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The Biologic Character of Donor Corneal Endothelial Cells Influences Endothelial Cell Density Post Successful Corneal Transplantation

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Purpose: Corneal endothelial cell density (ECD) gradually decreases after corneal transplantation by unknown biologic, biophysical, or immunologic mechanism. Our purpose was to assess the association between donor corneal endothelial cell (CEC) maturity in culture and postoperative endothelial cell loss (ECL) after successful corneal transplantation.

Design: Prospective cohort study.

Participants: This cohort study was conducted at Baptist Eye Institute, Kyoto, Japan, between October 2014 and October 2016. It included 68 patients with a 36-month follow-up period who had undergone successful Descemet stripping automated endothelial keratoplasty (DSAEK) or penetrating keratoplasty.

Methods: Human CECs (HCECs) from remaining peripheral donor corneas were cultured and evaluated for maturity by surface markers ($CD166^{+}$, $CD44^{-/dull}$, $CD24^{-}$, and $CD105^{-}$) using fluorescence-activated cell sorting. Postoperative ECD was assessed according to the mature-differentiated HCEC contents: high-maturity group: $>$ 70%, middle-maturity group: 10% to 70%, low-maturity group: $<$ 10%. The successful rate of ECD maintained at 1500 cells/mm^2 at 36 months postoperative was analyzed using the log-rank test.

Main Outcome Measures: Endothelial cell density and ECL at 36 months postoperative.

Results: The 68 included patients (mean [standard deviation] age 68.1 [13.6] years, 47.1% women, 52.9% DSAEK). The high, middle, and low-maturity groups included 17, 32, and 19 eyes, respectively. At 36 months postoperative, the mean (standard deviation) ECD significantly decreased to 911 (388) cells/mm^2 by 66% in the low-maturity group, compared with 1604 (436) by 40% and 1424 (613) cells/mm^2 by 50% in the high and middle-maturity groups ($P < 0.001$ and $P = 0.007$, respectively) and the low-maturity group significantly failed to maintain ECD at 1500 cells/mm^2 at 36 months postoperative ($P < 0.001$). Additional ECD analysis for patients who underwent DSAEK alone displayed a significant failure to maintain ECD at 1500 cells/mm^2 at 36 months postoperative ($P < 0.001$).

Conclusions: The high content of mature-differentiated HCECs expressed in culture by the donor peripheral cornea was coincident with low ECL, suggesting that a high-maturity CEC content predicts long-term graft survival. Understanding the molecular mechanism for maintaining HCEC maturity could elucidate the mechanism of ECL after corneal transplantation and aid in developing effective interventions.

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The development of corneal endothelial transplantation, such as Descemet stripping automated endothelial keratoplasty (DSAEK) and Descemet membrane endothelial keratoplasty, has dramatically improved visual prognosis after corneal transplantation.[1] The midterm graft survival rate has also improved because of a reduction in allograft rejection compared with conventional full-thickness corneal transplantation.[2,3] However, corneal endothelial cell density (ECD) still decreases substantially and continuously with years after DSAEK, Descemet membrane endothelial keratoplasty, and penetrating keratoplasty (PK), even if no apparent complications, such as allograft rejection, occur after transplantation. For example, Lass et al[4] reported that the ECD at 5 years post-DSAEK declined by $>$ 70% of the baseline ECD. Furthermore, the Cornea Donor Study and other reports have demonstrated continuous yearly

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decreases in ECD after corneal transplantation, with graft survival or endothelial cell loss (ECL) associated with factors that included donor ECD, as well as donor age, sex, preservation time, and donor size.[5–9] In addition, Yamaguchi et al[10] proposed that iris damage was a risk factor for early corneal endothelial decompensation because of the elevation of inflammatory cytokines in the anterior chamber.[11,12] However, no clear reasons can yet explain why transplanted donor corneal endothelial cells (CECs) show this continuous decrease in ECD as compared with cataract surgery and so forth, and that some cases after transplantation eventually fall into chronic graft failure without obvious allograft rejection.

Of particular interest is that ∼10% of donor corneal endothelium shows > 2000 cells/mm^2 after 5 years post PK without any association of donor factors or host disease,[13] implying unsolved biologic aspects of the donor cornea may be involved in this event. In addition, there is evidence showing that donor corneas contained some dead CECs that were not related to storage period or donor age.[14] These suggest that the viability and longevity of CECs may vary among individual donor corneas. Based on these pieces of evidence, we hypothesize that a biologic precommitment of cell longevity already exists in the donor corneas before transplantation, although we cannot detect such factors using known biologic markers or clinical parameters, including donor ECD.

In the present study, to explore the possibility mentioned above, we used 1 biologic cell character obtained from our basic experiment of human CEC (HCEC) culture for HCEC-injection therapy, which is the proportion of CD166$^+$, CD44$^{-/dull}$, CD24$^-$, and CD105$^-$ in the cultured HCECs at confluency. They are mature-differentiated cells without cell-state transition and disposed to mitochondria-dependent oxidative phosphorylation. We surmise from our previous experiments that high proportion of HCECs with CD166$^+$, CD44$^{-/dull}$, CD24$^-$, and CD105$^-$ possibly possess longer longevity with good mitochondrial function than those with its low proportion.[15,16] Based on this hypothesis, we investigated the relationship between the postoperative ECD after successful corneal transplantation and the biologic character mentioned above, using cultured HCECs from the peripheral cornea of the same donor used for corneal transplantation, possibly indicating the healthiness and longevity of the donor CECs (Fig 1).

