Cultivated Corneal Endothelial Cell Sheet Transplantation in a Primate Model

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Purpose. To examine the feasibility of cultivated corneal endothelial cell transplantation in a primate model.

Methods. Monkey corneal endothelial cells (MCECs) obtained from three cynomolgus monkeys were cultivated, with subcultures grown on collagen type I carriers for 4 weeks. The corneal endothelium of the right eye of six monkeys was mechanically scraped, after which a cultivated MCEC sheet was brought into the anterior chamber of four eyes and fixed to Descemet's membrane by air. As the control, a collagen sheet without MCECs was transplanted into one eye of one monkey, and a suspension of cultivated MCECs was injected into the anterior chamber in one eye.

Results. Cultivated MCECs produced a confluent monolayer of closely attached hexagonal cells that showed both ZO-1 and Na^+-K^+ ATPase expression. In the early postoperative period MCEC sheets were attached to Descemet's membrane and corneal clarity was recovered. The recovered clarity was accompanied by a decrease in corneal thickness. Fluorescein DiI labeled donor corneal endothelial cells were detected on the host cornea on postoperative day 7. Six months after transplantation MCEC-transplanted corneas remained clear, and hexagonal cells were observed by in vivo specular microscopy with a density of 1992 to 2475 cells/mm^2. Control eyes showed irreversible bullous keratopathy that precluded pachymetry and specular microscopy.

Conclusions. A model of cultivated corneal endothelial transplantation for corneal endothelial dysfunction was established in primates whose corneal endothelial cells have less proliferative capacity in vivo. Our results suggest that this is a useful model for long-term observation in advance of the future clinical application of cultivated corneal endothelial transplantation. (*Invest Ophthalmol Vis Sci.* 2007;48:4519–4526) DOI:10.1167/iovs.07-0567

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The human corneal endothelium is essentially nonregenerative in vivo. Because endothelial cell loss due to dystrophy, trauma, or surgical intervention is followed by a compensatory enlargement of the remaining endothelial cells, the outcome is often irreversible corneal endothelial dysfunction. Penetrating keratoplasty for corneal endothelial dysfunction is not risk free, and alternative methods for replacing the endothelium without corneal trephination and sutures have been developed, which include posterior lamellar keratoplasty, deep lamellar endothelial keratoplasty, and Descemet's stripping endothelial keratoplasty.[1–3] Irrespective of the selected keratoplasty procedure, fresh donor corneas are necessary to treat corneal endothelial dysfunction, and because their availability is limited, the replacement of endothelial cells with cultivated corneal endothelial cells (CECs) constitutes an important alternative treatment method for corneal endothelial dysfunction.

Although human CECs have poor proliferative capacity in vivo and are arrested before the mid-G_1 phase, they do retain the capacity to proliferate.[4,5] Techniques for growing human CECs in culture have been reported,[6,7] and attempts have been made to develop transplantation models of cultivated human CEC sheets using carriers such as collagen sheets,[8] amniotic membrane,[9] or no carrier matrix.[10] In these studies,[8–10] the transplant recipient was the rabbit, an animal in which the corneal endothelium retains high-proliferation ability throughout most of its life, and in which residual peripheral CECs proliferate rapidly after injury and regenerate a clear cornea.[11] The eventual establishment of cultivated CEC transplants into the clinical realm requires in vivo confirmation of their long-term efficacy. As in humans, the ability of monkey CECs to proliferate is severely limited,[12–15] rendering this a representative model for corneal endothelial cell research.

Previously, Gospodarowics et al.[16] transplanted cultivated bovine CECs into feline eyes. Insler and Lopez[17] replaced CECs in eye bank donor corneas with cultivated human neonatal CECs and transplanted the corneas into rhesus monkeys. However, as these studies involved xenogeneic transplantation, the ultimate applicability of their results to the treatment of human corneal endothelial dysfunction is limited and the need for an allogeneic transplantation model remains.

Ours is the first study undertaken to investigate the feasibility of cultivated corneal endothelial sheet transplantation in a primate model, in which corneal endothelial cells have low proliferative potential. We undertook this investigation to establish an allogeneic transplantation model that constitutes an intermediate step toward the development of new surgical treatments for patients with corneal endothelial dysfunction. To this end, we produced a cultivated monkey corneal endothelial cell (MCEC) monolayer on a type I collagen sheet and studied its ultrastructure and function-related protein expression. We next transplanted the MCEC sheets into monkey eyes with corneal endothelial dysfunction and conducted a long-term follow-up of corneal clarity, corneal thickness, and in vivo corneal endothelial observation by noncontact specular microscopy.

Materials and Methods

Animal Experiment Approval

In all experiments, animals were housed and treated with in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Cell sheet transplantations were performed at the Research Center for Animal Life Science, Shiga University of Medical Science. The experimental procedures were approved by the Animal Care and Use Committee of Shiga University of Medical Science (approved No. 2005-1-5).

Primary Cultures

We used six corneas from three cynomolgus monkeys (3–5 years old; estimated human age, 5–20 years) housed at Nissei Bilis Co., Ltd. (Ohtsu, Japan), and Keari Co., Ltd. (Wakayama, Japan). The corneas were harvested at euthanatization for other research purposes, and the cells were placed in culture within 24 hours. We cultivated MCECs according to a modified protocol of human CEC culture reported previously.[9] Descemet's membrane was stripped of intact MCECs and transferred to 1.2 U/mL dispase II (Roche Applied Science, Penzberg, Germany). After a 20-minute incubation at 37°C, the MCECs obtained from individual corneas were resuspended in culture medium and were plated in one well of a 12-well plate coated with cell attachment reagent (FNC coating mix; Athena ES, Baltimore, MD). All primary cell cultures and serial passages of MCECs were performed in growth medium comprising DMEM supplemented with 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 ng/mL bFGF (Invitrogen-Life Technologies, Carlsbad, CA). MCECs were cultured in a humidified atmosphere at 37°C in 5% CO_2. The culture medium was changed every 2 days. When cells reached confluence in 10 to 14 days, they were rinsed in Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline, trypsinized with 0.05% Trypsin-EDTA (Invitrogen-Life Technologies) for 3 minutes at 37°C, and passaged at ratios of 1:3. At the first passage, MCECs derived from two corneas of one animal were mixed and equally divided to new plates.

