Cultured Human Corneal Endothelial Cell Transplantation with a Collagen Sheet in a Rabbit Model

Tatsuya Mimura,1 Satoru Yamagami,2 Seiichi Yokoo,2 Tomobiko Usui,1 Keisuke Tanaka,3 Shunji Hattori,3 Shinkichi Irie,3 Kazunori Miyata,4 Makoto Araie,1 and Shiro Amano1

PURPOSE. To evaluate the function of cultured human corneal endothelial cells (HCECs) in vivo and the feasibility of HCEC transplantation with a collagen sheet as the substitute carrier of HCECs.

METHODS. Adult human donor cornea derived from cultured HCECs was labeled with the fluorescent tracker Dil (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) and seeded on a collagen sheet. The pump function of the HCEC sheet was evaluated by measurement of the potential difference and short-current circuit. A 6-mm sclerocorneal incision and Descemetorhexis were performed on rabbit eyes. The HCECs on a collagen sheet was brought into the anterior chamber and fixed to the posterior stroma (HCEC group). Rabbit corneas with collagen sheet transplantation after Descemetorhexis (collagen group) and with only Descemetorhexis (no-transplantation group) were the control. Each group, observed for 28 days after surgery, underwent histologic and fluorescence microscopic examinations.

RESULTS. Pump function parameters of the HCEC sheets were 76% to 95% of those of human donor corneas. Mean corneal thickness in the HCEC group was significantly less than in the collagen and no-transplantation groups 1, 3, 7, 14, 21, and 28 days ($P < 0.05$) after surgery. DiHabeled cells were spread over the rear corneal surface in the HCEC group. Marked stromal edema was present within the collagen and no-transplantation groups with hematoxylin-eosin staining, but none in the HCEC group with collagen sheets bearing monolayer cells.

CONCLUSIONS. The findings indicate that cultured HCECs transplanted from adult human donor cornea by means of a collagen sheet can retain their function of corneal dehydration in a rabbit model and suggest the feasibility of transplantation for CEC dysfunction using cultured HCECs with a collagen sheet. (Invest Ophtalmol Vis Sci. 2004;45:2992–2997) DOI: 10.1167/iovs.03-1174

From the Departments of 1Ophthalmology and 2Corneal Tissue Regeneration, University of Tokyo Graduate School of Medicine, Tokyo, Japan; the 3Nippi Research Institute of Biomatrix, Tokyo, Japan; and the 4Miyata Eye Hospital, Miyakonojo, Japan.

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Corresponding author: Satoru Yamagami, Department of Corneal Tissue Regeneration, Tokyo University Graduate School of Medicine, Hongo 7-3-1, Bunkyo-ku, Tokyo, Japan 113-8655; syamagami-tky@umin.ac.jp.

Researchers have demonstrated the feasibility of transplanting cultured corneal endothelial cells (CECs) in various animal models. In initial studies of cultured CECs, corneal transplantations were made in animal models by means of reconstructed corneal grafts bearing cultured animal CECs seeded on feline,1 rabbit,2,3 bovine,4 and murine5 Descemet’s membranes, or a gelatin membrane.6 A similar transplantation model reported for primates used cultured human neonatal and infant CECs.8–10 Cultured CECs were attached to a corneal graft ex vivo after full-thickness trephination and then transplanted to the host’s corneal bed by corneal surface circular sutures.1–10 Whether cultured human CECs (HCECs) derived from adult donor cornea can maintain the function of corneal dehydration in an in vivo model and whether artificial material bearing HCECs can be used to avoid corneal trephination and corneal button sutures has yet to be determined.

A culture technique for HCECs derived from adult human donor corneas recently was established, and transplantation onto denuded Descemet’s membrane of cultured HCECs ex vivo provided morphologic findings.11–16 We evaluated the function of cultured HCECs derived from adult human donor cornea in an in vivo rabbit model and propose a cultured HCEC transplantation method that uses a collagen sheet as a substitute carrier of HCECs.