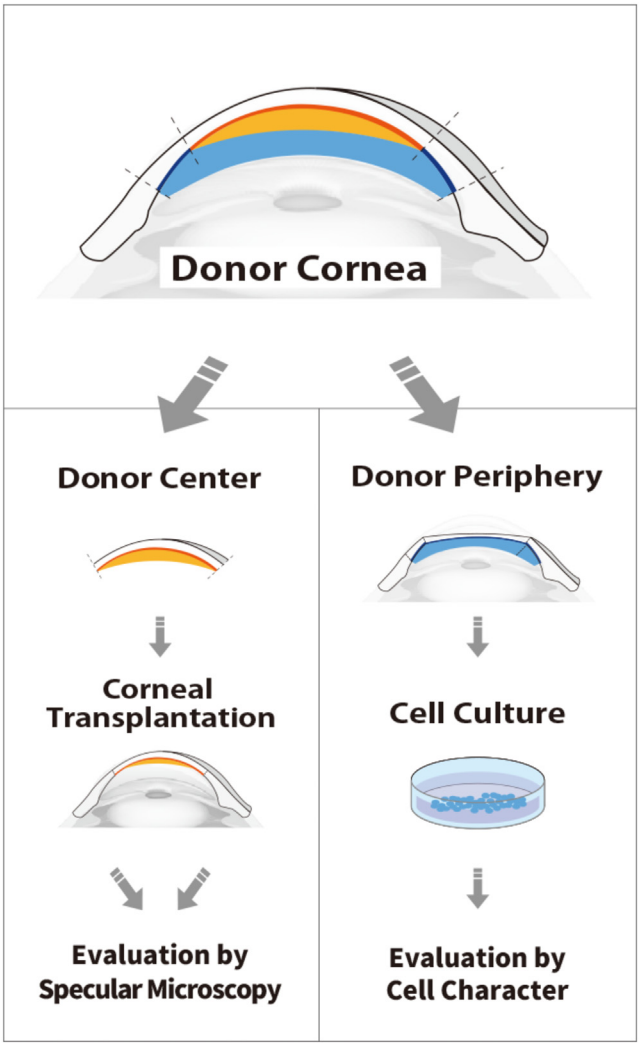

Figure 1. Schematic figure of the biologic evaluation of the donor corneas. The relationship between the postoperative endothelial cell density and the maturity of cultured corneal endothelial cells from the peripheral region of the donor cornea was investigated.

Methods

Patients

This cohort study of consecutive patients who underwent DSAEK or PK was conducted at the Baptist Eye Institute, Kyoto, Japan, and was approved by the Research Ethics Committee of Kyoto Prefectural University of Medicine (Approval #ERB-C 1006). Before surgery, written informed consent was obtained from all subjects in accordance with the tenets set forth in the Declaration of Helsinki. The study included 68 patients who underwent DSAEK or PK at the Baptist Eye Institute between October 2014 and October 2016. All surgeries were performed by 1 expert corneal surgeon (S.K.). The indications were corneal stromal opacity in 21 eyes, glaucoma-related bullous keratopathy (BK) in 17 eyes, pseudophakic/aphakic BK in 15 eyes, Fuchs endothelial corneal dystrophy in 6 eyes, keratoconus in 4 eyes, and other-type BK in 5 eyes (i.e., iridocorneal endothelial syndrome in 1 eye, pseudoexfoliation keratopathy in 1 eye, and unknown causes in 3 eyes). None of the patients experienced graft failure throughout the 36 months postoperative follow-up period.

Cell Culture of Donor CECs

The HCECs obtained from the peripheral rims of 68 human donor corneas were individually cultured according to published protocols,[17] with some modifications.[18] Briefly, the Descemet membranes with the CECs at the peripheral rims were stripped from donor corneas without contamination of trabecular meshwork tissues and digested at 37° C with 1 mg/mL collagenase A (Roche Diagnostics GmbH) for 2 hours. The HCECs obtained from a single donor corneal peripheral rim were seeded in a single well of a Type-I collagen-coated 24-well plate (Corning, Inc). The culture medium was prepared according to published protocols.[17] Briefly, basal medium was

prepared with Opti-MEM I (Thermo Fisher Scientific, Inc), 8% fetal bovine serum, 5 ng/mL epidermal growth factor (Thermo Fisher Scientific, Inc), 20 μg/mL ascorbic acid (Sigma-Aldrich), 200 mg/L calcium chloride (Sigma-Aldrich), 0.08% chondroitin sulfate (Fujifilm Wako Pure Chemical Corporation), 10 μM Y-27632 (Fujifilm Wako Pure Chemical Corporation), 10 μM SB203580 (Cayman Chemical Co), and 50 μg/mL gentamicin. The HCECs at passage 0 were cultured at 37° C in a humidified 5% CO_2 atmosphere with the change of culture medium twice weekly. After reaching confluency at 5 weeks, the cultured cells were passaged at the cell density of 800 cells/mm^2, continuing each culture using the same media for 5 weeks until confluency at passage (P) 1 (P1). The cultured donor CECs at P1 confluency was used for the subsequent flow cytometric analysis. Those cells at P0 confluency were not able to be used for analysis because of the shortage of cell amount.

