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Elevation of preoperative recipient aqueous cytokine levels in eyes with primary graft failure after corneal transplantation

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Purpose: Primary graft failure after corneal transplantation is caused by dysfunction of corneal endothelial cells. Recently, we demonstrated that preoperative recipients' aqueous cytokine levels are associated with rapid corneal endothelial cell loss after corneal transplantation. In the present study, we evaluated the preoperative inflammatory cytokine levels in the aqueous humor (AqH) of eyes with primary graft failure following corneal transplantation.

Methods: Among the prospective consecutive case series (273 eyes), this study included patients who developed primary graft failure (eight eyes) and patients who underwent corneal transplantation for the treatment of bullous keratopathy (108 eyes) or cataract surgery (30 eyes). AqH samples were collected at the beginning of each surgery. The levels of the cytokines (interleukin [IL]-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, interferon [IFN]-γ, monocyte chemotactic protein [MCP]-1, E-selectin, P-selectin, and soluble intercellular adhesion molecule [sICAM]-1) in the AqH were measured with multiplex beads immunoassay.

Results: In eyes with primary graft failure, the preoperative levels of aqueous protein (4.6-fold), interleukin (IL)-6 (179-fold), IL-17A (7.1-fold), MCP-1 (2.6-fold), IFN-γ (4.3-fold), E-selectin (2.3-fold), P-selectin (2.0-fold), and sICAM-1 (5.5-fold) were statistically significantly higher compared to the cataract controls ($p<0.0021$). There was no primary graft failure among the recipients who received corneal grafts of fellow eyes from the same donors.

Conclusions: Preoperative levels of AqH cytokines, such as IL-6, IL-17A, MCP-1, IFN-γ, and sICAM-1, increased in eyes with primary graft failure after corneal transplantation. These cytokine levels could be prognostic biomarkers to predict primary graft failure after corneal transplantation.

Corneal transplants are among the most successful solid tissue transplants. The primary cause of graft failure is corneal endothelial decompensation, even in eyes without evidence of immunologic rejection [1]. The corneal endothelial cell density (ECD) decreases with age [2,3] and in various conditions, including uveitis [4,5] and post-intraocular surgeries [6-9]. Loss of endothelial cells following corneal transplantation has been shown to be associated with graft failure [10,11]. Chronic loss of ECD is the major challenge for the improvement of prognosis of corneal transplantation [10]. Risk factors for postoperative corneal endothelial cell loss after corneal transplantation include donor age, the recipient bed's endothelial reservoir, graft diameter, lens status, glaucoma, graft rejection, and presence of peripheral corneal diseases [12-14]. However, the exact mechanism of chronic endothelial cell loss is still poorly understood.

Recently, we reported that preexisting iris damage was one of the clinical factors for graft failure and rapid endothelial cell loss after Descemet stripping automated endothelial keratoplasty (DSAEK) [15]. Moreover, we showed elevated cytokine levels in the aqueous humor (AqH) of eyes with bullous keratopathy (BK) and reduced ECD [16]. The reason behind the decreased ECD in eyes with severe iris damage remains elusive. Anatomically, the 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 [17-19]. In an in vitro study, a combination of proinflammatory cytokines synergistically induced apoptosis of corneal endothelial cells [20]. In eyes with severe iris damage, the levels of aqueous cytokines were associated with the extent of iris damage [21]. Furthermore, in eyes with preoperative higher levels of aqueous cytokines, the ECD decreased rapidly after penetrating keratoplasty (PKP) [22] and DSAEK [23]. Recent clinical studies reported that recipient factors, not donor factors, had significant effects on graft failure after corneal transplantation [24,25]. Collectively, inflammatory factors in the recipient AqH may directly result in loss of corneal endothelial cells.

Primary graft failure (PGF) occurs in 0.5% to 2% of corneal grafts [26,27]. Despite the low incidence, PGF remains an important issue in corneal transplantation. Causes include preexisting abnormality of the donor cornea [28], damage during retrieval or storage [29], and surgical trauma [30]. We hypothesized that the high levels of AqH cytokines

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Table 1. Demographics of the subjects.

Study groups	Primary graft failure +	Primary graft failure -	Cataract control
No. of subjects	8	108	30
Age (years)	76.5±9.5	71.5±11.2	75.6±7.0
Gender (Male/ Female)	3 /5	49 / 59	16/14

can cause PGF due to the rapid loss of ECD after corneal transplantation. The purpose of this study was to evaluate the preoperative cytokine levels in eyes with PGF following corneal transplantation.

