Transplantation of a Tissue-Engineered Corneal Endothelium Reconstructed on a Devitalized Carrier in the Feline Model

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PURPOSE. To evaluate the functional outcome of tissue-engineered corneal endothelium reconstructed on a devitalized carrier and transplanted in the living feline model.

METHODS. Eighteen healthy adult cats underwent full-thickness corneal transplantation. In 11 animals, the donor cornea was reconstructed from cultured allogeneic feline corneal endothelial cells seeded on the denuded Descemet’s membrane of a devitalized human cornea. The reconstructed corneal endothelium was cultured for 2 weeks before transplantation. Five control animals received autologous (n = 1), allogeneic (n = 3), or human xenogeneic (n = 1) native cornea. Two other control animals were grafted with the devitalized carrier only (no cells). Animals were observed daily by slit lamp until euthanatization on day 7. Postmortem analysis included optical coherence tomography (OCT), alizarin red staining, histology, fluorescence microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM).

RESULTS. Nine of the 11 reconstructed corneal endothelial grafts and all five native (autologous, allogeneic, xenogeneic) control grafts were clear and thin 7 days after grafting. In contrast, the two control grafts consisting of the carrier only (without endothelium) remained thick and opaque. Alizarin red staining, histology, SEM, and TEM showed that the transplanted reconstructed endothelium maintained a normal morphology and ultrastructure and expressed the function-related proteins Na+/K+-ATPase α1, Na+/HCO3−, and ZO-1.

CONCLUSIONS. This study provides evidence for the short-term (7-day) anatomic and functional success of corneal transplantation with a tissue-engineered corneal endothelium reconstructed on a devitalized carrier. (Invest Ophthalmol Vis Sci. 2009;50:2686–2694) DOI:10.1167/iovs.08-2793

The Eye Bank Association of America (EBAA) statistical report on Eye Banking Activity for 2007 reported 50,122 corneal transplants in 2007, an increase of 11% from 2006. Pseudophakic bullous keratopathy and primary endotheliopathies (including Fuchs’ endothelial dystrophy), which are responsible for 28.2% to 37.8% of all corneal transplants, represent the leading indications for corneal transplantation. As the population ages, the number of patients needing corneas for endothelial dysfunction is expected to increase. The EBAA reports that the most significant difference in tissue demand from 2006 to 2007 is a 134.9% increase in the number of tissue provided for endothelial keratoplasty (EK; 6027 corneas were provided for EK in 2006 compared with 14,159 in 2007). In addition, the increasing severity of the eye banking exclusion criteria for donor tissue is expected to further reduce accessibility to corneal transplantation and to increase waiting times.

Recent progress in tissue engineering has made it possible to consider new solutions to the problems of donor tissue quality and tissue shortage. For many years, researchers have evaluated the feasibility of reconstructing a corneal endothelium from cultured corneal endothelial cells seeded on a carrier for transplantation in humans. Studies have shown that cultured cells adhere on the carrier and form a high-cell-density endothelium that expresses the function-related proteins Na+/K+-ATPase and ZO-1. However, the choice of the carrier and its suitability for in vivo studies is an important step for transplantation success. Synthetic polymers, such as hydrogel 

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The goal of this study was to assess the functionality of these reconstructed corneas when transplanted in vivo in the feline eye. This work constitutes the first evidence that cultured endothelial cells seeded on a devitalized stromal carrier can recover an active pump function and can restore and maintain normal corneal thickness and crystal-clear transparency over a 7-day observation period after transplantation. This successful outcome represents an additional step toward the concept of tissue-engineered corneal substitutes for the replacement of eye bank native donors.

Materials and Methods

All experiments described in this article were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Declaration of Helsinki. The research protocol was approved by the Committee for Animal Protection of Maisonneuve-Rosemont Hospital (Montreal, QC, Canada) and by the Committee for the Protection of Human Subjects (Hôpital du St-Sacrement du CHA, Quebec, QC, Canada) and followed our local eye bank (Banque d’Yeux Nationale du CHUL, Quebec, QC, Canada) regulations.

