

Treatment of Rabbit Bullous Keratopathy with Precursors Derived from Cultured Human Corneal Endothelium

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PURPOSE. To establish a method for the mass production of human corneal endothelium (HCE) precursors and the therapeutic application of these cells in a rabbit CE-deficiency model.

METHODS. A sphere-forming assay was performed to produce precursors from cultured HCE. Various marker expressions were examined in the sphere colonies, and their progenies by immunocytochemistry and reverse transcription-polymerase chain reaction (RT-PCR). The transport activity of the sphere-derived cell sheet was evaluated by the Ussing chamber system. DiI-labeled precursors obtained from cultured HCE were injected the anterior chamber of the eye in a rabbit CE-deficiency model, and the eye-down position was maintained for 24 hours for attachment to Descemet's membrane (sphere eye-down group). The sphere eye-down and control groups, observed for 28 days after surgery, underwent histologic and fluorescence microscopic examinations.

RESULTS. Cultured HCE formed primary and secondary sphere colonies. The spheres expressed α -smooth muscle actin and nestin, and progeny expressed α -smooth muscle actin, confirmed by RT-PCR. The progeny showed an HCE-like hexagonal shape, were confluent, and had adequate transport activity. Mean corneal thickness in the sphere eye-down group was significantly less than in the other control groups 14, 21, and 28 days ($P < 0.006$) after surgery. The HCE-like hexagonal cells detected on the Descemet's membrane are DiI-positive in the sphere eye-down group.

CONCLUSIONS. The findings demonstrate that culture of HCE can promote mass production of HCE precursors, determined by sphere-forming assay. Injection of precursors derived from cultured HCE into the anterior chamber is an effective treatment strategy for CE deficiency in a rabbit model. (*Invest Ophthalmol Vis Sci.* 2005;46:3637-3644) DOI:10.1167/iovs.05-0462

Adult stem cells, or precursors, have been detected in various human tissues by the sphere-forming assay, including the central nervous system,¹ bone marrow,² skin,³ retina,⁴

corneal stroma,⁵ and corneal endothelium (CE).⁶ Use of such cells can provide an ideal strategy for tissue regeneration and engineering.⁷ However, clinical transplantation of spheres derived from neural tissue or bone marrow stromal cells by a sphere-forming procedure has been limited to attempts at regenerating parts of the nervous system, such as the spinal cord and brain.⁸⁻¹¹ Moreover, a method of mass production of adult stem or precursor cells to compensate for the lack of organ donors has not been established.

The CE is formed by a single layer of hexagonal cells that separate the corneal stroma from the aqueous humor of the anterior chamber. Corneal transparency is maintained by regulation of stromal hydration through the barrier and pump functions of the CE. Corneal transplantation has traditionally been used for defects of the CE, and more than half of the patients undergoing full-thickness corneal transplantation have decreased visual acuity due to a CE defect alone.¹²⁻¹⁴ Corneal transplantation requires a fresh human cornea, but there is a worldwide shortage of donors.

We have isolated precursors with the propensity to develop into CE-like cells from the CE of human donor corneas.⁶ Moreover, we have reported that rabbit CE-derived precursors for CE deficiency are an effective cell source for a rabbit CE-deficiency model.¹⁵ Because the number of CE precursors that can be isolated from a cornea is insufficient for corneal transplantation, establishment of a mass production method for precursor cells is needed for clinical use. In this study, we isolated spheres from cultured human corneal endothelium (HCE) and investigated whether the cells obtained had CE-like functions. We also tested the effect of anterior chamber injection of HCE spheres (instead of full-thickness corneal transplantation) in a rabbit model of bullous keratopathy, which represents a state of CE deficiency.

METHODS

Isolation of Sphere Colonies from Cultured HCE

A cornea was obtained from the Rocky Mountain Lions' Eye Bank. The age of the donor was 41 years. The tissue was managed according to the guidelines of the Declaration of Helsinki. Primary culture of HCE has been described elsewhere.¹⁶ Cells at the fourth or fifth passages were used in the study. HCE cells were incubated in 0.2% EDTA at 37°C for 5 minutes and then were dissociated into single cells by pipetting with a flame-polished Pasteur pipette. The viability of the isolated HCE was >90% as shown by trypan blue staining (Wako Pure Chemical Industries, Osaka, Japan). The sphere-forming procedure was used for primary culture.¹⁷ Basal medium containing a methylcellulose gel matrix (1.5%; Wako) was used, as described previously,^{18,19} to prevent reaggregation of the cells. Cells were plated at a density of 10 viable cells/ μ L (40,000 cells per well or 1420 cells/cm²) in the uncoated wells of 60-mm culture dishes. The basal medium was Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with B27, epidermal growth factor (EGF, 20 ng/mL), and basic fibroblast growth factor (bFGF, 20 ng/mL). To distinguish growing spheres from dying cell clusters, only those with a diameter of more than 50 μ m were

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counted. For passaging, primary spheres (day 7) were treated with 0.5% EDTA and dissociated into single cells, which were plated in 24-well culture plates at a density of 10 cells/ μ L. Culture was continued for another 7 days in basal medium containing a methylcellulose gel matrix. To measure sphere diameters, cultures were observed under an inverted phase-contrast microscope (model ELWD 0.3; Nikon, Tokyo, Japan) with a 10 \times objective lens, and images were analyzed by the NIH image program (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

Differentiation of Sphere Colonies

Individual primary spheres (day 7) were transferred to 13-mm glass coverslips coated with 50 μ g/mL poly-L-lysine (PLL; Sigma-Aldrich, Tokyo, Japan) and 10 μ g/mL fibronectin (BD Biosciences, Billerica, MA) in separate wells, as described elsewhere.¹⁵ To promote differentiation, 1% or 15% FBS was added to the basal medium, after which culture was continued for another 7 days.

