Extended Incubation Times Improve Corneal Endothelial Cell Transplantation Success

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To investigate the ability of extended incubation times to improve the success of endothelial cell transplantation, eight human donor corneas were denuded of their native endothelium, seeded twice during a 1-hr interval with a suspension of cultured infant human corneal endothelial cells, and then incubated for 144 hr under standard conditions. Subsequently the corneas were transplanted into African green monkeys using routine penetrating keratoplasty techniques. Rotational autografts and corneas devoid of endothelial cells served as controls. The seeded corneas appeared hazy at the time of surgery (mean pachymetry 48 hr postoperatively, 0.794 mm). Six corneas (75%) subsequently cleared, yielding a mean corneal thickness of 0.541 ± 0.040 and 0.554 ± 0.040 at 6 and 12 postoperative months, respectively. All control eyes showed advanced edema (thickness, > 1.0 mm) and developed extensive neovascularization. Clinically, the extended postseeding incubation corneas were observed to clear more rapidly and stabilize their thickness earlier than corneas incubated for only 24–48 hr. Scanning electron microscopy of extended postseeding incubation corneas revealed an intact monolayer of contact-inhibited cells with the hexagonal mosaic typical of corneal endothelium in vivo and improved intercellular contact compared with corneas incubated for only 24–48 hr. Invest Ophthalmol Vis Sci 32:1828–1836, 1991

It has been well established that the corneal endothelium is essential for the maintenance of normal corneal hydration, thickness, and transparency.1,2 Corneal transparency is dependent on healthy endothelial cells sufficient in number to maintain deturgescence of the corneal stroma, with the normal level of corneal thickness and hydration maintained by the barrier function and active fluid pump of these cells.3 The corneal endothelium of most primates, including humans, has essentially no regenerative capacity4–6; typically, in vivo cell loss is followed by compensatory hypertrophy of the remaining cells. This lack of regenerative capacity poses a significant clinical problem because many corneal diseases are accompanied by substantial endothelial cell loss, resulting in irreversible corneal edema. Corneal transplantation is currently the only available means of replacing diseased or damaged endothelium.

The current state of corneal transplant surgery requires the use of whole donor material for those conditions requiring endothelial cell replacement due to corneal endothelial cell dysfunction. Presently, the largest number of cases requiring keratoplasty include those of Fuchs’ hereditary endothelial dystrophy and aphakic or pseudophakic bullous keratopathy—two diseases primarily affecting the corneal endothelium.7 Penetrating keratoplasty is one of the most common ophthalmic surgical procedures; more than 36,500 transplants were done in the US in 1988.8 Although the success rate is high, a chronic shortage of human donor corneas (over 4650 in 1989)8 and the postoperative complications (eg, graft rejection and postoperative astigmatism) frequently plague the corneal transplant surgeon. Worldwide, the corneal donor shortage is acute; there are literally hundreds of thousands of patients who would benefit from a corneal transplant.8 Additionally, the mean age of cornea donors has been increasing, due to increases in life span, resulting in donor tissue that is often inadequate for use in keratoplasty.10

Previous studies revealed varied success in the maintenance and transplantation of rabbit and bovine corneal endothelium in both allogeneic and xenogeneic situations.11–18 One study19 had short-term success in
transplanting human corneal endothelium cells into rabbits. Although adult human corneal endothelium is known to have only minimal regenerative capacity in vivo, recent studies show that human corneal endothelium can be cultivated successfully in vitro.\textsuperscript{20-22} In pilot studies with nonhuman primates, human eye-bank corneas seeded with tissue-cultured neonatal corneal endothelium showed a favorable response in transplanted eyes, with thinning and clearing of the donor tissue.\textsuperscript{23} By using standard tissue-culture techniques, we investigated the feasibility of transplanting human corneal endothelium as an alternative method for endothelial cell replacement.