Reconstruction of the Corneal Endothelium

For cell culture, intact cat eyes were obtained from other research laboratories after completion of their research within 24 hours of death ($n = 6$). Cat corneal endothelial cells were isolated and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Burlington, ON, Canada) containing 20% FBS (Hyclone, Logan, UT), 100 IU/mL penicillin (Sigma, Oakville, ON, Canada), and 25 μg/mL gentamicin (Schering Canada, Pointe-Claire, QC, Canada), as previously described. In some cases ($n = 5$), cells were labeled with a fluorescent dye tracker (SP-DioC18, Invitrogen), as described by the manufacturer. Briefly, the cell suspension was incubated for 5 minutes at 37°C and for 15 minutes at 4°C in a 2 mM solution of fluorescent dye tracker (SP-DioC18, Invitrogen), rinsed in PBS, and resuspended in fresh medium before seeding onto Descemet’s membrane.

The protocol for the reconstruction of the endothelium was previously described. Briefly, native human corneas unsuitable for transplantation in humans were obtained from our local eye bank (Banque d’Yeux Nationale du CHUL, Quebec, Canada). Twenty-six corneas from 16 donors (age range, 42–88 years; mean ± SD, 65 ± 14) were used. Native cells were killed through three freeze (−20°C)/thaw cycles and were stored at −20°C until used (range, 7–154 days; mean, 49 ± 38 days). Devitalized human corneas were then placed in a six-well plate, denuded Descemet’s membrane facing up. Feline corneal endothelial cells (250,000 [n = 2] or 285,000 [n = 9]) in 100 μL culture medium were placed on top of the denuded Descemet’s membrane and allowed to adhere for 4 hours before their complete immersion in culture medium. They were further cultured for 8 to 24 days (mean, 13 ± 5 days) before they were placed in transport medium (minimal essential medium [MEM]/M199 medium 1:1; both from Invitrogen), 26 mM NaHCO$_3$ (Fisher, Ottawa, ON, Canada), 25 mM HEPES (MP Biomedicals, Montreal, QC, Canada), 55 mM 2-mercaptoethanol (Bio-Rad Laboratories, Mississauga, ON, Canada), 0.02 mg/mL ascorbic acid (Sigma), 25 mg/mL chondroitin sulfate (Sigma), 1% dextran (Dextran T500; Fisher), 100 IU/mL penicillin (Sigma), and 25 μg/mL gentamicin (Schering Canada) at 4°C (range, 2–4 days; mean, 3.6 ± 0.8 days). On the day of surgery, the reconstructed endothelium was warmed at room temperature 2 hours before transplantation. Two devitalized stromal carriers were reconstructed for each experiment; one was transplanted, and the other was used for histopathologic examination.

Population

Eighteen healthy adult cats (weight range, 3–7 kg; mean ± SEM, 4.4 ± 0.3 kg) underwent full-thickness corneal transplantation. Eleven animals were grafted with reconstructed corneal endothelium on a devitalized stromal carrier. Five control animals received native grafts consisting of autologous ($n = 1$), allogeneic ($n = 3$), or xenogeneic human ($n = 1$) native cornea. Two other controls were grafted with the stromal carrier only, without endothelial cells. One eye per animal was randomly assigned to surgery, and the contralateral unoperated eye was used as control.

Preoperative Management of the Animals

Animals were obtained from a certified supplier. All animals were found to be healthy after complete physical checkup. Standard ophthalmic examination included slit lamp examination (Haag-Streit, Bern, Switzerland), intraocular pressure measurement with a handheld veterinary tonometer (Tonovet, TV01; Tiolat Oy, Helsinki, Finland), and central corneal pachymetry (Ultrasound Pachymeter SP 3000; Tomey, Nagoya, Japan). Oral prednisone (5 mg/d) was given for 3 days preceding surgery.