Immunocytochemistry

Immunocytochemical examination of the spheres and their progeny was performed after 7 days of adherent culturing on glass coverslips. Cells were fixed with 4% paraformaldehyde (Wako) in PBS for 10 minutes. After they were washed in PBS, the cells were incubated for 30 minutes with 3% bovine serum albumin (BSA; Sigma-Aldrich) in PBS containing 0.3% Triton X-100 (BSA/PBST; Rohm & Haas, Philadelphia, PA) to block nonspecific binding. Next, the cells were incubated for 2 hours at room temperature with the after specific primary antibodies diluted in BSA/PBST: mouse anti-cytokeratin three monoclonal antibody (mAb; AE-5; Progen Biotechnik GmbH, Heidelberg, Germany), mouse anti- α -smooth muscle actin (α -SMA) mAb (1:400; Sigma-Aldrich), mouse anti-nestin mAb (1:400; BD Biosciences, Franklin Lakes, NJ), mouse anti-neurofilament 145 mAb (NFM, 1:400; Chemicon, Temecula, CA), rabbit anti- β -III tubulin polyclonal (pAb, 1:2000; Covance Research Products, Denver, PA), rabbit anti-GFAP pAb (1:400; Dako, Temecula, CA), and mouse anti-5-bromo-2'-deoxyuridine (BrdU)/fluorescence mAb (1:100; Roche Diagnostics, Basel, Switzerland). Mouse IgG (1:1000; Sigma-Aldrich) or normal rabbit serum (1:1000; Dako) was used as the control in place of the primary antibody. After being washed in PBS, the cells were reacted for 1 hour at room temperature with fluorescence-labeled goat anti-mouse IgG (Alexa Fluor 488, 1:2000; Molecular Probes, Eugene, OR) and fluorescence-labeled goat anti-rabbit IgG (Alexa Fluor 594, 1:400; Molecular Probes) as the secondary antibody. Finally, fluorescence was detected by observation with a fluorescence microscope (model BH2-RFL-T3 and BX50; Olympus, Tokyo, Japan).

Extraction of Total RNA and RT-PCR

Total RNA was isolated from primary sphere colonies and their progenies by using a kit (Isogen; Nippon Gene, Tokyo, Japan). The isolated total RNA was treated with RNase-free DNase I (Stratagene, La Jolla, CA) for 30 minutes, and then cDNA was obtained with reverse transcriptase (Super Script II; Invitrogen-Gibco, Grand Island, NY). T12VN primer (25 ng/ μ L) was used to make the first-strand cDNA. RT-PCR was performed in the absence of reverse transcriptase as the negative control. The PCR primers were based on the sequences of nestin, keratin-3, GFAP, α -SMA, and G3PDH. The nestin primers were 5'-CACCTGTGCCAGCCTTCTTAA-3' (sense) and 5'-CCACCGGATTCTC-CATCCTTA-3' (antisense), the keratin-3 primers were 5'-CATCATT-GCTGAAGTTGGTGC-3' (sense) and 5'-TCTTGGAGCTTGGCATTGG-3' (antisense), and the GFAP primers were 5'-CTGGGCTCAAGCAGTC-TACC-3' (sense) and 5'-AATTGCCTCCTCCTCATCT-3' (antisense). In addition, the α -SMA primers were 5'-CACCTGTGCCAGCCTTCT-TAA-3' (sense) and 5'-CCACCGGATTCTC CATCCTTA-3' (antisense), and the G3PDH primers were 5'-GGTGAAGTCCGG TGTGAACGGA-3' (sense) and 5'-TGTTAGTGGGCTCGCTCCTG-3' (antisense). Prod-

ucts were separated by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide.

Measurement of the Pump Function of Cells Derived from HCE Spheres

The pump function of four collagen sheets seeded with cells derived from HCE spheres was measured in a Ussing chamber, as reported previously with some modifications.²⁰⁻²³ The collagen sheets²³ were obtained from the Nippi Research Institute of Biomatrix (Tokyo, Japan). Cells from HCE spheres were suspended at 5.0×10^6 cells in 1.5 mL of culture medium and transferred to individual circular collagen sheets (10 mm in diameter). Each sheet was placed in one well of a 24-well plate, and the plate was centrifuged at 1000 rpm (176 g) for 10 minutes to enhance cell attachment. The sheets were incubated in the culture medium for 2 days, after which nonadherent cells and debris were removed. Human donor corneas with the epithelium removed mechanically ($n = 4$), plain collagen sheets ($n = 4$), or HCE-coated collagen sheets ($n = 4$), were mounted in the Ussing chamber.

