that some cytokines (MCP-1 and IFN- γ) that significantly correlated with post-DSAEK ECD are denoted by green circles with color gradients.

inflammation,⁴⁴ and its soluble form, sICAM-1 has been shown to be increased in the AqH of patients with bullous keratopathy.^{20,45} Richer et al.⁴⁴ reported strong correlations between sICAM-1 and soluble apoptotic factors, such as soluble Fas and Fas ligand in rhegmatogenous retinal detachment. Recently, Dohlman et al.⁴⁶ found that blockade of E-selectin suppressed graft rejection in murine corneal transplantation model, whereas both levels of E-selectin and P-selectin were upregulated in the rejected graft. Although there were no eyes with graft rejection after DSAEK in the case series, future studies on the immunologic mechanism of ECD loss after DSAEK will be valuable.

The preoperative levels of IL-4, IL-6, IL-8, IL-10, IL-12p70, and P-selectin were elevated in the eyes that underwent DSAEK compared with control eyes, although there were no correlations between the preoperative levels of these cytokines and ECD after DSAEK. Thus, given that the combination of cytokines synergistically induces apoptosis of endothelial cells,¹⁹ not only MCP-1, IFN- γ , and sICAM-1 but also other cytokines may have affected ECD after DSAEK. The figure shows the preoperative protein and cytokine levels, as well as the correlations among them and ECD at 12 months. There were complicated correlations among the cytokines; the correlations among IL-17A, MCP-1, IFN- γ , E-selectin, and sICAM-1 seem to be important, as the levels were strongly correlated with those of other cytokines and ECD at 12 months after DSAEK.

Regarding cytokine levels in other organ transplants, previous reports showed the association between the serum cytokine levels and prognosis. Allen et al.⁴⁷ reported that a preoperative inflammatory state in the recipient, as indicated by high levels of serum IL-10 and MCP-1 has an important impact for early lung allograft function. Kim et al.⁴⁸ showed an association of combined detection of serum IL-10, IL-17, and IP-10 with acute rejection following adult liver transplantation. Crescioli et al.⁴⁹ reported that pretransplant serum CXCL10 can be a predictive marker for cardiac acute rejection. As in the other organ transplants, it can be potentially valuable to examine preoperative cytokine levels prior to corneal transplantation as predictive biomarkers to prevent and treat the chronic loss of ECD after DSAEK.

The aqueous protein levels in eyes undergoing DSAEK were significantly higher than those in the healthy control group, which reflects the breakdown of the blood-ocular barrier (BAB). Ambrose et al.⁵⁰ measured aqueous flare using a fluorophotometer, and reported that the breakdown of BAB due to iris chafing by anterior chamber IOL influences the progression of endothelial cell loss. The breakdown of BAB can induce not only elevated cytokine levels, but also extensive alterations in the other kinds of proteins in the AqH. In the current study, a history of glaucoma and trabeculectomy was shown to be a risk factor for low ECD, however, there were no significant differences in cytokine levels between DSAEK eyes with and those without history of glaucoma or trabeculectomy. This may be attributable to the limited number of subjects with glaucoma in the present study, because the aqueous levels of IL-1 α , IL-4, IL-8, IL-10, IFN- γ , and MCP-1 elevate in eyes after trabeculectomy.^{20,36} Further studies are necessary to elucidate the exact mechanism of endothelial cell loss after corneal transplantation, using proteomics analysis of the aqueous humor to specify the alteration of the aqueous environment.

The ECD count can cause measurement error. We used the EM-4000 automated software for ECD in the current study. Price et al.²⁸ reported ECD was most accurate even in DSAEK eyes when they were measured using EM-3000, whereas ECD differed by more than 1000 cells/mm² when measured with other type of specular microscope. Using Bland-Altman plots analysis, Luft et al.⁵¹ compared four specular microscopes in healthy eyes and eyes after DSAEK, which showed that EM-3000 automated software (a former type of specular microscope from Tomey) provided quantitative endothelial measurements that were well comparable to those obtained with the manual gold standard method even in post-DSAEK eyes.