METHODS

This prospective study was performed in accordance with the Declaration of Helsinki, and the ARVO statement of human subjects. The study was approved by the institutional ethics review board of Tokyo Dental College Ichikawa General Hospital (I-15–42). Written informed consent was obtained from all participants before the interventions.

Study participants: Primary graft failure occurred in eight of 273 eyes (2.9%) undergoing corneal transplantation at Tokyo Dental College Ichikawa General Hospital from October 2015 to April 2017. This prospective study included a total of 146 eyes of 146 patients: eight eyes that developed PGF following penetrating PKP (three eyes) and DSAEK (five eyes), 108 BK eyes undergoing corneal transplantation without PGF, and 30 eyes that underwent cataract surgery as the control group (Table 1). PGF failure was defined as (1) the presence of a diffusely edematous corneal graft on the first postoperative day, (2) failure of the cloudy graft to clear at any time postoperatively, and (3) lack of an identifiable cause of corneal graft failure [27]. None of the patients with PGF recovered despite the follow-up treatment, which lasted longer than 6 months. Among 273 patients who underwent corneal transplantation during the study period, we excluded patients with non-BK eyes (58 eyes; keratoconus, corneal scar, etc.), eyes with ECD less than 1,000 cells/mm^2 at 6 months after corneal transplantation (31 eyes), and Fuchs endothelial corneal dystrophy (28 eyes), as well as patients with a follow-up period less than 6 months (19 eyes), endotheliitis (four eyes), and birth injury (three eyes). We excluded eyes with ECD less than 1,000 cells/mm^2 at 6 months after corneal transplantation because preoperative cytokine levels have been reported to be high in eyes with such rapid ECD loss after corneal transplantation [22,23]. We excluded seven patients who underwent Descemet's membrane endothelial keratoplasty (DMEK) because the surgical outcome was poor as we started performing DMEK during the study period. We also excluded one eye with PGF following DSAEK performed by corneal fellows, because ECD loss could have been caused by iatrogenic reasons. As a result, 108 BK eyes were included in the group without PGF.

Aqueous humor samples: The AqH samples were 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 sample was obtained using a 27-gauge needle taking care not to touch the iris, lens, or corneal endothelium. The samples were centrifuged at 3,000 ×*g* for 5 min. The soluble factions were collected and stored at −80 °C until the cytokine levels were measured.

Surgical technique: We performed PKP and DSAEK according to our standard technique as previously published [31]. Briefly, PKP was performed under retrobulbar anesthesia. The donor button was cut with a Baron punch trephine. A Hessburg-Baron suction trephine was used to cut a partial-depth, circular incision in the cornea, centered at the geometric center of the cornea. Excision of the recipient corneal button was completed with curved corneal scissors. The graft was sutured in place with a single-running 10–0 nylon suture with 24 bites in all eyes. The donor corneas were obtained from domestic or American eye banks. Histocompatibility matching was not performed. The typical trephination size was 7.5 mm for recipient eyes and 7.75 mm for the donor grafts.

DSAEK surgery was performed using the double-glide technique [32]. After sub-Tenon anesthesia with injection of 2% lidocaine, a 5.0-mm temporal corneoscleral incision was made. An AC 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). The recipient's endothelium and Descemet's membrane were carefully removed using forceps. Precut donor grafts were trephinated at a diameter of 7.0–8.5 mm, and the corneal 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. The fluid between the recipient's

stroma and the graft was drained from small incisions in the midperipheral recipient cornea. At 10 min after air injection, half of the air was replaced with balanced salt solution (BSS, Alcon, Fort Worth, TX).

At the end of the surgery, 2 mg subconjunctival betamethasone was administered. Postoperatively, the patients were treated five times per day with topical antibiotics levofloxacin and 0.1% betamethasone. The betamethasone eye drop was administered for at least up to 6 months after surgery in all eyes.

Protein concentration measurements: The protein concentrations of the AqH samples were determined using the DC protein assay (Bio-Rad, Hercules, CA). The reactions were based on the Lowry assay and measured according to the manufacturer's instructions. In brief, bovine albumin serum (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 min of incubation at room temperature in the dark, the microplates were read at 690 nm and 405 nm using a microplate reader (Model 550; Bio-Rad). Concentrations were calculated by the subtraction method using the microplate manager system (Bio-Rad).

