Transplantation of Tissue-Engineered Epithelial Cell Sheets after Excimer Laser Photoablation Reduces Postoperative Corneal Haze

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PURPOSE. To apply tissue-engineered cell sheet transplantation after excimer laser keratectomy as a novel approach for the reduction of postoperative corneal haze.

METHODS. Limbal biopsy specimens were obtained, and epithelial cells were cultured on temperature-responsive culture inserts without the use of feeder cells. Laser keratectomy (7.0-mm ablation zone and 160-μm depth) was performed in the contralateral eye, and autologous epithelial cell sheets were transplanted to the ablated corneal stroma. Transplant and control group eyes were assessed by slit lamp biomicroscopy, and corneal haze was scored in a masked fashion, according to the Fantes grading scale. For further examination histologic and immunohistochemical analyses were performed.

RESULTS. Tissue-engineered cell sheets produced stable attachment to the laser-ablated sites, resulting in epithelialization, 5 minutes after transplantation. Conversely, control corneas required 3 to 5 days for complete re-epithelialization. At both 1 and 2 months after surgery, corneal haze was significantly inhibited in the transplant group. Histologic analyses showed that the number of keratocytes undergoing apoptosis was decreased in the transplant group at 3 days after surgery. Similarly, the expression of both collagen III and α-smooth muscle actin, which may enhance corneal haze, were diminished in the transplant group at 2 months.

CONCLUSIONS. The transplantation of tissue-engineered epithelial cell sheets can successfully prevent the development of corneal haze after excimer laser keratectomy. (Invest Ophtalmol Vis Sci. 2006;47:552–557) DOI:10.1167/iovs.05-04995

For more than 20 years, the excimer laser has been an effective surgical tool for the treatment of corneal diseases, allowing for the safe and effective removal of superficial corneal opacities for improvement of optical transparency (photorheologic keratometry [PTK]),1 or for the correction of low to moderate refractive errors (photorefractive keratometry [PRK]).2–5 However, even with its widespread use, there are still drawbacks to excimer laser keratometry, notably postoperative eye pain, late recovery of vision, the requirement of prolonged use of steroids, and the risk of postoperative haze after treatment of high ametropia.6 Laser in situ keratomileusis (LASIK) effectively addresses the problems associated with PRK by the creation of a corneal flap with laser ablation performed on the exposed stromal bed.7 However, there are several possible flap-related complications,8 such as irregularities, striae, epithelial ingrowth,7 and corneal ectasia, which can develop after deep ablations.8 In addition, LASIK cannot be used as a replacement for PTK in removing superficial corneal opacities.

A major problem with the application of PRK is the subepithelial stromal opacity or "haze" that can develop in the visual axis and therefore affect the patient's postoperative visual acuity.9 Variable degrees of corneal haze after PRK can reduce visual acuity and may in some cases, persist for long periods after laser photoablation.2,10,11 In some cases of corneal dystrophy, treatment becomes increasingly difficult, due to a high rate of recurrence or subsequent corneal erosion that can lead to corneal opacity. In cases of high myopia that require a large refractive change, this recurrent corneal haze resists medical treatment and may require repeated laser photoablation. However, this method can often result in a failure to restore corneal transparency. In addition, although recent progress has been made in reducing the overall incidence of haze after PRK with the use of mitomycin C,12–15 the possibility of mitomycin C-induced cell apoptosis in normal tissues cannot be excluded, and therefore postoperative consequences from its application after PRK are not well understood.

The causes of the corneal haze that occurs during wound-healing have been attributed to processes that occur at the interface between the epithelium and stroma.16 In the present study, we therefore sought to enhance postkeratectomy wound healing by applying the transplantation of tissue-engineered corneal epithelial cell sheets directly after excimer laser ablation. We have recently described a novel method of ocular surface reconstruction using carrier-free transplantable cell constructs harvested from temperature-responsive cell culture surfaces. For clinical applications, because the epithelial cells are expanded ex vivo, multiple cell sheets can be fabricated on temperature-responsive surfaces from a single donor eye. These tissue-engineered cell sheets consist of stratified epithelial layers that closely resemble the native corneal epithelium and can be transplanted without the use of any carrier substrates.17,18 Because cultured cells are harvested as intact
sheets along with their deposited extracellular matrix (ECM), they attach directly to the corneal stroma without the need for sutures. This method, if applied after excimer laser keratectomy, seemingly allows for the treatment of both refractive errors and superficial corneal opacities with reduced postoperative stromal haze, thereby improving the patients’ quality of life. In comparison to LASIK treatment, which has become increasingly popular, these tissue-engineered epithelial cell sheets, which can be directly attached to the corneal stroma, may provide an advantage by avoiding the occurrence of flap-related complications and allowing for the treatment of superficial opacities that cannot be treated by LASIK.