Subculture on the Collagen Carrier

We used collagen type I sheets (Vitrigel; Asahi Techno Glass, Tokyo, Japan) as carriers for the cultivated MCECs. Vitrigel is a thin collagen type I gel membrane prepared by gelation and vitrification of a traditional collagen gel.[18] The collagen sheet was attached to the bottom of the 35-mm dish supported with a nylon membrane ring. Before seeding the MCECs, Vitrigel was rehydrated in a 10-minute incubation at 37°C with 2 mL of culture medium. Confluent subculture cells derived from each of the three monkey corneas at passages 3 to 5 were trypsinized, centrifuged, and resuspended in a culture medium. They were then seeded onto rehydrated Vitrigel-sheets in 35-mm dishes at a cell-seeding concentration of 5 to 10 $\times$ 10^2 cells/mm^2 and cultivated for 4 weeks, with the medium changed every other day. In some experiments, to investigate the survival of cultivated MCECs in vivo after

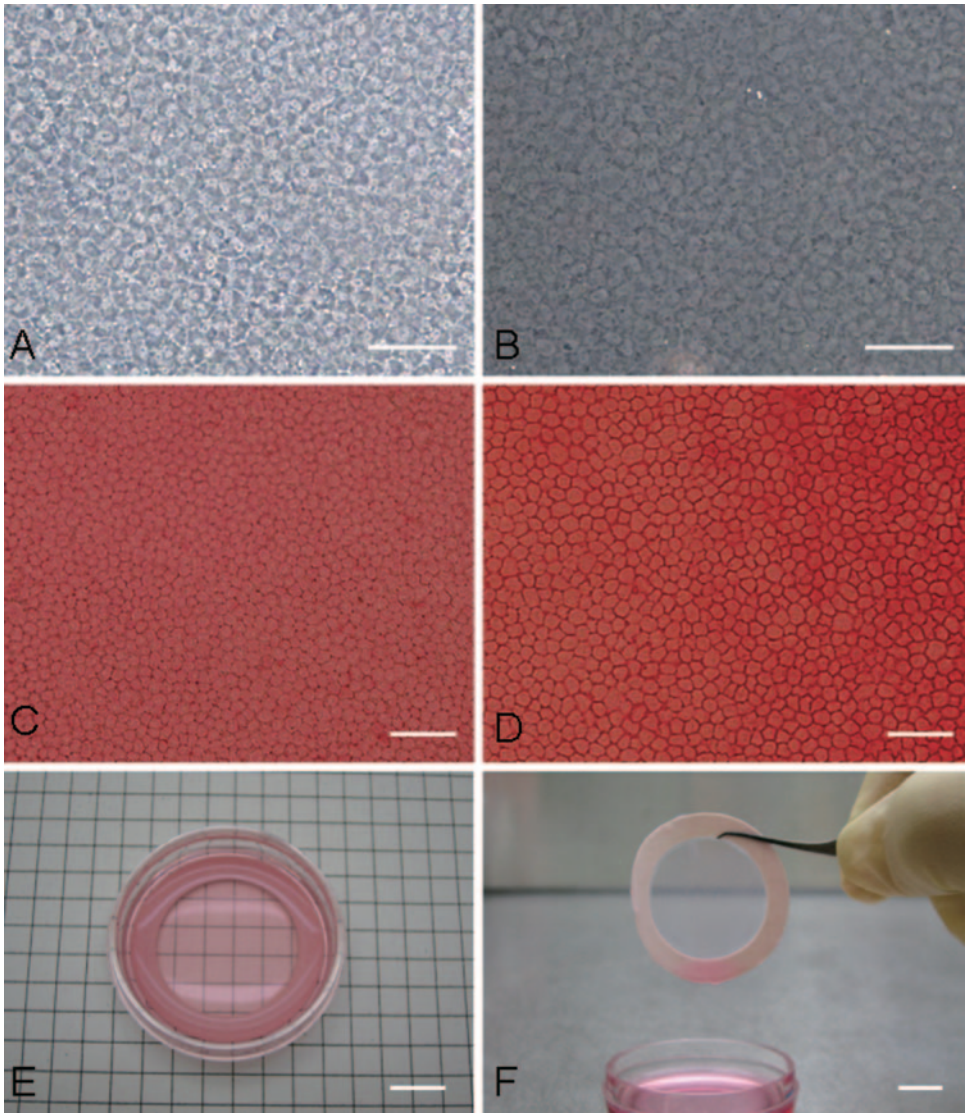

Figure 1. Primary culture and subculture of MCECs. (**A**, **B**) Primary culture of corneal endothelial cells from 4-year-old cynomolgus monkeys showing a confluent monolayer of homogeneous hexagonal cells with densities of 2509 and 3013 cells/mm^2, respectively. (**C**, **D**) Subculture of MCECs at passage 3 grown on a collagen type I carrier; staining was with alizarin red S. Endothelial cell densities were 2776 and 2221 cells/mm^2, respectively. (**E**) Macrographic image of cultivated MCECs grown on a collagen type I matrix attached to the bottom of a 35-mm dish and supported by a nylon membrane ring. (**F**) The sheet was easily peeled from the dish, and its flexibility and transparency were adequate for transplantation. Scale bars: (**A–D**) 100 μm; (**E**, **F**) 10 mm.

