ORIGINAL ARTICLE

Injection of Cultured Cells with a ROCK Inhibitor for Bullous Keratopathy

Shigeru Kinoshita, M.D., Ph.D., Noriko Koizumi, M.D., Ph.D., Morio Ueno, M.D., Ph.D., Naoki Okumura, M.D., Ph.D., Kojiro Imai, M.D., Ph.D., Hiroshi Tanaka, M.D., Ph.D., Yuji Yamamoto, M.D., Takahiro Nakamura, M.D., Ph.D., Tsutomu Inatomi, M.D., Ph.D., John Bush, B.A., Munetoyo Toda, Ph.D., Michio Hagiya, Ph.D., Isao Yokota, Ph.D., Satoshi Teramukai, Ph.D., Chie Sotozono, M.D., Ph.D., and Junji Hamuro, Ph.D.

ABSTRACT

BACKGROUND

Corneal endothelial cell (CEC) disorders, such as Fuchs's endothelial corneal dystrophy, induce abnormal corneal hydration and result in corneal haziness and vision loss known as bullous keratopathy. We investigated whether injection of cultured human CECs supplemented with a rho-associated protein kinase (ROCK) inhibitor into the anterior chamber could increase CEC density.

METHODS

We performed an uncontrolled, single-group study involving 11 persons who had received a diagnosis of bullous keratopathy and had no detectable CECs. Human CECs were cultured from a donor cornea; a total of 1×10^6 passaged cells were supplemented with a ROCK inhibitor (final volume, 300 μl) and injected into the anterior chamber of the eye that was selected for treatment. After the procedure, patients were placed in a prone position for 3 hours. The primary outcome was restoration of corneal transparency, with a CEC density of more than 500 cells per square millimeter at the central cornea at 24 weeks after cell injection. Secondary outcomes were a corneal thickness of less than 630 μm and an improvement in best corrected visual acuity equivalent to two lines or more on a Landolt C eye chart at 24 weeks after cell injection.

RESULTS

At 24 weeks after cell injection, we recorded a CEC density of more than 500 cells per square millimeter (range, 947 to 2833) in 11 of the 11 treated eyes (100%; 95% confidence interval [CI], 72 to 100), of which 10 had a CEC density exceeding 1000 cells per square millimeter. A corneal thickness of less than 630 μm (range, 489 to 640) was attained in 10 of the 11 treated eyes (91%; 95% CI, 59 to 100), and an improvement in best corrected visual acuity of two lines or more was recorded in 9 of the 11 treated eyes (82%; 95% CI, 48 to 98).

CONCLUSIONS

Injection of human CECs supplemented with a ROCK inhibitor was followed by an increase in CEC density after 24 weeks in 11 persons with bullous keratopathy. (Funded by the Japan Agency for Medical Research and Development and others; UMIN number, UMIN000012534.)

From the Departments of Frontier Medical Science and Technology for Ophthalmology (S.K., T.N., M.T., M.H.), Ophthalmology (M.U., K.I., H.T., Y.Y., T.I., J.B., C.S., J.H.), and Biostatistics (I.Y., S.T.), Kyoto Prefectural University of Medicine, and the Department of Biomedical Engineering, Doshisha University (N.K., N.O.) — both in Kyoto, Japan. Address reprint requests to Dr. Kinoshita at the 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, or at shigeruk@koto.kpu-m.ac.jp.

Drs. Kinoshita, Koizumi, and Ueno contributed equally to this article.

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The corneal endothelium is a single layer of cells that line the posterior surface of the cornea. This cell layer maintains corneal transparency by regulating the flow of water from the aqueous humor into the cornea.[1] Corneal endothelial damage or disorder and subsequent loss of corneal endothelial cells (CECs) owing to pathologic conditions such as Fuchs's endothelial corneal dystrophy is compensated by the natural spread of the remaining CECs.[1] However, when CEC density, which typically exceeds 2000 cells per square millimeter in healthy persons, diminishes to fewer than 400 cells per square millimeter, corneal endothelial dysfunction and abnormal corneal hydration occur, resulting in corneal thickening and haziness known as bullous keratopathy[2]; this condition can ultimately lead to loss of vision.

