AMERICAN ACADEMY OF OPHTHALMOLOGY®

Five-Year Follow-up of First 11 Patients Undergoing Injection of Cultured Corneal Endothelial Cells for Corneal Endothelial Failure

Kohsaku Numa, MD,[1] *Kojiro Imai, MD, PhD,*[1] *Morio Ueno, MD, PhD,*[1] *Koji Kitazawa, MD, PhD,*[1] *Hiroshi Tanaka, MD, PhD,*[1] *John D. Bush, BA,*[1] *Satoshi Teramukai, PhD,*[2] *Naoki Okumura, MD, PhD,*[3] *Noriko Koizumi, MD, PhD,*[3] *Junji Hamuro, PhD,*[1] *Chie Sotozono, MD, PhD,*[1] *Shigeru Kinoshita, MD, PhD*[4]

Purpose: To report the safety and efficacy of a novel cell injection therapy using cultured human corneal endothelial cells (hCECs) for endothelial failure conditions via the report of the long-term 5-year postoperative clinical data from a first-in-humans clinical trial group.

Design: Prospective observational study.

Participants: This study involved 11 eyes of 11 patients with pseudophakic endothelial failure conditions who underwent hCEC injection therapy between December 2013 and December 2014.

Methods: All patients underwent follow-up examinations at 1 week, 4 weeks, 12 weeks, and 24 weeks and 1 year, 2 years, 3 years, 4 years, and 5 years after surgery. Specific corneal endothelial cell parameters (i.e., corneal endothelial cell density [ECD], coefficient of variation of area, and percentage of hexagonal cells) and central corneal thickness, best-corrected visual acuity (BCVA) on a Landolt C eye chart, and intraocular pressure (IOP) were recorded.

Main Outcome Measures: The primary outcome was the change in central ECD after cell injection therapy, and the secondary outcome was corneal thickness, BCVA, and IOP during the 5-year-postoperative follow-up period.

Results: At 5 years after surgery, normal corneal endothelial function was restored in 10 of the 11 eyes, the mean ± standard deviation central corneal ECD was 1257 ± 467 cells/mm^2 (range, 601−2067 cells/mm^2), BCVA improved significantly in 10 treated eyes, the mean visual acuity changed from 0.876 logarithm of the minimum angle of resolution before surgery to 0.046 logarithm of the minimum angle of resolution after surgery, and no major adverse reactions directly related to the hCEC injection therapy were observed.

Conclusions: The findings in this study confirmed the safety and efficacy of cultured hCEC injection therapy for up to 5 years after surgery. *Ophthalmology 2020;■:1−11* © *2020 by the American Academy of Ophthalmology.*

Supplemental material available at www.aaojournal.org.

The current surgical treatments for corneal endothelial failure, classically termed *bullous keratopathy*, are penetrating keratoplasty,[1,2] Descemet stripping automated endothelial keratoplasty (DSAEK),[3−5] and Descemet membrane endothelial keratoplasty (DMEK),[6,7] and they all require the use of a donor cornea.[8,9] Although recent developments in endothelial keratoplasty techniques have provided for faster and more predictable visual rehabilitation,[10] they are invasive and can result in an early surge of endothelial cell loss resulting from surgical intervention[4] and graft detachment or dislocation.[11] Moreover, the corneal shape is sometimes not well restored, possibly because of the alteration of corneal thickness,[12] posterior corneal curvature,[13] increased scattering of the graft−host interface,[14] and induced high-order aberrations.[15,16] Descemet membrane stripping only (DSO), also known as descemetorhexis without endothelial keratoplasty, is a procedure that involves the removal of a small area of the failed central endothelium and Descemet membrane without the need for transplantation of a donor corneal tissue graft. Reportedly, it has been performed in select patients with Fuchs endothelial corneal dystrophy (FECD) in an attempt to restore the cornea with a normal corneal shape.[17] Although DSO is an interesting procedure because it involves no use of donor corneal tissue, and thus no risk of corneal endothelial graft rejection or possible depletion of the donor pool, its use is currently limited to early-phase cases of corneal endothelial failure and its long-term efficacy has yet to be elucidated fully. Theoretically, an ideal therapy would be a surgical

https://doi.org/10.1016/j.ophtha.2020.09.002
ISSN 0161-6420/20

procedure that reproduces the normal shape of a healthy cornea accompanied by high corneal endothelial cell density (ECD) with no structural irregularity or distortion, thus resulting in good postoperative visual acuity (VA) and proper corneal function for all types of corneal endothelial failure.

In previous studies, we reported the novel surgical procedure and early postoperative results of the injection of cultured human corneal endothelial cells (hCECs) for the treatment of several endothelial failure conditions,[18] which typically result from FECD, corneal graft failure, corneal-endothelial surgical trauma, and pseudoexfoliation syndrome (PEX) keratopathy,[19] and revealed that our new method for the injection of hCECs involves the simultaneous injection of a Rho-associated protein kinase inhibitor used as an adjunctive drug to promote corneal endothelial cell (CEC) engraftment.[18,20,21] In our previously published report,[18] we presented the clinical outcomes of our novel hCEC injection therapy at 2 years after surgery. However, to investigate the safety and efficacy of our new therapy further via the clinical research aspect, with the ultimate goal of government-approved clinical application, we deemed that it was vital for us to assess the long-term clinical data to demonstrate that the procedure is consistently safe and effective in the clinical setting. To that end, it is important to note that to verify the safety and efficacy of corneal transplantation procedures such as penetrating keratoplasty, DSAEK, and DMEK, the data obtained at 5 years after surgery are usually cited as the long-term clinical data. Thus, the purpose of the current prospective observational study was to present the 5-year follow-up results on the first 11 treated eyes to demonstrate the safety and efficacy of the hCEC injection therapy via the presentation of more long-term clinical data.

Methods

Patients

This first-in-humans clinical trial was initially approved in 2013 by the institutional review board of Kyoto Prefectural University of Medicine, Kyoto, Japan (approval no., RBMR-R-31-4) and by the Special Committee of the Japanese Ministry of Health, Labour and Welfare to observe the guidelines on clinical research using human stem cells in Japan (approval no., 0329-23). The trial was registered with identifier UMIN000036422 at www.umin.ac.jp/english/, and the data up to 2 years after surgery were previously reported elsewhere.[18]

The protocol of this 5-year prospective observational study was approved separately by the institutional review board of Kyoto Prefectural University of Medicine (approval no., 1604) and conducted in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all patients before their participation in the study. This study involved 11 eyes of 11 patients who underwent hCEC injection therapy between December 2013 and February 2014 and between September 2014 and December 2014. All 11 treated eyes had endothelial failure conditions, that is, FECD (n = 7 eyes), argon laser iridotomy-induced corneal edema (n = 2 eyes), PEX-related corneal edema[19] (n = 1 eye), and intraocular surgery-related corneal edema with intraocular lens suturing (n = 1 eye), and all 11 treated eyes were pseudophakic with a posterior chamber intraocular lens (Table 1). Before surgery, all 11 treated eyes demonstrated total, not partial, corneal edema, and none of the patients had previously undergone glaucoma drainage procedures.