The potential applications of these results have far-reaching clinical implications. The replacement of damaged or diseased endothelium with a viable cell-cultured endothelium to repopulate aging or diseased corneas before, during, or perhaps in place of penetrating keratoplasty, would dramatically increase the number of corneas suitable for transplantation, provide a means of standardizing the established population of donors, and increase the success of future human corneal transplant surgery.

**Materials and Methods**

**Infant Corneal Tissue**

Infant human corneas were obtained from regional eyebanks. Donor age ranged from 1 day to 3 yr, and all donors were full-term, except where noted (Table 1). Corneas were harvested within 15 hr of death (mean, 4.40 ± 4.45 hr) and were normal by slit-lamp examination. Each cornea was excised with a 3-4-mm scleral rim, placed in either Dexsol, chondroitin sulfate corneal storage medium, or McCarey-Kaufman medium (Chiron Ophthalmics, Irvine, CA), and stored at 4°C during shipment and before use.

**Human Corneal Endothelial Cell Culture**

Primary cultures of human corneal endothelial cells were established from infant corneas as previously detailed.\textsuperscript{23,24} In the past we dealt primarily with the culture of infant human corneal endothelium and the mechanism by which it repopulates human corneal buttons that have been mechanically denuded of their native endothelium.\textsuperscript{25-27} In using infant donor tissue to establish primary cultures of corneal endothelium, we found that, in vitro, infant human corneal endothelial cells have proliferative mitotic activity and form a contact-inhibited monolayer composed of flattened, polygonal cells.\textsuperscript{22,23,28} Using sterile technique, Descemet's membrane was micro-dissected meticulously, intact, from the stroma and placed in a culture dish containing 2 ml of Versene (0.2 mg/ml ethylenediaminetetraacetic acid in isotonically buffered saline solution, pH 7.4; Whittaker, Walkersville, MD). The Versene was removed after 30 min and the tissue incubated in a 0.20% collagenase solution (Grade III; Worthington, Freehold, NJ) at room temperature until cell separation was observed. The cells were rinsed in Eagle's minimum essential medium (E-MEM) with Earle's balanced salt solution/4% calf serum (Whittaker) and gently centrifuged. The supernatant was removed and the cells resuspended in a supplemented version of E-MEM containing essential and nonessential amino acids, MEM vitamin mixture, L-glutamine (560 \( \mu \)g/ml), gentamicin sulfate (50 \( \mu \)g/ml), sodium bicarbonate, and 15% of a formulated serum substitute (NuSerum IV; Collaborative Research, Bedford, MA). Endothelial cells from paired infant corneas were pooled and cultured in 25-cm\( ^2 \) tissue culture flasks (Corning, Corning, NY) at 37°C, in 95% air/5% CO\(_2\) with 95% relative humidity, with a final medium volume of 4 ml per flask. Cultures were fed initially after 72 hr and thereafter twice weekly with half-volume medium changes.

**Table 1. Infant cornea donor summary**

<table>
<thead>
<tr>
<th>Eye bank no.</th>
<th>Donor age</th>
<th>Cause of death</th>
<th>Storage medium</th>
<th>Death to preservation time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-89-02-053</td>
<td>5 wk</td>
<td>SIDS*</td>
<td>Dexsol</td>
<td>15</td>
</tr>
<tr>
<td>CD-89-03-007</td>
<td>1 hr†</td>
<td>Anencephaly</td>
<td>M-K</td>
<td>4</td>
</tr>
<tr>
<td>CL-89-03-036</td>
<td>3 mo</td>
<td>SIDS</td>
<td>Dexsol</td>
<td>8</td>
</tr>
<tr>
<td>K89132</td>
<td>7 mo</td>
<td>Microcephaly</td>
<td>CSM</td>
<td>2</td>
</tr>
<tr>
<td>CD-89-03-094</td>
<td>6 mo</td>
<td>Cardiac anomaly</td>
<td>M-K</td>
<td>2</td>
</tr>
<tr>
<td>CD-89-04-001</td>
<td>1 day</td>
<td>Anencephaly</td>
<td>M-K</td>
<td>2</td>
</tr>
<tr>
<td>CD-89-04-022</td>
<td>4 day</td>
<td>Hypoplastic heart</td>
<td>M-K/Dexsol†</td>
<td>1</td>
</tr>
<tr>
<td>89-148A/B</td>
<td>7 day</td>
<td>IUA§</td>
<td>M-K</td>
<td>2</td>
</tr>
<tr>
<td>CD-89-04-045</td>
<td>3 yr</td>
<td>Meningitis</td>
<td>Dexsol</td>
<td>1</td>
</tr>
<tr>
<td>CD-89-04-077</td>
<td>3 mo</td>
<td>SIDS</td>
<td>Dexsol</td>
<td>7</td>
</tr>
</tbody>
</table>