The current treatments for bullous keratopathy include penetrating keratoplasty, Descemet's stripping automated endothelial keratoplasty, and Descemet's membrane endothelial keratoplasty, all of which involve the use of a donor cornea.[3-9] Although these surgical procedures are widely used, they are invasive, and their long-term clinical results remain unclear. Visual acuity after corneal transplantation is sometimes unsatisfactory because of a surgery-induced irregularity of the corneal structure. A surgical procedure that results in a healthy cornea with a normal shape and function that permit good visual acuity is therefore desired. Two procedures — one that involved the simple peeling of the Descemet's membrane from the central cornea[10] and one that involved the removal of localized corneal endothelium by freezing combined with the topical application of a rho-associated protein kinase (ROCK) inhibitor[11] — were reported to be effective in the treatment of early-stage corneal endothelial dysfunction but not in the treatment of advanced-stage diffuse corneal endothelial dysfunction.[11]

Successful procedures for culturing CECs have been reported in studies in humans and in nonhuman primates.[12-23] Taking into account this line of research, we developed an approach to treat severe corneal endothelial dysfunction through injection of cultured human CECs combined with a ROCK inhibitor into the anterior chamber. Although the ROCK inhibitor was used as an adjunctive drug to promote engraftment of CECs, we could not rule out the possibility that the ROCK inhibitor would have a therapeutic effect through its action on cells in situ.[23] In previous studies in rabbits and monkeys, we reported that CECs supplemented with a ROCK inhibitor, when injected into the anterior chamber, repopulated and self-organized[24,25] on the posterior surface of the cornea, had the functional properties of healthy corneal endothelium, and produced a normal cornea with no structural alteration.[26,27] Through this work, we determined that human CEC cultures comprise a plurality of subpopulations, some of which are unsuitable and unsafe for injection into human eyes.[28,29] We therefore devised a method[30] to consistently produce differentiated cultured functional human CECs. Here we report a case series of 11 consecutive patients with bullous keratopathy who were treated successfully by injection of human CECs supplemented with a ROCK inhibitor.

METHODS

STUDY DESIGN AND OVERSIGHT

We conducted a nonrandomized, single-group study involving a small number of participants. The protocol and amendments (available with the full text of this article at NEJM.org) and other relevant study documentation of this clinical trial were reviewed for compliance with the guidelines on clinical research with human stem cells in Japan and were approved by the institutional review board at Kyoto Prefectural University of Medicine and by the Special Committee of the Japanese Ministry of Health, Labor, and Welfare. The authors vouch for the accuracy and completeness of the data and analyses and the reporting of adverse events and for the fidelity of the study to the protocol.

PATIENTS

Eligible patients had received a diagnosis of bullous keratopathy; were between 20 and 90 years of age; and had an eye with no detectable CECs on specular microscopy (Fig. S1 in the Supplementary Appendix, available at NEJM.org), a corneal thickness greater than 630 μm and the presence of corneal epithelial edema, and a best corrected visual acuity below 0.5 (decimal visual acuity). Decimal visual acuity, which is widely used in Japan and Europe, is an alternative system to Snellen visual acuity and logMAR (logarithm of the minimum angle of resolution) visual acuity.[31] A decimal visual acuity of 0.5 corresponds to 20/40 (Snellen) and 0.30 (logMAR);

a decimal visual acuity of 0.1 corresponds to 20/200 (Snellen) and 1.00 (logMAR). All the patients provided written informed consent.

HUMAN CEC CULTURE AND QUALITY CONTROL

Corneas from deceased donors (range of age at death, 7 to 29 years) were used for the in vitro subculture material; the donor corneas were provided by SightLife. Human CECs were cultured at the Cell Processing Center of Kyoto Prefectural University of Medicine with the use of a standard operating procedure that conformed to the guidelines of Good Manufacturing Practices. Details of the culture procedure are described in the Supplementary Appendix. Cell lots for clinical application were examined to verify that they met criteria for surgical use (Table S1 in the Supplementary Appendix), and enzyme-linked immunosorbent assay or fluorescence-activated cell-sorting analysis (or both) was performed to elucidate the biologic characteristics of the cells. The cells were recovered by means of enzyme treatment with TrypLE Reagent (Thermo Fisher Scientific) and were washed with modified Opti-MEM I Reduced Serum Media (Thermo Fisher Scientific). The human CECs were then dispensed into a Proteosave container (Sumitomo Bakelite) to block nonspecific absorption of protein (1.5×10^6 cultured human CECs were distributed per 450 μl of modified Opti-MEM I supplemented with the ROCK inhibitor Y-27632 [Wako Pure Chemical Industries] at a final cell concentration of 100 μM).