Human Corneal Endothelial Cell Culture, Surgical Procedure, and Postoperative Care

The methods used in this study for the culture of the hCECs, surgical application, and postoperative care were the same as previously described.[18] Briefly, fluorescence-activated cell sorting analysis and immunohistochemical analyses of the cultured cells, as well as enzyme-linked immunosorbent assay of the cultured media, were performed to elucidate the biological characteristics of the cells.[22,23] Bacterial and viral testing were also performed. At a few hours before surgery, the unique fluid-based cell suspension in a Proteosave (Sumitomo Bakelite, Co, Ltd, Tokyo, Japan) container was created from the cells in a culture flask, and the container held 1.5×10^6 cells/450 μl in modified Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific, Waltham, MA) supplemented with Rho-associated protein kinase inhibitor Y-27632, with a final sample concentration of 100 μM used for the cell injection procedure.

At surgery, after abnormal materials on the host Descemet's membrane were mechanically removed in an 8-mm diameter of the central cornea by use of a silicone cannula irrigation needle in all patients, and a 5-mm diameter descemetorhexis was additionally performed in patients 5 and 9 because of partial Descemet's membrane rupture that occurred during the mechanical removal, the cultured hCECs (1×10^6 cells, except in patient 1) suspended in 300 μl aspirated into a dead-space–free syringe were injected into the anterior chamber, with the patients then being placed in a face-down position for 3 hours to enhance the adhesion and engraftment of the injected cells. After surgery, all patients underwent the administration of systemic and topical steroids to inhibit any innate immunity response, as described in the regimens in the detailed study protocol in our previous report (Supplemental Video, available at www.aaojournal.org).[18]

Collection of Clinical Data

All patients were examined at 1 week, 4 weeks, 12 weeks, and 24 weeks and 1 year, 2 years, 3 years, 4 years, and 5 years after hCEC injection therapy, and all data points, that is, central corneal ECD, central corneal thickness, best-corrected VA (BCVA) on a Landolt C eye chart (a measure of decimal VA), intraocular pressure (IOP), and other CEC parameters (coefficient of variation [CV] of area, and the percentage of hexagonality) were recorded at each follow-up examination. In vivo CEC images were obtained via the use of a slit-scan contact specular microscope and its computer algorithm by the center method (CellChek; Konan Medical, Co, Ltd, Nishinomiya, Japan). The central corneal thickness was measured by Scheimpflug imaging with focus on the thickness at the pupillary center (Pentacam HR; Oculus Optikgerate GmbH, Wetzlar, Germany).

Statistical Analysis

The preoperative and postoperative data were collected independently, monitored by a third-party organization, and analyzed by the Department of Biostatistics, Kyoto Prefectural University of Medicine. The outcomes were assessed as the change of the measurement from that at baseline to that at 5 years after surgery. SAS software version 9.4 (SAS Institute, Inc, Cary, NC) was used for all statistical analyses.

Table 1. Preoperative and Postoperative Clinical Summary of the Patients

Patient No.	Before Cell Injection			3 Years after Cell Injection			5 Years after Cell Injection		
	Corneal Endothelial Cell Density (cells/mm²)	*Central Corneal Thickness (μm)*	*Best-Corrected Visual Acuity**	*Corneal Endothelial Cell Density (cells/mm²)*	*Central Corneal Thickness (μm)*	*Best-Corrected Visual Acuity**	*Corneal Endothelial Cell Density (cells/mm²)*	*Central Corneal Thickness (μm)*	*Best-Corrected Visual Acuity**
1	<DL	760	0.04 (1.40)	859	508	1.20 (−0.08)	863	504	1.50 (−0.18)
2	<DL	964	0.05 (1.30)	1238	536	1.00 (0.00)	1089	540	1.20 (−0.08)
3	<DL	727	0.20 (0.70)	1984	536	1.20 (−0.08)	2011	536	1.50 (−0.18)
4	<DL	792	0.10 (1.00)	<DL	797	0.10 (1.00)	<DL	693	0.15 (0.82)
5	<DL	637	0.40 (0.40)	1035	546	1.00 (0.00)	1044	547	1.50 (−0.18)
6	<DL	775	0.10 (1.00)	1440	515	1.50 (−0.18)	1138	523	1.20 (−0.08)
7	<DL	750	0.30 (0.52)	1258	532	0.50 (0.30)	1228	547	0.60 (0.22)
8	<DL	657	0.20 (0.70)	2104	496	0.40 (0.40)	2067	510	0.70 (0.15)
9	<DL	649	0.40 (0.40)	1476	569	0.60 (0.22)	1080	576	0.80 (0.10)
10	<DL	741	0.20 (0.70)	1707	531	1.00 (0.00)	1455	530	1.20 (−0.08)
11	<DL	725	0.03 (1.52)	746	583	0.80 (0.10)	601	605	1.00 (0.00)

DL = detection limit of the instrument.
*In decimal visual acuity with logarithm of the minimum angle of resolution units in the parentheses.

Results

Characteristics of the Cultured Human Corneal Endothelial Cells

Seven lots of cultured hCECs (at passage 2 or 3) were used for the hCEC injection therapy. For the first 3 patients, 1 lot per patient was used (n = 3 lots), and for the next 8 patients, 1 lot was used for every 2 patients (n = 4 lots), per the safety regulation set by the Special Committee of the Japanese Ministry of Health, Labour and Welfare. Most of the cultured hCECs were small cobblestone-like cells (Fig 1) and met the preset quality control for this study.[18] The cultured corneal ECD ranged from 1835 to 2530 cells/mm^2 (Table S1, available at www.aaojournal.org).

Clinical Outcomes

Over the 5-year-postoperative follow-up period, excellent corneal restoration with good BCVA was continuously well maintained in 10 of the 11 treated eyes. However, in the one patient with PEX-related endothelial failure with PEX accumulation on the iris, we found that there was clinical improvement with an ECD of 871 cells/mm^2 until 2 years after surgery, gradually seen in the early-stage corneal stromal edema observed at 3 years after surgery, which did not progress by 5 years (patient 4; Table 1). Two representative patients with successful surgical outcomes are shown in Figure 2: patient 3, with FECD, and patient 6, with argon laser iridotomy-related endothelial failure. In both patients, corneal transparency and thickness were well maintained. Moreover, no local or systemic adverse events, such as immunologic endothelial rejection, uveitis, or infection, were observed in any treated eyes during the 5-year follow-up period. However, a short-term increase in IOP induced by steroid administration did occur in 1 patient that later was treated successfully.

Corneal Endothelial Cell Integrity

The primary end point of this study, an ECD of more than 500 cells/mm^2 at 5 years after surgery, was obtained in 10 of the 11 treated eyes (91%; 95% confidence interval, 59%−100%). Of those 10 eyes, 8 had a corneal ECD of more than 1000 cells/mm^2 and 2 had a corneal ECD of more than 2000 cells/mm^2 at 5 years after surgery (Fig 3; Table 1). The postoperative time course of the corneal ECD is shown in Figure 4. The mean ± standard deviation corneal ECD at 3 years, 4 years, and 5 years after surgery was 1384 ± 451 cells/mm^2 (range, 746−2104 cells/mm^2), 1268 ± 472 cells/mm^2 (range, 552−2105 cells/mm^2), and 1257 ± 467 cells/mm^2 (range, 601−2067 cells/mm^2), respectively (Fig 4; Table 1). With regard to the specific time course of the other CEC parameters, such as CV and percentage of hexagonality, the CV improved from 0.46 ± 0.076 to 0.37 ± 0.088, and the percentage of 6A also improved from 47 ± 8.7% to 54 ± 6.2%, thus indicating that the CECs stabilized over the 5-year postoperative period (Fig S1, available at www.aaojounal.org).

