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Prospects for Descemet Stripping Automated Endothelial Keratoplasty Using Cultured Human Corneal Endothelial Cells

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Abstract

Study background: Descemet stripping automated endothelial keratoplasty (DSAEK) allows selective replacement of the diseased corneal endothelium. However, DSAEK requires a donor cornea and the worldwide shortage of corneas limits its application. In this review, we introduce our recent work on tissue engineering for DSAEK using cultured human corneal endothelial cells (HCEC) and recently published experimental data on HCEC precursors.

Methods and Results: We seeded DiI-labeled cultured HCECs onto collagen sheets, yielding HCEC sheets. The pump function parameters of these sheets were 76% to 95% of those for human donor corneas. Then HCEC sheets were transplanted onto the posterior stroma of New Zealand white rabbits after Descemetorhexis (DSAEK group). Rabbit corneas with only Descemetorhexis were the control group. The mean corneal thickness was significantly smaller in the DSAEK group than in the untransplanted control group throughout the observation period. DiI-labeled cells covered the posterior corneal surface in the DSAEK group. Severe stromal edema was detected in the control group by microscopy with hematoxylin-eosin staining, but not in the DSAEK group. Next, we isolated HCEC precursors from human donor corneas. Cultured precursor cells formed sphere colonies that expressed neural and mesenchymal proteins. The progeny of these colonies were HCEC-like hexagonal cells. Cell sheets constructed using HCEC precursors showed stronger staining for BrdU and nestin than cell sheets constructed with differentiated cultured HCECs.

Conclusions: These findings indicate that cultured HCECs transplanted from adult human donor corneas retain their corneal dehydration function and suggest the feasibility of performing DSAEK with HCECs to treat endothelial dysfunction. Adult human corneal endothelium contains precursors that can differentiate into corneal endothelial cells. HCEC precursors may become a powerful tool for the construction of HCEC-coated collagen sheets.

Keywords: Review; Corneal endothelium; Descemet stripping automated endothelial keratoplasty (DSAEK); Precursors; Transplantation

Abbreviations: CEC: Corneal Endothelial Cell; HCEC: Human CEC; DSAEK: Descemet's stripping with automated endothelial keratoplasty; DiI: 1,1-Dioctadecyl-3,3,3,3-Tetramethylindocarbocyanine Perchlorate

Introduction

The cornea is composed of a multilayered epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium. The corneal endothelium is a single layer of hexagonal cells that separates the corneal stroma from the aqueous humor of the anterior chamber. Transparency of the cornea is maintained by regulation of stromal hydration through the barrier and pump functions of the corneal endothelium. Corneal transplantation has long been used to treat defects of corneal endothelial cells (CECs). In fact, more than half of the patients who receive full-thickness corneal transplantation have decreased visual acuity due to corneal endothelial problems alone and have normal corneal epithelium [1-3]. Corneal transplantation requires a fresh human cornea, but there is a worldwide shortage of donors [4-7].

As a step toward to clinical application of human CEC sheet transplantation as a substitute for full thickness corneal transplantation, the feasibility of using cultured human CECs (HCECs) has been reported [8-15]. Cultured HCECs derived from adult human donor corneas have been transplanted onto denuded Descemet's membrane [8-13], collagen matrix [14], amniotic membrane [15], and human corneal stromal discs [16] *ex vivo*. Culture of differentiated HCECs yields cells with an HCEC-like morphology and function, but the cells become increasingly heterogeneous with older donor age and more passaging [17-19]. The number of HCECs, a pivotal factor in maintaining corneal transparency over the long term, decreases after transplantation [14]. Thus, a high cell density and normal hexagonal cells with adequate endothelial function are crucial requirements for producing cultured HCEC sheets that are comparable with or better than donor CECs.

Over the last few years, Descemet stripping with automated endothelial keratoplasty (DSAEK) has become a standard procedure for corneal transplantation in patients with endothelial dysfunction [20-23]. This procedure improves postoperative visual function and reduces the risks associated with penetrating keratoplasty, such as severe astigmatism and expulsive hemorrhage. However, DSAEK requires a donor cornea, so the worldwide shortage of donor corneas limits the application of this procedure [4-7]. If cultured HCECs could be used in corneal transplantation, many patients with corneal endothelial dysfunction could be treated by using cells from a single donor cornea. Therefore, we have been investigating the feasibility of DSAEK using cultured HCECs [14,16]. In this review, we introduce our recent work on tissue engineering of corneal endothelium with cultured HCECs and progenitor cells.