Ex Vivo Migration and Proliferation of HCE Spheres on Rabbit Descemet's Membrane

All animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Primary HCE spheres (cultured for 7 days) were labeled with a fluorescent cell tracker (CM-DiI; C-7000; Molecular Probes), as described elsewhere,²³ to trace their localization. Rabbit CE cells were gently scraped off four freshly excised rabbit corneas with a sterile cotton swab, and HCE spheres were applied to the posterior surface of each cornea, after which the corneas were placed in 24-well plates and maintained in culture medium for 7 days. Fluorescence of the HCE that migrated onto the corneas was viewed under a fluorescence microscope, and the area occupied by cells migrating from the spheres was measured by the NIH image program ($n = 10$).

Cryoinjury and Labeled HCE Sphere Injection into the Anterior Chamber

New Zealand White rabbits (weighing 2.0-2.4 kg, $n = 24$) were anesthetized with an intramuscular injection of ketamine hydrochloride (60 mg/kg; Sankyo, Tokyo, Japan) and xylazine (10 mg/kg; Bayer, Leverkusen, Germany). To detach the CE from Descemet's membrane, a brass dowel cooled in liquid nitrogen was brought in contact with the rabbit cornea nine times, at the center and at eight peripheral sites on the corneal epithelium. This procedure was repeated twice. Then the anterior chamber was washed three times with PBS through a 1.5-mm width paracentesis, and 150 DiI-labeled HCE spheres or 1.0×10^7 HCE were injected into the anterior chamber of each right eye. Thereafter, the cryo (cryoinjury alone), HCE (cryoinjury and HCE injection), and sphere eye-down (cryoinjury and sphere injection) groups of rabbits were kept in the eye-down position (Descemet's membrane down), whereas the sphere eye-up group (cryoinjury and sphere injection) was in the eye-up position (Descemet's membrane up), for 24 hours under deep anesthesia. Cultured HCE-injection and the eye-up position group were not included in this study, because corneal edema was not decreased by the treatment in our preliminary study (Mimura T, unpublished observation, 2003). Each surgical eye was checked twice or three times a week by external examination and was photographed on postoperative days 7, 14, 21, and 28. Central corneal thickness was measured with an ultrasound pachymeter (range, 0-1200 μ m; Tomey, Nagoya, Japan) and intraocular pressure was determined with a pneumatic tonometer (model 30 Classic; Mentor O & O, Norwell, MA) at 1, 3, 7, 14, 21, and 28 days after surgery. The average of three readings was obtained each time. One-way analysis of variance and Scheffé's multiple comparison test were used to compare the mean data. $P < 0.05$ was considered significant.

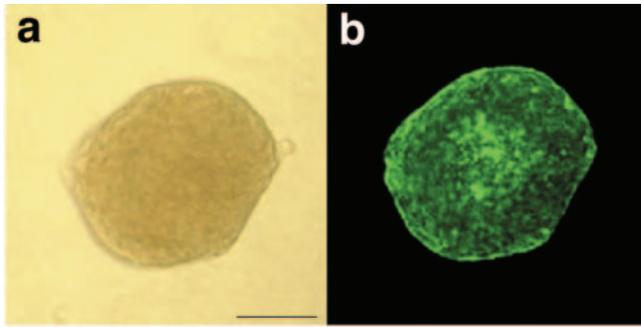


FIGURE 1. Sphere-forming procedure for cultured HCE cells. Cultured HCE cells were disaggregated into single cells and plated at a density of 10 viable cells/ μL in basal medium containing a methylcellulose gel matrix to prevent reaggregation. (a) A representative day-7 sphere is shown. (b) Each colony was labeled by BrdU on day 7. There were 44 ± 10 primary spheres generated per 10,000 cells (mean \pm SD). Scale bar, 50 μm .

Histologic Examination

To examine DiI fluorescence, the eyes were removed 1 month after transplantation and viewed as whole mounts under a fluorescence microscope. Then the corneas were excised and bisected. Postoperative endothelial morphology was evaluated using half of the divided cornea after staining with 3% trypan blue and 5% alizarin red to determine the CE density. The number of cells in a 0.1×0.1 -mm square was counted at four different sites on each of the six treated corneas. Next, the corneas were immersed in a fixative composed of 4% paraformaldehyde in 0.1 M PBS (pH 7.4), and were embedded in OCT compound (Tissue-Tek; Miles Laboratories, Naperville, IL) at -20°C . Frozen sections were cut at a thickness of 8 μm . The other half of each cornea was immediately fixed in 10% formalin and used for HE staining.

RESULTS

Isolation of Sphere Colonies

Spheres formed after 7 days of culture (Fig. 1a), whereas nonproliferating cells were eliminated. Many of the cells in each colony were BrdU positive (Fig. 1b), indicating that such cells were proliferating. These findings suggest that spheres were derived from single isolated cultured HCE and that the sphere-forming cells had proliferative activity. The number of sphere colonies obtained from the HCE after 7 days of culture was 44 ± 10 per 10,000 cells (mean \pm SD). Replating of primary spheres to generate secondary sphere colonies was less efficient, indicating that the cells had only limited self-renewal capacity.