At 5 years after surgery, cornea guttae were still observed in the FECD eyes (patients 2, 3, 7, 8, 10, and 11). However, the guttae in those eyes had not changed or decreased to any extent in relation to the size and area as compared with that observed at 6 months and 2 years after surgery. The BCVA was more than 1.0 (Snellen equivalent, 20/20) in 4 of those 6 patients and more than 0.5 (Snellen equivalent, 20/40) in all 6 eyes.

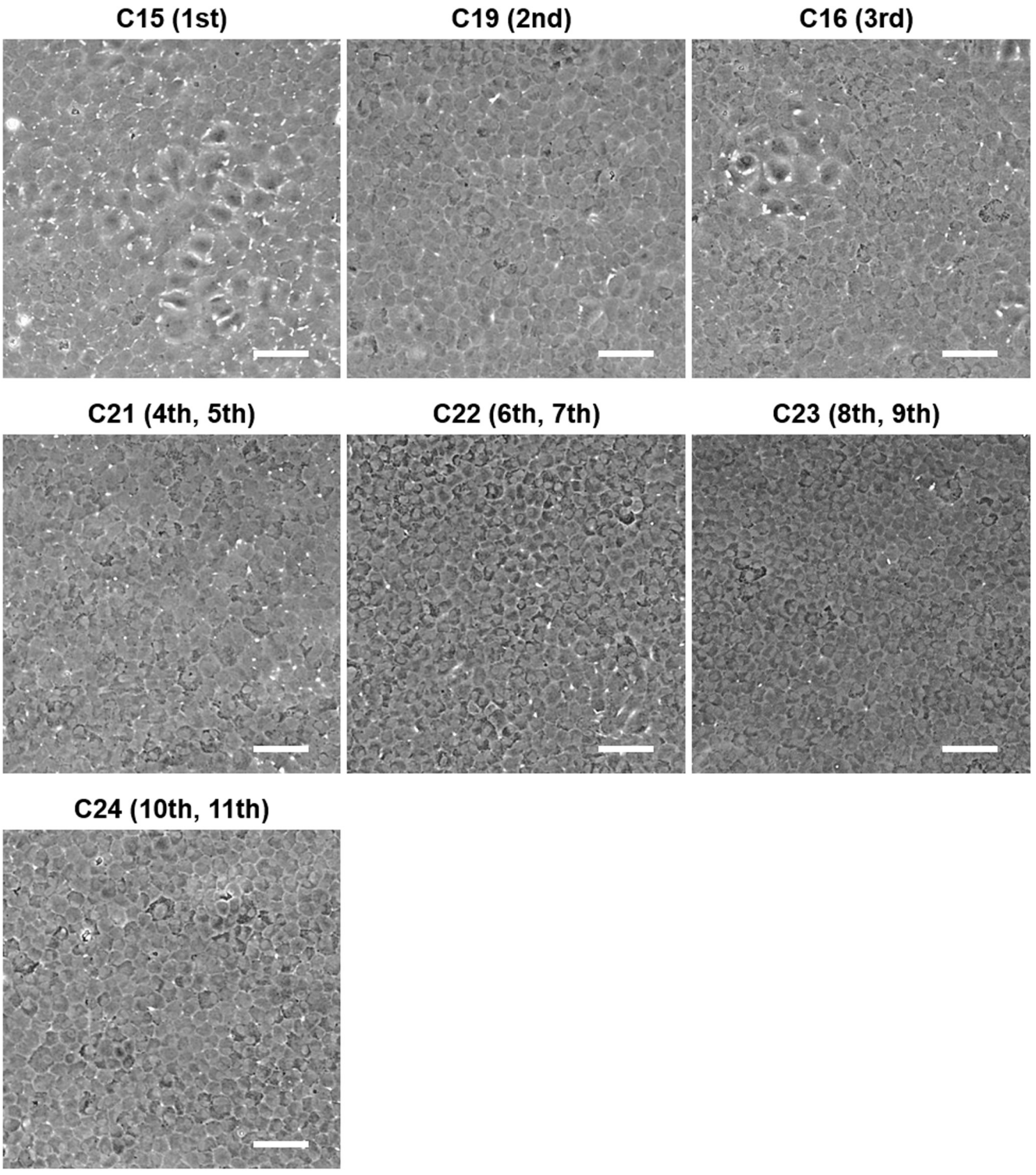

Figure 1. Inverted microscopy images of the cultured human corneal endothelial cells (hCECs) used for hCEC injection therapy. As shown in the images, most of the cultured hCECs for clinical use are high density and uniform and have a cobblestone-like shape. However, C15 and C16 contained a small cluster of cell-state transition cells. Seven lots of cultured hCECs (at passage 2 or 3) were used for the hCEC injection therapy. For the first 3 patients, 1 lot per patient was used (n = 3 lots), and for the next 8 patients, 1 lot was used for every 2 patients (n = 4 lots) per the safety regulation suggested by the Special Committee of the Japanese Ministry of Health, Labour and Welfare. Scale bars, 200 μm.