MATERIALS AND METHODS

Media and Culture Conditions for HCECs

All the primary cultures and serial passages of HCECs were in growth medium consisting of low-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 2.5 mg/L amphotericin B (Invitrogen-Gibco, Grand Island, NY), 2.5 mg/L doxycycline, and 2 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich, St. Louis, MO). Cells were maintained in a humidified incubator at 37°C and 10% CO2. Medium used to produce bovine extracellular matrix (ECM) consisted of low-glucose DMEM with 10% FBS, 5% calf serum (Invitrogen-Gibco), 2.5 mg/L amphotericin B, 2.5 mg/L doxycycline, 2 ng/mL bFGF, and 2% dextran (Sigma-Aldrich).

Primary Culture of HCECs

Primary HCEC cultures were made as described elsewhere.16 Briefly, cultures were established from the remainder of donor corneas after full-thickness corneal transplantation. All the donor corneas were obtained from the Rocky Mountain Lions’ Eye Bank. Small explants from the endothelial layer, including Descemet’s membrane, were removed with sterile surgical forceps. The approximately 0.5-mm2 explants were made of a cornea and placed endothelial cell-side down onto four 35-mm tissue culture dishes coated with bovine ECM and the dishes carefully placed in an incubator. This coating dish was prepared by primary bovine CEC culture and CEC removal with trypsin-EDTA, because the ECM derived from bovine CECs is essential for primary culture of HCECs.16 The medium was exchanged after 3 days and thereafter replaced every other day. When a sufficient proliferating cell density was reached, the HCECs were passaged at ratios ranging from

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1:1 to 1:4. Subsequent passages were done by the same method, but at a ratio of 1:16. Cultured cells from the fourth and fifth passages were used in the study.

**Seeding of Cultured HCECs on Collagen Sheets**

The cell carriers were collagen sheets obtained from Nippi Research Institute of Biomatrix (Tokyo, Japan). Each sheet, composed of a network of loosely cross-linked type 1 collagen, was treated with alkaline solution, dried, and sterilized for 2 hours under ultraviolet light, as reported previously. Before use, the desiccated sheets were immersed for 10 minutes in sterilized saline. A 6.0-mm trephine was used as the biopsy punch (Kai Medical, Gifu, Japan). Each sheet was approximately 40 to 50 μm thick. To observe HCEC localization after transplantation, cultured HCECs were labeled with the fluorescent tracker DiI 1,1'-diocadecyl-3,3',3'',3''-tetramethylindocarbocyanine perchlorate; Cell tracker CM-Dil, C-7000; Molecular Probes, Eugene, OR) before reconstruction. Immediately before labeling, Dil was diluted to 10 μg/mL with phosphate-buffered saline (PBS). Trypsinized 1.0 × 10⁶ HCECs were incubated in 5 mL Dil solution on ice for 5 minutes at 37°C and then for an additional 15 minutes at 4°C. After labeling, the HCECs were washed twice with PBS and resuspended in 300 μL low-glucose DMEM containing 6% dextran (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

An HCEC suspension of 1.0 × 10⁶ cells in 300 μL culture medium was transferred to each sheet, and the sheet placed in individual wells of 96-well plates. The plates then were centrifuged at 1000 rpm (176g) for 10 minutes to enhance cell attachment to the sheets. Sheets were maintained in the cell culture medium for 2 days, after which nonadhering cells and debris were removed.

**Measurement of the Pump Function of the HCEC Collagen Sheet**

Pump functions of four HCEC collagen sheets were measured, with some modification, in a Ussing chamber as reported previously. 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; NaHCO₃, 24; KCl, 4; Na₂HPO₄, 1; MgSO₄, 1; glucose, 4.45; reduced glutathione, 1; and CaCl₂, 2.54 and bubbled with a 5% CO₂–7% O₂, 88% N₂ 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.