Surgical Protocol

Surgery was performed under general anesthesia by a single surgeon (IB). Anesthesia was induced and maintained by inhalation of isoflurane 2%. Atracurium (0.25 mg/kg, followed by 0.1 mg/kg every 20–30 minutes as needed) was used to induce paralysis of the extracocular muscles and to prevent third eyelid prolapse. The donor button was cut with a 9-mm Weck punch (Solan Medtronics, Jacksonville, FL), and the recipient cornea was cut with an 8-mm trephine (Hessburg-Barron; Katena Products, Denville, NJ). The anterior chamber was filled with viscoelastic (Viscoat; Alcon, Mississauga, ON, Canada). The donor disc was gently rinsed with balanced salt solution (BSS; Alcon) and secured to the recipient bed with four cardinal sutures, followed by a 10–0 nylon single running suture mounted on a needle (CU1; Alcon Surgical, Fort Worth, TX). The viscoelastic was then rinsed with BSS, and a recombinant tissue plasminogen activator (Cathflo, Alteplase, 100 μg in 0.2 mL; Roche, Mississauga, ON, Canada) was injected into the anterior chamber. Knots were cut short and buried, and the wound was checked for leaks.

Postoperative Follow-up

Tobramycin 0.3% and dexamethasone 0.1% ointment (TobraDex; Alcon) was applied twice daily in the operated eye, and prednisone (5 mg/d) was administered orally for 7 days. Animals were examined on a daily basis to check for graft clarity, signs of infection, or other complications. A grading scheme adapted from Bourges et al. was used to assess the transparency of the graft (4+, clear graft; 3+, slight opacity with iris/lens details easily visible; 2+, mild opacity, iris/lens details still visible; 1+, moderate opacity with no iris/lens details; 0, opaque cornea).

Postmortem Assessment

Animals were humanely killed with pentobarbital sodium (2 mL/4.5 kg administered intravenously) 7 days (±12 hours) after surgery to avoid the acute immune reaction known to occur 9 to 14 days after grafting. Operated and control eyes were enucleated and examined. Optical coherence tomography (OCT) was performed (OCT III; Carl Zeiss Meditec, Dublin, CA) to assess graft thickness and to document the fine structures in the anterior chamber susceptible to washing out during tissue processing for histology.

Corneal Endothelial Cell Density

The endothelium was stained for 45 seconds with the intercellular stain alizarin red S (0.2%; Sigma), rinsed in PBS, and photographed with a stereomicroscope (Nikon SMZ800 [Mississauga, ON, Canada] or SteREO Discovery V12 [Carl Zeiss Canada, Toronto, ON, Canada]). Endothelial cell densities and morphometric analyses were made with the software available on a noncontact specular microscope system (KSS-409SP, version 2.10; Cellchek XL; Konan Medical USA, Torrance, CA). The percentage of hexagonal cells was used as an index of pleomorphism, and the coefficient of variation in cell area was used as a measure of polymegethism. Endothelial cell densities and morphometric analyses were made preoperatively (using the contralateral donor).
reconstructed endothelium, not grafted, cornea) and postoperatively on the same cases (n = 3; 2 reconstructed and 1 control in which only the carrier was grafted). A minimum of 100 cells per cornea was counted.

**Histology, Electron Microscopy, and Fluorescence Analysis**

Corneas were then fixed as follows: one third was fixed in 10% formaldehyde for light microscopy (Masson trichrome staining), scanning electron microscopy\(^6\) (SEM; 6360LV; JEOL, Tokyo, Japan), and visualization of fluorescent DiOC-labeled cells. One third was frozen in optimal cutting temperature solution (Somagen; Edmonton, AB, Canada) for immunodetection. The last third of the cornea was fixed in glutaraldehyde 2.5% and processed for transmission electron microscopy (TEM\(^5\); JEM-1230; JEOL). Corneal endothelial specimens labeled with a fluorescent dye tracker (DiOC; n = 5) were further fixed in acetone and observed with an inverted microscope (Eclipse TE-2000U; Nikon) and a confocal microscope (C1; Nikon). Two of those cases were counterstained with Hoechst reagent 33258.