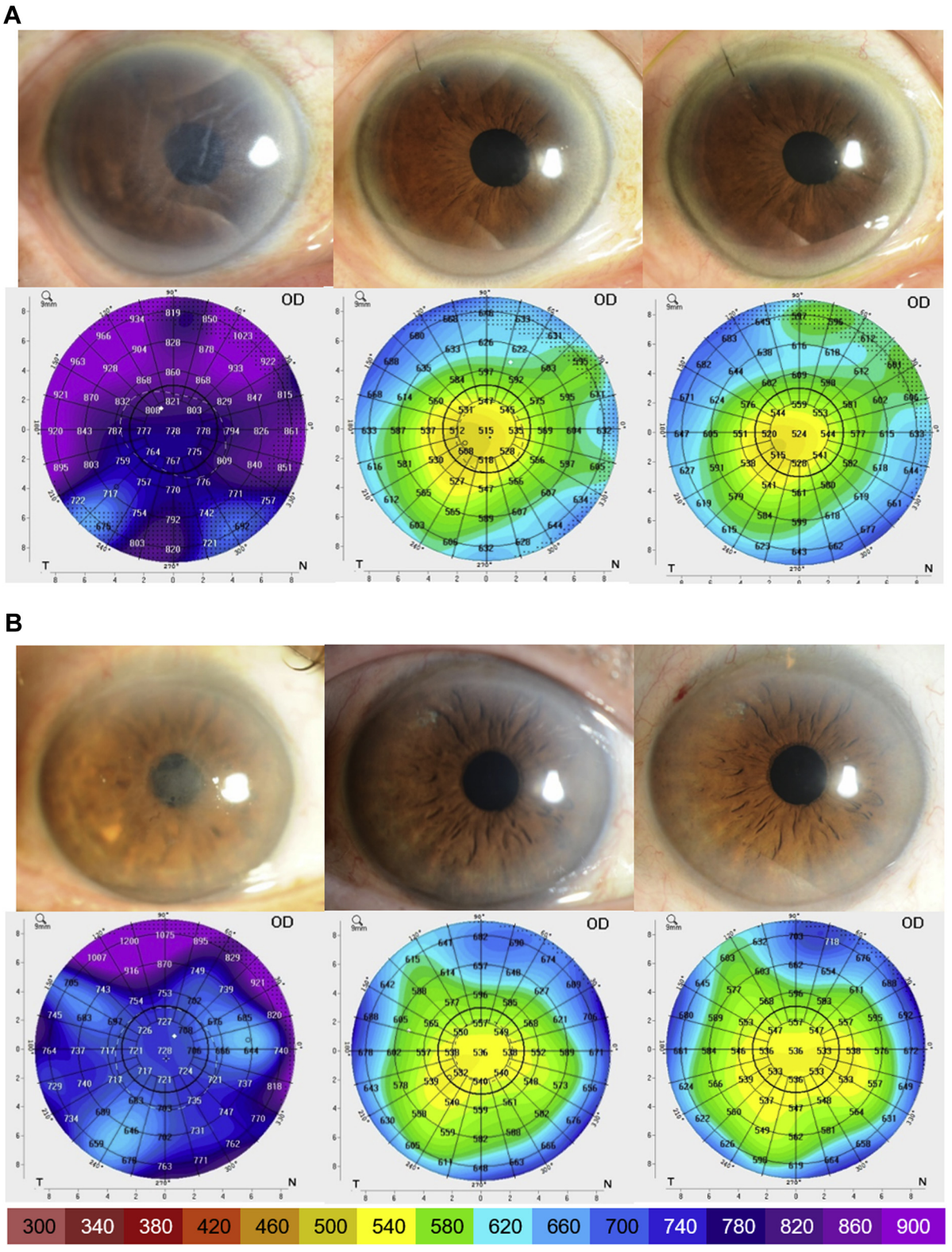

Figure 2. Slit-lamp microscopy images (top rows) and Scheimpflug camera images (bottom rows) from 2 representative patients obtained before surgery and at 3 and 5 years after cultured human corneal endothelial cell injection therapy: (**A**) patient with argon laser iridotomy-induced bullous keratopathy (patient 6) and (**B**) patient with Fuchs endothelial corneal dystrophy (patient 3). Images were obtained before surgery (left column), 3 years after injection (middle column), and 5 years after injection (right column). The color maps shown below each slit-lamp microscopy image illustrate the corneal thickness at each representative area of the corneal image above. The color bar located below (**B**) indicates the approximate corneal thickness of each of the colors shown in the maps. N = nasal; OD = right eye; T = temporal.

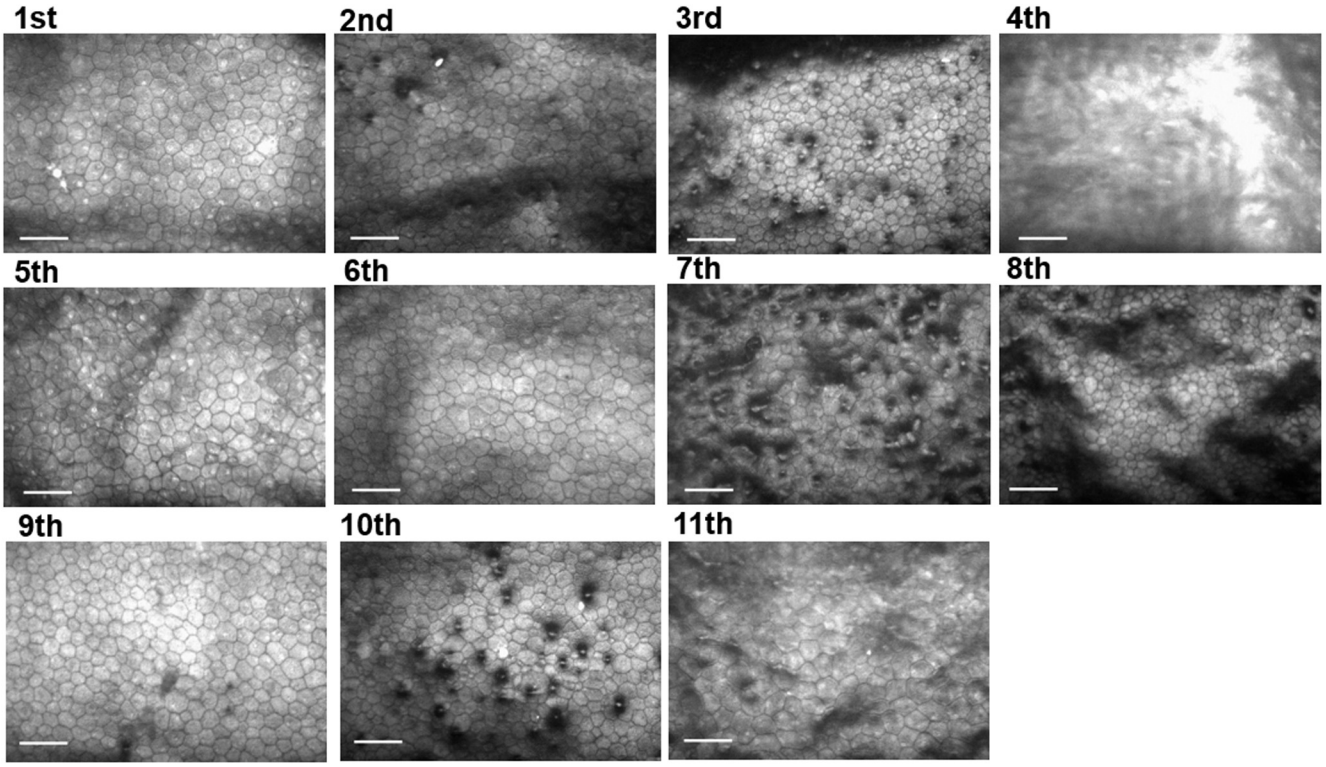

Figure 3. Contact specular microscopy images of the central cornea in each of the 11 patients at 5 years after surgery. The endothelial cells are clearly visible, and a reasonable corneal endothelial cell density can be seen in 10 of the 11 treated eyes. The image of the eye of patient 4 shows some cells yet is not clear, suggesting that the eye shows borderline corneal edema. Fuchs endothelial corneal dystrophy cases (patients 2, 3, 5, 7, 8, 10, and 11) still show corneal guttae; however, the density of corneal guttae in those cases was found to have tended to decrease. Scale bars, 100 μm.

Corneal Thickness

Corneal thickness at the center of the cornea at 5 years after surgery was within the normal range (i.e., < 630 μm) in 10 of the 11 treated eyes (Table 1). The recorded change of corneal thickness indicated a rapid decrease of corneal thickness within 4 weeks after surgery, followed by a gradual decrease of corneal thickness that was maintained up to 5 years after surgery (Fig 5A).

Best-Corrected Visual Acuity

At 5 years after surgery, BCVA improved in 10 of the 11 treated eyes (91%), a VA recovery of 0.5 or more was attained in 10 of the 11 eyes (91%; Table 1), and the mean BCVA improved to 0.046 logarithm of the minimum angle of resolution from 0.876 logarithm of the minimum angle of resolution before surgery (Fig 5B).