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Origin and Development of the Corneal Endothelium

Neural crest cells, from which the corneal endothelium is derived [24,25], migrate and differentiate in two waves during corneal development [26,27]. In the first wave, the corneal epithelium is formed by periocular mesenchymal cells of neural crest origin and it synthesizes the primary stroma, after which neural crest cells migrate to the margin of the optic cup and then migrate between the lens and corneal epithelium to contribute to development of the corneal endothelium and trabecular meshwork. In the second wave, neural crest cells invade the primary stroma and differentiate into corneal keratocytes.

Culture of HCECs

Several groups have established HCEC culture techniques [9,12,19,28]. Various growth factors have been reported to influence the proliferation of cells cultured from human corneal endothelium, including fibroblast growth factor [9,12,28-31] epidermal growth factor [12,28,31,32], nerve growth factor [12], and endothelial cell growth supplement [28,33]. In addition, cell attachment and growth can be supported by seeding cells onto an artificial matrix, such as chondroitin sulfate and laminin [29], laminin-5 [34], extracellular matrix secreted by bovine corneal endothelial cells [19,33], or fibronectin plus type I collagen coating mix [18].

In our studies, human donor corneas were handled according to the tenets of the Declaration of Helsinki of 1975 and its 1983 revision. All donor corneas were obtained from the Rocky Mountain Lion's Eye Bank. The age of the donors ranged from 42 to 67 years. HCECs were isolated and cultured according to the published protocols of Joyce and our laboratory with some modifications [12,18,19]. Briefly, Descemet's membrane with intact endothelium was carefully dissected. After centrifugation, the strips were incubated in 0.02% ethylenediamine tetraacetic acid disodium salt solution at 37°C for 1 hour to loosen intercellular junctions. Isolated cells were plated in 6-well tissue culture plates that had been precoated with undiluted fibronectin plus type I collagen coating mix. The plates were then incubated at 37°C in a humidified atmosphere with 5% carbon dioxide. After primary cultures reached confluence, cells were subcultured at a 1:4 ratio. Subsequent passages were done by the same method, but at a ratio of 1:16 and cells from the fourth, fifth, or sixth passages were used in this study.

Construction of a Cell Sheet from Cultured HCECs

Seeding of cultured HCECs on collagen sheets

As a cell carrier, collagen sheets obtained from Nippi Research Institute of Biomatrix (Tokyo, Japan) were employed. These sheets were composed of a network of loosely arranged cross-linked type I collagen fibers that had been treated with an alkaline solution, dried, and sterilized for 2 hours under ultraviolet light [35,36]. Before use, the desiccated sheets were immersed in sterile saline for 10 minutes. A 6.0-mm trephine was used as the biopsy punch. Each sheet was approximately 40 to 50 μm thick. To allow observation of the cells after transplantation, cultured HCECs were labeled with a fluorescent tracker (1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate: DiI). Immediately before labeling the cells, DiI was diluted to 10 g/mL with phosphate-buffered saline. Then 1.0×10^6 trypsinized HCECs were incubated in 5 mL of DiI solution for 5 minutes at 37°C, followed by an additional 15 minutes on ice at 4°C. After labeling, HCECs were washed twice with phosphate-buffered saline and resuspended in 300 μL of low-glucose Dulbecco's modified Eagle's medium containing 6% dextran. Then 1.0×10^6 cells in 300 μL of culture medium were transferred to sheets in each well of a 96-well plates. The plates were centrifuged at 1,000 rpm (176 g) for 10 minutes to promote attachment of cells to the sheets. After culture for 2 days, nonadherent cells and debris were removed.

Density of cultured HCECs on collagen sheets

We succeeded in achieving a mean endothelial cell density>3,000 cells/mm^2 for HCECs cultured on collagen sheets by improving the cell seeding technique. Adhesion of the cells was promoted by centrifugation after seeding using the methods of Jamblatt [37] and Engelmann et al. [10,11] with modifications. Engelmann and associates recommended that each cell suspension was centrifuged at 33g for 5min in their report [10]. In our study, cells were centrifuged at 1,000 rpm (176g) for 10 minutes to promote cell adhesion. Preoperative endothelial densities was around 1,200 cells/mm^2 without centrifugation, but the density was increased to about 3,500 × cells/mm^2 by centrifuging in the reconstructed corneal endothelial sheet. Application of fibronectin before cell seeding and a longer centrifugation time were found to prevent detachment of HCECs from Descemet's membrane.