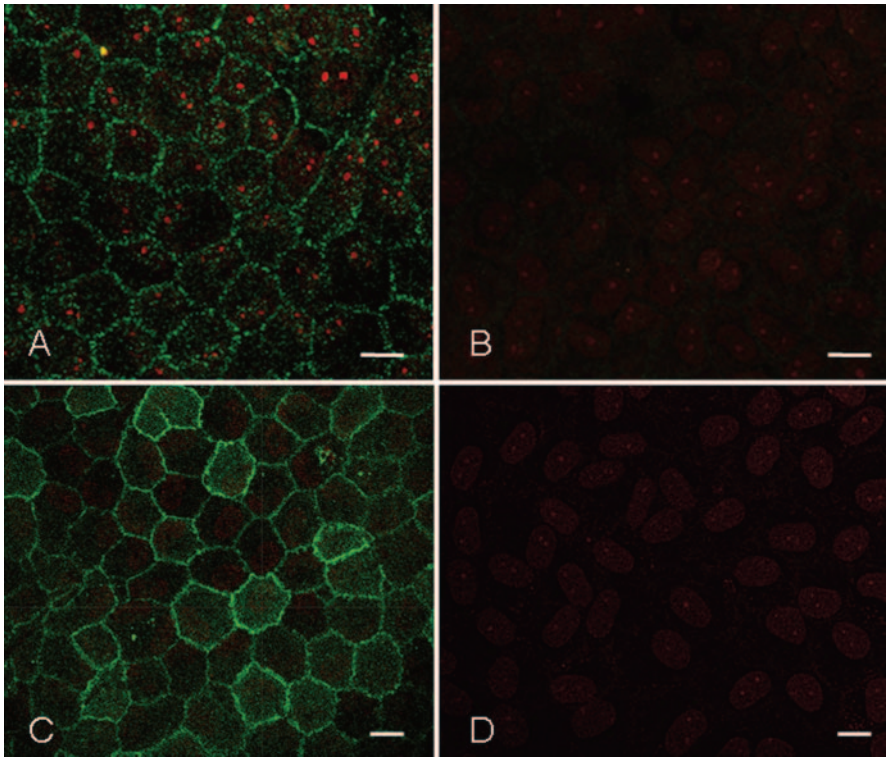

FIGURE 2. Function-related protein expression in cultivated MCECs grown on a collagen type I sheet. The cells expressed ZO-1 (**A**) and Na^+-K^+-ATPase (**C**) at their lateral cell membranes (*green*). (**B**, **D**) Negative controls without primary antibodies. Propidium iodide was used to visualize the cell nuclei (*red*). Scale bars, 15 μm.

transplantation, MCEC sheets on collagen type I carriers were labeled with fluorescein membrane DiI (1,1- dioctadecyl-3, 3, 3, 3-tetramethylindocarbocyanine perchlorate; Invitrogen-Molecular Probes, Eugene, OR) before transplantation. The cultivated confluent MCEC sheet was incubated for 20 minutes at 37°C with DiI solution diluted 1:200.

Light and Electron Microscopy

Three cultivated MCEC sheets on collagen type I carriers derived from three monkeys were stained with 0.1% alizarin red (Wako Pure Chemical Industries, Osaka, Japan) in deionized water to aid in examining the morphology and density of the cells under a light microscope. Cell density was calculated as the average cell density of five areas, each measuring 0.25 $mm,^2$ per sheet. For electron microscopy, a cultivated MCEC sheet on collagen type I was fixed in 2.5% glutaraldehyde in PBS, washed three times in PBS, postfixed for 2 hours in 2% osmium tetroxide, and washed another three times in PBS. Dehydration was through a graded ethanol series. For scanning electron microscopy (SEM) specimens were exposed twice to hexamethyldisilazine (Agar Scientific, London, UK), air-dried, mounted on aluminum stubs, and sputter-coated with gold before examination on a microscope (JSM