Cytokine level measurements: The cytokine levels of interleukin [IL]-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, interferon [IFN]-γ, monocyte chemotactic protein [MCP]-1, E-selectin, P-selectin, soluble intercellular adhesion molecule [sICAM]-1, macrophage inflammatory protein [MIP]-1β, and interferon gamma-induced protein [IP]-10 in the AqH samples were measured using Luminex (ProcaPlex kit, Luminex, San Antonio, TX) beads-based multiplex immunoassay according to previous reports [16]. Briefly, 50 µl of AqH samples were incubated with antibody-coated capture beads in an incubation buffer at room temperature. After 2-h incubation, the beads were washed three times using washing buffer, and phycoerythrin-labeled streptavidin was added for 30 min in the dark at room temperature. After being washed three times with washing buffer, the plates were resuspended in 150 µl of reading buffer, and the assays were performed using a Luminex 200.

Data analysis: The preoperative iris damage score (IDS) was assessed using slit-lamp microscopy and anterior segment optical coherence tomography (AS-OCT, SS-1000, CASIA, TOMEY, Nagoya, Japan) as reported previously [15]. The ECD was measured by masked orthoptists using a specular microscopy system (EM-4000, TOMEY). Approximately 50 cells were analyzed for mean cell density.

Statistical analysis: Data were analyzed using Prism for Windows software (version 6.04, GraphPad Software, Inc., San Diego, CA). The D'Agostino-Pearson omnibus normality test was used to assess whether the data showed a normal distribution. To compare differences in protein and cytokine concentrations across the groups, the Kruskal–Wallis test with Dunn's multiple comparisons test was used. The data are expressed as the mean ± standard deviation (SD). Statistical significance was considered at a p value of less than 0.05 except cytokine data. Cytokine data were controlled with Bonferroni correction. Because there were 14 different comparisons (one protein and 13 cytokines), p values of less than 0.0035 (i.e., p=0.05/14) were considered statistically significant after Bonferroni correction.

RESULTS

Patients with primary graft failure: Details of the eyes with PGF are shown in Table 2. Despite the absence of intra- and postoperative complications, eight eyes developed PGF. Six eyes had a history of trabeculectomy. The mean preoperative IDS was 3.1±1.1 in eyes with PGF, 1.6±0.7 in eyes without PGF, and 0 in the cataract control.

Aqueous humor protein and cytokine levels: In eyes with PGF, the preoperative levels of aqueous protein (4.6-fold), IL-4 (3.7-fold), IL-6 (179-fold), IL-17A (7.1-fold), MCP-1 (2.6-fold), IFN-γ (4.3-fold), E-selectin (2.3-fold), P-selectin (2.0-fold), and sICAM-1 (5.5-fold) were statistically significantly higher compared to those in the cataract control group (Table 3, all, p<0.0035). In eyes with PGF, the preoperative levels of IL-17A (1.5-fold, p=0.0465), E-selectin (1.5-fold, p=0.0273), and sICAM-1 (1.4-fold, p=0.0298) were higher compared to the BK eyes without PGF, although there were no statistically significant differences when Bonferroni correction was applied.

Absence of primary graft failure in eyes receiving donor fellow eyes: Primary graft failure can still be caused by donor-related issues even with the preoperative elevation of aqueous cytokine levels in recipients' eyes with PGF. Thus, we investigated the clinical courses of corneal transplantation of donor fellow eyes (Table 4). There was no PGF among recipients who received corneal grafts of fellow eyes from the same donors.

DISCUSSION

Primary graft failure was present in eight eyes (2.9%) that underwent PKP or DSAEK. The preoperative levels of aqueous protein, IL-4, IL-6, IL-17A, MCP-1, IFN-γ, E-selectin, P-selectin, and sICAM-1 were statistically

Table 2. Demographics of the subjects with primary graft failure following corneal transplantation.

Case	Age/ gender	Etiology	Past ocular surgery	Glaucoma	Phakia /IOL	Preop IDS	NV	Current surgery	Donor age (years)	Graft ECD (cells/ mm^2)	Preop VA	Preop IOP (mm/ Hg)	Preop steroid use
1	63M	PBK	**TLO, TLE, PKP x3**	+	**IOL**	**4**	**0**	**PKP**	**69**	**2618**	**0.03**	**8**	-
2	69F	PBK	**PEA+IOL, COMET**	-	**IOL**	**3**	**3**	**PKP**	**51**	**3244**	**HM**	**15**	-
3	92F	PBK	**ECCE+IOL suturing**	-	**IOL**	**3**	**0**	**PKP**	**93**	**2914**	**0.01**	**7**	-
4	79F	BK	**ALI**	+	**cata-ract**	**1**	**0**	**DSAEK+PEA+IOL**	**57**	**2681**	**HM**	**8**	-
5	69F	PBK	**PEA+IOL, TLE**	+	**IOL**	**2**	**0**	**DSAEK**	**81**	**2896**	**0.03**	**12**	-
6	75M	PBK	**PEA+IOL, TLE**	+	**IOL**	**4**	**0**	**DSAEK**	**49**	**3457**	**0.01**	**16**	-
7	84F	PBK	**PEA+IOL, TLE**	+	**IOL**	**4**	**0**	**DSAEK**	**58**	**3348**	**0.01**	**10**	-
8	81F	PBK	**PEA+IOL, TLE**	+	**IOL**	**4**	**0**	**DSAEK**	**66**	**2695**	**0.01**	**12**	-