**MATERIALS AND METHODS**

**Primary Culture of Corneal Epithelial Cells on Temperature-Responsive Culture Surfaces**

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with experimental procedures approved by the Committee for Animal Research of Osaka University Medical School. Corneal limbal biopsy specimens (5 × 5 mm) were taken from 40 New Zealand White rabbits (male, 2.0 kg) under deep anesthesia induced by intramuscular injection of xylazine hydrochloride (5 mg/mL) and ketamine hydrochloride (50 mg/mL). Isolated corneal epithelial cells were cultured on temperature-responsive culture inserts (23 mm diameter; CellSeed, Inc., Tokyo, Japan) without feeder cells. After 1 to 2 weeks, the cultured epithelial cells formed a confluent monolayer, and cells were cultured for an additional week to allow for stratification. These cultured cell sheets were finally noninvasively harvested from the temperature-responsive culture inserts by incubation at 20°C for 30 minutes.

**Excimer Laser Photoablation and Transplantation of Tissue-Engineered Epithelial Cell Sheets**

Excimer laser photoablation was performed unilaterally in 40 rabbits with a 7.0-mm diameter ablation zone and a depth of 160 μm, using a 193-nm argon-fluoride excimer laser (EC-5000; Nidek, Gamagori, Japan). Ablated eyes were then divided into two experimental groups with 19 eyes receiving autologous epithelial cell sheet transplantation and the other 21 eyes acting as the control, receiving only laser ablation. For transplantation, autologous tissue-engineered epithelial cell sheets were harvested from temperature-responsive culture inserts using a polyvinylidene difluoride (PVDF) donut-shaped support membrane (outer diameter: 23 mm; inner diameter: 16 mm) and placed directly onto the exposed stromal bed, immediately after laser ablation (Fig. 1). Within 5 minutes, the cell sheets produced stable attachment to the laser-ablated stromal bed and the PVDF membranes were carefully removed with scissors. The peripheral areas of the cell sheets that covered the unablated portions of the host corneas were then trimmed and removed, and intact corneal epithelial barrier function was confirmed by fluorescein staining (Movie 1, available online at http://www.iovs.org/cgi/content/full/47/2/552/DC1). The entire ocular surface was finally covered with a custom-made hard contact lens for postoperative healing protection. One drop of each levofloxacin and diclofenac was applied to the eyes immediately after the procedures and topical levofloxacin was administered three times a day for 2 weeks. Both steroids and nonsteroidal anti-inflammatory drugs (NSAIDs) were not used during the follow-up period. For histologic analyses, each animal was killed by an overdose of pentobarbital at the appropriate postoperative time.

To observe the presence of the transplanted cell sheets, cultured cells were stained with a fluorescent cell-tracer dye, Dil (Sigma-Aldrich, St. Louis, MO), 1 day before surgery. Three additional rabbits were killed 1 week after transplantation to allow for fluorescence examination by confocal laser scanning microscopy. Three rabbits were used as control subjects without cell sheet transplantation after laser keratectomy and examined at 1 week.

**Quantification of Corneal Haze**

Slit-lamp microscopic examinations (Topcon Co. Ltd., Japan) were performed daily until corneal re-epithelialization was completed and then weekly up to 2 months after surgery. The evaluation of corneal haze was graded by slit lamp examination according to a previously described method, and corneal haze was scored by three corneal specialists (KN, YH, NM) in a masked fashion according to the Fantes grading scale. To compare grades of corneal haze between transplant and control groups, the Mann-Whitney U-test was applied. For further examination, rabbits were then killed by an overdose of pentobarbital sodium, and the surgically altered eyes were enucleated and the corneas bisected for processing into frozen and paraffin-embedded sections, respectively.