Flow Cytometry Analysis of the Cultured Donor CECs

Cultured donor HCECs at P1 confluency were collected from the culture dish by treatment with 10 × TrypLE Select (Thermo Fisher Scientific, Inc) at 37° C for 12 minutes. The cells were then suspended at a concentration of 4 × 10^6 cells/mL in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline containing 1% bovine serum albumin and 0.05% sodium azide). An equal volume of antibody solution was added and incubated at 4° C for 2 hours. The antibodies were the following: E-conjugated antihuman CD166 mAb, PerCP-Cy 5.5 conjugated antihuman CD24 mAb, PE-Cy 7-conjugated antihuman CD44 mAb (all from BD Biosciences), and Allophycocyanin-conjugated antihuman CD105 mAb (Thermo Fisher Scientific, Inc). After washing with FACS buffer, the cultured HCECs were analyzed with a FACS-Canto II Flow Cytometry Analyzer System (BD Biosciences). The content of mature-differentiated HCECs (CD166$^+$, CD44$^{-/dull}$, CD24$^-$, and CD105$^-$) was measured by FACS. The cultured HCECs were classified into the following 3 groups: (1) high-maturity group: a > 70% content of mature-differentiated HCECs, (2) middle-maturity group: a 10% to 70% content of mature-differentiated HCECs, and (3) low-maturity group: a < 10% content of mature-differentiated HCECs, based on the top 25%, the middle 50%, and the bottom 25% of the entire donors, respectively, as the overall ECD, as well as the ECL, at 36 months postoperative was normally distributed (Kolmogorov−Smirnov test; $P > 0.100$) (Fig S1A−D, available at www.ophthalmologyscience.org).

Surgical Technique

The donor corneas in this study were obtained from CorneaGen Eye Bank. All DSAEK flaps used for implantation were prepared by CorneaGen before shipping to Japan. The patients underwent general anesthesia (or retrobulbar anesthesia if they had any previous history of respiratory, heart, or kidney problems). The host cornea was trephined using a Hessburg-Barron Vacuum Trephine (Katena Products, Inc) or a Moria One Single-Use Adjustable Vacuum Trephine (Moria, Inc) for PK, and the donor cornea was then cut with a Barron Vacuum Donor Cornea Punch (Katena Products) or a Moria One Corneal Vacuum Punch (Moria, Inc) for PK and DSAEK. The techniques used for all DSAEK and PK surgeries were as previously described.[19,20] Briefly, for DSAEK, the Descemet membrane at the central posterior cornea was removed using a reverse Sinskey hook (Bausch & Lomb Inc), the prepared DSAEK flap was inserted into the anterior chamber using a Busin glide, and air was then injected into the anterior chamber to sufficiently increase the intraocular pressure to ensure firm attachment of the graft to the host cornea. For PK, after trephination, the donor corneal graft was fixed to the host eye with 8 interrupted sutures, followed by a continuous suture. Cataract extraction was performed by phacoemulsification and aspiration, followed by intraocular lens implantation or transscleral suture of intraocular lens with 10-0 polypropylene if necessary.

Postoperative Management

As we previously reported,[19] after corneal transplantation, each patient received a systemic dose of 4 mg betamethasone for 2 days, followed by 1 mg betamethasone for 5 days, together with topical application of 0.3% gatifloxacin and 0.1% betamethasone eye drops 4 times daily. An adequate systemic dose (i.e., 125 mg) of methylprednisolone was administered immediately before surgery. The topical 0.1% betamethasone eye drops were continued for 6 postoperative months for patients who underwent DSAEK and PK, and then tapered to 0.1% fluorometholone eye drops 2- to 4-times daily.

Clinical Evaluation

To investigate the relationship between the postoperative ECD and the biologic indicator in maturity of donor CECs, ECD in each patient was measured every 6 months with a noncontact specular microscope (EM-3000; Tomey Corporation) and the biological quality of the donor cornea was evaluated by HCEC culture (Fig 1). Donor CECs were cultured from the peripheral area of the donor transplant remaining after puncture removal, according to our previously reported method,[17] and HCEC morphology and maturity were determined according to the expression pattern of surface markers at 5 weeks after the P1 culture. CD166$^+$, CD44$^{-/dull}$, CD24$^-$, and CD105$^-$ cultured donor CECs, with a high maturity, were considered to represent healthy in vivo donor CECs,[15] as described in our previous paper.[18] In this study, the donor characteristics included donor age, sex, trephination size, cause of death, donor-cell preservation time, and the number of days postmortem of the donor cornea. Donor cause of death was classified into the following 2 groups: (1) acute (e.g., heart disease, cerebrovascular disease, or acute respiratory failure [e.g., asphyxia]), and (2) chronic (e.g., a malignant tumor or chronic liver disease).