Pump function of HCEC sheets

The pump function of HCEC collagen sheets was measured in an Ussing chamber by the method reported previously with some modifications [38-40]. Six relaxing radial incisions were made in the peripheral area of donor corneas from which epithelium had been scraped mechanically. The donor corneas (n=4), collagen sheets only (n=4), and HCEC collagen sheets (n=4) were mounted in the Ussing chamber. Corneas were incubated in Ringer solution containing (in mM): NaCl, 117.5; $NaHCO_2$, 24; KCl, 4; Na_2HPO_4, 1; $MgSO_4$, 1; glucose, 4.45; reduced glutathione, 1; and $CaCl_2$, 2.54 and bubbled with a 5% CO_2–7% O_2, 88% N_2 gas mixture to pH 7.38. After steady state levels of the potential difference and short-circuit current were reached, ouabain (0.1 mM), an Na^+, K^+ -ATPase inhibitor, was added to the chamber, and the potential difference and short-circuit current redetermined. The mean potential difference of the HCEC collagen sheets at 1, 5, and 10 minutes was respectively 85%, 80%, and 95% of that for human donor corneas (Figure 2A). The average short-circuit current of the HCEC collagen sheets at 1, 5, and 10 minutes was respectively 76%, 78%, and 82% of that for human donor corneas denuded of epithelium. The potential difference and short-circuit currents of the collagens sheets and human donor corneas denuded of epithelium and endothelium were 0 mV and 0 A at each time of assessment (Figures 2A and 2B). After the Na^+, K^+-ATPase inhibitor ouabain was added to the chambers, the potential difference and short-circuit currents reached 0 mV for all test samples within 5 minutes. These results indicate that CEC pump function, which mainly depends on Na^+, K^+-ATPase, was satisfactory with our HCEC sheets.

Transplantation of DSAEK Grafts in a Rabbit Model

Transplantation technique

All procedures were done in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rabbits were obtained from Saitama Experimental Animals, Inc. (Saitama, Japan). Eight New Zealand White rabbits weighing 2.0 to 2.4 kg were anesthetized with intramuscular ketamine hydrochloride (60 mg/kg) and xylazine (10 mg/kg). HCEC sheets were prepared as shown in Figure 3A. An HCEC sheet (with the HCEC side up) was placed on a foldable silicone plate for transplantation (Figure 3A). After disinfection and sterile draping, a 6-mm sclerocorneal incision centered at 12 o'clock was made with a slit knife, and a viscoelastic agent was infused

into the anterior chamber (Figure 3B). After the corneal surface had been ruled with a marking pen, a circular Descemetorhexis (6.0-mm in diameter) was created in the center of the cornea with a 30-gauge needle (Figure 3C) or a Price hook (Moria), and Descemet's membrane was removed from the anterior chamber. An HCEC sheet was brought into the anterior chamber with the forceps (Figure 3D) or using a foldable silicone plate (Figure 3E) and then was fixed to the posterior stroma that had been stripped of Descemet's membrane (Figure 3F).

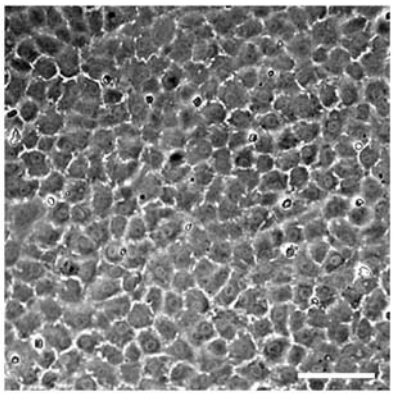

Figure 1: P6 cultured human corneal endothelial cells (HCECs) derived from a 65-year-old donor. Confluent cells show the characteristic hexagonal shape of corneal endothelial cells. Scale bar=100 µm.

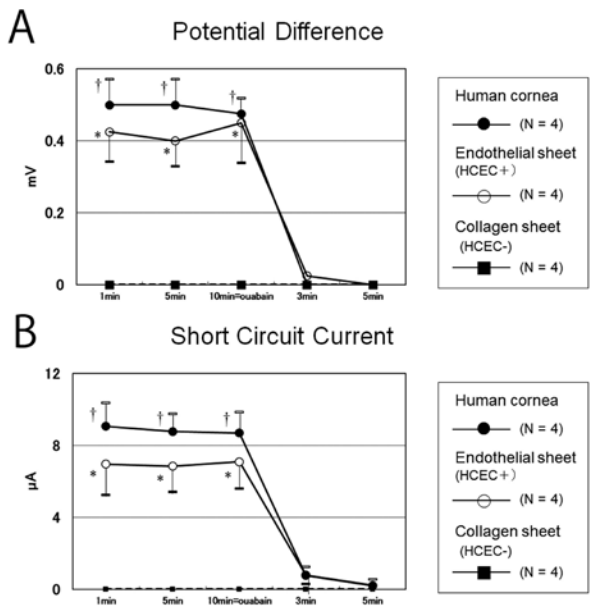

Figure 2: Changes of the potential difference and short circuit current in cultured HCEC collagen sheets. (A) The average potential difference for HCEC sheets is 80-95% of that for human donor corneas denuded of epithelium. Addition of ouabain (a Na^+-K^+ ATPase inhibitor) causes the potential difference to become 0 mV in all samples tested. (B) The average short circuit current for HCEC sheets is 76-82% of that for human donor corneas denuded of epithelium. Addition of ouabain causes the short circuit current to become 0 µA in all samples tested. Closed circles, open circles, and squares indicate human donor corneas denuded of epithelium (n=4), HCEC sheets (n=4), and bare collagen sheets (n=4), respectively. Data are the mean±SD. *p<0.001, HCEC sheet vs. collagen sheet, †p<0.001, human donor cornea vs. collagen sheet.