Intraocular Pressure

The postoperative time course of IOP is shown in Figure 5C. In 10 of the 11 treated eyes, no increase of IOP was observed during the 5-year postoperative follow-up period. However, at 8 months after surgery, the IOP in 1 eye (patient 8) increased because of a response to topical steroids. Gonioscopy examination of that eye revealed a normal anterior chamber angle. Thus, at 12 months after surgery, we performed ab externo trabeculotomy, and the IOP in that eye returned to normal and has remained within the normal range up to 5 years after surgery without the need for antiglaucoma eye-drop medication.

Discussion

The findings at 5 years after surgery in this current first-in-humans clinical trial support the indication that our novel hCEC injection therapy, which is a minimally invasive surgery, is an overall safe and effective treatment for complete corneal restoration in patients afflicted with severe

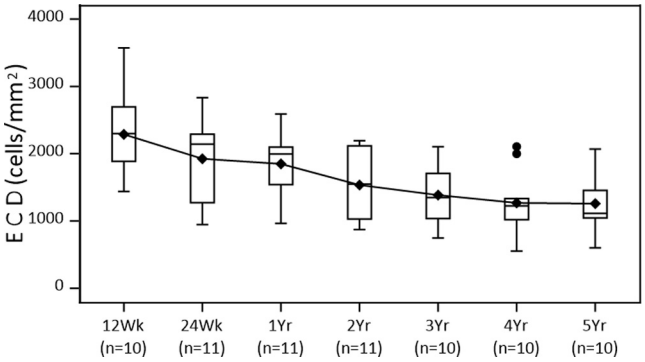

Figure 4. Box-and-whisker plot showing clinical data of corneal endothelial cell density (ECD) obtained via contact specular microscopy. The mean ECD ± standard deviation at 3 years, 4 years, and 5 years after surgery was 1384 ± 451 cells/mm^2 (range, 746–2104 cells/mm^2), 1268 ± 472 cells/mm^2 (range, 552–2105 cells/mm^2), and 1257 ± 467 cells/mm^2 (range, 601–2067 cells/mm^2), respectively.

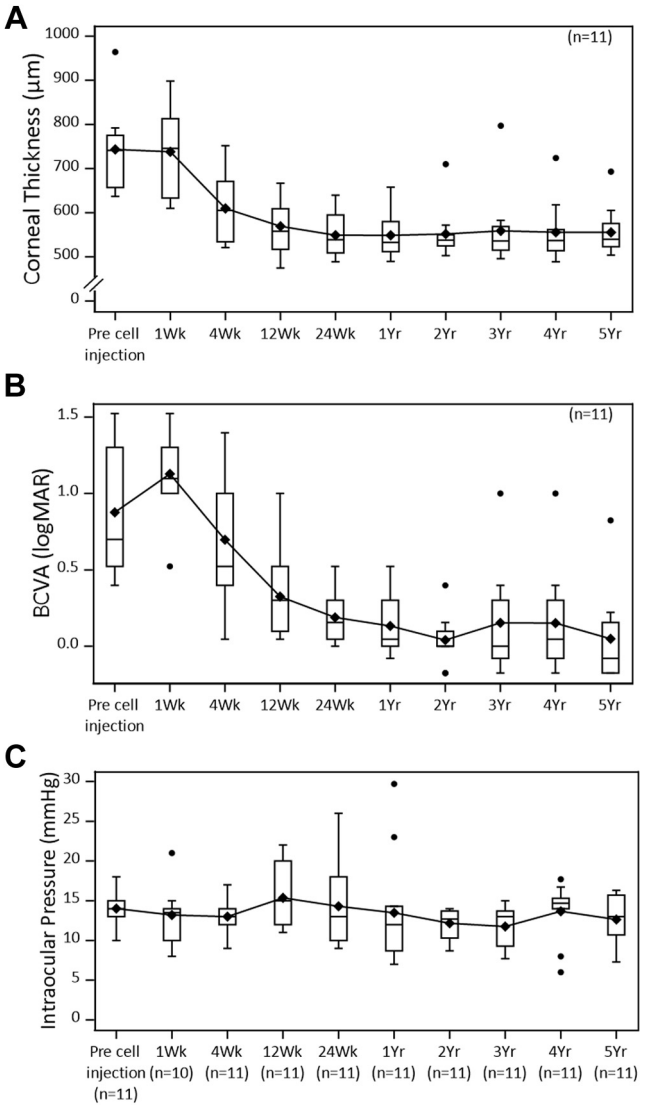

Figure 5. Box-and-whisker plots showing postoperative follow-up clinical data of (**A**) corneal thickness, (**B**) best-corrected visual acuity (BCVA), and (**C**) intraocular pressure. logMAR = logarithm of the minimum angle of resolution.

corneal endothelial failure conditions, similar to the conclusion obtained from the assessment of the findings of our 2-year postoperative data in 2018.[18] In this study, the clinical findings revealed that normal corneal thickness was attained in 10 of the 11 treated eyes during the 5-year-postoperative follow-up period and that corneal epithelial and stromal edema completely disappeared. The hCECs produced by our first-generation culture protocol were repopulated successfully, that is, on the Descemet's membrane or the bare posterior surface of the corneal stroma, or both, thus illustrating that they are biologically functional with excellent longevity. Contact specular microscopy imaging performed at 5 years after surgery revealed a relatively high corneal ECD at the center of the posterior corneal surface in 10 eyes (i.e., ranging from 601 to 2067 cells/mm^2), a decrease in CV, and an increase in the hexagonality of the cells, thus suggesting that the CECs at the posterior surface at 5 years after surgery tended to be more biophysically stable[24] than those at the early postoperative period.

An overall comparison, with 5-year-postoperative outcomes, among DSAEK, DMEK, and hCEC injection therapy eyes presented in the present study for the treatment of corneal endothelial failure is shown in Table 2. Compared with the previously published data regarding the surgical outcomes of DSAEK and DMEK at 5 years after surgery,[25–33] the findings in this pilot study showed that our novel hCEC injection therapy seems to be equivalent to the various reported clinical outcomes, including the graft survival rates and immunologic rejection rates, as well as the ECD and BCVA after surgery. Of critical importance is the finding that the clinical results in the present study were obtained via the use of cultured hCECs produced by our first-generation culture protocol.[18] Those cultured cells comprised approximately 70%, not 95% to 100%, matured cells. One PEX-related endothelial failure eye (patient 4) with severe PEX accumulation on the iris was clinically borderline or failed, probably because of an abnormal microenvironment of the anterior chamber, as previously reported by us and others.[19,34] Thus, our findings imply that the variations of CEC density after surgery, as well as postoperative clinical success, depend on either the quality of the injected cells (i.e., the so-called seeds), the corneal-endothelial disease itself, including the anterior-chamber microenvironment (i.e., the so-called soil), or both. The findings in several previous reports have provided evidence that the overall healthiness of CECs after corneal transplantation can be affected by an impaired microenvironment (i.e., bad "soil"), that is, data regarding cytokine profiles and iris damage in the anterior chamber,[35,36] as well as the size and height of the cornea guttae.[37] In addition, 1 report suggested that the biological healthiness of donor CECs has an influence on CEC density after penetrating keratoplasty.[38] Thus, a deeper understanding of these issues is critical to make marked progress in obtaining excellent clinical outcomes of both our cultured hCEC injection therapy and currently used corneal transplantation procedures.