Statistical Analysis

Statistical analyses were performed using the Prism 9 version 9.2.0.283 software (GraphPad Software). The ECD graphs are presented as the median ± 25 to 75 percentile. Normality assumption for samples was examined with Kolmogorov−Smirnov test and normal quantile−quantile plot. Dunn's multiple comparison test analyzed the ECD and ECL after PK or DSAEK among the maturity groups at each assessment. According to the maturity of the donor cornea, the postoperative ECD over time was analyzed with the mixed effects model for repeated measures. Each patient was determined as a random effect, and time and maturity were determined as fixed effects. According to the donor maturity, the statistical significance was analyzed with a log-rank test in cases maintained at > 1000 cells/mm^2, 1500 cells/mm^2, and 2000 cells/mm^2 at 36 months postoperative. The Spearman rank correlation coefficient test examined the correlation between the maturity of donor HCECs and postoperative ECL. Differences in recipient characteristics, donor characteristics corresponding to each recipient, and recipient and postoperative ECDs among the 3 groups were analyzed using the Kruskal−Wallis test or the chi-square test with Bonferroni correction. A P value of < 0.05 was considered statistically significant.

Table 1. Characteristics of Donor and Recipient According to Maturity of Cultured Corneal Endothelial Cells

	High Maturity	Middle Maturity	Low Maturity	*P* Value
Donor				
Age, mean (SD), y	60.5 (8.0)	61.1 (10.1)	61.7 (9.6)	0.731
Range	42–71	23–74	30–73	
Female, n (%)	12 (71)	12 (38)	8 (42)	0.080
ECD, mean (SD), cells/mm^2	2695 (194)	2832 (336)	2734 (236)	0.403
Range	2505–3094	2506–3543	2503–3296	
Cause of death (acute/chronic)	12/5	22/10	11/8	0.666
Death to preservation mean (SD), min	817 (343)	808 (310)	709 (398)	0.426
Range	345–1424	375–1427	210–1430	
Postmortem days mean (SD), d	6.0 (0.8)	6.0 (0.9)	6.4 (1.1)	0.248
Range	5–8	4–7	4–9	
Trephination sizes (SD), mm	7.90 (0.21)	7.94 (0.25)	7.92 (0.40)	0.730
Range	7.5–8.5	7.25–8.5	7.0–9.0	
Recipient				
Age, mean (SD), y	62.5 (16.8)	69.6 (12.5)	70.5 (11.3)	0.343
Range	26–85	44–94	49–88	
Female, n (%)	10 (59)	13 (41)	9 (47)	0.483
Primary indication, n (%)				
Corneal opacity	5 (29)	12 (38)	4 (21)	0.470
Glaucoma-related BK	5 (29)	7 (22)	5 (26)	0.837
PBK/ABK	3 (18)	7 (22)	5 (26)	0.824
FECD	1 (6)	3 (9)	2 (11)	0.878
KC	2 (12)	2 (6)	0 (0)	0.329
Other BK	1 (6)	1 (3)	3 (16)	0.242
Surgical procedure, n (%)				
DSAEK	4 (24)	13 (41)	8 (42)	0.429
DSAEK+IOL	3 (18)	4 (13)	4 (21)	0.716
PK	6 (35)	9 (28)	2 (11)	0.202
PK+IOL	4 (24)	6 (19)	5 (26)	0.811
Total	17	32	19	

ABK = aphakic BK; BK = bullous keratopathy; DSAEK = Descemet stripping automated endothelial keratoplasty; ECD = endothelial cell density; FECD = Fuchs endothelial corneal dystrophy; IOL = intraocular lens; KC = keratoconus; PBK = pseudophakic BK; PK = penetrating keratoplasty; SD = standard deviation.
Statistical analysis for multiple comparison was performed with Kruskal–Wallis test.

Results

Maturity of the Cultured Donor CECs

Phase-contrast microscopy revealed a nonfibroblastic phenotype with a characteristic polygonal shape and monolayer in the maturity groups; however, HCECs in the low-maturity group showed the contamination of fibrotic phenotype, including endothelial-mesenchymal transition and senescence cells, and were more variable in size than in the high-maturity group and middle-maturity group. These morphologic phenotypes suggested a substantial number of nonfunctional CECs existed. Assessment of the content of mature-differentiated HCECs in the cultured donor CECs by FACS revealed 17 eyes in the high-maturity group, 32 eyes in the middle-maturity group, and 19 eyes in the low-maturity group (Table 1, Figs S2A, B, available at www.ophthalmologyscience.org).

Examination of the variable donor factors that could affect the quality of the donor-eye CECs, including donor age, donor sex, donor ECD, trephination size, cause of death, elapsed time from death to preservation, and the number of days postmortem of the donor cornea, revealed no significant differences among the 3 groups (Table 1). In addition, the recipient characteristics, including age, sex, primary indication, and surgical procedure, were examined according to the maturity of the donor CECs. No significant differences were found among the 3 groups in regard to any of the clinical factors (Table 1). Moreover, the overall ECD/ECL was normally distributed (Kolmogorov–Smirnov test; $P > 0.100$) (Figs S1A–D); however, the proportion of mature CECs displayed the right skew distribution, indicating a nonnormal distribution, as shown in the normal quantile–quantile plot (Kolmogorov–Smirnov test; $P = 0.007$), (Figs S3A, B, available at www.ophthalmologyscience.org).