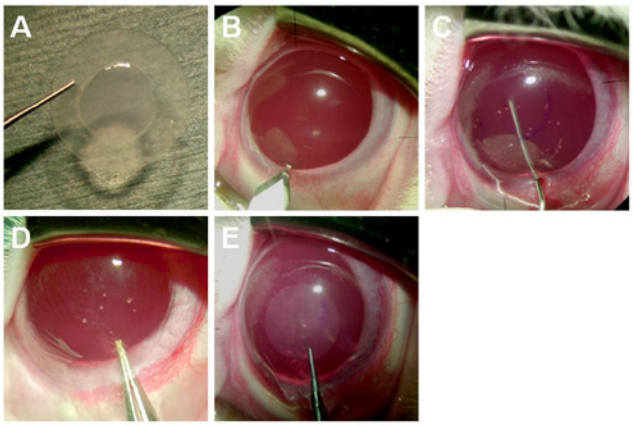

Figure 3: Procedure for HCEC sheet transplantation. (A) HCEC collagen sheet on a silicon plate. (B) A 6-mm sclerocorneal incision centered at 12 o'clock is made with a slit-knife. (C) A 6.0-mm diameter circular Descemetorhexis was performed with a 30-gauge needle and Descemet's membrane was removed from the anterior chamber. (D, E, F) The HCEC sheet was inserted into the anterior chamber using the forceps (D) or a foldable silicone plate (E) and attached to the posterior stroma (F).

When the HCEC sheet was brought into anterior chamber using the silicone, the plate was settled on a wound, but was not brought into the anterior chamber. After the rolled HCEC sheet was pushed into anterior chamber through the folded silicone plate using forceps, the plate was removed from the wound. Cultured HCEC sheet was fixed to the rear surface of cornea by air. The air bubble that fills the anterior chamber was replaced with balanced salt solution after transplantation of HCEC sheet. If the sheet was difficult to attach, the anterior chamber was completely filled with air at the end of the surgery. The sclerocorneal wound was closed with two to three interrupted sutures of 10-0 nylon. The rabbits were divided into two groups, which were the DSAEK group (rabbits with peeling of Descemet's membrane and transplantation of an HCEC sheet) and the control group (rabbits with peeling of Descemet's membrane only). Each group comprised four rabbits (four eyes). No immunosuppressive agents were administered either topically or systemically.

Observation after surgery

Corneal edema decreased much earlier after HCEC sheet transplantation in the DSAEK group than in the control group (Figure 4A). In the control group, mean corneal thickness remained at approximately 1,000 µm throughout the 28-day observation period. In contrast, it decreased rapidly in the DSAEK group, and the cornea was significantly thinner than in the control group at 1 ($P<0.05$), 3, 7, 14, 21, and 28 days ($P<0.001$) after surgery (Figure 4A). Figure 4B and 4C show representative anterior segment photographs from each group. The cornea is opaque with severe stromal edema in the control group, while the cornea transplanted with a cultured HCEC sheet is clear and has no stromal edema on day 28 in the DSAEK group (Figure 4). Slit lamp examination showed only mild opacity of the collagen sheet.

In the DSAEK group, grafts remained transparent for 1 month after surgery and the corneas with HCEC sheets were significantly thinner than the corneas of the control group. These results suggest the feasibility of performing corneal reconstruction by using HCEC cultured from adult donor corneas.