**Transplantation of Cultured HCECs into Rabbits**

Treatment was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All the rabbits were from Saitama Experimental Animals, Inc. (Saitama, Japan). Eight New Zealand White rabbits, weighing 2.0 to 2.4 kg, were anesthetized intraocularly with ketamine hydrochloride (60 mg/kg; Sankyo, Tokyo, Japan) and xylazine (10 mg/kg; Bayer, Munich, Germany). After disinfection and sterile draping of the operation site, a 6.0-mm sclerocorneal incision made with a slit knife was centered at 12 o’clock. A 6.0-mm diameter circular Descemetorhexis was performed with a 30-gauge needle. (C) Descemet’s membrane was removed from the anterior chamber of the eye. (D) The reconstructed HCEC sheet was brought into the anterior chamber and attached to the posterior stroma. (E) A silicone plate was used for HCEC sheet insertion. The plate with adherent HCEC sheet was folded by forceps and inserted into the anterior chamber. The plate was then removed.

**Figure 1. Surgical procedure for HCEC sheet transplantation.** (A) A 6-mm sclerocorneal incision made with a slit knife was centered at 12 o’clock. (B) A 6.0-mm diameter circular Descemetorhexis was performed with a 30-gauge needle. (C) Descemet’s membrane was removed from the anterior chamber of the eye. (D) The reconstructed HCEC sheet was brought into the anterior chamber and attached to the posterior stroma. (E) A silicone plate was used for HCEC sheet insertion. The plate with adherent HCEC sheet was folded by forceps and inserted into the anterior chamber. The plate was then removed.
Clinical Observations

Each surgically treated eye was checked daily by external examination with a slit lamp biomicroscope and photographed 7, 14, and 28 days after surgery. Central corneal thickness was measured with an ultrasound pachymeter (Tomey, Nagoya, Japan), and intraocular pressure with a pneumatic tonometer (model 30 Classic; Mentor, Norwell, MA) 1, 5, 7, 14, 21, and 28 days after surgery. An average of three readings was taken.

Histologic Examination and Localization of HCECs

One month after transplantation, the rabbits, under deep anesthesia, were killed by an intravenous overdose of pentobarbital sodium (Dainippon Pharmaceutical, Osaka, Japan). Their corneas were excised and bisected. HCEC morphology was evaluated on one side of the divided corneas. All the plates were examined under a light microscope (model BX50; Olympus, Tokyo, Japan) and the images saved to a computer. The number of cells in a 0.1-mm² area was counted at four different sites in the four reconstructed corneas. Wholemount samples of the corneas of the HCEC group also were examined for DiI-labeled HCEC sites in the four reconstructed corneas. Wholemount samples of the corneas deprived of their epithelia. The short-circuit currents measurement were 85%, 80%, and 95% of the value for human epithelium-deprived donor corneas. Addition of ATPase inhibitor, produced a short-circuit current of 0 mA in all the samples tested. Data shown are for human epithelium-deprived donor corneas, (●) HCEC sheets, and (○) collagen-only sheets; n = 4 in each group. Data are the mean ± SD.

Results

Pump Function of the HCEC Sheet

Time-course changes in the average and SD of the differences in potential differences in the donor corneas and in the collagen sheets, are shown in Figure 2A (collagen sheet and corneal stromal sides, positive). Average potential differences in the HCEC collagen sheets 1, 5, and 10 minutes after measurement were 85%, 80%, and 95% of the value of human donor corneas deprived of epithelia. The potential difference for the collagen sheets only (Fig. 2A) and for human donor corneas deprived of the epithelium and endothelium (data not shown) was 0 mV at each time point. After the Na⁺,K⁺-ATPase inhibitor ouabain was added to the chambers, within 5 minutes the potential difference became 0 mV in all the test samples.