Immunocytochemistry and RT-PCR of Sphere Colonies and Their Progeny

The spheres were immunostained with nestin, which is a marker of immature cells,²⁴ and with α -SMA as a mesenchymal myofibroblast marker. Some cells in the spheres were immunoreactive for nestin (Fig. 2a) and α -SMA (Fig. 2b). We have previously demonstrated that primary spheres derived from human donor CE express β -III tubulin and a mature glial cell marker, glial fibrillary acidic protein (GFAP), as well as nestin and α -SMA,⁶ but these markers were negative in the spheres derived from cultured HCE. The spheres were next transferred to PLL/fibronectin-coated glass coverslips in 24-well plates and cultured in a differentiation medium containing 1% and 15% fetal bovine serum (FBS). After 7 days, many cells were found

to have migrated from the colonies. Whether cultured with 1% or 15% FBS, less than 5% of these cells were α -SMA-positive (Fig. 2c), whereas all were negative for the control IgG (Fig. 2d) and the differentiated epithelial cell marker cytokeratin 3, as well as for nestin, β -III tubulin, and GFAP (not shown). These findings indicated that a single sphere colony can give rise to a small population of mesenchymal cells under clonogenic conditions. Nestin and α -SMA expression by the spheres, as well as α -SMA expression by their progenies, was confirmed using RT-PCR (Fig. 3). Positivity for β -III tubulin mRNA was detected only in cultures with 1% FBS.

HCE Morphology, and Transport Activity of HCE Sheets Reconstituted with Cultured Spheres

We differentiated spheres by culture in DMEM containing 10% FBS and investigated whether the differentiated cells showed an HCE-like hexagonal morphology, contact inhibition as an antiproliferative mechanism,²⁵ and or possessed transport activity. Confluent cells derived from cultured HCE spheres were found to show the single layered characteristic hexagonal shape (Fig. 4a) and contact inhibition during proliferation. We also measured the potential difference and short-circuit current in a standard Ussing chamber system,²⁰⁻²³ because HCE should have significant transport activity. Changes of the potential difference (Fig. 4b) and short-circuit current (Fig. 4c) are compared in Figure 4 between normal human donor corneas and HCE sheets constructed with cells from spheres. The average potential difference and short-circuit current of the sphere-derived HCE sheets at 1, 5, and 10 minutes ranged from 81% to 100%, which corresponds to the range for normal human donor corneas denuded of epithelium. These findings suggested that the cultured HCE spheres could generate HCE-like cells with adequate transport activity.

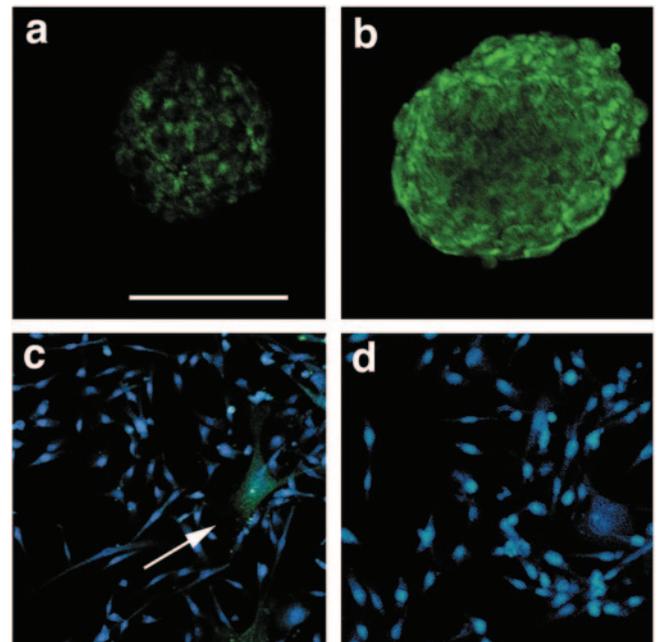


FIGURE 2. Immunocytochemistry of a sphere colony and progeny. A day-7 sphere shows nestin (a) and α -SMA staining (b). Less than 5% of the progeny cells are immunostained by the mesenchymal cell marker α -SMA (c, arrow), and there is no staining for the control IgG (d). Scale bar, 100 μm .

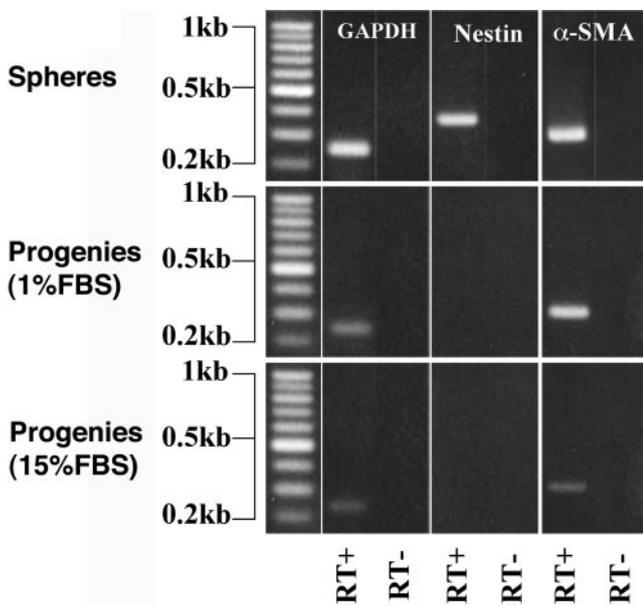


FIGURE 3. RT-PCR analysis of spheres and progenies. cDNA was obtained from cultured HCE spheres and progenies in 1% FBS or 15% FBS. GAPDH was detected in all samples, except those without reverse transcriptase (RT). Nestin mRNA expression can be detected in the cultured HCE spheres, but not in progenies cultured in either 1% or 15% FBS. cDNAs of all three samples yielded α -SMA-mRNA.