Indirect immunofluorescence assay was performed on acetone-fixed (10 minutes at −20°C) cryosections (5-μm thick).\(^5\) Antibodies were diluted in PBS containing 1% bovine serum albumin (BSA; Sigma). After three rinses in PBS-1% BSA, sections were incubated for 45 minutes at room temperature with primary antibodies. Primary antibodies used were mouse monoclonal anti-Na\(^+\)/K\(^+\)-ATPase α1 (1:200; clone C464.6; Millipore, Billerica, MA), rabbit polyclonal anti-Na\(^+\)/HCO\(_3\) cotransporter (1:50; NBC aa 928-1035; Chemicon, Temecula, CA), rabbit polyclonal anti-ZO-1 (1:20; clone ZMD-436; Invitrogen), and mouse monoclonal anti-Ki67 (1:400; clone B56; BD Pharmingen, Mississauga, ON, Canada). After three rinses in PBS, sections were incubated with secondary antibodies for 30 minutes at room temperature. Goat anti-mouse IgG antibodies and chicken anti–rabbit antibodies conjugated with Alexa 594 (Invitrogen) were used as secondary antibodies. Negligible background was observed for controls (primary antibodies omitted). Cell nuclei were counterstained with Hoechst reagent 33258. To determine the percentage of proliferating cells, the ratio of the number of Ki67-positive nuclei per total nuclei was calculated.

**RESULTS**

**Preoperative Characterization of the Reconstructed Corneal Endothelium**

Feline corneal endothelial cells were seeded at high density (3000 cells/mm\(^2\)) on the denuded Descemet’s membrane of the devitalized human corneal carrier. Confluence was achieved within 24 hours. Cells were further cultured for 8 to 24 days to promote cell-cell contact. As seen in Figure 1A, the cultured cells formed a continuous monolayer of flattened cells entirely covering the Descemet’s membrane. Alizarin staining showed that the rounded or polygonal cultured cells entirely covered the denuded Descemet’s membrane. Note the absence of keratocytes in the devitalized stroma. (B) Alizarin red staining showed that the rounded or polygonal cultured cells entirely covered the denuded Descemet’s membrane. Dark spots are fluorescent cell tracker precipitates. (C) SEM of a reconstructed corneal endothelium showed polygonal cells with few intercellular spaces. (D, E) TEM showed a monolayer in close apposition to Descemet’s membrane in which endothelial cells were close to each other. Scale bars: (A) 50 μm, (B) 100 μm, (C) 10 μm, (D, E) 2 μm.

**Post-transplantation Follow-up**

Nine of the 11 reconstructed grafts were clear 7 days after transplantation (excellent transparency, grade 3.5/4 or 4/4). As seen in Figures 2A–D, slit lamp photographs on day 7 showed a crystal-clear reconstructed graft through which the fine details of iris and lens could easily be seen. Slit lamp and OCT photographs showed uniform and thin transplants, with tight sutures and minimal inflammation of the anterior segment.