Postoperative ECD and ECL According to the Maturation of Donor CECs

The postoperative ECD and the proportion of mature-differentiated HCECs in the donor corneas were examined for a potential association. The overall mean (standard deviation [SD]) ECDs at baseline and at 6, 12, 24, and 36 months postoperative in 68 eyes were 2770 (282), 2137 (477), 1926 (502), 1636 (592), and 1325 (577) cells/mm^2,

Table 2. ECD and ECL Over Time after Successful Corneal Transplantation

Characteristic	Mean ECD (SD), cells/mm^2			Mean ECL (SD), %			ECD, *P* Value	ECL, *P* Value
	High Maturity	*Middle Maturity*	*Low Maturity*	*High Maturity*	*Middle Maturity*	*Low Maturity*		
Overall groups								
Baseline	2695 (194)	2832 (336)	2734 (236)				0.403	
6 m	2268 (200)	2157 (524)	2000 (531)	17 (9)	24 (15)	26 (18)	0.432	0.281
12 m	2034 (286)	1999 (535)	1711 (542)	24 (13)	29 (18)	35 (17)	0.164	0.211
24 m	1878 (437)	1770 (579)	1226 (530)	30 (18)	38 (18)	53 (17)	0.003	0.006
36 m	1604 (436)	1424 (613)	911 (388)	40 (18)	50 (21)	66 (15)	< 0.001	< 0.001
Subgroup (DSAEK)								
Baseline	2811 (235)	2833 (320)	2722 (204)				0.611	
6 m	2293 (97)	2079 (611)	1877 (543)	20 (7)	27 (17)	31 (20)	0.452	0.570
12 m	1954 (263)	1854 (572)	1683 (443)	31 (12)	35 (19)	38 (17)	0.400	0.630
24 m	1949 (293)	1638 (613)	1278 (473)	31 (12)	46 (23)	53 (17)	0.034	0.053
36 m	1639 (373)	1373 (541)	1026 (381)	41 (15)	51 (19)	62 (14)	0.025	0.072

DSAEK = Descemet stripping automated endothelial keratoplasty; ECD = endothelial cell density; ECL = endothelial cell loss; SD = standard deviation. Statistical analysis for multiple comparison was performed with Kruskal–Wallis test.

respectively. No allograft rejection occurred in this study, and all of the grafts retained their transparency throughout the follow-up period.

The mean (SD) ECD at baseline and at 6, 12, 24, and 36 months postoperative was 2695 (194), 2268 (200), 2034 (286), 1878 (437), and 1604 (436) cells/mm^2, respectively, in the high-maturity group, 2832 (336), 2157 (524), 1999 (535), 1770 (579), and 1424 (613) cells/mm^2, respectively, in the middle-maturity group, and 2734 (236), 2000 (531), 1711 (542), 1216 (530), and 911 (388) cells/mm^2, respectively, in the low-maturity group (Table 2). At 6 months postoperative, the ECD in each group was found to have declined sharply from that at baseline. Thereafter, the ECD decrease became slow and gradual in the high- and middle-maturity groups, whereas a steady decline remained in the low-maturity group. A comparison of the 3 groups revealed that there was a significant decrease of ECD in the low-maturity group at 24 and 36 months postoperative (Kruskal–Wallis test, $P = 0.003$ and $P < 0.001$, respectively), and a significantly lower ECD in the low-maturity group than in the high-maturity group at 24 and 36 months postoperative (Dunn's multiple comparison test, $P = 0.007$ and $P < 0.001$, respectively) (Fig 2 and Table 2) and the middle-maturity group at 24 and 36 months postoperative (Dunn's multiple comparison test, $P = 0.011$ and $P = 0.007$, respectively). Endothelial cell loss in the low-maturity group displayed a significant decrease of 53% at 24 months postoperative and 66% at 36 months postoperative, compared with the 24 and 36 months postoperative ECL of 38% and 50%, respectively, in the middle-maturity group and 30% and 40%, respectively, in the high-maturity group (Kruskal–Wallis test, $P = 0.006$ and $P < 0.001$, respectively) (Table 2). In addition, the mixed effects model revealed that all 3 groups displayed a significant decrease in ECD ($P < 0.001$) throughout the time of the examination period. In this context, there was a significant difference in ECD change over time among all 3 maturity groups ($P < 0.001$) (Fig 2). Generally, the middle-maturity group tended to show an intermediate level of ECL and ECD change between the high- and low-maturity groups.

Postoperative ECD and the Maturity of Donor CECs

A high retention of ECD throughout the postoperative follow-up period is a clinically useful hallmark of a successful corneal transplantation. Endothelial cell density that was maintained at a density of > 1000 cells/mm^2, 1500 cells/mm^2, and 2000 cells/mm^2 at 36 months postoperative was analyzed with the Kaplan–Meier survival curve according to the maturity of the donor CECs. Unlike the high-maturity group, the low-maturity group significantly failed to maintain ECD at 1500 cells/mm^2 at 36 months postcorneal transplantation, which is thought to be one of the hallmark indicators of long-term graft survival (Fig 3A). The high-maturity group was found to be more likely to maintain an ECD > 1500 cells/mm^2 for over 3 postoperative years (log-rank test; $P < 0.001$), as well as an ECD of 2000 cells/mm^2 and 1000 cells/mm^2 (log-rank test; $P = 0.021$ and $P = 0.001$, respectively) (Fig 3A and Figs S4A, B, available at www.ophthalmologyscience.org).