Histologic examination

Fluorescence microscopy of whole mounted corneas showed

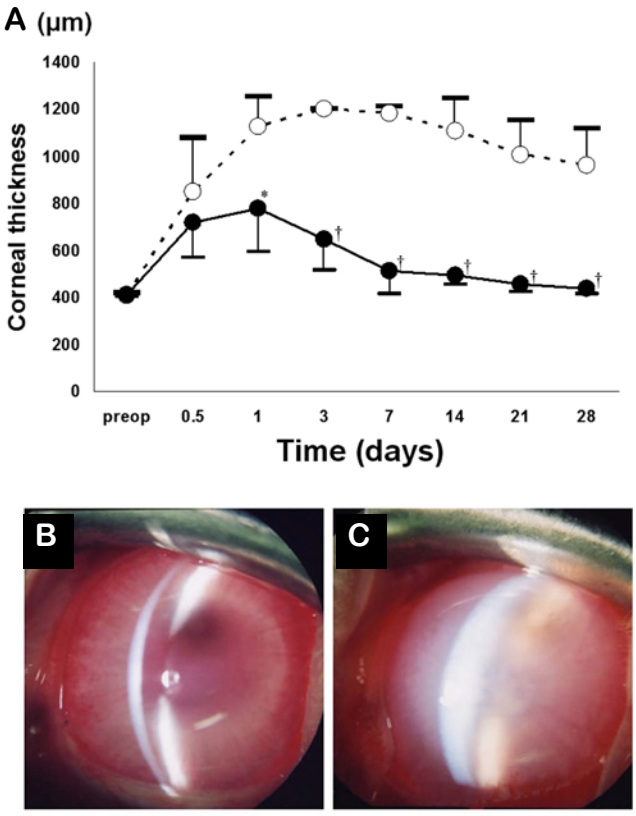

Figure 4: Central corneal thickness in the control group (open circles) and the DSAEK group (closed circles) (A) and anterior segment photographs obtained with a slit-lamp microscope at 28 days after surgery (B, C). In the control group, the mean corneal thickness remains at around 1,000 μm for 28 days (A). In contrast, the mean corneal thickness gradually decreases in the DSAEK group and becomes significantly less than in the control group (A). There are significant differences of corneal thickness between the DSAEK and control groups on days 1, 3, 7, 14, 21, and 28. *p<0.05, †p<0.01 (A). (B) Representative anterior segment from the DSAEK group, showing a thin cornea without stromal edema. The margin of the pupil is clearly observed. (C) Severe corneal edema is observed in the control group. Details of structures in the anterior chamber cannot be visualized (C).

DiI-positive cells localized on the transplanted collagen sheet and a clear margin of the sheet at 28 days after transplantation (Figure 5A). HCECs on the collagen sheets had a fairly regular morphology with well-defined boundaries (Figure 5B). No defects were detected on the collagen sheets. Most cells on the collagen sheets transplanted to the posterior surface of the cornea were DiI-positive in the DSAEK group (Figure 5C). Since endocytosed DiI cannot be transferred to adjacent cells [41], it is probable that the cultured HCECs remained on the sheet.

The endothelial cell density of the four grafts in the DSAEK group was around 2,500 cells/mm^2 at 28 days after surgery, whereas the preoperative endothelial density was around 3,500 cells/mm^2. In the control group, no CECs were detected on the stroma at the site of Descemetorhexis. HE-stained sections obtained 28 days after transplantation are shown in Figure 6. There is edema and diffuse cellular infiltration of the stroma in the control group (Figure 6A). Fibrous tissue and fibroblast-like cells were observed in the posterior stroma of the control group (Figure 6B). In contrast, there was no edema of the transplanted HCEC collagen sheets in the DSAEK group (Figures 6C, D).

Immune Privilege of the Anterior Chamber

The anterior chamber of the eye is an immune-privileged site and anterior chamber-associated immune deviation allows the long-term acceptance and survival of histoincompatible tissue grafts that would be rejected if transplanted to other sites [42,43]. In our study, no evidence of an inflammatory reaction, such as massive cell infiltration, keratic precipitates, or fibrin deposition, was detected in the anterior chamber by slit lamp microscopy, indicating that there was no notable acute rejection. When HCEC sheets are grafted, the transplanted HCECs face the anterior chamber may induce anterior chamber-associated immune deviation, thereby avoiding rejection, as evidenced by the lack of any immune reaction at one month after human to rabbit HCEC sheet [14] or precursor cell [44] xenotransplantation. Another possible reason is that the collagen sheet does not permit cell infiltration.

Current Limitations and Challenges

Autologous CEC transplantation is undoubtedly an ideal strategy to completely negate the possibility of rejection. Because CECs from the peripheral cornea contain a higher density of precursors than CECs from

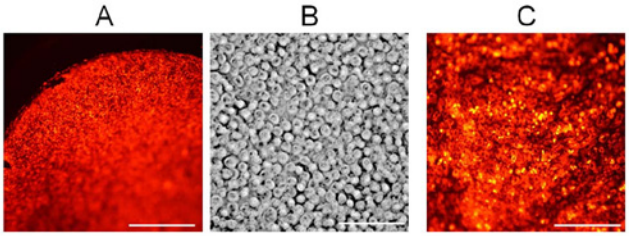

Figure 5: Fluorescein microscopy of the cornea in the DSAEK group on day 28. Fluorescein microscopic examination (A and C) and bright field micrograph (B) of flat mounts of the cornea obtained 28 days after transplantation in the DSAEK group. (A) DiI-positive cells on the HCEC collagen sheet clearly show the margin of the transplanted sheet. (B) At a higher magnification, HCECs on the collagen sheet have a regular morphology and are confluent. (C) Most cells on the collagen sheet are DiI-positive (same magnification as Figure 6B). Bars=1,000 μm (A) and 100 μm (B and C).