Of the two semioaqueous grafts at day 7, one had an almost complete detachment of Descemet’s membrane (mean transparency grade, 2.5). Although this was not noticed at the time of the preoperative in vitro examination of the reconstructed donor tissue, it was obvious at the time of surgery; the endothelial layer waved gently with every movement of the aqueous or cornea. Surgery was otherwise smooth and uneventful. To evaluate whether this endothelium would reattach by itself, the decision was made not to replace this transplant and not to try to reposition the detached endothelium (no air bubble, injection of viscoelastic, or suture). After surgery, this endothelial layer never entirely reattached; rather, it remained in close contact with the donor stroma. The space trapped between the stroma and the detached Descemet’s was optically denser than the aqueous. The other unclear graft had a transparency grade of 2.5 (periphery) to 3 (center) on day 7. This case was characterized by positive vitreous pressure during surgery, without vitreous loss, and intraoperative formation of gelatinous fibrinous stands. The postoperative follow-up was also more inflammatory than usually seen, with +2 flare persisting at day 7 and fluffy fibrinous precipitates preferentially but not exclusively located against the nasal superior quadrant.
This transplant was only 60% reepithelialized by day 7. Examination of the donor rim and that of the reconstructed mate cornea did not reveal any sign of preoperative endothelial damage. Otherwise, no signs of anterior segment inflammation were seen other than a mild and transient flare, a few cells, and a fine weblike fibrinous remnant from surgery in seven cases (xenograft, 1; allogeneic, 1; reconstructed, 4; carrier only, 1). No specific signs of immunologic reaction (keratic precipitates, rejection line) were encountered in any of the operated eyes. No corneal neovascularization developed in any of the grafted corneas during the observation period.

Native control grafts consisting of autologous, allogeneic, or xenogeneic tissue were all clear at day 7 (autologous and allogeneic grafts were graded 4/4; xenogeneic graft was graded 3.5/4). Significantly more scarring was noted, however, in the native grafts at the level of the wound and sutures. Figures 2E-H illustrate the outcome of one of the native grafts. In this case, some degree of mild scarring was seen at the level of the folds in Descemet’s membrane and deep stroma. No difference in OCT imaging was found between the native and the reconstructed grafts.

The two control corneas grafted with the carrier only (no endothelial cells) remained opaque and edematous until the end of the study (transparency grade, 0.5–1). Slit lamp photographs at day 7 (Figs. 2I-K) show the typical severe graft edema observed in the absence of corneal endothelium, with deep folds in Descemet’s membrane and total loss of visibility of iris and lens details (Figs. 2J, 2K) through the graft, whereas the recipient cornea remained crystal clear. OCT showed a thick cornea with folds in Descemet’s membrane and posterior stroma (Fig. 2L).

Reepithelialization

Early reepithelialization after corneal transplantation is important because it ensures restoration of a natural protection against infection, inflammation, ulceration, and eventually graft failure. Reepithelialization was carefully monitored on a daily basis during the entire study period. None of the reconstructed grafts were epithelialized at the time of surgery. Seven days after surgery, reepithelialization was complete in 9 of 11 reconstructed grafts and reached 60% and 75% of the graft surface in two cases. Despite the importance of stromal edema, no epithelial deficit remained in the two grafts consisting of the carrier only (without endothelial cells). The epithelium of the five native grafts remained intact before, during, and after surgery.

Pachymetry

All reconstructed grafts were edematous at the time of surgery, with central thicknesses beyond the limits of measurement of the pachymeter. Corneal thickness decreased rapidly after surgery (Fig. 3A), with mean values (±SEM) of 712 ± 24 μm at day 3 and 659 ± 35 μm at day 7. The native grafts showed thicknesses of 696 ± 36 μm and 675 ± 27 μm, and the unoperated contralateral eyes showed thicknesses of 651 ± 14 μm and 637 ± 18 μm at 3 and 7 days, respectively. The two grafts consisting of the carrier only (without endothelial cells) remained thicker than 1000 μm during the entire postoperative period. Corneal thickness was highly correlated with the transplant subjective transparency score (Pearson’s r = -0.86; P < 0.001).
2 in the carrier-only group; the other animal had normal findings. The small size of this group explains the heavy weight of these high IOP measurements on the group mean IOP and SE values.

**Endothelial Cell Counts and Morphometry**

Mean (±SEM) endothelial cell count of the reconstructed corneas before surgery was 2563 ± 314 cells/mm² (mean of two cases; Table 1). Seven days after transplantation, the same cases had a mean endothelial cell density of 1774 ± 95 cells/mm², which corresponded to a 31% cell loss. Preoperative morphometric analysis in the reconstructed corneas showed rounded endothelial cells for which cell corners were difficult to identify reliably. However, 7 days after transplantation, 44% of the cells were clearly hexagonal. The preoperative reconstructed corneas had 23% fewer cells than the unoperated native controls (3334 ± 322 cells/mm²).