Postoperative ECD in Patients Who Underwent DSAEK and the Maturity of Cultured Donor CECs

The patients in this present study underwent 2 different surgical procedures, DSAEK and PK, which can influence, to some extent, the postoperative ECD validation because of the primary indication and the amount of surgical invasion. Thus, to minimize the amount of surgical bias, ECD and ECL were analyzed in the patients who underwent DSAEK alone. The overall mean (SD) ECD after DAESK at baseline and at 6, 12, 24, and 36 months preoperative was 2792 (272), 2039 (548), 1813 (488), 1566 (564), and 1290 (499) cells/mm^2, respectively, and the mean (SD) ECL was 27 (17), 35 (17), 46 (20), and 53 (18)%, respectively (Figs S5A, B, available at www.ophthalmologyscience.org),

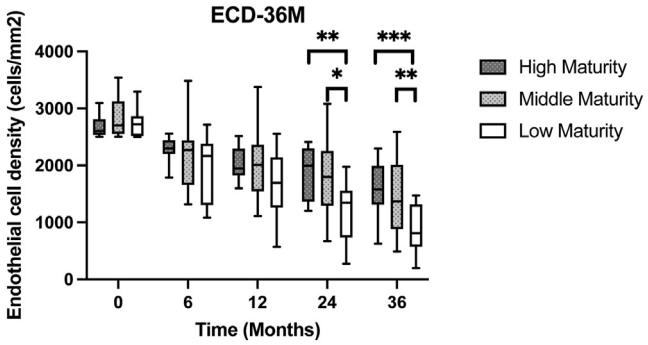

Figure 2. Postoperative endothelial cell density (ECD) over time in patinets transplanted with donor corneal grafts consiting of corneal endothelial cells (CECs) of differing maturity. Overall, the ECD in the groups included Descemet stripping automated endothelial keratoplasty and patients who underwent penetrating keratoplasty according to the maturity of the donor CECs. The upper and lower edges of each box represent the interquartile range (25th–75th percentile). The line inside each box is the median. The upper bar indicates the maximum value and the lower bar indicates the minimum value. ***$P < 0.001$, ** $P < 0.01$, and * $P < 0.05$.

whereas following PK, the mean (SD) ECD was 2746 (300), 2241 (368), 2049 (494), 1713 (622), and 1344 (657) cells/mm^2, respectively, and the mean (SD) ECL was 18 (10), 23 (15), 38 (23), 51 (24)%, respectively (Figs S5C, D).

Previous reports have indicated that short-term ECL is higher post-DSAEK than post-PK,[21] even though the ECL was found to be comparable for both DSAEK and PK by 10-years postoperative.[22] In fact, there was a greater decline of ECD at 6 months post-DSAEK because of predicted surgical trauma, and the ECL was much lower in patients who underwent DSAEK than in patients who underwent PK until 24 months postoperative. The maturity of the donor CECs was assessed further for patients who underwent DSAEK alone, because the recipients of DAESK are considered to have a better-controlled background than the recipients of PK.

In regard to the donor factors and the recipient backgrounds in this present study, no significant differences were found among the 3 groups. The mean (SD) ECDs at 6, 12, 24, and 36 months postoperative was 2.811 (235), 2293 (97), 1954 (263), 1949 (293), and 1639 (373) cells/mm^2, respectively, in the high-maturity group, 2833 (320), 2079 (611), 1854 (572), 1638 (613), and 1373 (541) cells/mm^2, respectively, in the middle-maturity group, and 2722 (204), 1877 (543), 1683 (443), 1278 (473), and 1026 (381) cells/mm^2, respectively, in the low-maturity group (Figs S6, available at www.ophthalmologyscience.org and Table 2). The ECD at 24 and 36 months postoperative was significantly higher in the high-maturity group than in the low-maturity group (Dunn's multiple comparison test, $P = 0.030$ and $P = 0.025$, respectively), and the trend for postoperative ECD in each group was similar to that shown in Figure 2 (Fig S6). When compared with the ECDs of the high and middle-maturity groups, the ECD in the low-maturity group continued to decline, and a greater decrease of ECD was found in the low-maturity group compared with the high-maturity group, which remained throughout the follow-up period (Fig S4). Kaplan–Meier survival curve analysis revealed that the low-maturity group displayed early failure to maintain ECD at 1500 cells/mm^2, and that the survival rate was significantly higher in correlation with the maturity of the donor HCECs (log-rank test, $P = 0.005$) as well as what was observed in cases with an ECD maintained at 2000 and 1000 cells/mm^2 ($P = 0.027$ and $P = 0.029$, respectively) (Fig 3B and Figs S7A, B, available at www.ophthalmologyscience.org).

Correlation Between Maturity of Donor CECs and ECL Post Corneal Transplantation

The association between the maturity of the CECs and postoperative ECL was analyzed with the Spearman rank test. The postoperative ECL clearly declined in direct relation to the proportion of mature CECs ($r = -0.365$, 95% confidence interval, -0.560 to -0.132, $P = 0.002$) (Fig S8A, available at www.ophthalmologyscience.org). The correlation analysis in DSAEK alone showed similar results ($r = -0.290$, 95% confidence interval, -0.572 to 0.05, $P = 0.086$) (Fig S8B). In fact, the histogram bar displayed that in accordance with the cell maturity, the high-maturity group included more patients with a lower ECL and the low-maturity group included more patients with a higher ECL, whereas the ECL in the middle-maturity group was in the middle (Figs S9A–C, available at www.ophthalmologyscience.org).