SURGICAL PROCEDURE AND FOLLOW-UP

All surgical procedures were performed under local anesthesia. After a 1.6-mm incision was made at the corneal limbus, a silicone needle (Inami) was used to remove the abnormal extracellular matrix on the patient's Descemet's membrane or the degenerated CECs in an 8-mm-diameter area of the central cornea (or both). After this procedure, a 26-gauge needle was used to inject 1×10^6 (Patients 2 to 11) or 5×10^5 (Patient 1) cultured human CECs suspended in 300 μl of modified Opti-MEM I medium containing the ROCK inhibitor Y-27632 into the anterior chamber. The patients were then immediately placed in a prone position for 3 hours to enhance the adhesion of the injected cells (Fig. 1). After cell injection, all patients were administered systemic and topical glucocorticoids to inhibit acute inflammation or immunologic reaction, and antimicrobial agents were administered as prophylaxis against infection in accordance with the drug regimen we use in our regular corneal transplantation procedures (Table S2 in the Supplementary Appendix).

PRIMARY, SECONDARY, AND EXPLORATORY OUTCOMES

The primary outcome was restoration of corneal transparency with a CEC density of more than 500 cells per square millimeter at the central cornea at 24 weeks after cell injection. The secondary outcomes were a corneal thickness of less than 630 μm at 24 weeks after cell injection (with a decrease in corneal thickness from the preoperative [baseline] measurement) and an improvement in best corrected visual acuity of two lines or more on a Landolt C eye chart (a measure of decimal visual acuity) at 24 weeks after cell injection. We converted decimal visual acuity to logMAR visual acuity to facilitate statistical analysis. A decrease of at least 0.2 in logMAR visual acuity corresponds to an improvement of two lines or more on a Landolt C eye chart, and an increase of at least 0.2 in logMAR visual acuity corresponds to a deterioration of two lines or more. For decimal visual acuity, a higher value indicates better visual acuity, whereas for logMAR visual acuity, a lower value indicates better visual acuity. The primary and secondary outcomes were also assessed as exploratory outcomes at 2 years after cell injection. We assessed the safety of the treatment by monitoring the treated eyes and the patients for adverse events (Table S3 in the Supplementary Appendix).