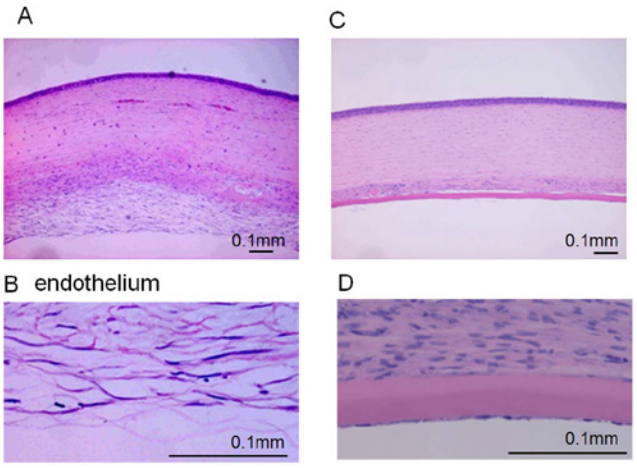

Figure 6: Histological examination of the cornea at 28 days after surgery. (A, B) In the control group, HCECs and Descemet's membrane are absent. Severe stromal edema and diffuse cell infiltration are observed. (C, D) HE staining shows a collagen sheet with HCECs on the posterior surface of the cornea and no stromal edema in the DSAEK group. Fibroblast-like cells are detected in the posterior corneal stroma attached to the collagen sheet. Bar=100 μm.

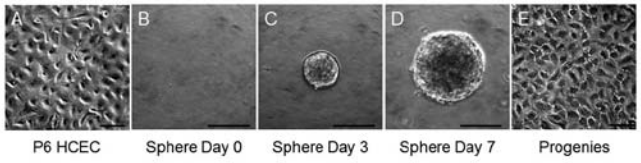

Figure 7: Representative sphere formation from passage 6 (P6) cultured human corneal endothelial cells (CECs). (A) P6 cultured CECs derived from a 43-year-old donor were grown as single cells in serum-free floating culture for 7 days. Dissociated cells are observed in medium containing methylcellulose gel matrix. (B) A sphere colony has grown from a single cell on day 3 (C) and has become larger on day 7. (D) Adherent progeny that migrated from the sphere colony (E). Scale bars=100 μm.

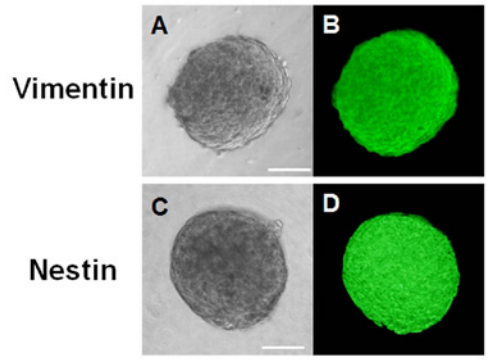

Figure 8: Immunocytochemistry of sphere colonies derived from cultured HCECs on day 7. Bright field images (A, C) and immunostaining (B, D) of spheres are shown. The spheres were stained for vimentin (a mesenchymal cell marker) and nestin (a neural stem cell marker). On day 7, a sphere is nestin and vimentin-positive. Scale bar=100 μm.

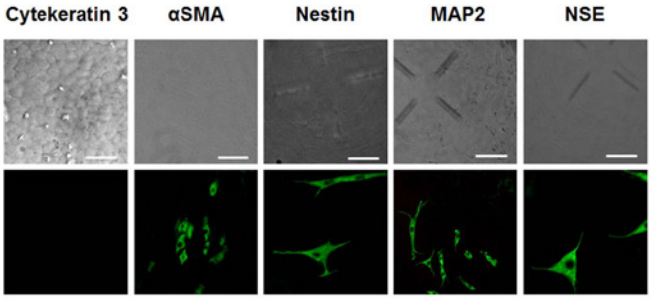

Figure 9: Immunocytochemistry of cells differentiated from spheres derived from cultured HCECs. Cells migrating out from the spheres express alpha-smooth muscle actin (α-SMA: a mesenchymal cell marker), neuron-specific enolase (NSE: a differentiated neural cell marker), microtubule-associated protein 2 (MAP2: a differentiated neural cell marker), and nestin (a neural stem cell marker), indicating that the colonies contain differentiated mesenchymal and neuronal cells. There is no staining for cytokeratin 3 (a differentiated epithelial cell marker). Scale bar=100 μm.