Discussion

The results of this present study indicate that the donor peripheral CECs that display a high proportion of mature-differentiated cultured HCECs at P1 (high-maturity group) correspond well to a higher postoperative ECD at the center of the transplanted donor cornea in the midterm postoperative period when compared with donors who contain a low proportion of mature-differentiated cultured HCECs (low-maturity group). In fact, the high-maturity group showed a slow decrease in ECD when compared with the steeper decline seen in the low-maturity group, and displayed a large number of patients with an ECD of > 1500 cells/mm^2 at 36 months post-DSAEK and PK. In our novel cultured HCEC-injection therapy, the proportion of mature-differentiated HCECs is used as a biologic marker of quality control.[17] In other words, there is accumulating evidence that a higher proportion of mature-differentiated cells in culture results in better-quality cell products. For example, 1 clinical trial that injected reasonably high-maturity cultured HCECs into the anterior chamber for the treatment of BK reported cell survival that extended over a 5-year follow-up period.[23] Furthermore, and as shown in our previous report, when a cell subpopulation consisting of $> 90\%$ mature-differentiated cells is used for cultured HCEC-injection therapy, it results in a better ECD and low-ECD attrition over a period of 3 postoperative years.[24] Moreover, we previously reported that the high-maturity HCECs that possess unique characteristics of cell-surface markers, such as CD166$^+$, CD44$^{-/dull}$, CD24$^-$, CD26$^-$, and CD105$^-$, were capable of growth, even when seeded at a low cell density in the culture dish, whereas low-

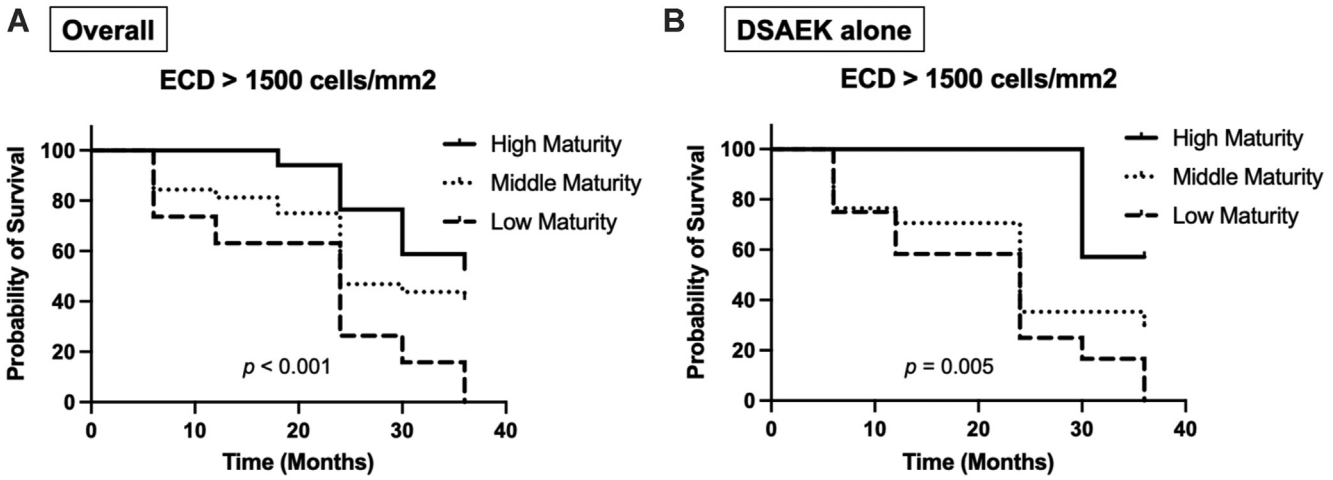

Figure 3. Kaplan–Meier survival curve graph of the cases in which an endothelial cell density (ECD) of 1500 cells/mm^2 was maintained post-transplantation of donor corneal grafts consiting of corneal endothelial cells of differing maturity. **A,** Overall, the group includes patients who underwent Descemet stripping automated endothelial keratoplasty (DSAEK) and penetrating keratoplasty. The surfival rate indicates the cases in which an ECD of > 1500 cells/mm^2 was maintained throughout the postoperative follow-up period. The log-rank test was used for statistical analysis. **B,** Subgroup that included DSAEK patients only.

maturity HCECs exhibited poor proliferative behavior even though they stained positive for ZO-1 and Na^+/K^+/ATPase,[18] 2 well-known markers of normal HCECs.[25] Furthermore, mature-differentiated HCECs have displayed a reasonable metabolic activity that can maintain their longevity and functions.[16,26] As shown in Figure S1, high-maturity HCECs maintain their hexagonal shape and are smaller in size than low-maturity HCECs, thus suggesting that high-maturity HCECs avoid epithelial-mesenchymal transition or a senescence phenotype undergoing cell death. In fact, we previously reported that the biologic status of mature-differentiated HCECs is controlled through the intracellular signaling pathway by positive regulation of p53 and miR34a and negative regulation of c-Myc, thus resulting in maintaining a healthy oxidative phosphorylation metabolism.[26–28] Taken as a whole, the above evidence may explain why the high-maturity group of HCECs was found to be associated with the extended longevity of graft survival post transplantation.