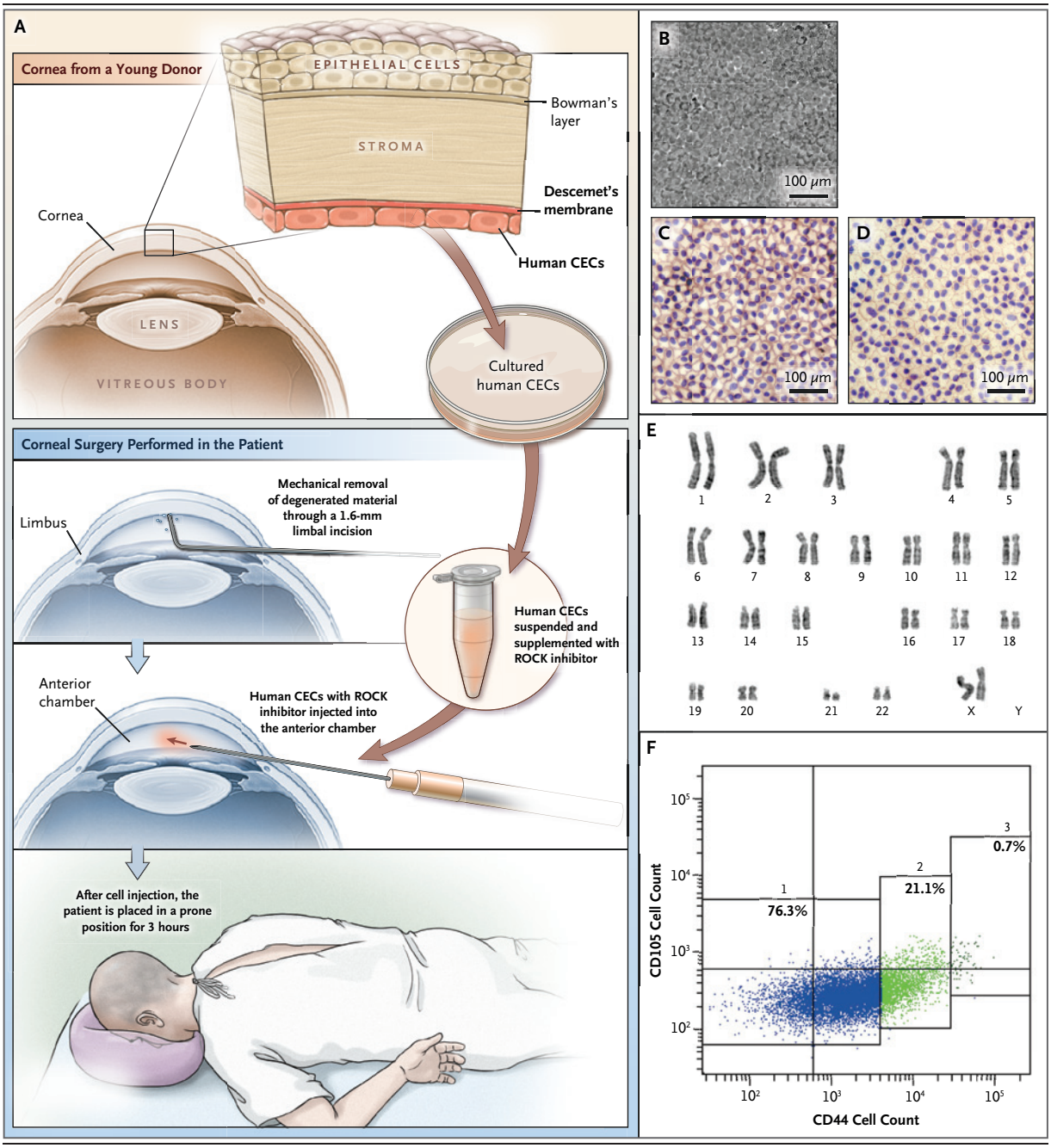
STATISTICAL ANALYSIS

We calculated the percentage (with 95% confidence intervals) of treated eyes that met the primary and secondary outcomes. The exploratory outcomes were assessed as the change from the baseline measurement to 2 years (see the Supplementary Appendix). We also recorded intraocular pressure as a safety measure and assessed this outcome as the change from the baseline measurement to 2 years.

RESULTS

PATIENT CHARACTERISTICS

We enrolled patients between December 10, 2013, and December 16, 2014. We injected human CECs supplemented with the ROCK inhibitor into 11 eyes, in 11 consecutive patients, during the



period from December 2013 through February 2014 (3 eyes) and September 2014 through December 2014 (8 eyes). The mean age of the patients was 64.4 years (range, 49 to 82), and 5 of the 11 patients (45%) were male. All the patients had pseudophakic bullous keratopathy (i.e., bullous keratopathy that develops after intraocular lens implantation in cataract surgery), with the following subtypes: Fuchs's endothelial corneal dystrophy (7 eyes), argon-laser iridotomy–induced bullous keratopathy (2 eyes), pseudoexfoliation syndrome–related bullous keratopathy (1 eye), and intraocular surgery–related bullous keratopathy (1 eye) (Table 1). The mean corneal thickness at baseline was 743 μm (range, 637 to 964). (Additional details are provided in the Supplementary Appendix.)

FEATURES OF THE CULTURED HUMAN CECS

We used seven lots of cultured human CECs (passaged two or three times). Each lot was derived from an independent donor. At the request of the Special Committee, for safety reasons we

Figure 1 (facing page). Preparation of Cultured Human Corneal Endothelial Cells.

Panel A shows the schema of cell-injection therapy, in which cultured human corneal endothelial cells (CECs) supplemented with a rho-associated protein kinase (ROCK) inhibitor are injected into the anterior chamber. After human CECs were removed from the Descemet's membrane of a cornea obtained from a young deceased donor, isolated human CECs free of any contamination were cultured and subcultured. A few hours before cell injection, the cells were recovered, suspended to obtain the appropriate number and density, and supplemented with the ROCK inhibitor. After mechanical removal of the abnormal extracellular matrix on the patient's Descemet's membrane or the degenerated CECs (or both), the cultured human CECs supplemented with the ROCK inhibitor were injected into the anterior chamber of one of the eyes of each participant. After the procedure, the patients were placed in a prone position for 3 hours. Panel B shows the cultured human CECs for clinical use. Panel C shows the expression of sodium–potassium ATPase by the cultured human CECs. Panel D shows the expression of ZO-1 by the cultured human CECs. Panel E shows the karyotype analysis of lot 5 of cultured human CECs. Panel F shows a representative result of the expression of CD44 and CD105 in the cultured human CECs using flow cytometry. The full vertical line indicates the boundary between the CD44-negative population and the CD44-positive population. The full horizontal line indicates the boundary between the CD105-negative population and the CD105-positive population. Rectangles 1, 2, and 3 indicate subpopulations of the cultured human CECs. The percentages of a negative to low level of CD44 expression (rectangle 1), a medium level of CD44 expression (rectangle 2), and a high level of CD44 expression (rectangle 3) were 76.3%, 21.1%, and 0.7%, respectively.