**Histology and Immunohistochemistry**

Hematoxylin and eosin staining was performed on 3-μm-thick, paraffin-embedded sections by conventional methods. For immunohistochemistry, 10-μm cryosections were treated with 50 mM Tris-buffered saline (TBS; pH 7.2) containing 0.4% Triton X-100 and 5% bovine serum albumin (BSA) for 60 minutes at room temperature to block nonspecific antibody reactions. Sections were then incubated with either a 1:200 dilution of rabbit polyclonal anti-cytokeratin 12 (K12) antibodies (kindly provided by Winston W. Kao, University of Cincinnati, OH), a 1:200 dilution of mouse monoclonal anti-cytokeratin 3 (AE3; Progen, Heidelberg, Germany), a 1:100 dilution of mouse anti-α-smooth muscle actin (α-SMA; 1A4, Dakopatts, Glostrup, Denmark), or a 1:100 dilution of mouse anti-type III collagen (3-53; Fuji Chemical Corp., Takaoka, Japan) overnight at 4°C. Sections incubated identically with normal mouse IgG or rabbit IgG were used as the negative control. After washing with TBS, sections were incubated with fluorescein isothiocyanate (FITC)-labeled secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 2 hours at room temperature. Sections were finally counterstained with propidium iodide (Sigma-Aldrich) to visualize cell nuclei, and observed by confocal laser scanning microscopy (LSM-410; Carl Zeiss Meditec, Jena, Germany).

**RESULTS**

**Corneal Epithelial Cell Culture on Temperature-Responsive Culture Inserts, without the Use of Feeder Cells**

Epithelial cells were isolated from corneal biopsy specimens containing both the limbus and peripheral cornea. Cell colonies proliferated to confluence on temperature-responsive culture inserts within 1 to 2 weeks without coculture with 3T3 feeder layers. After an additional week of culture, tissue-engineered epithelial cell sheets were harvested by incubation at 20°C for 30 minutes, and hematoxylin and eosin (H&E) staining demonstrated that the fabricated cell sheets consisted of three to five stratified and well-differentiated cell layers (Fig. 2a), with a morphology similar to both the native corneal epithelium (Fig. 2b) and corneal epithelial sheets fabricated from limbal stem cells cultured with 3T3 feeder cells (Fig. 2c).
Effect of Transplantation of Tissue-Engineered Epithelial Cell Sheets on Postkeratectomy Corneal Haze

Control eyes without cell sheet transplantation showed positive fluorescein staining, indicative of epithelial defects, up to 4 days after laser ablation. In contrast, transplanted cell sheets adhered directly to the ablated stromal bed, with epithelial defects created by excimer laser ablation healed immediately after the transplantation of the tissue-engineered epithelial cell sheets, as demonstrated by negative fluorescein staining (Figs. 2d–g). Whereas eyes receiving conventional laser ablation required 3 to 5 days to become completely re-epithelialized, corneal surfaces of transplanted eyes demonstrated a complete and intact epithelial layer immediately after cell sheet attachment, with no defects present. Cells stained with the fluorescent tracer dye, Dil showed that 1 week after transplantation, the cell sheet remained stably on the stromal bed at the ablation site (Fig. 2h), as red fluorescence was detectable only in the central 7.0-mm diameter areas and not in the peripheral regions (Fig. 2i). These results imply that the engineered cell sheets can form stable attachment to create an epithelial barrier, even on the ablated stromal beds.

Corneal haze was measured in a masked fashion for both transplant-recipient and control eyes at 1, 2, 4, 6, and 8 weeks according to the Fantes grading scale.20 Whereas eyes that received cell sheet transplantation were scored as having generally mild haze at 1 (Fig. 3a) and 2 (Fig. 3c) months after surgery, control corneas showed more severe degrees of corneal haze throughout the follow-up period (Figs. 3b, 3d). Statistical analysis of haze scores demonstrated that the development of postoperative corneal haze was significantly inhibited, in comparison to the control group, both 1 and 2 months after transplantation (Fig. 3e).

Effect of Cell Sheet Transplantation on Host Stromal Responses to Laser Ablation

Three days after surgery, during the early phase of the wound healing, epithelial hyperplasia, along with a subepithelial acellular area suggestive of keratocyte apoptosis, was observed in the control group, but not in eyes having received the cell sheet transplants (Fig. 4a). At 1 week, control eyes showed increased hyperplasia, with 7 to 10 epithelial layers, as well as an accumulation of keratocytes and inflammatory cells, in the central ablation zone. In contrast, the epithelium of the transplant group consisted of five to six layers, closely resembling the native corneal epithelium, throughout the follow-up period (Fig. 4b). Two months after laser keratectomy, control eyes began to demonstrate a decrease in the previously observed epithelial hyperplasia, as well as a reduction in the number of spindle-shaped stromal cells in the subepithelial areas of the ablation zone, beginning to more closely resemble a normal corneal surface (Fig. 4c).
Upon complete re-epithelialization, both the transplant and control groups express the cornea-specific markers K3 and K12 in all epithelial layers of the central ablated site (data not shown), similar to previously reported results. Two months after laser keratectomy, control eyes showed strong positive staining of both α-SMA (Fig. 5a) and collagen type III (Fig. 5b) in the subepithelial stromal areas of the ablation site, whereas neither of these proteins was detected in the cell sheet transplant groups.