Observations in the Rabbit Model

Our findings suggest that cultured HCE precursors have a limited self-renewal capacity and mainly differentiate into HCE-like cells. Therapeutic use of precursors derived from cultured HCE was investigated in a rabbit model of CE deficiency. To

estimate the number of spheres needed to cover the inner surface of the cornea (Descemet's membrane), DiI-labeled spheres were prepared and seeded onto the denuded Descemet's membrane in culture. Figure 5a shows that cells migrated from DiI-labeled spheres and that the mean area covered per sphere was $1.2 \pm 0.2 \text{ mm}^2$ on day 7 (Fig. 5b). Therefore, the number of spheres needed per cornea was calculated to be 75. To allow for loss of spheres that failed to adhere, 150 spheres were injected to the anterior chamber of rabbit eyes subjected to cryoinjury, and the rabbits were maintained in the eye-down position (Descemet's membrane down) for 24 hours to allow attachment (sphere eye-down group, $n = 6$). Cryoinjury alone (cryo group, $n = 6$), injection of cultured HCE with the eye-down position for 24 hours (HCE group, $n = 6$), and injection of spheres with the eye-up position (Descemet's membrane up; sphere eye-up group, $n = 6$) was the control. In the cryo and HCE groups, the mean corneal thickness ranged from 953 ± 182 to $1200 \pm 0 \mu\text{m}$ (mean \pm SD) as shown in Figure 6. The mean corneal thickness of the sphere eye-up group ($704 \pm 174 \mu\text{m}$) was significantly less than that of the cryo ($1011 \pm 190 \mu\text{m}$; $P = 0.006$) and HCE ($953 \pm 182 \mu\text{m}$; $P = 0.022$) groups after 28 days of observation, but the corneas were still edematous. In contrast, the corneal thickness decreased rapidly in the sphere eye-down group, and the cornea was significantly thinner than in the other three groups after 14 ($672 \pm 90 \mu\text{m}$), 21 ($483 \pm 84 \mu\text{m}$), and 28 ($394 \pm 26 \mu\text{m}$) days ($*P < 0.006$; Fig. 6a). As shown in the representative anterior segment photographs from the cryo (Fig. 6b), HCE (Fig. 6c), and sphere eye-up (Fig. 6d) groups, the corneas were edematous with stromal opacity. In contrast, corneas from the sphere eye-down group corneas became clear and the anterior chamber was clearly visible (Fig. 6e). No apparent inflammatory reactions suggesting immunologic rejection were observed with a slit lamp microscope during the follow-up period. On day 14, intraocular pressure in the sphere eye-up group was

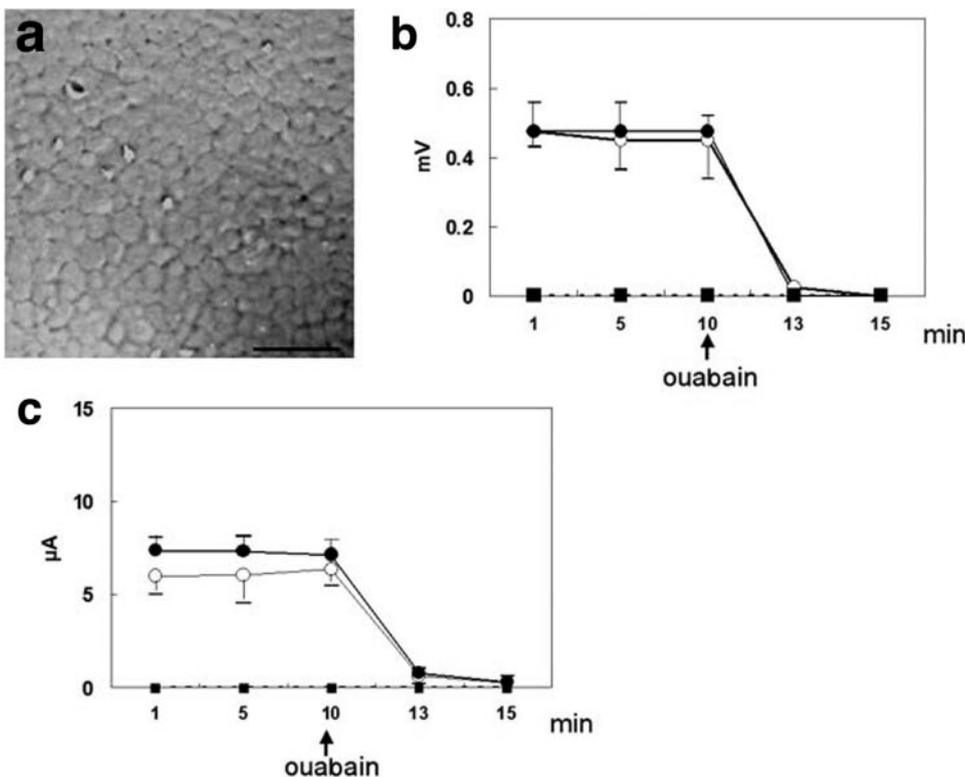


FIGURE 4. Morphology and transport activity of cells from cultured HCE spheres. (a) Confluent cells cultured in DMEM containing 10% FBS show the characteristic hexagonal shape of CE cells. Time-course changes (mean \pm SD) of the potential difference (b) and short-circuit current (c) in studies of human donor corneas and cultured HCE-like collagen sheets (coated with HCE spheres). The mean potential difference and short-circuit current after 1, 5, and 10 minutes ranged from 81% to 100% in the HCE sheets, similar to the range for human donor corneas denuded of epithelium, indicating that the HCE-like cells had adequate transport activity. After the $\text{Na}^+\text{-K}^+$ ATPase inhibitor ouabain was added to the chamber, the potential difference fell to 0 mV, and the short-circuit current to 0 μA in all cases.