Mean death to preservation time, 4.4 hours.

* Sudden infant death syndrome.
† Thirty-one-week estimated gestational age.
‡ OS, M-K medium; OD, Dexsol.
§ In utero asphyxia (cord prolapse).
Cell viability was determined by trypan blue exclusion during harvest of the cells from culture. 

Trypan blue solution (Sigma, St. Louis, MO) was prepared in phosphate-buffered saline, and the pH was adjusted to 7.3-7.4. A sample of released cells (0.9 ml) was mixed with 0.1 ml of trypan blue solution and allowed to stand at room temperature for 5 min. The cells were counted in a modified Neubauer hemocytometer (Reichert-Jung, Buffalo, NY), and the ratio of viable (unstained) to nonviable (stained) cells was determined. Cell viability was greater than 94% in all cell transplantation experiments.

Seeding of Cultured Cells on Corneal Buttons

The cell-coating technique originally detailed by Gospodarowicz and Greenberg and successfully utilized by Alvarado et al was used essentially without modification. Paired adult human donor corneas with attached scleral rims were obtained from regional eye banks. The donor corneas were rated unsuitable for human transplantation because of donor age or low endothelial cell density but were acceptable by other criteria. Using a sterile, moistened cotton-tipped applicator (CIDA, Overland Park, KS), the native endothelium was removed from the donor corneas. Care was taken not to disrupt the integrity of Descemet's membrane. The efficacy of this technique of endothelial cell removal has been previously demonstrated (Fig. 1).16-22,26 The corneas were rinsed thoroughly in serum-free medium and placed in corneal storage chambers (CooperVision, Irvine, CA), endothelial side up, in the presence of supplemented E-MEM containing 5% dextran (average molecular weight, 4.0 x 10^5 D; Sigma) to reduce stromal hydration during culture.11,30 The seeded corneas were incubated at 37°C in corneal storage chambers for 144 hr (Fig. 2). After incubation, one cornea of each pair was fixed promptly for scanning electron microscopic evaluation of cell density, plating efficiency, and monolayer morphology.

Fig. 1. Mechanically denuded Descemet's membrane, prior to seeding of cultured neonatal human corneal endothelial cells. (SEM, bar indicates 10 μm; original magnification ×1500).
Keratoplasty Technique

After incubation, the corneas were transplanted into adult African green monkeys (Cercopithecus aethiops) by a method detailed previously, using a combination of eight interrupted and one continuous 16-bite running 10-0 nylon monofilament suture (Alcon, Fort Worth, TX, Fig. 3). After sedation with intramuscular ketamine HCl (10 mg/ml, Ketaset; Aveco, Fort Dodge, IA), the animals were intubated and general anesthesia maintained with halothane. The selected eye was prepared with povidone-iodine solution and topical acetylcholine chloride (Miochol; IOLAB, Claremont, CA); a surgical drape was placed over the field. A pediatric wire speculum was inserted and the recipient cornea trephined (6.0 mm) until anterior chamber penetration occurred. Removal of the cornea was completed subsequently with corneal scissors. The donor cornea populated with cultured infant human endothelium was rinsed gently to remove nonadherent cells and then punched (6.5 mm) from the endothelial surface on a Teflon corneal cutting block (Storz Ophthalmic Instruments, St. Louis, MO) and sutured into the monkey’s eye. Peripheral iridectomies were done in all eyes receiving grafts to prevent pupillary block and iris synechiae. Endothelial cell integrity was protected by the use of sodium hyaluronate (Healon; Pharmacia, Pasadena, CA). After completion of the surgery, all knots were buried, and the anterior chamber was formed with BSS Plus (Alcon). Gentamicin sulfate (40 mg/ml; Elkins-Sinn, Cherry Hill, NJ), dexamethasone sodium phosphate (24 mg/ml, Decadron; Quad, Indianapolis, IN), and methylprednisolone acetate (40 mg/ml, Depo-Medrol; Upjohn, Kalamazoo, MI) were injected subconjunctivally. The lids were closed with 2% lidocaine, and the animals were extubated and transferred to their holding quarters. Keratoplasty was done on only one eye of each animal, using standard microsurgical instruments and an operating microscope.