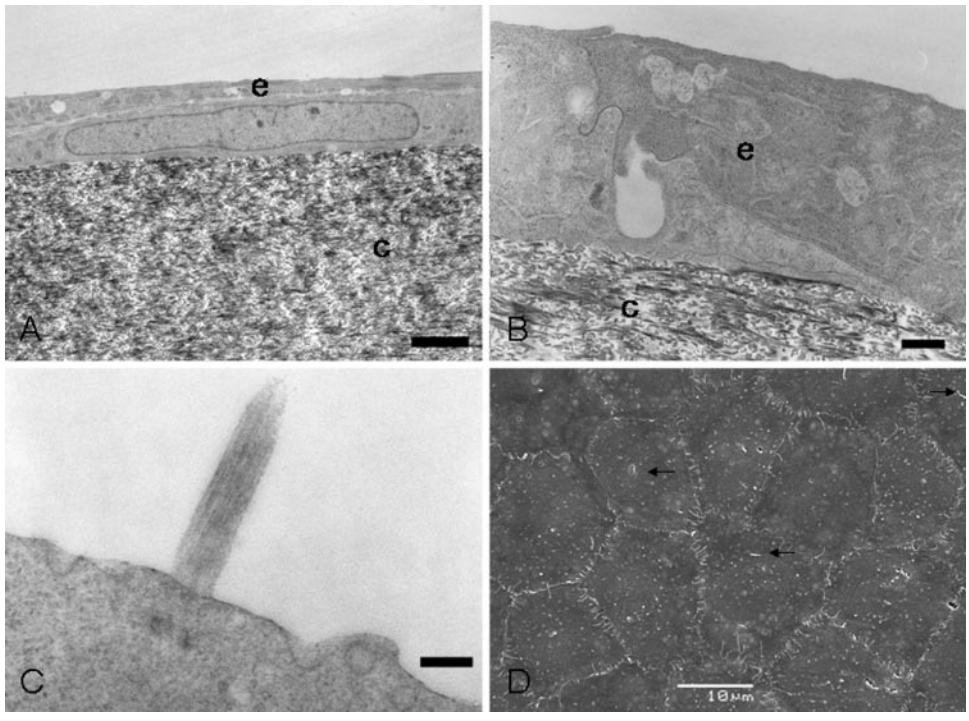

FIGURE 3. Transmission (**A–C**) and scanning (**D**) electron micrographs of cultivated MCECs (e) grown on a collagen type I matrix (c). Note the monolayer of endothelial cells similar in appearance to cells found in normal corneal endothelium in vivo. Adjacent cells are closely attached to each other and cilia are seen on the center surface of many cells (*arrows*) Scale bars: (**A**) 2 μm; (**B**) 500 nm; (**C**) 200 nm; (**D**) 10 μm.

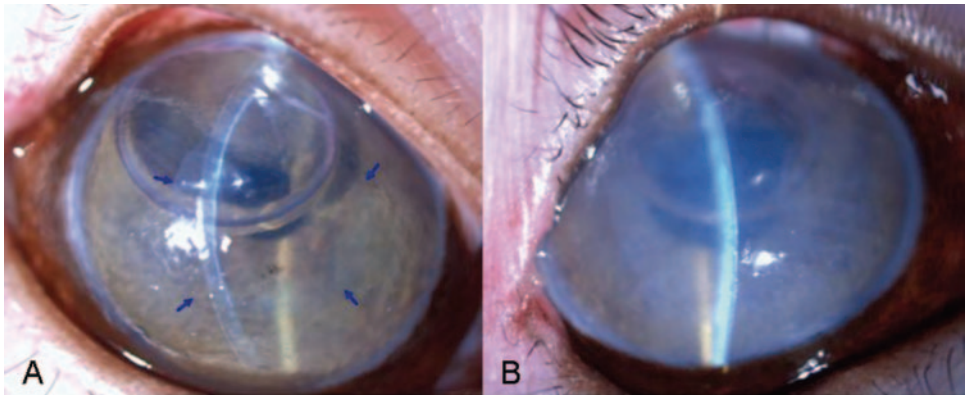

FIGURE 4. Anterior segment photographs made with a slit lamp microscope 24 hours after surgery. (**A**) The MCEC sheet was attached to Descemet's membrane (*arrows*), with slight swelling of the cornea observed in the MCEC group. Inflammation in the anterior chamber was minimal, and neither fibrin nor synechia were detected. (**B**) Severe corneal edema was observed in the control eye, which had a collagen sheet without MCECs implanted. The collagen sheet was visible, but no details of the anterior chamber were visible.

5600; JEOL, Tokyo, Japan). For transmission electron microscopy (TEM), specimens were embedded in epoxy resin and ultrathin sections (50–70 nm) were collected on copper grids. Counterstaining was with aqueous uranyl acetate and phosphotungstic acid (1 hour each) followed by Reynolds' lead citrate (20 minutes). Specimens were examined on a transmission electron microscope (JEM 1010; JEOL).

Immunocytochemistry

ZO-1, a tight-junction-associated protein, and Na^+-K^+ ATPase, the protein associated with pump function, were studied by indirect immunocytochemistry. For ZO-1 studies, a cultivated MCEC sheet on a collagen type I carrier was fixed at room temperature for 10 minutes in 3.3% formaldehyde in PBS containing 1.0 mM $MgCl_2$ and 0.1 mM $CaCl_2$. After several PBS washes, the specimens were permeabilized by a 10-minute incubation in PBS containing 0.1% Triton X-100, washed, and incubated for 30 minutes with 1% bovine serum albumin to block nonspecific binding. This was followed by 1-hour incubation at room temperature with 1:25-diluted rabbit anti-ZO-1 polyclonal antibody from Zymed Laboratories (South San Francisco, CA) and six washes in PBS. The MCEC sheets were then incubated for 1 hour in a 1:2000 dilution of FITC-conjugated goat anti-rabbit IgG antibody (Invitrogen-Molecular Probes) and again washed three times. For Na^+-K^+-ATPase studies, we fixed a cultivated MCEC sheet on collagen type I in ice-cold methanol (10 minutes) and cut it into six pieces. After several changes with the wash buffer (0.3% Triton X-100 in PBS), the specimens were incubated with a blocking buffer (1% BSA and 0.3% Triton X-100 in PBS) for 30 minutes to block nonspecific binding. They were then incubated for 1 hour with 1:200-diluted mouse anti- Na^+-K^+-ATPase monoclonal antibody (Upstate Biotech, Lake Placid, NY) and washed three times in wash buffer. The specimens were subsequently incubated for 1 hour with a 1:2000 dilution of FITC-conjugated goat anti-mouse IgG antibody (Invitrogen-Molecular Probes) and again washed three times. Negative control specimens for ZO-1 and Na^+-K^+-ATPase immunocytochemistry were obtained by the use of the same blocking buffer without the primary antibody. During all steps, the endothelial side was face-up to avoid damage. After they were washed with PBS in the dark, the specimens were mounted on glass slides with anti-fading mounting medium containing propidium iodide (Vector Laboratories, Burlingame, CA), and the slides were inspected with a laser confocal microscope (TCS SP2 AOBS; Leica Microsystems, Wetzlar, Germany).