**Postmortem Histopathology**

Histologic examination of all grafts with a reconstructed corneal endothelium showed a thin graft (Fig. 4A) with a continuous monolayer of tightly packed and flattened endothelial cells (Fig. 4B). Few keratocytes started to migrate to the peripheral stroma of the graft (Fig. 4C). On day 7, the denuded Bowman membrane of the reconstructed grafts was completely reepithelialized by the host epithelium in 9 of 11 cases (Fig. 4D). Control grafts transplanted with the carrier only (no endothelial cells seeded) still had no cells on their Descemet’s membrane on day 7 after transplantation (Fig. 4E), indicating that no migration had occurred from the recipient’s endothelium. Histologic examination showed a Descemet's membrane detachment in one case (the one that had a detached Descemet’s membrane at slit lamp; Fig. 4F).

Alizarin staining of the control grafts transplanted with the carrier only (no cells) confirmed that there was no evidence of host cells migrating onto the denuded Descemet’s membrane of the reconstructed corneal endothelium (Fig. 5A).

SEM of the successful grafts showed a polygonal morphology of the reconstructed endothelium (Fig. 5B). Cells were flat and had distinct interdigitations at the cell boundaries. Microvilli at the apical surface could also be observed.

TEM of the reconstructed corneal endothelium showed thickness and ultrastructure (Fig. 6B) made out of an intact continuous monolayer of cells closely adherent to Descemet’s membrane and to each other, as in the native feline corneal endothelium (Fig. 6A). Nuclei showed focal areas of dense heterochromatin and often contained a large nucleolus, indicating active mRNA synthesis. Many mitochondria, lysosomes, abundant endoplasmic reticulum (Fig. 6C), and a few microvilli were also present. Large loose vacuoles were sometimes present between cells and Descemet’s membrane (Fig. 6D). In contrast to the preoperative recon-

### Table 1. Endothelial Cell Density and Morphometry

<table>
<thead>
<tr>
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<th>Reconstructed Grafts</th>
<th>Mate Endothelial Donors</th>
<th>Grafts with Carrier Only (no cells)</th>
<th>Unoperated Contralateral Corneas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7 (n = 2 corneas)</td>
<td>(not transplanted) (n = 2 corneas)</td>
<td>Day 7 (n = 2 corneas)</td>
<td>Day 7 (n = 2 corneas)</td>
</tr>
<tr>
<td>Average (±SEM) cell count</td>
<td>1774 ± 95</td>
<td>2563 ± 314</td>
<td>0</td>
<td>3334 ± 322</td>
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<td>Average cell area (μm²)</td>
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<td>315</td>
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<td>211</td>
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<td>N/A</td>
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<tr>
<td>6-sided cells, %</td>
<td>44</td>
<td>Round corners</td>
<td>N/A</td>
<td>69</td>
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N/A, not available.
structed corneal endothelium, no intercellular spaces were present, and interdigitations of the lateral membranes of adjacent cells were observed, a structure similarly seen in native feline corneal endothelium.

**Fluorescence Microscopy**

After grafting, fluorescence microscopy of whole mount corneas showed that the reconstructed endothelium was covered with DiOC-labeled cells (Figs. 7A, 7B). No fluorescence was observed on the recipient’s endothelium (Fig. 7A). In two cases, fluorescence was observed within the recipient’s stroma, trapped into a suture track. A few fluorescent cells were also found on the recipient’s trabeculum in 3 of 5 cases grafted with DiOC-labeled cells (Fig. 7C). Pachymetry of the reconstructed corneal endothelium, no intercellular spaces were present, and interdigitations of the lateral membranes of adjacent cells were observed, a structure similarly seen in native feline corneal endothelium.
DISCUSSION

This study constitutes the first evidence that cultured endothelial cells seeded on a devitalizedstromal carrier can recover active pump function, restore and maintain normal corneal thickness, and yield crystal-clear corneal transparency for 7 days after transplantation in the living feline eye. The reconstructed endothelium had a normal morphology and ultrastructure and expressed the function-related proteins Na^+/K^+-ATPase α1, Na^+/HCO_3^−, and ZO-1.