In this study, because the host Descemet's membrane with cornea guttae was kept at the posterior corneal surface after mechanical scraping, except for that in patients 5 and 9, who additionally underwent descemetorhexis, the treated FECD eyes (i.e., patients 2, 3, 7, 8, 10, and 11) still exhibited some cornea guttae up to 5 years after surgery. However, the postoperative BCVA in those patients was reasonably good. Of particular interest is whether the cornea guttae in those treated eyes decreased or increased in size and area within the 5-year postoperative period. Although the findings of our brief observation implied that the size and area of the guttae did not increase and rather decreased, probably because of the repopulation of non-FECD healthy hCECs on the Descemet's membrane, further investigation is needed to answer this fundamental question. One important emerging topic is the comparison of the clinical outcomes among hCEC injection therapy, hCEC injection therapy with descemetorhexis, DSO, and DSO with Rho-

Table 2. Overall Comparison among Descemet Stripping Automated Endothelial Keratoplasty, Descemet Membrane Endothelial Keratoplasty, and Human Corneal Endothelial Cell Injection Therapy Eyes

Study	Year Published	Surgery	No. of Eyes	% of Fuchs Endothelial Corneal Dystrophy	Graft Survival Rate (%)	Graft Rejection Rate (%)	Endothelial Cell Density at 5 Years	Best-Corrected Visual Acuity at 5 Years			
								Logarithm of the Minimum Angle of Resolution	*Snellen > 20/20*	*Snellen > 20/25*	*Snellen > 20/40*
Wacker et al[25]	2016	DSAEK	49	100	88	2	1322	0.09	—	56	91
Ang et al[26]	2016	DSAEK	423	39	79	5	1464	—	—	—	—
Fuest et al[27]	2017	DSAEK	423	39	—	—	—	0.46	—	—	70
Fajgenbaum et al[28]	2017	DSAEK	210	81	94	11.4	870	—	—	—	—
Price et al[29]	2018	DSAEK	1312	100	93	7.9	1604	—	—	—	—
Wakimasu et al[30]	2020	DSAEK	130	13	85	5.4	1096	—	—	—	—
Madi et al[31]	2019	UT-DSAEK	354	62	94	6.9	1204	0.09	53	—	97
Schlögl et al[32]	2016	DMEK	97	91	95	1	1460	0.18	—	48	88
Price et al[29]	2018	DMEK	705	100	93	2.6	1550	—	—	—	—
Birbal et al[33]	2020	DMEK	500	89	90	2.8	1140	0.05	54	82	99
Current study	2020	Cell injection	11	64	91	0	1257	0.04	64	73	91

DMEK = Descemet membrane endothelial keratoplasty; DSAEK = Descemet stripping automated endothelial keratoplasty; UT-DSAEK = ultrathin Descemet stripping automated endothelial keratoplasty; — = not available.

associated protein kinase inhibitor topical application. Although DSO can be applied for the treatment of early-phase FECD, it probably cannot be applied for the treatment of advanced-phase FECD or cases of non-FECD endothelial failure. The preliminary data for hCEC injection therapy with descemetorhexis have shown positive results. Thus, in the future, the choice of the treatment to be applied will probably be made by the final outcome of the ECD with biophysical morphologic features on a long-term basis, that is, multiple treatment choices will be selected on a case-by-case basis. With regard to the potential adverse effects, we focused on 3 significant pathologies: increased IOP, allogeneic immune reaction, and anterior uveitis. In this 5-year prospective observational study, elevated IOP occurred in 1 eye (patient 8) at 8 months after surgery as a result of steroid-induced glaucoma and was controlled successfully via trabeculotomy. Since then, IOP was found to be within the normal range in all 11 treated eyes. Neither allogeneic immune reaction nor anterior uveitis occurred during the 5-year observation period. Because the risk of immunological rejection was reportedly relatively low with ultrathin DSAEK and DMEK compared with conventional DSAEK,[31,33] and because no case of antigen recognition after cell injection was reported in an animal model experiment,[39] we theorize that the risk in our novel hCEC injection therapy is extremely low. The preliminary results of enzyme-linked immune absorbent spot assay against interferon γ showed that no antigen recognition occurred in the patients who underwent our cultured hCEC injection therapy. Because the rate of corneal endothelial rejection is low in patients who undergo DSAEK and DMEK, we speculate that corneal transplantation-related chronic corneal endothelial dysfunction occurs mainly because of either the biologically inadequate quality of the donor CECs (although the ECD is high),[38] a low-grade chronic innate immune response on the donor CECs,[40] or both. A similar event can be imagined easily in cell injection therapy, and producing cultured hCECs of the highest quality may minimize these events.[22,23,41] Furthermore, because corneal transplantation requires one donor cornea to treat one diseased eye, and because there is a continuous annual shortage of donor corneas worldwide, a novel surgical procedure that eliminates these problems would be highly beneficial.[42]

Limitations and Future Challenges

It should be noted that this study did include some limitations. First, the number of eyes enrolled was small. Second, the number of cells injected into the anterior chamber was based on the presumption derived from the findings regarding the appropriate number of cells required in our previous animal model experiments[21] and measurement of the posterior corneal surface area in humans.[43] Third, although the quality of the injected cultured hCECs used in this study was very acceptable, it was not perfect. Thus, further refinement is necessary.

It should also be noted that we now understand that cultured hCECs are diverse and that they can be classified into several subpopulations,[41] some of which are frequently

accompanied by cell-state transition and epithelial-mesenchymal transition. Although most of the cultured hCECs used in the current study (i.e., the first-generation cell culture protocol) were of acceptable quality for clinical application, our goal is to improve the quality of the cultured hCECs used for injection further. Thus, we have been working diligently on creating high-density, maturely differentiated cultured hCECs, not stem-like cells, for a clinical trial, because our intention is to develop in vitro CECs that precisely mimic in vivo CECs with an enormous energy-dependent metabolism using abundant mitochondria.[23,44] Currently, we are able to create cultured CECs containing nearly 100% matured cells successfully.[22,23] Moreover, our preliminary findings revealed that the corneal ECD after the injection of high-density, maturely differentiated cultured hCECs was higher than that after the injection of the cells used in the current 11 treated eyes (Ueno M, et al. Effectiveness of injecting differentiated subpopulations of cultured corneal endothelial cells for bullous keratopathy. Poster presented at: American Academy of Ophthalmology Annual Meeting; October 12, 2019; San Francisco, CA).

In conclusion, we firmly believe that our new cultured hCEC injection therapy is a paradigm shift in corneal regenerative medicine, with potential clinical application to patients worldwide because it allows for enough hCECs to treat at least 300 diseased eyes to be cultured from just 1 donor cornea. Further improvement of the biological quality of the cultured hCECs (the seeds) via maximum quality control of the cell-processing method, as determined by several biological markers, as well as a deep understanding of its supplemental treatment on each specific corneal-endothelial disease pathologic feature and its microenvironment (the soil), will improve the clinical outcomes of our current hCEC injection therapy drastically.