Transplantation of Cultivated MCEC Sheets in a Monkey Model

Monkey surgeries for this study were conducted by a research group of fully trained ophthalmologists and veterinarians who hold proper licenses.

Six female cynomolgus monkeys (2.0–2.5 kg; Keari Co., Ltd.) were anesthetized intramuscularly with a mixture of ketamine hydrochloride (5 mg/kg; Sankyo, Tokyo, Japan) and xylazine (1 mg/kg; Bayer, Munich, Germany) followed by inhalation anesthesia with isoflurane. Transplantation was performed on the right eye of each animal only and was performed in an animal surgery room at the same levels of cleanliness as for human keratoplasty. During surgical procedures, animals were observed by veterinarians monitoring pulse, blood pressure, and Pao_2. To induce endothelial dysfunction 3-mm limbal–corneal incisions were made in six eyes of six monkeys, and the corneal endothelia were mechanically scraped with a 20-gauge silicone needle (soft tapered needle; Inami, Tokyo, Japan). The scraped area measured at least 9 mm in diameter (the diameter of the cornea is ~10 mm), and the denuded area was confirmed by 0.04% trypan blue staining during surgery. In preliminary experiments we confirmed that the mechanically scraped area had no cells on Descemet's membrane, and residual corneal endothelial cells were detected in only a 500- to 600-μm area at the edge of Descemet's membrane. At surgery, the limbal–corneal incision was spread to 6-mm, and a 6-mm diameter disc of a cultivated MCEC sheet was brought into the anterior chamber in four eyes of four animals on a supportive carrier (Lens Glide; Alcon, Tokyo, Japan) with the corneal endothelial side facing the anterior chamber. In one of the surgeries a DiI-labeled cultivated MCEC sheet was used. In all cases the limbal–corneal incision was closed with 10-0 nylon interrupted sutures, and the cultivated MCEC sheet attached to Descemet's membrane by air injection. As the control, a collagen sheet without MCECs was transplanted in one eye of one animal with endothelial dysfunction, and a suspension of cultivated MCECs (8×10^4 cells in 50 μL intraocular irrigating solution; OpeGuard Neo Kit; Senju, Osaka, Japan) was injected into the anterior chamber in one eye of another. The number of injected MCECs was the same as the number of MCECs on one 6-mm diameter disc of cultivated MCEC sheet. After surgery, all animals had a subconjunctival injection of dexamethasone (1.0 mg),

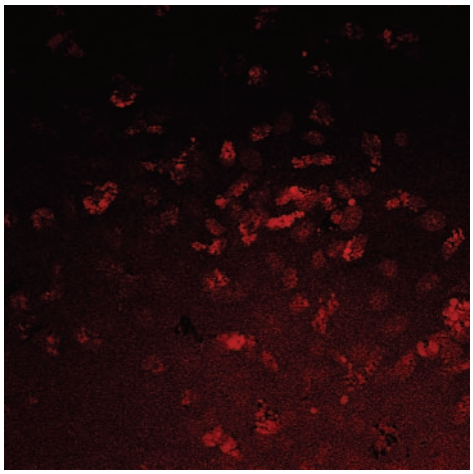

FIGURE 5. DiI-labeled MCECs (*red*) detected on the host Descemet's membrane. On day 7, one monkey which had a DiI-labeled MCEC sheet was killed and wholemount cornea was observed by fluorescein microscope. Original magnification, ×200.

followed by 0.1% betamethasone ointment once a day for 10 days. No systemic immunosuppression was used.

Evaluation of Corneal Appearance, Thickness, Histology, and Specular Microscopy after Transplantation

After transplantation, the corneal appearance was examined by slit-lamp biomicroscopy everyday for the first week, once a week thereafter for the first month, then once a month afterward up to 6 months. One animal that had a DiI-labeled MCEC sheet was killed at day 7 to observe the survival of the transplanted donor corneal endothelial cells. In the other five animals, corneal thickness was determined with an ultrasound pachymeter (model SP-2000; Tomey, Nagoya, Japan), and the mean of 10 measured values calculated. Six months after transplantation, in vivo corneal endothelial cells of two eyes which had cultivated MCEC sheet transplantation were examined using a noncontact specular microscope (Noncon Robo, SP-8800; Konan Medical, Nishinomiya, Japan). Finally, 6 months after transplantation, two animals that had undergone cultivated MCEC sheet transplantation were killed, after which the surgically treated eyes were enucleated and processed for electron microscopy. The other animals—one MCEC sheet transplantation and two control subjects—were kept for long-term observation.

Results

Primary Culture of MCECs

Primary cultures were made from 1.5 to 2.5 $\times$ 10^5 MCECs from individual monkey corneas that were plated in one well of a 12-well plate. Primary cultures from all three donor animals formed confluent layers of hexagonal cells within 14 days, with a cell density that ranged from 2125 to 3013 cells/mm^2 (n = 3, mean $\pm$ SD = 2549 $\pm$ 445.3 cells/mm^2; Figs. 1A, 1B). All these primary cultures were subcultured in new wells at a ratio of 1:3. Passage-3 cells were transferred to collagen sheets for further experiments.