used one lot per patient in the first three patients (a total of three lots) and one lot for every two patients for the following eight patients (a total of four lots). All cultured human CECs were small, hexagonally shaped cells (no epithelial-to-mesenchymal transition–like cells) expressing ZO-1 and sodium–potassium ATPase. CEC density ranged from 1835 to 2530 cells per square millimeter. All seven lots used in this study met the prespecified quality-control requirements (Fig. 1, and Table S1 in the Supplementary Appendix), and no chromosomal abnormalities were observed. Enzyme-linked immunosorbent assay of the culture supernatant was performed at the final culture stage and revealed a type I collagen level lower than 15 ng per milliliter (lots 1 through 3) and an interleukin-8 level lower than 500 pg per milliliter and a platelet-derived growth factor BB level higher than 30 pg per milliliter (lots 4 through 7). In lots 2 through 7, the percentage of CECs with high levels of CD44 expression was below 10%. The viral antigens of herpes simplex virus type 1 and type 2, varicella–zoster virus, cytomegalovirus, Epstein–Barr virus, and parvovirus B19, as measured by polymerase-chain-reaction assay and standard bacterial testing, were found to be negative in the cultured cells and supernatant of all seven lots.

PRIMARY OUTCOME

At 24 weeks after cell injection, contact specular microscopic examination (Fig. S2 in the Supplementary Appendix) revealed that the primary outcome (i.e., restoration of corneal transparency with a CEC density of >500 cells per square millimeter) was met in 11 of the 11 treated eyes (100%; 95% confidence interval [CI], 72 to 100). CEC density ranged from 947 to 2833 cells per square millimeter in the 11 eyes (mean density, 1924 cells per square millimeter [95% CI, 1537 to 2312]) and exceeded 1000 cells per square millimeter in 10 eyes and 2000 cells per square millimeter in 6 eyes (Table 1, and Table S4 in the Supplementary Appendix).

SECONDARY OUTCOMES

At 24 weeks after cell injection, a corneal thickness of less than 630 μm was attained in 10 of the 11 treated eyes (91%; 95% CI, 59 to 100), with a mean corneal thickness of 549 μm (95% CI, 515 to 583). A significant improvement in best corrected visual acuity of two lines or more was attained at 24 weeks in 9 of the 11 treated eyes (82%; 95% CI, 48 to 98) (Table 1, and Table S5 in the Supplementary Appendix).

EXPLORATORY OUTCOMES

At 2 years after cell injection, corneal thickness was less than 600 μm in 10 eyes, and the cornea was thinner than the baseline measure in all 11 eyes (Table 1). There was a rapid decrease in corneal thickness within 4 weeks after cell injection, followed by a more gradual decrease over the next 5 months (Fig. 2A). The mean best corrected logMAR visual acuity decreased from 0.88 at baseline to 0.04, indicating an improvement (Fig. 2B). (Additional details are provided in Table S5 in the Supplementary Appendix.)

At 2 years after cell injection, each of the 11 eyes maintained corneal transparency (mean CEC density, 1534 cells per square millimeter [95% CI, 1213 to 1855]) (Fig. 3). The duration of corneal clearing (i.e., the amount of time that it took for the cornea to clear) differed slightly

Table 1. Patient Characteristics and Clinical Summary before and after Cell Injection.*