The average and standard deviations of the short-circuit current in the donor corneas, collagen sheet, and HCEC collagen sheet are shown in Figure 2B. The average short-circuit current in the HCEC collagen sheets 1, 5, and 10 minutes after measurement was 76%, 78%, and 82% that of the human donor corneas deprived of their epithelium. The short-circuit currents of the collagen sheets and human donor corneas deprived of the epithelium and endothelium was 0 μA at each time point. After ouabain was added to the chambers, within 5 minutes the short-circuit current became 0 μA in all the test samples.

Clinical Observations after Surgery

Corneal edema decreased much earlier after HCEC sheet transplantation in the HCECs than in the collagen and no-transplantation groups. In the collagen and no-transplantation control group, mean corneal thickness was approximately 1000 μm throughout the 28 days of observation. In contrast, it decreased rapidly in the HCEC group, being significantly less than in the control group 1 (P < 0.05), 3, 7, 14, 21, and 28 days (P < 0.001) after surgery (Fig. 3). Figure 4 shows representative anterior segment photographs for each group. Compared with the opaque cornea with severe stromal edema seen in the collagen and no-transplantation groups, in the HCEC group on day 28, the reconstructed cornea transplanted by means of a cultured HCEC sheet was clear with no stromal edema (Fig. 4). In the slit lamp examination, mild stromal opacity was discernible on the layer attached to the collagen sheet. There was no intraocular pressure increase in each group throughout the observation period (data not shown).
corneal thickness remained approximately 1000 μm throughout the 28-day observation period. In contrast, mean corneal thickness in the HCEC group gradually decreased, becoming significantly less than that in the no-transplantation or collagen group. There were significant differences in corneal thickness (*P < 0.05, †P < 0.001) between the HCECs and no transplantation or collagen group on days 1, 3, 7, 14, 21, and 28.

**Histologic Examination**

HE-stained sections 28 days after transplantation are shown in Figure 6. Stromal edema and diffuse cell infiltration into the stroma were present in the no-transplantation group (Fig. 6A). In the posterior corneal stroma without Descemet’s membrane, there was marked edema (Fig. 6B). Similar findings were obtained for the posterior stroma of reconstructed corneas transplanted with a collagen sheet without HCECs (collagen group, Fig. 6C). Fibrous tissue with fibroblast-like cells was observed in the posterior stroma (Fig. 6D). In contrast, histologic findings for the HCEC group show no apparent stromal edema on the collagen sheet bearing HCECs (HCEC group, Fig. 6E). Corneal thickness in the HCEC group was clearly less than that in the no-transplantation or collagen group. Histologic examinations showed no apparent stromal edema on the collagen sheet bearing HCECs (HCEC group, Fig. 6F). An HCEC monolayer was present on the posterior surface of the collagen sheet. Fibroblast-like cells can be seen in the posterior stroma attached to the collagen sheet in the HCEC group. Periodic acid-Schiff (PAS) staining was performed to reveal whether HCECs produces a basement membrane in the HCEC group. PAS-positive staining was not detected on the rear surface of collagen sheet with HCECs 28 days after transplantation (data not shown).

**DISCUSSION**

Full-thickness corneal transplantation has been performed clinically for such CEC diseases as Fuchs dystrophy and pseudophakic or aphakic bullous keratopathy. This technique, however, has the severe complications associated with open-sky surgery as well as frequent complications of high or irregular astigmatism, refractive error, and suture-related problems. Deep lamellar endothelial keratoplasty (DLEK), which is the replacement of the posterior corneal layer with a graft consisting of the posterior stroma, Descemet’s membrane, and CECs, has been used to treat endothelial dystrophy.\(^\text{22,23}\) Moreover, Melles et al.\(^\text{24}\) have introduced a surgical technique for transplanting Descemet’s membrane derived from donor cornea ex vivo. DLEK and Descemet’s membrane transplantation solve the problems that accompany corneal surface circular incision and suturing. These techniques use human donor cornea, but worldwide there is a problem of an inadequate supply of donor corneas.