Cultivated Monkey Corneal Endothelial Sheets

All cultures reached confluence after 1 week on collagen type I sheets. After this time, they were kept in culture for an additional 3 weeks to promote the formation of cell–cell contacts and the deposition of extracellular matrix. Alizarin red staining revealed mainly hexagonal, homogeneous cells with a density that ranged from 2221 to 2776 cells/ mm^2 (n = 3; mean $\pm$ SD = 2240 $\pm$ 30.9 cells/mm^2; Figs. 1C, 1D). On manipulation, we found that the endothelial cell sheets were

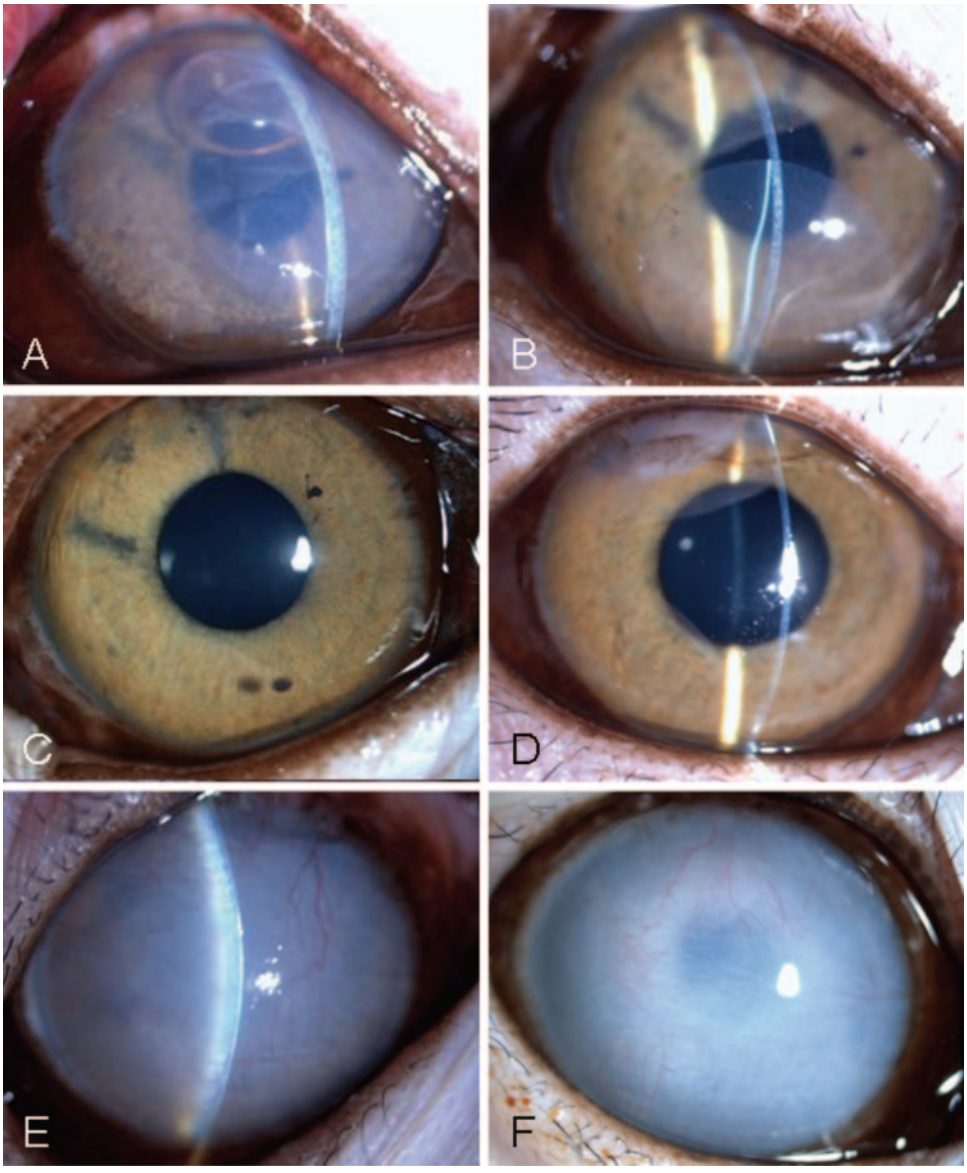

Figure 6. Time-course changes in the anterior segment slit-lamp photographs after cultivated MCEC sheet transplantation. (**A**) Up to 5 days after surgery, the transplanted sheets were attached to Descemet's membrane with no severe loss of corneal transparency in the MCEC sheet group. (**B**) At 14 days, MCEC sheets were detached from the Descemet's membrane in the anterior chamber. The corneas had recovered complete clarity, even when the detached MCEC sheet was located in the anterior chamber on the 14th day. (**C**) The eye maintained a clear cornea up to 6 months after surgery (the detached MCEC sheet was surgically removed from this eye). (**D**) The other eye from the MCEC sheet group 6 months after surgery. The MCEC sheet had shrunk and was located in the peripheral anterior angle area. (**E**) A control eye which had a collagen sheet transplantation without cultivated MCECs. (**F**) A control eye in which a cell-suspension of cultivated MCECs was injected. In control eyes, severe corneal edema developed and persisted after surgery, accompanied by neovascularization similar to advanced bullous keratopathy in humans.

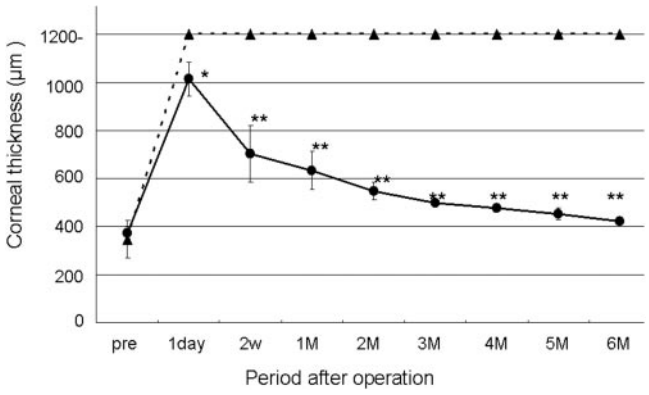

FIGURE 7. Central corneal thickness in the MCEC sheet (●; $n = 3$) and control (▲; $n = 2$) groups. In the MCEC sheet group, the mean corneal thickness gradually decreased and reached almost preoperative levels 6 months after the operation. In the control group, the corneas showed severe corneal edema and thickness measurement was impossible (over the pachymeter detection level; 1200 μm) throughout the 6-month observation period. Data are the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

easily peeled from the dishes by following the outline of the nylon ring with microforceps under a phase-contrast microscope, and that their flexibility and transparency were suitable for transplantation (Figs. 1E, 1F).