IOL: intraocular lens, IDS: iris damage score, NV: neovascularization, ECD: endothelial cell density, VA: visual acuity, IOP: intraocular pressure, PBK: pseudophakic bullous keratopathy, BK: bullous keratopathy, TLO: trabeculotomy, TLE: trabeculectomy, PKP: penetrating keratoplasty, COMET: cultivated oral mucosal epithelial transplantation, HM: hand motion, ECCE: extracapsular cataract extraction, ALI: argon laser iridotomy, DSAEK: Descemet's automated endothelial keratoplasty, PEA: phacoemulsification and aspiration

Table 3. Aqueous cytokine levels in each group.

Aqueous cytokine	Primary graft failure (+) n=8	Primary graft failure (-) n=108	Cataract control n=30	P value*	P value†	P value‡
Protein	1.25±0.21 (1.08)	1.11±0.08 (1.02)	0.27±0.04 (0.25)	**0.0006**	0.473	0.0035
IL-4	71.8±23.1 (41.5)	45.4±7.0 (27.7)	19.2±0.74 (20.1)	**0.0007**	0.199	0.018
IL-6	1148±271 (1527)	780±100 (364)	6.4±0.9 (4.5)	**<0.0001**	0.109	**<0.0002**
IL-8	57.9±18.5 (34.2)	72.0±12.0 (39.5)	19.3±1.2 (19.8)	0.115	0.946	0.0002
IL-10	16.9±13.2 (3.2)	4.7±1.2 (2.9)	1.7±0.1 (1.7)	0.0183	0.324	**0.0011**
IL-12p70	15.2±3.4 (16.4)	16.2±3.0 (8.3)	6.2±0.1 (6.3)	0.041	0.533	**0.0007**
IL-17A	26.3±8.4 (20.6)	17.4±4.0 (7.1)	3.7±0.2 (3.4)	**0.0002**	0.0465	**<0.0001**
MIP-1β	73.6±27.1 (46.7)	218±39.2 (46.8)	315.1±11.8 (314)	0.0073	0.690	**<0.0001**
MCP-1	1173±168 (1245)	802±36.9 (735)	449±24 (457)	**0.0007**	0.0895	**<0.0001**
IFN -γ	228.7±83.0 (96.8)	188±43.2 (66.6)	53.4±1.6 (54.0)	**0.0035**	0.108	0.0086
E-Selectin	4891±618 (4770)	3351±447 (3274)	2093±37 (2135)	**<0.0001**	0.0273	**0.0001**
P-Selectin	7315±808 (7227)	10,343±1471 (4717)	3658±137 (3533)	**0.0003**	0.164	**0.0007**
sICAM-1	7683±1796 (6648)	5537±772 (3440)	1397±119 (1287)	**<0.0001**	0.0298	**<0.0001**
IP10	543±318 (141)	210±27.4 (136)	132.7±22.0 (95.6)	0.634	0.735	0.151

Mean ± SEM (median) Protein (mg/mL), cytokines (pg/mL) IL: interleukin, MIP: macrophage inflammatory protein, MCP: monocyte chemotactic protein, IFN: interferon, sICAM: soluble intracellular adhesion molecule, SE: standard error * p values: compared between eyes with primary graft failure (+) and the cataract control group, Kruskal–Wallis test with Dunn's multiple comparisons test †P values: compared between eyes with primary graft failure (+) and primary graft failure (-), Kruskal–Wallis test with Dunn's multiple comparisons test ‡P values: compared between eyes with primary graft failure (-) and the cataract control group, Kruskal–Wallis test with Dunn's multiple comparisons test

Table 4. Absence of primary graft failure in eyes receiving the donor fellow eyes.