The survival rate at 12 months was 87.5% (56/64 eyes), which is low compared with that reported in the previous studies.^{4,5} This study included complicated eyes with a history of multiple intraocular surgeries and trabeculectomy. Stratifying the subjects based on the etiologies, the survival rate at 12 months was 100% (11/11) in eye with FECD, 90.9% (10/11) in post-LI eyes, 92% (23/25) in eyes with pseudophakic bullous keratopathy, and 50% (5/10) in eyes after trabeculectomy,

which was comparable to that reported in previous studies.^{4,5,11,12} Thus, we postulated that the ECD reduction can be due to inflammatory alteration of the AqH microenvironment, not due to surgical mal-manipulation or measurement error.

Multivariate analyses showed that preoperative steroid use was not correlated with postoperative ECD, which is consistent with the results reported in our previous studies, in which we showed that there were no significant differences in cytokine levels between patients who used and did not use topical steroids preoperatively.²⁰⁻²² Therefore, the translational impact of the current study into clinical practice may be poor. Moreover, the current study might have been biased in that patients with severe conditions used steroid eye drops, whereas some of the patients with mild bullous keratopathy did not. Thus, a prospective study will be required to evaluate the efficacy of topical steroid in reducing preoperative aqueous cytokine levels, which in turn may prevent ECD loss after DSAEK. Further, the detailed response of endothelial cell against the chronic inflammatory condition remains elusive. What types of pathways are activated inside corneal endothelial cells in inflamed AqH, “oxidative stress,”^{52,53} “mitochondrial damage,”⁵⁴ “ER stress,”⁵⁵ or “cell senescence?”^{56,57} If we uncover the abnormal cell responses against inflamed AqH, it could lead to prophylactic therapy. Transcriptomic analyses of human endothelial cells derived from inflamed AqH using microarray or next generation sequencing could specify the implicated pathway in the future.

This study had some limitations. First, the different graft sizes (7.5–8.5 mm) may have had some effect on the ECD results.⁶ A larger graft size can cause a more severe immunologic reaction after DSAEK because it loads more antigen. Multivariate analyses showed that the correlation coefficients between graft size and postoperative ECD were positive at multiple time points (i.e., Table 4: $\beta = 0.185$, $P = 0.122$, Supplementary Table S6: $\beta = 0.192$, $P = 0.105$ at 12 months), suggesting that the larger the graft size, the more ECD after DSAEK, though there were no significant correlations. Thus, we think its influence is minimal. Second, postoperative inflammation due to immune response against the donor stromal tissue or surgical trauma can increase the cytokine levels in the AqH, which may affect ECD after DSAEK. In the future, we will have to evaluate the correlations between pre- and postoperative cytokine levels after obtaining the approval from our institutional review board. This study will show the direct correlations between postoperative cytokine levels and ECD after DSAEK. Third, the nature of the underlying disease was heterogeneous in the current study. The analyses excluding FECD eyes showed stronger correlations between preoperative cytokines and ECD after DSAEK, suggesting that the mechanism involved in ECD reduction in pseudophakic bullous keratopathy may be different from that in FECD. Another limitation is the statistical analyses we performed. In the current study, due to the limited number of subjects, we used Spearman correlation analyses at 6 and 12 months. However, to evaluate the correlation between ECD and the clinical/AqH factors comprehensively as previously reported,^{34,58} longitudinal repeated measures analyses are more appropriate. We will increase the number of subjects and conduct longitudinal repeated measures analyses in the future.

In conclusion, we showed that the preoperative levels of specific aqueous cytokines, such as MCP-1, IFN- γ , IL-17A, E-selectin, and sICAM-1, had a significant correlation with ECD after DSAEK for bullous keratopathy.

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