Patient No., Sex, and Age	Eye	Disease Subtype	Before Cell Injection†		24 Weeks after Cell Injection			2 Years after Cell Injection		
			Central Corneal Thickness	BCVA‡	CEC Density	Central Corneal Thickness	BCVA‡	CEC Density	Central Corneal Thickness	BCVA‡
			μm	*decimal visual acuity (logMAR)*	*cells/mm²*	*μm*	*decimal visual acuity (logMAR)*	*cells/mm²*	*μm*	*decimal visual acuity (logMAR)*
1, F, 68 yr	Right	Argon-laser iridotomy–induced BK	760	0.04 (1.40)	947	511	0.70 (0.15)	1029	532	1.00 (0.00)
2, M, 60 yr	Right	Fuchs's endothelial corneal dystrophy	964	0.05 (1.30)	1965	525	1.00 (0.00)	2193	538	1.00 (0.00)
3, M, 58 yr	Right	Fuchs's endothelial corneal dystrophy	727	0.20 (0.70)	2146	540	0.70 (0.15)	2115	546	1.50 (–0.18)
4, M, 71 yr	Right	Pseudoexfoliation syndrome–related BK	792	0.10 (1.00)	1271	640	0.40 (0.40)	871	710	0.40 (0.40)
5, M, 49 yr	Left	Fuchs's endothelial corneal dystrophy	637	0.40 (0.40)	1134	509	1.00 (0.00)	959	529	1.50 (–0.18)
6, F, 74 yr	Right	Argon-laser iridotomy–induced BK	775	0.10 (1.00)	2232	505	0.80 (0.10)	1447	525	1.00 (0.00)
7, F, 72 yr	Left	Fuchs's endothelial corneal dystrophy	750	0.30 (0.52)	2288	626	0.30 (0.52)	1316	550	0.80 (0.10)
8, F, 57 yr	Left	Fuchs's endothelial corneal dystrophy	657	0.20 (0.70)	2833	489	0.50 (0.30)	2188	503	0.70 (0.15)
9, M, 63 yr	Left	Intraocular surgery–related BK	649	0.40 (0.40)	1880	595	0.50 (0.30)	1546	543	0.80 (0.10)
10, F, 82 yr	Right	Fuchs's endothelial corneal dystrophy	741	0.20 (0.70)	2141	539	0.80 (0.10)	1600	523	0.90 (0.05)
11, F, 56 yr	Left	Fuchs's endothelial corneal dystrophy	725	0.03 (1.52)	2331	561	0.90 (0.05)	1610	572	1.00 (0.00)

* BK denotes bullous keratopathy, BCVA best corrected visual acuity, CEC corneal endothelial cell, and logMAR logarithm of the minimum angle of resolution.
† Before cell injection, CEC density was below the detection limit of the specular microscope in all 11 patients.
‡ Decimal visual acuity is widely used in Japan and Europe as an alternative system to Snellen visual acuity and logMAR visual acuity.[31] For decimal visual acuity, higher values indicate better visual acuity, whereas for logMAR visual acuity, lower numbers indicate better visual acuity: logMAR visual acuity=−log(decimal visual acuity).