Clinical Analysis

Postoperative slit-lamp examinations of the anterior segment were done biweekly on anesthetized animals for 4 months and monthly for 8 months. Graft evaluation criteria included corneal clarity, thickness, vascularity, epithelial healing, and anterior chamber status. Subconjunctival injections of dexamethasone (24 mg/ml) and methylprednisolone acetate (40 mg/ml) were administered at each examination and any loose sutures removed. Topical applications of neomycin sulfate, polymyxin b sulfate, and dexamethasone ointment (Maxitrol; Alcon) were administered as needed. Corneal thickness was measured by ultrasonic pachymetry using a Storz Corneo-Scan II (Storz) pachymeter equipped with an aqueous probe; scan velocity was 1.640 km/sec. Central corneal thickness was derived from the average of two consecutive measurements with a standard deviation of less than 0.004 mm.

Postoperative endothelial cell densities were determined from photomicrographs of the central corneal endothelium obtained with a Keeler-Konan Pocklington SP-1 specular microscope (Konan, Nishinomiya, Japan) 1 yr after corneal transplantation. Cell counts were obtained from a minimum of four fixed frames analyzed by a skilled examiner. The reliability of the counting was confirmed by the random inclusion of previously counted photographs.

The animals were removed from further clinical examination in the event of graft failure, as evidenced by immunologic rejection or prolonged elevated corneal thickness (>1.0 mm). All surgery and postoperative examinations were done at Tulane Regional Primate Research Center (Covington, LA). This study adhered to the ARVO Resolution on the Use of Animals in Research and US Public Health Service Policy for Humane Care and Use of Laboratory Animals.

Scanning Electron Microscopy

The tissues were rinsed in balanced salt solution, transferred into 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and stored at 4°C for 3 hr. After fixation, the tissue was rinsed three times with 0.1 M sodium cacodylate buffer supplemented with 5% sucrose (w/v), postfixed for 1 hr in 1% phosphate-buffered osmium tetroxide, and again rinsed three times with 0.1 M

Fig. 3. Experimental human corneal endothelial cell-lined donor in African green monkey. The small (6.5 mm) graft contains a combination of interrupted and running 10-0 nylon sutures. The transplant has thinned to 0.520 mm centrally and is crystal clear 2 months postoperatively. Endothelial cell density, as determined by specular microscopy 1 year after surgery, was 1200 cells/mm².
sodium cacodylate buffer. They were dehydrated through a graded ethanol series (30–100%) terminating in absolute acetone. The samples were critical-point dried from carbon dioxide, sputter-coated with gold–palladium alloy to a depth of 15–20 nm, and examined using a Zeiss DSM-950 scanning electron microscope (Carl Zeiss, Oberkochen, West Germany).

Results
Human corneal endothelial cell cultures were established from paired infant corneas harvested no later than 15 hr postmortem (Table 1). Pure endothelial cell growth was obtained from all donor tissue, with no evidence of fibroblast contamination. At confluence the cells had the morphology of corneal endothelial cells in vivo, forming a highly ordered, contact-inhibited, flattened monolayer, with a density of approximately 2000–2200 cells/mm² (Fig. 4). The cells produced a basement membrane in vitro.