the central cornea in rabbits [45] and humans [46], culture of peripheral cells obtained by resecting a small piece of Descemet's membrane may eventually allow HCEC sheet transplantation for unilateral bullous keratopathy. However autologous CEC transplantation could not be used in patients with bilateral bullous keratopathy. For patients with bilateral CEC deficiency, use of heterologous CECs, embryonic stem cells, and induced pluripotent stem cells (iPSCs) are valuable option. Heterologous CECs can be isolated from the donor cornea or healthy patients' corneal limbal region. However there is still the requirement to select corneal endothelial precursor cells with their potential to differentiate into corneal endothelial lineage committed cells from these undifferentiated embryonic stem cells, adult stem cells, or iPSCs in final cell preparations.

Precursor Cells Derived From HCECs

Schimmelpfennig and Amann et al. reported that the density of HCECs is higher in the peripheral cornea than the central cornea [47,48]. It is also well known that the density of HCECs gradually decreases throughout life. Several groups have studied HCEC replication and have measured cell densities at the peripheral and central cornea [49,50]. Tissue culture studies by Bednarz et al. have shown that HCECs from the peripheral cornea are able to replicate, while cells from the central cornea exhibit little to no mitotic activity [49]. We previously reported that the percentage of replication-competent HCECs is higher for cells from the peripheral cornea than the central cornea. Significantly fewer central HCECs from older donors retain the ability to replicate compared with cells from the central cornea of younger donors, and HCECs, particularly those from the central cornea, undergo senescence-like changes with advancing donor age [51].

In various fields of regenerative medicine, precursor cells have been isolated by the neurosphere assay and utilized to regenerate tissues. Precursor cells are immature and have a greater potential to proliferate. Using the neurosphere assay, we tried to obtain precursor cells from CECs to explore the possibility of reversing senescence [44-46,52-57]. The CEC and Descemet's membrane were peeled away in a sheet from the periphery to the center of the inner surface of the cornea with fine forceps. The separated CEC and Descemet's membrane were incubated at 37°C for 3 hours in basal medium containing 0.02% collagenase. The tissues next were incubated in 0.2% EDTA at 37°C for 5 minutes, then dissociated into single cells by trituration with a fire-polished Pasteur pipette. Basal medium containing a methylcellulose gel matrix (1.5%) was used to prevent cell reaggregation. Cells were plated at the density of 1.0 viable cell/μL (5000 cells/well; 250 cells/cm^2) in 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). For passaging, primary spheres (day 7) were treated with 0.5% EDTA, dissociated into single cells, and the cells plated in 24-well culture plates at a density of 1 cell/μL.

After spheres formed, gene expression was examined by immunostaining and semiquantitative real-time polymerase chain reaction [52]. To differentiate HCECs, spheres adherent to dishes coated with poly-l-lysine and fibronectin were incubated in medium containing bovine serum. Gene expression of adherent cells that differentiated from the spheres was examined by immunostaining and real-time polymerase chain reaction.

Primary and secondary spheres were obtained from human corneal endothelium. Bromodeoxyuridine labeled most of the cells within each sphere, indicating that the colonies contained proliferating cells [52,56]. When the primary colonies were trypsinized and incubated in floating culture, some secondary colonies were generated, suggesting that HCECs have the capacity for self-renewal as sphere colonies. Cultured cells derived from the spheres had a polygonal shape at confluence. The mean potential difference and short circuit current for cell sheets derived from spheres were around 70% of those for normal corneas, suggesting that the spheres generated cells with considerable transport activity. Immunostaining showed that the sphere colonies expressed nestin (a marker of immature cells), α-smooth muscle actin

(a marker of mesenchymal myofibroblasts), β3-tubulin (an immature neuronal marker), and glial fibrillary acidic protein (an astroglial marker), whereas their progenies expressed β3-III tubulin and nestin [52]. These results suggest that individual spheres and their progeny produced mesenchymal cell lineage marker-positive cells. Although we cannot show direct evidence that the spheres give rise to HCEC, the characteristic hexagonal morphology and the CEC like transport activity determined in the Ussing chamber suggest that the spheres generated HCEC-like cells with sufficient transport activity to modulate corneal hydration. Taken together, these findings indicate that spheres isolated from HCEC contain bipotential precursors and that their progenies display essential HCE functions.