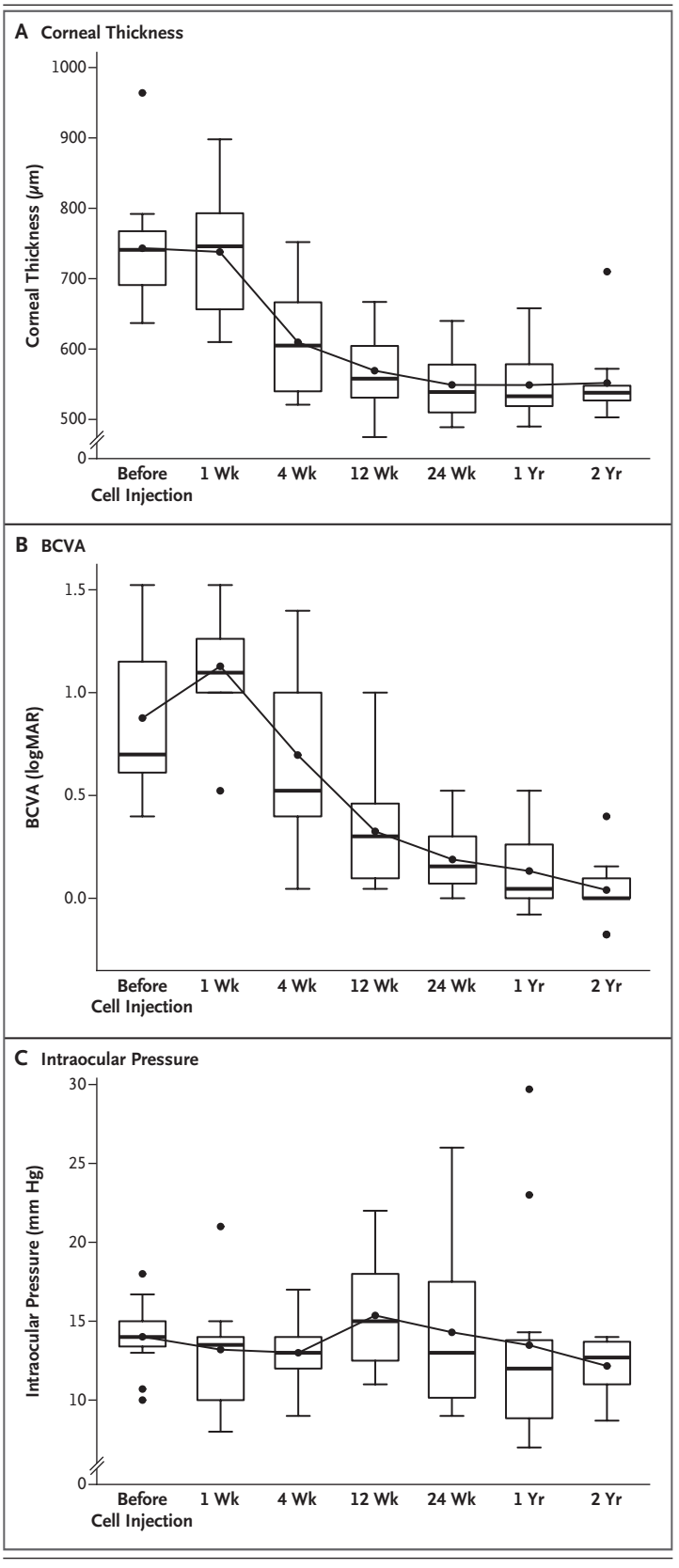


Figure 2. Corneal Thickness, Best Corrected Visual Acuity, and Intraocular Pressure.

Measures of corneal thickness (Panel A), best corrected visual acuity (BCVA) (Panel B), and intraocular pressure (Panel C) are provided in box plots. The results with respect to the secondary outcomes of a corneal thickness of less than 630 μm and an improvement in BCVA of two lines or more on a Landolt C eye chart (a measure of decimal visual acuity) at 24 weeks, as compared with baseline, were significant (Table S5 in the Supplementary Appendix). BCVA is expressed as logMAR visual acuity, which was converted from decimal visual acuity to facilitate statistical analysis (a decrease of at least 0.2 in logMAR BCVA corresponds to an improvement of two lines or more on a Landolt C eye chart). There was no significant increase in intraocular pressure during each evaluation period. The horizontal line in the boxes represents the median, and the bottom and top of the boxes represent the lower and upper quartiles, respectively. I bars indicate 1.5 times the lower and upper quartiles, and the dots represent outliers.

from patient to patient owing to variations in the quality of the injected cells and in the subtype of disease. (Additional details are provided in Table S4 and Figs. S3 through S5 in the Supplementary Appendix.)

SAFETY

We did not observe anterior uveitis, intraocular infection, or immunologic reaction in any of the patients after cell injection. The postoperative time course of intraocular pressure is shown in Figure 2C. In 10 of the 11 eyes, there was no increase in intraocular pressure during the 2-year period of evaluation. The treated eye in Patient 8 showed an increase in intraocular pressure (to 27 mm Hg) at 8 months after cell injection; the pressure resolved after trabeculotomy without the use of antiglaucoma eyedrop medication. At 2 years after cell injection, intraocular pressure was within the normal range in each of the 11 eyes. No abnormal findings from general health evaluations or blood tests were observed at 4, 12, and 24 weeks after cell injection. (Additional details are provided in Tables S5 and S6 in the Supplementary Appendix.)

DISCUSSION

Our findings showed that injection of human CECs supplemented with a ROCK inhibitor in patients with bullous keratopathy was followed by corneal restoration, with attainment of normal corneal thickness and resolution of corneal