Previous reports have demonstrated that donor age,[4,29] donor sex,[5] and corneal preservation time[8,30,31] were associated with graft survival and ECL. It is true that sex differences can affect the longevity of the CECs, even though that factor was not found to be statistically significant in this present study. In previous studies, it has been reported that the female donor factor has a positive effect on ECD during the postoperative follow-up period.[5,6] However, findings in other reports have demonstrated that female sex is known to be a risk for Fuchs endothelial corneal dystrophy, and that estrogen metabolites cause oxidative stress, thus leading to CEC apoptosis.[32,33] Hence, it remains controversial as to whether or not female sex has a positive effect on CECs posttransplantation.

In this study, we highlighted evidence that the biologic maturity of cultured HCECs from donor corneas is also a potential predictor for graft survival posttransplantation, even though the differences in surgical procedures should be taken into account. Overall, our data and close analysis of the patients after undergoing DSAEK showed similar ECD trends among the groups, and no donor factors were related to the maturity of HCECs. Our present findings seem to suggest that both the primary indication and the specific surgical procedure used has no influence on the postoperative ECD. We previously reported the presence of dead cells in donor corneal endothelium preserved in storage medium, and presented our finding that the number of those cells declined after incubation.[14] Those findings suggest that an endothelial cell count alone is not sufficient for a proper judgment of the quality of donor corneas and may ultimately result in cell death after keratoplasty. Considering these findings, cell maturity in culture would seem to be an independent donor factor that could be used to predict longer HCEC survival post transplantation. This biologic character may be influenced by either the intrinsic factor of the donor CEC itself or the specific method used for donor cornea storage, possibly because of the oxidative stress environment.

Postoperative ECL can be affected by baseline recipient characteristics and donor factors. Unlike Fuchs endothelial corneal dystrophy, both pseudophakic/aphakic BK and glaucoma are reportedly associated with an increased risk of graft failure.[34,35] Several previous reports have shown that the graft survival rate at 5 years postoperative declines to < 50% in DSAEK or Descemet membrane endothelial keratoplasty patients with previous history of glaucoma,[36–38] and the potential effect of the recipient host environment because of the primary indication for surgery should be addressed. However, in the present study, 4 of the 5 patients with glaucoma (80%) in the high-maturity group were found to have maintained an ECD of >1000 cells/mm^2 at 36 months postoperative, whereas over that

same postoperative period, ECD was maintained in only 40% of the patients with glaucoma in the low-maturity group even though there was no significant difference between the 2 groups because of underpowering. The mechanism underlying ECL remains unclear, and it seems to be unrelated to donor age, sex, trephination size, cause of death, elapsed time from death to preservation, and the number of days postmortem of the donor cornea. Thus, it seems that in future studies it would be beneficial to try to elucidate the biomarkers associated with the high/low maturity of endothelial cells, as they can be assessed noninvasively.

Limitations

It should be noted that in this present study, the evaluation of donor CECs was performed with cell culture, so some technical experimental bias may have occurred. However, we adhered to the previously published protocols for HCEC culture applied to clinical trials,[17] which should have minimized this bias to a large degree. Moreover, an additional argument could be raised in regard to the difference in the region of the cornea in which CECs were obtained; that is, the central versus peripheral region. However, from our experience, there has been no difference in cell growth in CECs obtained from those 2 regions. Another limitation in this study was that the low-maturity group included 12 cases that contained none of mature HCECs, in which of cases did not even grow the CECs in culture and the sample population displayed right-skewed distribution. However, that did not seem to influence the present results showing the lower ECD and higher ECL in the low-maturity group postsurgery. In addition, the previously reported evidence that the injection of cultured HCECs with high maturity survived throughout a 5-year follow-up period[23] also supports the present findings. Another possible limitation is that the baseline recipient characteristics varied. Although the number of cases analyzed in this study was limited, the ECD trend after DSAEK for the treatment of BK showed that the HCECs had a more prolonged survival in the high-maturity group than in the low-maturity group, thus suggesting that the present findings were reasonable.

Donor corneas with higher-maturity HCECs in culture were found to contribute to higher postoperative ECD and lowered postoperative ECL, thus suggesting that a high content of mature HCECs in culture can result in an extended longevity and healthiness of primary in vivo HCECs, which could be a predictive indicator of long-term graft survival. A better understanding of the molecular mechanism underlying the maintenance of HCEC maturity will hopefully lead to the elucidation of the mechanism of ECL after successful corneal transplantation and the successful development of effective interventions.

Footnotes and Disclosures

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HUMAN SUBJECTS: Human subjects were included in this study. This cohort study of consecutive patients who underwent DSAEK or PK was conducted at the Baptist Eye Institute, Kyoto, Japan, and was approved by the Research Ethics Committee of Kyoto Prefectural University of Medicine (Approval #ERB-C 1006). Before surgery, written informed consent was obtained from all subjects in accordance with the tenets set forth in the Declaration of Helsinki.

No animal subjects were used in this study.

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Obtained funding: Kinoshita

Overall responsibility: Kitazawa, Toda, Sotozono, Kinoshita

Abbreviations and Acronyms:
BK = bullous keratopathy; **CEC** = corneal endothelial cell; **DSAEK** = Descemet stripping automated endothelial keratoplasty; **ECD** = endothelial cell density; **ECL** = endothelial cell loss; **FACS** = fluorescence-activated cell sorting; **HCEC** = human CEC; **P** = passage; **PK** = penetrating keratoplasty; **SD** = standard deviation.

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Corneal endothelial cell density, Corneal transplantation, Cultured corneal endothelial cells, DSAEK, Endothelial cell loss.

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