Next, the distribution and self-renewal capacity of corneal endothelial precursor cells were examined in rabbits [45] and humans [46]. CECs were obtained from the peripheral or central region of the cornea and the sphere-forming assay was performed. Primary spheres were isolated from both the peripheral and central regions, but the rate of primary sphere formation was significantly higher with peripheral cornea than central cornea. The rate of secondary sphere formation was also significantly higher with peripheral than central cornea, suggesting a higher self-renewal capacity of cells from the peripheral region.

We then tried to construct cell sheets using HCEC precursors derived from cultured HCECs [56]. We estimate that at least 2,000 8-mm HCEC sheet can be made using the passage 5 HCECs from one donor cornea. Alternatively, we estimate that we can get approximately 10,000 spheres containing HCEC precursors derived from the cultured passage 5 HCECs from one donor cornea.

Adherent and proliferating precursor cells achieved a high density. Immunocytochemistry demonstrated that staining of precursor sheets for Bromodeoxyuridine and nestin was stronger compared with P6-cultured HCECs. Progeny derived from the spheres showed little staining for the senescence marker SA-β-gal and displayed a regular morphology and grew at a higher density compared with passaged HCECs from the same source. These findings suggest that the sphere-forming assay can enrich young precursor cells from HCECs.

Because only 0.5% of cultured HCECs are sphere-forming cells, it may be that cells with long telomeres are selectively isolated by sphere-forming assay and senescent cells with shorter telomere are eliminated [56]. Cultured HCECs are an attractive source for regenerative medicine because currently the supply of donor corneas is insufficient to meet demand in most countries other than the USA, while cultured HCECs could create a number of corneal sheets for transplantation. Passaging of cultured HCECs, however, leads to senescence, and the cultured cells become larger and more senescent with older donor age and increasing passage number [17-19]. Our data suggest that the sphere-forming assay could be a useful tool to maximize the number of HCECs from a donor cornea and it may even become a standard technique to obtain cells for regenerative medicine.

Endless proliferation of transplanted precursors in the anterior chamber may cause glaucoma, because excessive numbers of HCECs would obstruct the trabecular meshwork and increase intraocular pressure. In vivo, HCECs are inhibited in the G1-phase of the cell cycle [58] and TGF-β2 in the aqueous humor suppresses entry into the S-phase [59]. Cell-cell contact inhibition also may be an important mechanism for inducing cell cycle arrest to maintain the mature monolayer in a nonproliferative state [60]. Moreover, the number of highly proliferative, cultured HECEs in vitro decreased after the HCEC sheet was transplanted to the anterior chamber [14], indicative that transplanted precursors may not proliferate continuously in the anterior chamber, because sphere progenies are exposed to aqueous humor containing TGF-β2 and reach cell confluence on Descemet's membrane for a short while.

Summary and Future Directions

New techniques that can replace full-thickness corneal transplantation have been tried both clinically and experimentally. HCEC transplantation with posterior stroma has been done clinically by procedures such as microkeratome-assisted deep lamellar keratoplasty [61-63], deep lamellar endothelial keratoplasty [64,65], and Descemet stripping endothelial keratoplasty [20-23]. HCEC transplantation without any stroma has been done clinically by Descemet membrane endothelial keratoplasty [66]. HCEC transplantation on carriers [14-16,67-71] has been investigated experimentally. Cultured HCECs should become a powerful tool for cell or tissue regeneration and transplantation. We have also demonstrated that sphere-forming precursors derived from HCECs largely give rise to HCEC-like cells with a hexagonal shape that possess essential functions such as pump activity. With the improvements in the ability to derive and purify HCEC precursors in vitro, HCECs can be now produced in sufficient quantities for in vitro experimental applications essential to clinical translation. However, several problems remain that will provide future challenges in the treatment of corneal endothelial dysfunction in the near future. As for experimental challenges, future work should address the way in which various type of stem cells are differentiated to HCECs with a focus on developmental physiology and cell culture microenvironments. Both approaches are essential step in improving the technology before clinical deployment. Before clinical trials, a very long-term investigation including histological observation will be necessary to evaluate the long-term viability of cultured HCEC after transplantation and clinically relevant postoperative complications such as graft rejection in animal model. Additionally, ethical and regulatory issues should be resolved for the cultured HCECs transplantation to be a routine clinical treatment. The culture medium contains some animal-derived purified proteins and the use of animal derived materials carries a risk of transmitting animal pathogens. Therefore, animal derived materials should be avoided. Several stem cell types such as embryotic stem cells, iPSCs, and adult stem cells may be a candidate for HCEC-replacement therapy. Isolation of human corneal endothelial precursors from peripheral portion of the cornea raises the possibility of autologous transplantation. This technique will circumvent the logistical, safety and ethical issues that arise with the transplantation of various other human stem cell types. Highly proliferative sphere-forming precursors may be employed for the treatment of corneal endothelial dysfunction at some point in the future.

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