ZO-1 and Na^+-K^+ ATPase Protein Expression

Immunocytochemical staining of ZO-1, a tight-junction–associated protein, revealed that it was located at the cell boundaries of the cultivated MCEC sheets, suggesting the formation of focal, tight junctional complexes (Fig. 2A). Na^+-K^+ ATPase, an integral membrane protein complex responsible for regulating pump functions, was located at the basolateral membrane of the MCECs on the collagen type I carrier (Fig. 2C). These findings indicate that MCECs cultivated on type I collagen carrier were well-differentiated and presumably functional in vivo.

Ultrastructure

Examination by TEM showed monolayers of endothelial cells similar to those in normal in vivo corneal endothelium (Fig. 3A). The cells were well-formed and adhered to the underlying collagen sheet (Fig. 3B). They were closely attached to each other and there was little intercellular space. An SEM observation of the apical surface of these MCEC sheets showed that they measured approximately 10 to 15 μm in diameter (Fig. 3D). Adjacent cells exhibited characteristic interdigitating junctions. SEM and TEM revealed a cilium on the center surface of each cell (Figs. 3C, 3D).

Transplantation of Cultivated MCEC Sheets

Twenty-four hours after surgery, in four eyes of four monkeys the MCEC sheet was attached to Descemet's membrane. Slight swelling of the cornea was observed (Fig. 4A), but inflammation in the anterior chamber was minimal and fibrin or synechia were not detected. In control eyes, severe corneal edema was observed at 24 hours after surgery (Fig. 4B). At day 7, a fluorescein microscope examination of the wholemount cornea which had received a DiI-labeled cultivated MCEC sheet showed DiI-positive cells on Descemet's membrane outside of the area on which the MCEC sheet was placed, though the MCEC sheet itself was detached during tissue preparation (Fig. 5). At least up to 5 days after surgery, the transplanted sheets were attached to Descemet's membrane, and corneal transparency was improved in all three experimental eyes of the MCEC group (Fig. 6A). From postoperative days 5 through 14, we observed that the MCEC sheets became detached from Descemet's membrane and dropped into the anterior chamber (Fig. 6B). Nevertheless, these corneas achieved full clarity, even when we observed the detached MCEC sheet in the anterior chamber on postoperative day 14, and maintained a clear cornea at least up to 6 months after surgery (Figs. 6C, 6D). From one eye of the MCEC group, we surgically removed the detached MCEC sheet because it floated in the anterior chamber just behind the corneal endothelium causing worries that it might induce corneal endothelial damage (Fig. 6C). In the other two eyes of the MCEC group, we left the detached MCEC sheets in the anterior chamber because they had shrunk and become located at the peripheral angle area (Fig. 6D).

Measurement of corneal thickness demonstrated that after surgery corneal edema was most prominent on day 1, after which corneal thickness gradually decreased with time (Fig. 7). In control eyes, severe, and persistent corneal edema was observed after surgery (Figs. 6E, 6F), such that corneal thickness measurements were not possible because the corneas were over the 1200-μm detection level of the pachymeter. Six months after transplantation, noncontact specular microscopy was used to image the two surgical eyes (Fig. 8), which had endothelial cell densities of 2475 and 1992 cells/mm^2, respectively.

By electron microscopy, the endothelial surface of a monkey cornea 6 months after MCEC sheet transplantation was found to be covered with a confluent layer of polygonal cells that ranged in size from 15 to 30 μm (Fig. 9A). Adjacent cells appeared morphologically normal and closely attached with interdigitating junctions similar to normal cornea (Fig. 9A, inset). The endothelial monolayer had little intercellular space between neighboring cells, and these cells seem to have produced a new basement membrane posterior to Descemet's membrane (Fig. 9B).

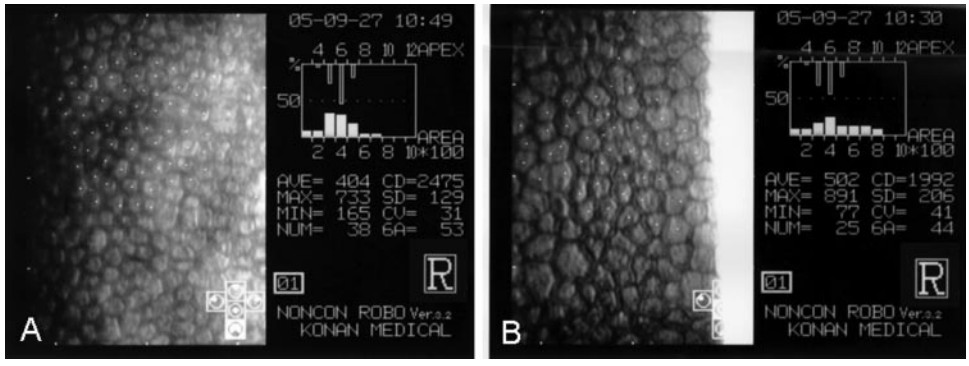

FIGURE 8. In vivo noncontact specular microscopy 6 months after MCEC sheet transplantation in two animals. Corneal endothelial densities were 2475 and 1992 cells/mm^2, respectively.