Acknowledgment

The authors thank Dr Munetoyo Toda and Dr Michio Hagiya for their valuable comments on the cultured cells, Dr Yoshitsugu Inoue and Dr Hidenobu Tanihara for their comments on clinical evaluation, Satomi Sakabayashi for excellent technical assistance, and SightLife Organization for providing the donor corneas used in this study.

References

1. Lass JH, Benetz BA, Gal RL, et al. Donor age and factors related to endothelial cell loss 10 years after penetrating keratoplasty: specular microscopy ancillary study. *Ophthalmology*. 2013;120:2428−2435.
2. Kitazawa K, Wakimasu K, Kayukawa K, et al. Moderately Long-Term Safety and Efficacy of Repeat Penetrating Keratoplasty. *Cornea*. 2018;37(10):1255−1259.
3. Gorovoy MS. Descemet-stripping automated endothelial keratoplasty. *Cornea*. 2006;25:886−889.
4. Price MO, Calhoun P, Kollman C, et al. Descemet stripping endothelial keratoplasty: ten-year endothelial cell loss compared with penetrating keratoplasty. *Ophthalmology*. 2016;123:1421−1427.
5. Lass JH, Benetz BA, Patel SV, et al. Donor, recipient, and operative factors associated with increased endothelial cell loss in the cornea preservation time study. *JAMA Ophthalmol*. 2019;137:185−193.
6. Melles GR, Ong TS, Ververs B, van der Wees J. Preliminary clinical results of Descemet membrane endothelial keratoplasty. *Am J Ophthalmol*. 2008;145:222−227.
7. Tourtas T, Laaser K, Bachmann BO, et al. Descemet membrane endothelial keratoplasty versus Descemet stripping automated endothelial keratoplasty. *Am J Ophthalmol*. 2012;153:1082−1090 e1082.
8. Tan DT, Dart JK, Holland EJ, Kinoshita S. Corneal transplantation. *Lancet*. 2012;379:1749−1761.
9. Mathews PM, Lindsley K, Aldave AJ, Akpek EK. Etiology of global corneal blindness and current practices of corneal transplantation: a focused review. *Cornea*. 2018;37:1198−1203.
10. Anshu A, Price MO, Tan DT, Price FW Jr. Endothelial keratoplasty: a revolution in evolution. *Surv Ophthalmol*. 2012;57:236−252.
11. Aldave AJ, Terry MA, Szczotka-Flynn LB, et al. Effect of graft attachment status and intraocular pressure on Descemet stripping automated endothelial keratoplasty outcomes in the cornea preservation time study. *Am J Ophthalmol*. 2019;203:78−88.
12. Tourabaly M, Chetrit Y, Provost J, et al. Influence of graft thickness and regularity on vision recovery after endothelial keratoplasty. *Br J Ophthalmol*. 2020;104(9):1317−1323.
13. Yazu H, Yamaguchi T, Dogru M, et al. Decreased visual acuity by an irregular corneal posterior surface after repeat Descemet stripping automated endothelial keratoplasty. *Eye Contact Lens*. 2018;44:S249−S254.
14. Hirabayashi KE, Chamberlain W, Rose-Nussbaumer J, et al. Corneal light scatter after ultrathin Descemet stripping automated endothelial keratoplasty versus Descemet membrane endothelial keratoplasty in Descemet Endothelial Thickness Comparison Trial: a randomized controlled trial. *Cornea*. 2020;39(6):691−696.
15. Rudolph M, Laaser K, Bachmann BO, et al. Corneal higher-order aberrations after Descemet's membrane endothelial keratoplasty. *Ophthalmology*. 2012;119:528−535.
16. Duggan MJ, Rose-Nussbaumer J, Lin CC, et al. Corneal higher-order aberrations in Descemet membrane endothelial keratoplasty versus ultrathin DSAEK in the Descemet Endothelial Thickness Comparison Trial: a randomized clinical trial. *Ophthalmology*. 2019;126:946−957.
17. Borkar DS, Veldman P, Colby KA. Treatment of Fuchs endothelial dystrophy by Descemet stripping without endothelial keratoplasty. *Cornea*. 2016;35:1267−1273.
18. Kinoshita S, Koizumi N, Ueno M, et al. Injection of cultured cells with a ROCK inhibitor for bullous keratopathy. *N Engl J Med*. 2018;378:995−1003.
19. Schlötzer-Schrehardt UM, Dörfler S, Naumann GOH. Corneal endothelial involvement in pseudoexfoliation syndrome. *Arch Ophthalmol*. 1993;111:666−674.
20. Okumura N, Ueno M, Koizumi N, et al. Enhancement on primate corneal endothelial cell survival in vitro by a ROCK inhibitor. *Invest Ophthalmol Vis Sci*. 2009;50:3680−3687.
21. Okumura N, Sakamoto Y, Fujii K, et al. Rho kinase inhibitor enables cell-based therapy for corneal endothelial dysfunction. *Sci Rep*. 2016;6:26113.
22. Toda M, Ueno M, Hiraga A, et al. Production of homogeneous cultured human corneal endothelial cells indispensable for innovative cell therapy. *Invest Ophthalmol Vis Sci*. 2017;58:2011−2020.