Case	Domestic / Imported	Donor age (years)	Graft ECD (cells/mm^2)	Recipients' Age / gender	PGF
1	Imported	69	2519	57F	No
2	Imported	51	3026	54M	No
3	Domestic	93	2519	51F	No
4	Imported	57	2950	81F	No
5	Domestic	81	3096	69F	No
6	Imported	49	3402	69F	No
7	Imported	58	3348	65F	No
8	Imported	66	2695	78F	No

ECD: endothelial cell density, PGF: primary graft failure

significantly higher in eyes with PGF than in those of the cataract control group. Among these cytokines, IL-6, IL-17A, and sICAM-1 were notably elevated compared to those of the controls up to 179-fold, 7.1-fold, and 5.5-fold, respectively. Furthermore, the recipients who received the donor cornea of fellow eyes did not develop PGF.

Primary graft dysfunction is a severe form of ischemia or reperfusion acute graft injury that is the leading cause of early morbidity and mortality after organ transplantation. Primary graft dysfunction affects an estimated 13.9% of heart [33], 10% to 25% of lung [34], and 26.5% of liver transplants [35]. In contrast, PGF has been regarded as a relatively rare complication (approximately 0.5% to 2%) in corneal transplantation [26,27]. Recently, we showed that aqueous cytokine levels were elevated in eyes with bullous keratopathy and low ECD [16]. In addition, we identified the risk factors for elevated aqueous cytokine levels as iris damage [21]. The preoperative higher levels of specific cytokines in the AqH were associated with the rapid reduction of ECD after PKP (IFN-γ and MCP-1) and after DSAEK (IL-17A and MCP-1) [22,23]. In the present study, there were no statistically significant differences in AqH cytokine levels between the PGF group and the without PGF group. However, the cytokine levels in the PGF group tended to be higher than those in the without PGF group. Collectively, these clinical results suggest that the inflammatory microenvironment in the AqH directly determines the survival of corneal endothelial cells after corneal transplantation. The results in the present study showed the extremely elevated levels of specific cytokines in the AqH, which suggests that PGF might be attributable to acute corneal endothelial cell damage.

In other organ transplantations, previous studies have reported that serum levels of specific cytokines are associated with PGF [36-38]. Allen et al. reported that the preoperative levels of IL-6, IL-8, IL-10, and MCP-1 were higher in patients with PGF after lung transplantation [36]. Kim et al. reported that the combination of serum levels of IL-10, IL-17, and CXCL-10 can predict acute graft rejection following liver transplantation [37]. Discovery of non-invasive prognostic and predictive biomarkers for acute rejection or PGF would help ensure the preservation of donor grafts and eventually contribute to improving graft survival. In the present study, the IDS was high in all eyes with PGF, ranging from 1 to 4. Although IDS is a potential clinical marker for elevated aqueous cytokine levels, there were no statistically significant differences in aqueous cytokine levels between mild and severe iris damage [21]. The measurement of preoperative aqueous cytokine levels, such as IL-6, IL-17A, MCP-1, and IFN-γ, can be powerful predictive/prognostic markers for PGF after corneal transplantation, especially in eyes with severe iris damage.

The present study has some limitations. First, it had a small sample size of eight eyes. In the future, we intend to conduct a study with more participants. Second, some of the patients included in the present study had complicated backgrounds, such as post-glaucoma surgery, which might have led to selection bias. Primary graft failure can occur in uncomplicated cases. Furthermore, in the present case series, anti-inflammatory treatment, such as steroid eye drops, were not used preoperatively in the PGF group. Future study on the effect of topical steroid on aqueous cytokine levels will be invaluable in eyes with severe iris damage to prevent PGF or rapid loss of ECD. Third, in general, PGF has been regarded to be due to donor-related reasons, although Yu et al. reported that preoperative recipient-related factors, such as anterior chamber lens or glaucoma-associated corneal edema, were associated with graft failure, whereas few donor-related factors had a statistically significant association with graft failure [24]. Therefore, we cannot exclude the possibility of donor-related graft failure. However, in the present study, preoperative assessment of the endothelium of donor grafts showed eligibility for corneal transplantation. Moreover, the results of corneal transplantation of the fellow eye of the same donor were favorable.

In conclusion, we have shown that the preoperative levels of specific aqueous cytokines, such as IL-6, IL-17A, IFN-γ, and MCP-1, were elevated in eyes with PGF after corneal transplantation. Further studies are necessary to elucidate the specific underlying molecular mechanisms of PGF to improve the prognosis of corneal transplantation.

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