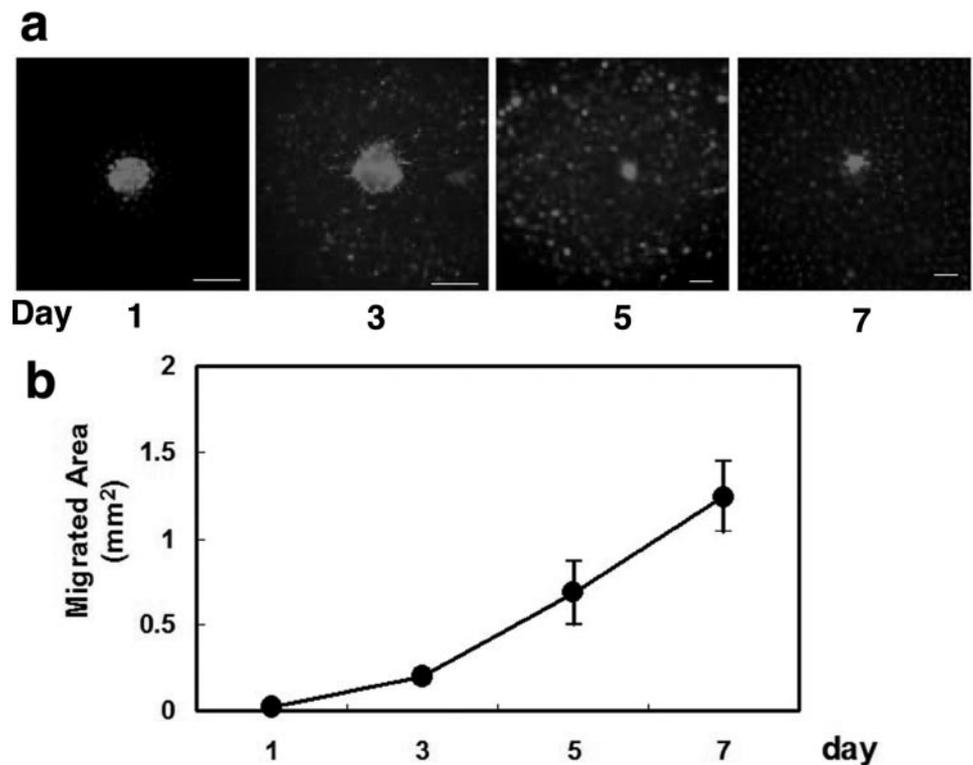


FIGURE 5. Migration of sphere-derived cells during culture for 7 days. DiI-labeled spheres were seeded onto the denuded Descemet's membranes of rabbit corneas in a humidified incubator with an atmosphere of 5% CO₂ and cultured for 1 week. The corneas were photographed, and cell migration was calculated on computer. (a) Representative photographs of the changes around an adherent DiI-labeled sphere. Scale bar, 100 μ m. (b) The mean area covered per sphere increased gradually and reached 1.2 ± 0.2 mm² on day 7 ($n = 10$).

significantly higher than that in the cryo group ($P = 0.013$). Except the day 14, however, there was no increase of intraocular pressure, a possible side effect, in any group during the observation period (Table 1).

Histologic Examination and Evaluation of HCE

Examination of HE-stained sections revealed that corneas in the cryo (Fig. 7a), HCE (Fig. 7b), and sphere eye-up (Fig. 7c) groups were thick, and there were no cells detectable on Descemet's membrane. In contrast, the corneas showed a monolayer of cells formed on Descemet's membrane, no edema, and no mononuclear cell infiltration of the posterior stroma in the sphere eye-down group (Fig. 7d).

In the cryo (Figs. 8a, 8e), HCE (Figs. 8b, 8f), and sphere eye-up (Figs. 8c, 8g) groups, no HCE (Figs. 8a–c) with DiI-positive staining (Figs. 8e–g) were found on Descemet's membrane at the central cornea in flatmount preparations. In contrast, HCE-like hexagonal cells were present at this location in the sphere eye-down group (Fig. 8d) and these cells were DiI-positive (Fig. 8h), indicating an origin from the injected spheres and not from the host rabbit. In the sphere eye-down group, DiI-negative cells were present in the peripheral cor-

nea, but cells in the central and paracentral cornea (8 mm in diameter) were all DiI-positive. The density of HCE in the six grafts of the sphere eye-down group at 28 days after surgery ranged from 2625 to 2875 cells/mm² (mean \pm SD, 2781 ± 92 cells/mm²). Before operation, CE density of rabbit cornea was from 3300 to 3500 cells/mm². In the sphere eye-down group, DiI-positive cells were barely detected in the inferior trabecular meshwork and iris surface, whereas a number of DiI-positive cells were attached thereto in the HCE and sphere eye-up groups (data not shown).