23. Hamuro J, Numa K, Fujita T, et al. Metabolites interrogation in cell fate decision of cultured human corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 2020;61(2):10.
24. Yee RW, Matsuda M, Schultz RO, Edelhauser HF. Changes in the normal corneal endothelial cellular pattern as a function of age. *Curr Eye Res*. 1985;4:671–678.
25. Wacker K, Baratz KH, Maguire LJ, et al. Descemet stripping endothelial keratoplasty for Fuchs' endothelial corneal dystrophy: five-year results of a prospective study. *Ophthalmology*. 2016;123:154–160.
26. Ang M, Soh Y, Htoon HM, et al. Five-year graft survival comparing Descemet stripping automated endothelial keratoplasty and penetrating keratoplasty. *Ophthalmology*. 2016;123:1646–1652.
27. Fuest M, Ang M, Htoon HM, et al. Long-term visual outcomes comparing Descemet stripping automated endothelial keratoplasty and penetrating keratoplasty. *Am J Ophthalmol*. 2017;182:62–71.
28. Fajgenbaum MA, Hollick EJ. Modeling endothelial cell loss after Descemet stripping endothelial keratoplasty: data from 5 years of follow-up. *Cornea*. 2017;36:553–560.
29. Price DA, Kelley M, Price FW Jr. Price MO. Five-year graft survival of Descemet membrane endothelial keratoplasty (EK) versus Descemet stripping EK and the effect of donor sex matching. *Ophthalmology*. 2018;125:1508–1514.
30. Wakimasu K, Kitazawa K, Kayukawa K, et al. Five-year follow-up outcomes after Descemet's stripping automated endothelial keratoplasty: a retrospective study. *BMJ Open Ophthalmol*. 2020;5:e000354.
31. Madi S, Leon P, Nahum Y, et al. Five-year outcomes of ultrathin Descemet stripping automated endothelial keratoplasty. *Cornea*. 2019;38:1192–1197.
32. Schlogl A, Tourtas T, Kruse FE, Weller JM. Long-term clinical outcome after Descemet membrane endothelial keratoplasty. *Am J Ophthalmol*. 2016;169:218–226.
33. Birbal RS, Ni Dhubhghaill S, Bourgonje VJA, et al. Five-year graft survival and clinical outcomes of 500 consecutive cases after Descemet membrane endothelial keratoplasty. *Cornea*. 2020;39:290–297.
34. Aoki T, Kitazawa K, Inatomi T, et al. Risk factors for corneal endothelial cell loss in patients with pseudoexfoliation syndrome. *Sci Rep*. 2020;10:7260.
35. Yagi-Yaguchi Y, Yamaguchi T, Higa K, et al. Association between corneal endothelial cell densities and elevated cytokine levels in the aqueous humor. *Sci Rep*. 2017;7:13603.
36. Yamaguchi T, Higa K, Yagi-Yaguchi Y, et al. Pathological processes in aqueous humor due to iris atrophy predispose to early corneal graft failure in humans and mice. *Sci Adv*. 2020;6:eaaz5195.
37. Kocaba V, Katikireddy KR, Gipson IK, et al. Association of the gutta-induced microenvironment with corneal endothelial cell behavior and demise in Fuchs endothelial corneal dystrophy. *JAMA Ophthalmol*. 2018;136:886–892.
38. Kayukawa K, Kitazawa K, Wakimasu K, et al. Cases with long-term high corneal endothelial cell density maintained after corneal transplantation. *Cornea*. 2020 May 29. https://doi.org/10.1097/ICO.0000000000002386. Online ahead of print.
39. Yamada J, Ueno M, Toda M, et al. Allogeneic sensitization and tolerance induction after corneal endothelial cell transplantation in mice. *Invest Ophthalmol Vis Sci*. 2016;57:4572–4580.
40. Okumura N, Inoue R, Nakano S, et al. Immune cells on the donor corneal endothelium after corneal transplantation. *Cornea*. 2018;37:1081–1086.
41. Hamuro J, Toda M, Asada K, et al. Cell homogeneity indispensable for regenerative medicine by cultured human corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 2016;57:4749–4761.
42. Gain P, Jullienne R, He Z, et al. Global survey of corneal transplantation and eye banking. *JAMA Ophthalmol*. 2016;134:167–173.
43. Kitazawa K, Yokota I, Sotozono C, Kinoshita S. Measurement of corneal endothelial surface area using anterior segment optical coherence tomography in normal subjects. *Cornea*. 2016;35:1229–1233.
44. Hamuro J, Deguchi H, Fujita T, et al. Polarized expression of ion channels and solute carrier family transporters on heterogeneous cultured human corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 2020;61(5):47.

Footnotes and Financial Disclosures

Originally received: July 1, 2020.
Final revision: August 26, 2020.
Accepted: September 1, 2020.
Available online: ■■■. Manuscript no. D-20-01855.

[1] Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan.

[2] Department of Biostatistics, Kyoto Prefectural University of Medicine, Kyoto, Japan.

[3] Department of Biomedical Engineering, Doshisha University, Kyotanabe, Japan.

[4] Department of Frontier Medical Science and Technology for Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan.

Financial Disclosure(s):
The author(s) have made the following disclosure(s): M.U.: Patents – WO2009028631, CA2697895, CN101835491, CN103931608, CN103937738, EP2193806, ES2659980, JP5657252, JP5843373, JP5969679, RU2474434, US9248125, WO2015016371, EP3029140, US2016266114, JP2018158937, JP2020078333, JP6548576

S.T.: Financial support – Nippon Boehringer Ingelheim, Daiichi Sankyo, Sanofi, Takeda Pharmaceutical, Bayer Yakuhin, Sysmex, Chugai Pharmaceutical, Solasia Pharma

N.O.: Financial support – ActualEyes, Inc.

N.K.: Financial support – Senju Pharmaceutical Co, Ltd, Kowa Company Ltd, Japan Innovative Therapeutics, Inc, ActualEyes, Inc, M's Science Corporation; Equity owner – ActualEyes, Inc; Patent – Senju Pharmaceutical Co, Ltd.

C.S.: Financial support – Santen Pharmaceutical Co, Ltd, Alcon Pharmaceutical Co, Ltd, Senju Pharmacoitical Co, Ltd, Otsuka Pharmacoitical Co, Ltd, HOYA Corporation, Nitto Medic Co, Ltd, Pfizer Inc, Sumitomo Dainippon Pharma Co, Ltd, NIDEK Co, Ltd, SEED Co, Ltd, Nitten Pharmaceutical Co, Ltd, Kowa Company, Ltd, Bayer Yakuhin, Ltd, CorneaGen; Lecturer – Otsuka Pharmaceutical Co, Ltd, Santen Pharmaceutical Co, Ltd, Senju Pharmaceutical Co, Ltd, Toa Pharmaceutical Co, Ltd.

S.K.: Financial support – Santen Pharmaceutical Co, Ltd, Otsuka Pharmaceutical Co, Ltd, Senju Pharmaceutical Co, Ltd, Kowa Co, Ltd, HOYA Corporation, Oncolys Biopharma, Inc, LION Corporation, CorneaGen, Alcon Japan, Ltd, Abbott Medical Optics, Inc, Novartis, Astellas Pharma; Patent – WO/2009/028631 (issued), WO/2013/100208 (pending)

Supported by the Research Project for Practical Applications of Regenerative Medicine from the Japan Agency for Medical Research and Development (S.K.); and the Japanese Ministry of Health, Labour and Welfare.

HUMAN SUBJECTS: Human subjects were included in this study. The human ethics committees at Kyoto Prefectural University of Medicine and the Japanese Ministry of Health, Labour and Welfare approved the study. All research adhered to the tenets of the Declaration of Helsinki. All participants provided informed consent.

No animal subjects were included in this study.

Author Contributions:

Conception and design: Numa, Imai, Ueno, Koizumi, Sotozono, Kinoshita

Analysis and interpretation: Numa, Imai, Teramukai, Hamuro, Sotozono, Kinoshita

Data collection: Numa, Ueno, Tanaka, Okumura, Koizumi, Kinoshita

Obtained funding: Ueno, Teramukai, Okumura, Koizumi, Sotozono, Kinoshita; Study was performed as part of regular employment duties at the Kyoto Prefectural University of Medicine. No additional funding was provided.

Overall responsibility: Numa, Kitazawa, Bush, Teramukai, Hamuro, Sotozono, Kinoshita

Abbreviations and Acronyms:
BCVA = best-corrected visual acuity; **CEC** = corneal endothelial cell; **CV** = coefficient of variation; **DMEK** = Descemet membrane endothelial keratoplasty; **DSAEK** = Descemet stripping automated endothelial keratoplasty; **DSO** = Descemet membrane stripping only; **ECD** = endothelial cell density; **FECD** = Fuchs endothelial corneal dystrophy; **hCEC** = human corneal endothelial cell; **IOP** = intraocular pressure; **PEX** = pseudoexfoliation syndrome; **VA** = visual acuity.

Keywords:
Cell injection, Corneal endothelial failure, Cultured corneal endothelial cells, Regenerative medicine, Rho-associated protein kinase inhibitor.

Correspondence:
Shigeru Kinoshita, MD, PhD, Department of Frontier Medical Science and Technology for Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Hirokoji-agaru, Kawaramachi-dori, Kamigyo-ku, Kyoto 602-0841, Japan. E-mail: fmstoph@koto.kpu-m.ac.jp.