The fluorescent-labeled cells confirmed that the reconstructed endothelial monolayer remained confined to the donor button, with no invasion of the recipient cornea or trabecular meshwork. The few labeled cells observed on the trabeculum of the inferior quadrant are believed to have resulted from the elimination of dead endothelial cells through aqueous filtration by the trabeculum. Ki67 staining showed that a small fraction of the reconstructed endothelial cells were proliferating 7 days after transplantation. However, no increase in cell density was observed in the reconstructed donors at 7 days. The 31% mean endothelial cell loss reported herein remains in the same order as that reported after corneal transplantation in human with native tissue (0%-28%; mean, 12% at 1 week).45

The choice of animal model was also an important factor to assess the regenerative potential of an endothelial graft in vivo. Contrary to corneal endothelial cells of animals such as rats44 and rabbits,45,46 in which the high natural regenerative capacity of the corneal endothelium is well recognized, those of cats and humans do not replicate under normal physiological conditions. For example, after the destruction of 50% of the endothelium by transcorneal freezing, the rabbit corneal endothelium heals in 10 days by extensive cellular division at the margin of the wound, restoring a monolayer of cells of normal size and morphology. In the cat, the corneal endothelium eventually covers the deficit after 1 month, but only by enlargement and migration of cells at the margin of the wound,46 leading to low endothelial cell counts, pleomorphism, and polymegethism. The cat is considered a better animal model than the rabbit31,46–48 for studies involving healing of the corneal endothelium because its limited regenerative capacity in vivo is closer to that observed in humans. In our study, no proliferation of the recipient’s endothelium was observed. However, the lack of proliferation did not preclude recolonization of the graft by cell migration. In this study, because no cells were observed on the denuded Descemet’s membrane of the two carrier-only (no cells) grafts, we concluded that there was no migration of the recipient’s endothelium toward the graft 7 days after surgery. Only long-term follow-up of recon-

grafts reconstructed, with and without fluorescent labeling at day 7, were similar (607 μm ± 35 [n = 5] and 702 μm ± 55 [n = 6], respectively).

To quantify endothelial cell proliferation, cryosections were immunostained for Ki67, a marker for proliferating cells in all active phases of the cell cycle from the late G1-phase through the M-phase.41,42 As seen in Figure 7D, basal cells were positive for Ki67 in the corneal epithelia. Of the 11 cases grafted with a reconstructed endothelium, Ki67 staining was detected in less than 1% of endothelial cells in seven cases, between 1% and 5% in three cases, and between 5% and 10% in one case: Ki67-positive cells were detected only on the graft (Fig. 7G). Ki67 staining was never detected on the recipient’s endothelium.

Immunofluorescence detection of the sodium-potassium pump Na^+/K^+-ATPase α1 and the Na^+/HCO_3^−cotransporter revealed that these proteins were found across the entire surface of the reconstructed corneal endothelium (Figs. 7H, 7J) and follow a pattern similar to that of native corneal endothelium (Figs. 7I, 7K). Immunofluorescence staining of ZO-1 revealed that this protein was located at the cell boundaries, suggesting the formation of focal tight junctional complexes in the reconstructed endothelium (Figs. 7L, 7L’). The same pattern was found in the native corneal endothelium (Figs. 7M, 7M’). The difference was that fluorescence of ZO-1 protein was more pronounced on the recipient’s endothelial cells.
structured grafts, however, will allow definitive assessment of endothelial cell migration.