DISCUSSION

We produced spheres from cultured HCE and evaluated their morphology and function. Spheres derived from cultured HCE showed high proliferative activity, as indicated by BrdU uptake. Self-renewal capacity was also demonstrated by the ability of progeny of individual spheres to form secondary spheres, but not tertiary spheres, suggesting that the self-renewal capacity was limited. Moreover, individual spheres and their progeny produced mesenchymal cell lineage marker-positive cells. Although we cannot show direct evidence that the spheres give

TABLE 1. Mean Intraocular Pressure after Surgery

Group	Days after Surgery				
	0	7	14	21	28
Cryo	12.7 \pm 1.9	10.8 \pm 1.8	10.5 \pm 4.3*	11.0 \pm 1.6	10.2 \pm 1.5
HCE	11.8 \pm 1.5	11.6 \pm 2.8	12.3 \pm 4.5	11.8 \pm 2.2	11.5 \pm 2.8
Sphere eye-up	13.2 \pm 2.0	9.9 \pm 1.9	17.2 \pm 4.0*	13.3 \pm 3.4	10.5 \pm 3.1
Sphere eye-down	13.5 \pm 3.2	8.7 \pm 2.1	14.8 \pm 2.2	13.7 \pm 1.7	11.7 \pm 1.9

$n = 6$ each, (mean \pm SD) mm Hg;

* $P = 0.013$

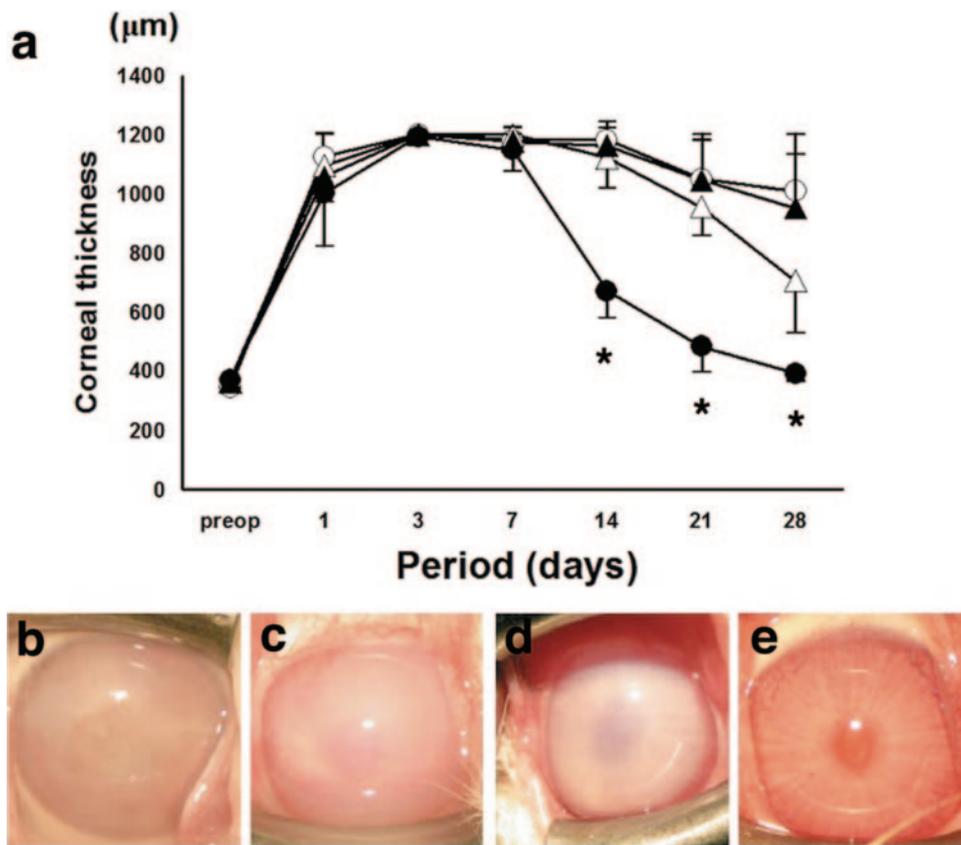


FIGURE 6. Changes of corneal thickness and other findings in the rabbit model. (a) Corneal thickness was measured with an ultrasound pachymeter. Mean corneal thickness decreased gradually in the sphere eye-down group (●, $n = 6$), being significantly less ($^*P < 0.001$) than in the cryo (○, $n = 6$), HCE (▲, $n = 6$), and sphere eye-up (△, $n = 6$) groups on days 14, 21, and 28 after treatment. (b–e) Representative photographs of corneas. Corneas were opaque in the cryo (b), HCE (c), and sphere eye-up (d) groups, and the anterior chamber was not clearly visible. In contrast, no corneal opacity was seen in the sphere eye-down group (e).

rise to HCE, the characteristic hexagonal morphology, contact inhibition as an antiproliferative mechanism,²⁵ and the HCE-like transport activity determined in the Ussing chamber^{20–23} suggest that the spheres generated HCE-like cells with sufficient transport activity to modulate corneal hydration. Taken together, these findings indicate that spheres isolated from cultured HCE contain bipotential precursors and that their progenies display essential HCE functions.