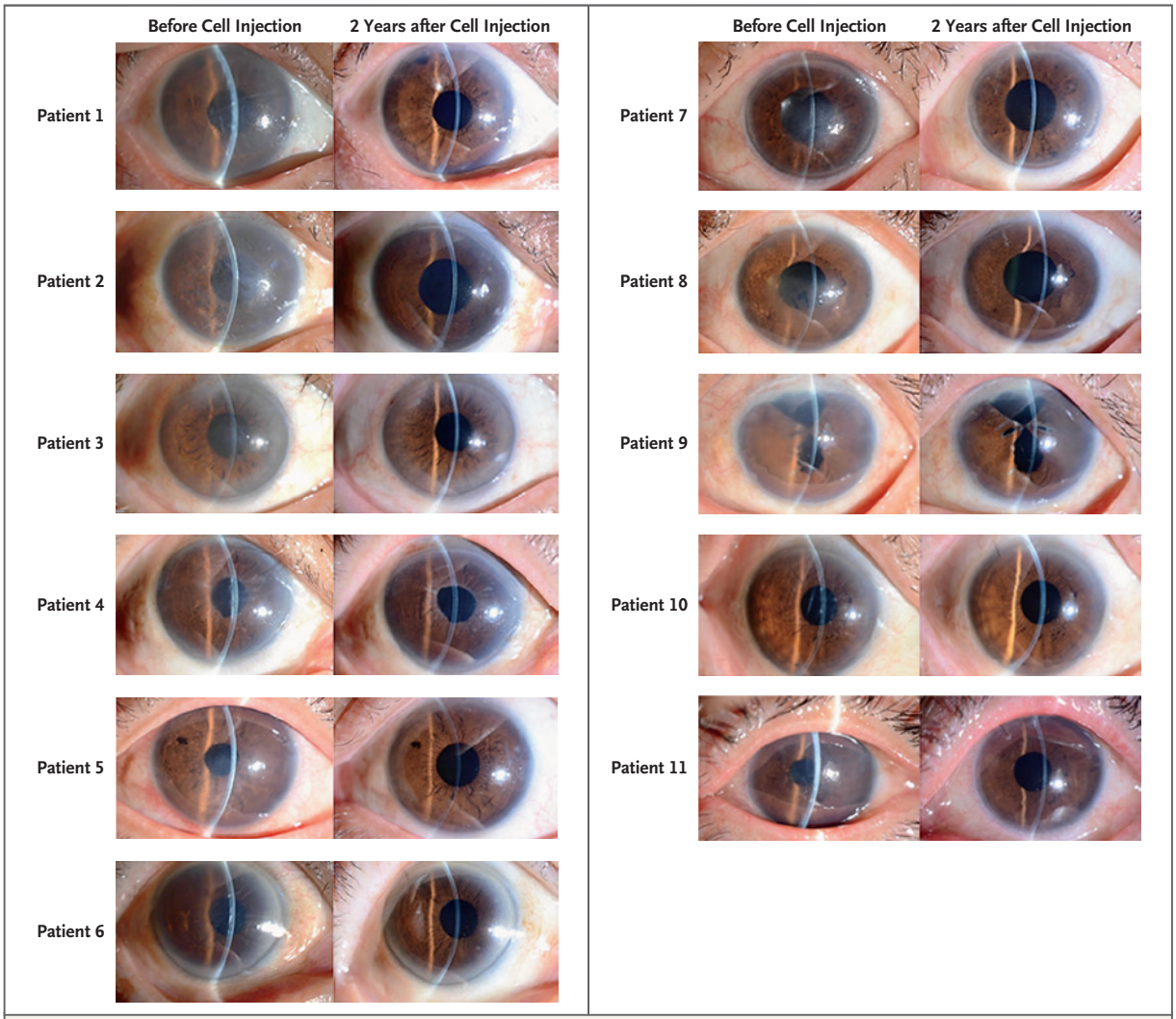


Figure 3. Slit-Lamp Microscopic Images of the Treated Eyes.

Slit-lamp microscopic photographs of all 11 treated eyes were obtained at baseline (before cell injection) and at 2 years after the injection of CECs supplemented with a ROCK inhibitor.

epithelial edema. Within the limits of this small, single-group study involving 11 patients who were followed for 2 years, the procedure was safe. We propose that injection of cultured human CECs supplemented with the ROCK inhibitor into the anterior chamber resulted in the repopulation of CECs on the Descemet's membrane and on the bare posterior surface of the corneal stroma. An alternative explanation is that the ROCK inhibitor or a factor released from the injected cells stimulated the CECs in the recipient to replicate; however, we consider this explanation to be unlikely because most of the eyes showed a high CEC density at 24 weeks after cell injection that mimics what would have been seen with ordinary corneal transplantation at 4 weeks.

We speculate that most of the injected CECs that did not attach to the cornea entered an adjacent vein through the trabecular meshwork and died or entered the systemic circulation. Because of the risk of ectopic tumor formation, we were particularly interested in the results of the blood tests and general health evaluations obtained from the participants after treatment; the normal findings were consistent with those from preclinical studies,[27] but we cannot rule out some potential for tumor formation over time or in future trials of this experimental intervention.

Elevated intraocular pressure occurred in one patient, in whom we diagnosed glucocorticoid-induced glaucoma, given the timing of presentation and the fact that there were no abnormal

findings associated with the trabecular meshwork. However, because of this observation, we plan to reevaluate the number and density of cells used in the injection in the event that further clinical trials are performed. Owing to the study design, we could not discern the extent to which the use of human CECs, the use of the ROCK inhibitor, and the decision to place patients in a prone position contributed to the clinical outcome. We observed no immune response to the human CECs during the follow-up period (the patients received topical glucocorticoids after the procedure), and previous studies have indicated induction of immune tolerance in the eye[32] and the anterior chamber–associated immune deviation of the eye.[33]

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