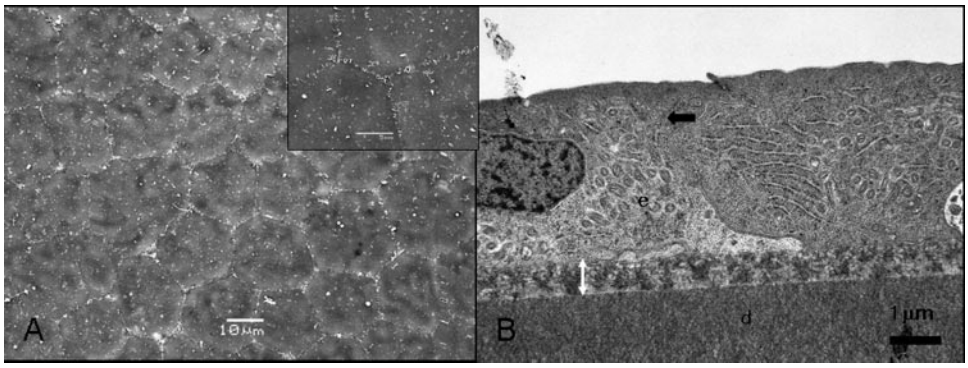

FIGURE 9. Electron microscopy of an MCEC sheet–recipient eye 6 months after surgery (same eye as in Fig. 8A). (**A**) The endothelial surface of the cornea was covered with a confluent layer of polygonal cells that ranged from 15 to 30 μm in diameter. Adjacent cells appeared closely attached with interdigitating junctions similar to normal cornea (*inset*). (**B**) The corneal endothelial cells seemed well developed with little intercellular space between neighboring cells. The cells (e) had produced a new basement membrane (*double arrow*) posterior to Descemet's membrane (d).

DISCUSSION

The transplantation of cultivated corneal endothelial cells has gained attention in efforts to treat patients with corneal endothelial dysfunction. Some groups have shown that human corneal endothelial cells can proliferate in vitro and that they function on transplantation into rabbit eyes.[8–10] However, unlike humans, rabbit corneal endothelial cells are highly proliferative in vivo, and transplantation models more akin to humans are needed to examine the long-term results of cultivated corneal endothelial transplants. In an effort to develop an appropriate animal model, we cultivated subcultured corneal endothelial cells derived from cynomolgus monkeys (MCECs) and transferred these cells onto a collagen type I carrier.

Other investigators have reported that isolated human CEC precursors injected into the anterior chamber regenerated the corneal endothelium in recipients.[19] Because the corneal endothelium is a thin monolayer, we consider sheet transplantation a better alternative, because it retains the polarity of the transplanted endothelial cells. Although Descemet's membrane is composed primarily of collagen type IV, in our experiments we used collagen type I sheets as the carrier for our cultivated MCECs because type IV sheets are not commercially available in Japan.

Our data show that MCECs grown on a collagen type I substrate produced well-formed cells with tight junctions and good cell-to-carrier contact in vitro and that they expressed ZO-1 and Na^+/K^+-ATPase protein. When the sheets were used to treat a monkey model of corneal endothelial dysfunction produced by intensive scraping of the corneal endothelium, we found that the irreversible corneal edema and corneal neovascularization similar to advanced bullous keratopathy in humans was avoided and that in the surgical eyes that received cultivated MCEC sheet transplants the cornea recovered its clarity. Of note, in these successful postsurgery animals, corneal endothelial cells were observed by specular microscopy, even though the transplanted collagen sheet was detached from Descemet's membrane during the observation period. Although longer term observation is necessary, we detected the survival of DiI-labeled donor corneal endothelial cells on the host Descemet's membrane in the early postoperative period. The mechanism of wound healing therefore was not as we planned, and we suspect that the procedure's success is due to cultivated MCECs migrating onto the host Descemet's membrane and proliferating in vivo. Our results lead us to speculate that once cultivated in vitro, monkey corneal endothelial cells may recover their proliferative ability and retain this ability even after they are returned to the in vivo environment. We are currently starting a series of experiments to study the in vivo proliferative mechanisms of transplanted cultivated monkey corneal endothelial cells.

One possible advantage of using cultivated CECs to treat patients with corneal endothelial dysfunction is the good productivity of the corneal endothelial sheets. With our culture protocol which uses cells from the third passage, we succeeded in producing a minimum of 12 35-mm diameter sheets from one monkey cornea. Moreover, and of particular interest, endothelial cells derived from one of six corneas used in this study maintained cell densities exceeding 2000 cells/m^2 at the 18th passage, theoretically, thus allowing the production of more than 100 cultivated MCEC sheets from one cornea. Advances in corneal endothelial stem cell research may result in a significant increase in the production of cultivated corneal endothelial sheets from a single cornea.

The surgical procedure for MCEC sheet transplantation presented in this study is not yet sophisticated enough for direct clinical application. Moreover, collagen type I carriers may not be appropriate for human transplantation; nevertheless, the results provide proof-of-principle for future investigations of MCEC sheet transplantation for the treatment of endothelial dysfunction.

The cynomolgus monkey is an adequate model for the study of the feasibility of this new surgical treatment, but in many developed countries animal experimentation, especially experimentation involving primates, is severely limited because of ethical and animal welfare considerations. Thus, research into the future clinical application of cultivated corneal endothelial sheet transplantation needs to be approved socially as well as scientifically, and should be conducted with the utmost adherence to animal welfare. Nevertheless, it is a reality that in some areas of medical research—such as in corneal endothelial cell research—the primate model provides invaluable information, and our allogeneic cultivated MCEC transplantation model constitutes an important step forward in regenerative medicine to treat patients with corneal endothelial dysfunction.

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