Our previous study demonstrated that spheres derived from donor corneal HCE express an immature cell marker (nestin), an immature neuronal marker (β -III tubulin), and a mature glial cell marker (GFAP), whereas their progenies express β -III tubulin and nestin.⁶ In contrast, the spheres and their progenies in this study did not express neuronal markers and showed decreased expression of immature cell markers. These findings

suggest that the precursors were closer in nature to the original tissue and underwent differentiation during culture. Thus, precursors obtained from cultured HCE may be a more appropriate cell source than those from donor corneal HCE, because precursors that efficiently differentiate into the original tissue are an ideal tool for tissue regeneration or cell transplantation.

Sphere injection in the eye-down position, but not injection of differentiated cultured HCE or sphere injection in the eye-up position, restored HCE function and decreased corneal edema in our rabbit bullous keratopathy model. Cells adhering to the inner surface of the cornea, Descemet's membrane, were DiI-positive in the sphere eye-down group, evidence that these were HCE derived from injected spheres and not residual host CE. DiI-positive spheres were barely detected in the trabecular meshwork and iris surface, and injected spheres mainly at

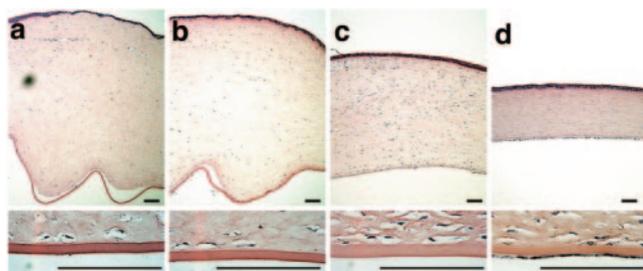


FIGURE 7. Histologic findings on HE staining. In the cryo (a), HCE (b), and sphere eye-up (c) groups, corneal stromal edema was prominent and no cells are present on Descemet's membrane at the central cornea. In contrast, a monolayer of cells can be detected on Descemet's membrane in the sphere eye-down group. No mononuclear cell infiltration is observed near Descemet's membrane, suggesting no immunologic rejection of the xenogeneic cells. Scale bar, 100 μ m.

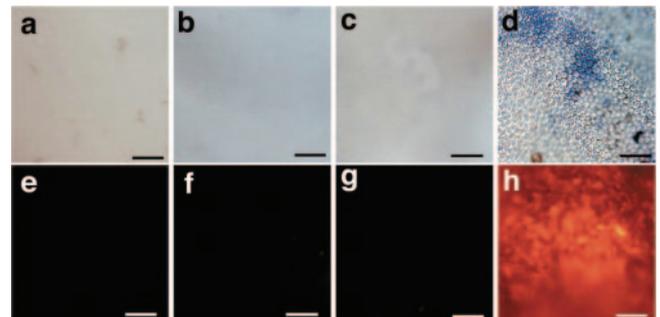


FIGURE 8. Flatmount preparation under phase-contrast (a–d) and fluorescence (e–h) microscopy. At the central cornea, there are no cells on Descemet's membrane in the cryo (a, e), HCE (b, f), and sphere eye-up (c, g) groups. In contrast, HCE-like hexagonal cells are present in the sphere eye-down group (d). These cells are DiI positive (h).

tached to and spread over the cornea in the sphere eye-down group, suggesting that injection of spheres derived from cultured HCE and maintenance of an eye-down position may have potential as a treatment strategy with minimal invasiveness for CE deficiency instead of conventional full-thickness corneal transplantation.

To perform regenerative medicine, amplification of stem cells is required for each tissue or organ. Although much attention has been paid to maintenance of the undifferentiated nature ("stemness") of stem cells and to promoting their amplification, the molecular mechanisms of stem cell replication and differentiation are still not fully understood.²⁶ In comparison with the amplification of adult stem cells, cultured cells may be more easily used to produce original tissue-committed precursors by the cell culture and sphere-forming method demonstrated in this study. Similar techniques to produce abundant precursors should be tested for various tissues as a method of obtaining cells for regenerative medicine.

Allograft rejection is a leading cause of the failure of conventional full-thickness human corneal allografting with local and/or systemic immunosuppressants.^{27,28} In a full-thickness murine corneal xenotransplantation model, corneal xenografts are rejected at approximately 2 to 16 days after transplantation.²⁹⁻³³ In contrast, mouse corneal allografts³⁴ and xenografts³⁵ containing CE that are inserted into the anterior chamber can survive indefinitely or for 4 weeks, respectively, without evidence of inflammation or rejection. No apparent inflammatory reaction suggesting immunologic rejection has been detected, even in the case of human-to-rabbit HCE sheet xenotransplantation²³ or HCE sphere xenotransplantation. These findings suggest that HCE transplantation may have some advantages over full thickness corneal grafting and that the transplanted cells may survive in the anterior chamber without rejection. This may be because antigen-presenting cells of the recipient cannot efficiently recognize HCE located between Descemet's membrane and the aqueous humor, or that the anterior chamber immune environment permits long-term acceptance and survival of histoincompatible cells.^{36,37} Long-term investigation of immunologic mechanisms is needed to determine whether allograft or xenograft rejection occurs after HCE transplantation.

We have established a method of mass production by isolation of precursors from cultured HCE using the sphere-forming assay. Transplantation of precursors into the anterior chamber and short-term maintenance of the eye-down position was a simple and effective treatment strategy for rabbit bullous keratopathy. This method of managing CE deficiency may replace conventional full-thickness corneal grafting and compensate for the worldwide shortage of donor corneas.

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