Of the eight experimental eyes receiving grafts (human corneas coated with cultured endothelial cells), six showed progressive stromal clearing and thinning of the transplanted corneas (Fig. 3). The grafts were initially thick (>0.81 mm) and opaque, requiring approximately 4 weeks before significant clearing was observed. In the six eyes with progressive thinning, central pachymetry measurements 6 months postoperatively ranged from 0.500–0.608 mm (mean thickness, 0.541 ± 0.040 mm). Central pachymetry measurements 1 yr postoperatively ranged from 0.503–0.612 mm (mean thickness, 0.554 ± 0.040 mm, Fig. 5). Reepithelialization occurred in all grafts within 1 week after surgery. Two transplanted corneas did not show initial clearing and remained cloudy throughout the postoperative period. Several of the eyes in the experimental group showed minor superficial vascularization and required selected removal of interrupted sutures. Postoperative intraocular pressure (IOP) dropped in one animal, yielding elevated corneal thickness for postoperative weeks 16–20; IOP subsequently rebounded, resulting in restoration of corneal clarity.

Endothelial cell densities of four of the six successful grafts 12 months postoperatively ranged from 1000–1600 cells/mm² (mean, 1225 ± 263 cells/mm², Table 2, Fig. 6). One animal was lost to follow-up immediately before specular microscopy; however, postoperative pachymetry of this animal at 12 months revealed a thickness of 0.593 mm, with a clear central cornea. Endothelial cell density on the remaining animal could not be determined due to moderate stromal edema and haze, which prevented a clear visualization of the endothelial cell mosaic pattern.

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Slit-lamp appearance</th>
<th>Thickness (mm)</th>
<th>Endothelial cell density (cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Central haze</td>
<td>0.612</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Crystal clear</td>
<td>0.503</td>
<td>1100</td>
</tr>
<tr>
<td>3</td>
<td>Clear, with peripheral haze</td>
<td>0.593</td>
<td>1000</td>
</tr>
<tr>
<td>4</td>
<td>Clear</td>
<td>0.551</td>
<td>1200</td>
</tr>
<tr>
<td>5</td>
<td>Clear</td>
<td>0.550</td>
<td>1600</td>
</tr>
<tr>
<td>6</td>
<td>Crystal clear</td>
<td>0.513</td>
<td>1600</td>
</tr>
<tr>
<td>7</td>
<td>Opaque (graft failure)</td>
<td>&gt;1.000§</td>
<td>1000</td>
</tr>
<tr>
<td>8</td>
<td>Opaque (graft failure)</td>
<td>&gt;1.000§</td>
<td>1000</td>
</tr>
</tbody>
</table>

* Postoperative month 12.
† Corneal haze prevented an accurate cell count.
‡ Animal was lost to follow-up prior to specular microscopy.
§ Failed by fourth postoperative week.

Postoperative weeks
Fig. 5 Central corneal thickness of extended postseed incubation corneal grafts. Six grafts cleared and continued to remain thin over the 52-week postoperative period while two grafts became edematous and failed within 4 weeks after transplant.
Fig. 6. Specular photomicrograph of transplanted neonatal human corneal endothelium 12 months after surgery. The endothelial cells demonstrate relatively uniform hexagonal shape with little evidence of pleomorphism; cell density is 1100 cells/mm² (original magnification X100).

Two human corneas lacking endothelium and two monkey corneas mechanically denuded of endothelium (rotational autografts) served as controls for native (monkey) endothelial cell mitosis or migration. These four eyes did not clear after transplantation (Fig. 7), indicating the limited capacity for endothelial cell mitosis in nonhuman primates after wounding.