This study highlights the importance of preserving the integrity of Descemet’s membrane attachment when transplanting a tissue-engineered corneal endothelium reconstructed from cultured endothelial cells seeded on a devitalized stromal carrier. The Descemet’s membrane that detached in one case never fully reattached, leading to severe graft edema. Endothelial cells remained attached to this Descemet’s membrane, as shown by histopathologic examination and SEM and as suspected by the increase density of the liquid trapped in the close spaces between Descemet’s and stroma, as if the endothelial cells had pumped some liquid out of these spaces but never enough to allow the membrane to fully reattach.

In this study, the morphologic and ultrastructural quality of the reconstructed endothelium improved continuously with time after seeding. In vitro, the round seeded endothelial cells rapidly adhered to Descemet’s membrane, yielding a monolayer of tightly packed polygonal cells after 2 weeks in culture. After transplantation, the residual intercellular spaces continued to close up, and SEM and TEM confirmed the normal and healthy shape and ultrastructure of the active endothelial cells. Cell size and shape evolved toward a more uniform pattern; 44% of the cells were hexagonal, a sign of increased endothelial stability.40 Cell shape analysis is known to be more sensitive to subtle changes in the endothelial monolayer than is cell density or cell area analysis.49,50 In vivo improvement in cell junctions might have been linked to the presence of factors in the aqueous humor that were absent in vitro. In vascular physiology, it has been shown that steady shear stress stimulates the cellular responses essential for vascular endothelial cell function.51 Similarly, aqueous humor flow and intraocular pressure may improve the function of corneal endothelial cells. The advantages of using a devitalized stroma as a carrier are multiple. First, the full reepithelialization of the demuced Bowman membrane, the progressive repopulation of the donor devitalized stroma by the recipient’s keratocytes, and the attachment of the cultured endothelium to Descemet’s membrane, along with restoration of its full function all demonstrate the high biocompatibility of devitalized stromal carriers. Biocompatibility is one of the major drawbacks of synthetic corneal substitutes.52–54 Second, the optical qualities of the normal stroma are preserved, as demonstrated by the crystal-clear transparency of the grafts. Freezing and thawing the corneal donor is known to be highly compatible with good visual outcome.55 Third, our results indicate that after 7 days, devitalized stromal carriers yield significantly less scarring at the level of the wound and along suture tracks than native transplants (Figs. 2A, 2E). Elimination of donor keratocytes may, in part, be responsible for this exceptional transparency given that the roles of keratocytes is to secrete extracellular matrix to repair the injured stroma.56 Although long-term assessment of these wounds is still needed (including biomechanical strength assessment), less scarring and clearer corneas would favor visual acuity, contrast sensitivity, and retinal image quality (higher order aberrations generated by the cornea). This would especially be interesting for the new generation of corneal transplantation techniques involving stromal dissection in the pupil area such as in Descemet-stripping automated endothelial keratoplasty.57–59 Fourth, because all three corneal cell types (epithelial, endothelial, keratocyte) contribute to graft rejection,60 it is expected that devitalization of the corneal carrier will reduce the antigenicity of the graft. Theoretically, the risk for rejection in our tissue-engineered model should essentially depend on the origin of the reconstructed endothelium; thus, it could be null if autologous endothelial cells are used. If this hypothesis is verified, in addition to the advantages shown, devitalized stromal carriers may become the most interesting carriers for reconstructed corneal endothelium transplantation in human. Fifth, eye banking advantages of frozen stromal carriers would also include the option of extended storage time,55–61 and elimination of all cells from the carrier would allow the alleviation of donor exclusion criteria, both of which will decrease tissue wastage. The 2007 Eye Banking Statistical Report of the EBAA1 states that 11.4% of donor corneas were discarded because of a low endothelial cell count and 3.6% because the tissue was not used within the acceptable time.

In conclusion, the successful outcome of our reconstructed living posterior transplants is attributed to a functional endothelium because grafts of devitalized carriers without endothelial cells primarily failed. Long-term functional outcome assessment in the feline model will be the next necessary step in the development of bioengineered living tissue. This represents a promising approach for the treatment of endothelial abnormalities.

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