To make up for the inadequate supply of donor corneas, studies have shown the feasibility of using cultured animal CECs as transplants in in vivo animal models.\(^\text{1,7}\) This type of transplantation, however, has yet to be achieved clinically because of difficulties in culturing HCECs from adult human donor corneas and the lack of thin, transparent HCEC carriers. We found that the potential difference and short-circuit current of a cultured HCEC collagen sheet are similar to those of donor corneas, indicative that the pump function, which depends mainly on Na\(^+-\)K\(^+-\)ATPase, and does not work completely, is satisfactory in reconstructed HCEC sheets. Rabbit corneas receiving cultured HCEC collagen sheets showed decreased corneal edema, whereas severe stromal edema was present throughout the follow-up period in corneas without cultured HCEC sheets. Results are supported by histologic examinations of corneas, with and without cultured HCECs. These findings and the residual Dil-labeled cells derived from HCECs indicate that transplantation of the cultured HCECs contributes to the maintenance of corneal stromal hydration, evidence of the feasibility of using this type of transplantation as a substitute carrier of HCECs.

Histologic examinations showed fibroblast-like cells at the stroma attached to the HCEC collagen sheets. These cells were induced by the collagen sheets with fibronectin as the adhesive, not by the cultured HCECs, as seen in similar histologic findings for the transplantation of collagen sheets without...
HCECs (collagen group). Because the HCEC sheet produced only mild opacity of the posterior stromal layer attached to the collagen sheet in the slit lamp microscope examination and no immunosuppressive agents had been administered during the follow-up period, topical immunosuppressants such as corticosteroid eye drops may easily prevent fibroblast-like cell infiltration. However, long-term follow-up studies are needed.

It is worth commenting on the immunologic rejection of human-to-rabbit xenotransplantation that occurs in this model. In the full-thickness murine corneal xenotransplantation model, no hyperacute rejection occurs, but corneal xenografts are rejected in concordant or discordant combination without exception, on the average over a period of 2 to 16 days after transplantation.\(^{25-28}\) In our study, no apparent inflammatory reactions, such as massive cell infiltration, keratic precipitates, or fibrin formation were present in the anterior chamber of rabbit eye under slit lamp microscopy, evidence that there was no notable episode of acute immunologic rejection. The anterior chamber of the eye is an immune-privileged site and anterior chamber-associated immune deviation (ACAID) permits the long-term acceptance and survival of histoincompatible tissue grafts that otherwise are rejected when transplanted to various anatomic sites.\(^{29,30}\) In HCEC sheet transplantation, the transplanted HCECs, which face the anterior chamber, may induce ACAID, thereby avoiding immunologic rejection, the leading cause of graft failure in full-thickness corneal transplantation. Another possible reason is due to the character of the collagen sheet, which does not permit cell infiltration. As shown in representative HE staining of the HCEC group, there were no infiltrating cells in the collagen sheet itself. No infiltrating cells were detected in the collagen sheet separated in 10% formalin for fixation in the collagen group (data not shown). These findings suggest that antigen-presenting cells derived from the recipient can recognize xenoantigens only through aqueous humor, but not corneal stroma. Therefore, xenoantigens may fail to sensitize fully the recipient’s secondary lymphoid tissue to induce immunologic rejection during the observation period. However, we cannot completely deny the possibility of mild subclinical immunologic rejection, because postoperative endothelial densities on an HCEC sheet decreased compared with preoperative densities.

In summary, using anterior chamber insertion of a collagen sheet bearing HCECs, we developed a transplantation technique that uses a collagen sheet as the substitute carrier of HCECs. We conclude that cultured HCECs derived from adult human donor corneas retain the essential CEC function of dehydration of the corneal stroma in vivo; an artificial collagen sheet can serve as the carrier of HCECs; and anterior chamber insertion of an HCEC collagen sheet with fine forceps does not require a corneal full-thickness trephination and corneal but-
ton sutures. Our findings indicate that transplantation of cultured HCECs by means of a collagen sheet can retain their function of corneal dehydration in a rabbit model.

References