Scanning electron microscopic analysis of cultured donor corneas 144 hr after seeding revealed a flattened, interdigitated monolayer similar to that observed in vivo (Fig. 8). Monolayer formation was not observed to be contiguous; cell-free areas could be seen at the periphery of the cornea. Tissue cultured control corneas, mechanically denuded of endothelium and then incubated for 144 hr had a bare Descemet’s membrane with no viable endothelial cells present; endothelial cell remnants were minimal, and no evidence of microgrooves was found.13

Fig. 7. Control human donor cornea, devoid of endothelium, transplanted to monkey eye. The tissue is opaque and thickened throughout, with deep and superficial stromal vascularization 4 weeks postoperatively. Denuded control corneas remained thick and opaque during the postoperative follow-up, demonstrating the nonregenerative capacity of the native monkey corneal endothelium.

Fig. 8. Endothelial cell monolayer formation in corneal buttons incubated for 144 hr after primary double seeding; the intact, contact-inhibited, interdigitated endothelial monolayer morphology resembles that typically found in vivo (SEM, original magnification X100).

Discussion

Previous studies show that cultured corneal endothelial cells can be seeded onto homologous and heterologous stroma and transplanted successfully, with the return of normal morphology and physiologic function for various periods. One study,15 using early-passage cultures of rabbit corneal endothelial cells, had a 25% success rate after 1 month when the cells were transplanted back onto rabbit corneas. Cultured bovine corneal endothelial cells seeded onto donor corneas denuded of endothelium were transplanted to rabbits12 and cats15 by others. In the latter study, bovine corneal endothelial cells completed monolayer formation in vivo in 8 days, with greater than 90% of the corneas remaining clear and edema free 6 months postoperatively.

Other investigators reported a high complication rate with heterologous corneas and transplanted endothelium. Bahn et al.,18,33 using feline corneal allografts populated with bovine corneal endothelial cells, had clinical findings compatible with endothelial rejection and retrocorneal membrane formation in a series of heterologous cell-lined transplants; they speculated on the role that subcultured endothelial cell surface
antigens might have in the rejection process. Others, using infant human cornea endothelial-lined rabbit homografts, reported an initial success of 67% (four of six) at 3 weeks but subsequently encountered severe rejection 30–40 days postoperatively, with only one graft remaining clear at 6 months. Xenograft rejection was postulated as the cause of delayed failure after initial clearing of the graft.

Our results, using human eyebank corneas coated with cultured infant corneal endothelial cells, showed a favorable response 1 year postoperatively in six of eight (75%) transplanted eyes. Our previous attempts at human corneal endothelial cell transplantation using short incubation periods (24–48 hr) yielded only a 63–65% success rate, with a mean pachymetry in excess of 0.57 mm. Current results suggest that increasing the incubation time after seeding to approximately 144 hr can moderately increase the success rate of human corneal endothelial transplantation, increasing the potential usefulness of endothelial cell transplants for therapeutic purpose.

In a study in which human donor corneas were transplanted into adult rhesus monkeys, the authors demonstrated an 83% success rate (five of six eyes) 6 months postoperatively, using a small graft-to-recipient bed size (6.5:6.0 mm). Five of the six eyes showed prompt corneal thinning, with pachymetry readings of 0.5 mm or less approximately 10 days after surgery. In this case the authors did not manipulate the endothelium of the donor human tissue. In our experimental group of extended postseeding incubation period corneas (in which we also used a 6.5:6.0 mm graft-to-bed size), 75% of the eyes had a gradual, progressive decrease in corneal thickness over time (Fig. 5). All corneas had a slight-to-moderate haze at the time of transplantation, attributable to the prolonged in vitro incubation period. These transplanted corneas eventually thinned to less than 0.56 mm, similar to the response of the adult human cornea after clinical penetrating keratoplasty, but they required almost 6 postoperative weeks before achieving this thickness.

Of the two experimental eyes that failed, no clearing was noted in the immediate postoperative period, and stromal neovascularization began by postoperative week 3. It is not clear whether this represents donor failure or graft rejection since the eyes were diffusely cloudy and the anterior chamber reaction could not be evaluated carefully. These eyes received weekly corticosteroid injections, and most likely the response was one of primary donor failure, due to the failure of the initial seeding.

Evaluation of the control corneas from which the native endothelium had been removed confirmed the failure of the host endothelial cells to migrate across the wound margin. These controls support earlier studies regarding the limited regenerative capacity of nonhuman primate corneal endothelium. By contrast, 7-mm rabbit corneal buttons, denuded of their endothelium and implanted in vivo, require 3 weeks before completing endothelial cell regeneration and subsequent corneal clearing.

Postoperative endothelial cell densities in the experimental group receiving prolonged incubation ranged from 1000–1600 cells/mm² (mean, 1225 ± 263 cells/mm²). Cell densities in this group were equal to or better than results from previous experiments using shorter incubation periods and a postoperative follow-up of only 5 months. They are similar to results obtained in another study, in which a primate model of human corneal transplantation yielded endothelial cell counts of 850–1600 cells/mm² (mean, 1240 ± 282 cells/mm²) 6 months postoperatively. Also, our present cell densities, measured 1 yr postoperatively, showed losses of a magnitude equivalent to endothelial cell loss after corneal transplantation in humans. 

By comparison, a clinical study by Piquot et al., using a 37°C organ culture technique, obtained a mean cell density of 1452 ± 437 cells/mm² in 36 human corneas 1 yr after transplantation. The corneas were incubated for an average of 12 days before transplantation. A total of 86% of the grafts were clear after 12 months, with a mean central corneal thickness of 0.48 ± 0.05 mm.

Primary cultures of infant human corneal endothelial cells were noted to have cell densities on the order of 2000–2200 cells/mm² at confluence (Fig. 4). In vivo, however, endothelial cell densities in infant corneas typically exceed 4000 cells/mm² and may range as high as 6000 cells/mm², suggesting the potential for achieving higher seeded cell densities on donor corneas using cultured infant cells. In our study, prolonged incubation of the seeded corneas improved the quality of the monolayer but did not increase endothelial cell density. Others also noted an improvement in cell morphology after extended periods of incubation and attributed it to basement membrane production by the transplanted cells. Based on scanning electron microscopic analysis of the seeded corneas and in vitro cell seeding experiments (unpublished data), we estimate that the initial preoperative cell densities on the seeded donor corneas were limited to 2000–2300 cells/mm². Although there were fewer cells than are found on neonatal corneas, these densities are typical of specular microscopic cell counts recorded in human donor corneas used in clinical corneal transplantation. Modifications of the donor seeding technique may be required to raise the seeded endothelial cell density. We have initiated experiments aimed at promoting cell junc-
tion separation in confluent cultures in an attempt to increase the endothelial cell reserve by the incorporation of exogenous cells into an existing monolayer.

Although the corneal thickness measurements (mean, 0.554 ± 0.004 mm) and endothelial cell counts (mean, 1225 cells/mm²) in our long-term animal experiments are no better than those presently being achieved in clinical situations, these experimental studies again prove that donor corneas seeded and incubated with endothelial cells grown in cell culture can result in clear grafts. Our results clearly demonstrate that infant human corneal endothelial cells maintained in cell culture remain functional when transplanted in vivo. Both homografts and xenografts of corneal endothelium have had short-term success in experimental animals, but clinical applications are still in the future.

Our preliminary results at cultivating and transplanting neonatal corneal endothelium appear promising, particularly because the active transport pump and barrier properties are functional over time in transplanted cultured endothelial cells. Successful transfer of human corneal endothelial cells into a heterologous host (nonhuman primate), with subsequent corneal thinning and clearing and minimal signs of an immune response, has been achieved. The relative ease with which infant corneal endothelium can be cultured and subsequently transplanted is an encouraging step forward in the investigation of endothelial cell replacement; yet, given the nonreplicating nature of these cells in vivo, further investigation of their growth characteristics is required. Additional gains are anticipated as advances in basic research illuminate our understanding of the corneal endothelial cell.

**Key words:** penetrating keratoplasty, corneal endothelium, cell culture, endothelial cell transplantation

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**References**