DISCUSSION

The transplantation of corneal epithelial cell sheets after excimer laser photoablation has several distinct advantages, by providing the intact epithelial barrier immediately after laser ablation. With cell sheet transplantation, the ablated corneal surfaces are completely re-epithelialized immediately after keratectomy, with a stratified epithelium, whereas control corneal surfaces generally required 3 to 5 days to become fully re-epithelialized by infiltrating host corneal epithelial cells. This delay in creating an intact epithelial barrier, probably contributes to both the postoperative pain and corneal haze that are associated with excimer laser photoablation.

Corneal haze after excimer laser photoablation can be the result of undesirable postoperative progression during corneal wound healing and significantly affects the patient’s quality of life. The use of cell sheet keratoplasty and its ability to inhibit postoperative corneal haze may therefore initiate a revolution-
nary change in our methods for the correction of refractive errors and the treatment of superficial corneal opacities.

In contrast to control corneas with significant epithelial hyperplasia with an accumulation of inflammatory cells in the subepithelial stroma 1 week after keratectomy, the eyes receiving cell sheet transplantation retained ocular surfaces that more closely resembled the native cornea, with three to five cell layers. In addition, whereas the observed hyperplasia in control corneas began to decrease at 2 months after photoablation, the accumulation of α-SMA and collagen III demonstrated the presence of myofibroblasts and activated keratocytes with enhanced ECM deposition in the central ablated zones. This appearance of myofibroblasts and increased extracellular matrix deposition in control eyes may be a key factor in the development of corneal haze that is observed in control eyes, suggesting that the interaction between epithelial cells and keratocytes is an important factor in corneal wound healing. Thus, the presence of an intact corneal epithelium directly after laser ablation may have an important role in curbing subepithelial haze and myofibroblast differentiation.

Similar to the popular technique of LASIK, which creates a corneal flap, the use of cell sheet keratotomy also allows for the formation of an intact epithelial barrier immediately after laser ablation and therefore may also provide several advantages over traditional PRK. However, although LASIK can only be used to correct refractive errors, the transplantation of epithelial cell sheets after excimer laser treatment can also be used to treat superficial corneal opacities, thereby creating a novel alternative treatment to both PRK and PTK.

An additional advantage of using transplantation of tissue-engineered epithelial cell sheets is the elimination of postoperative epithelial hyperplastic changes and accumulation of ECM, therefore allowing for the stable correction of refractive errors in comparison to other techniques. As the transplantation of tissue-engineered epithelial cell sheets recreates an intact and smooth ocular surface during the early postoperative period, the positive refractive effects from laser ablation may be prolonged. Similarly, in cases when retreatment of refractive errors is required, ocular surfaces can receive another cell sheet keratoplasty, even without the use of mitomycin C, which is commonly required for prevention of postoperative corneal haze.

With the use of temperature-responsive surfaces, cultured corneal epithelial cells can be directly transplanted to the ablated stromal bed without the use of any sutures, due to the presence of adhesive proteins on the basal surface. The presence of this deposited ECM is likely to be a critical factor in promoting cell sheet integration by allowing for direct interactions between the cell sheet and the ablated stromal bed. It has been observed that an unhealthy corneal epithelium generally demonstrates a defective cell membrane, with improper cellular alignment and insufficient intercellular tight junctions may contribute to an inadequate barrier function. In a similar fashion, with the use of LASIK, the risk of flap-related complications due to improper reattachment, such as flap striae and corneal ectasia can result in abnormal corneal epithelial function. However, the stable attachment of tissue-engineered corneal epithelial cell sheets presents an intact epithelial barrier function that is required and probably aids in the recovery process after excimer laser ablation.

FIGURE 4. Host corneal responses were modified after cell sheet transplantation. (a) H&E staining revealed that 3 days after ablation during the early phase of wound healing, eyes undergoing transplantation had an intact epithelial layer, but that control corneas had an acellular area representing kerocyte apoptosis, with epithelial hyperplasia. (b) One week after surgery, the transplanted corneal epithelium consisted of five to six layers and closely resembled the native cornea, whereas control eyes have hyperplastic changes with an epithelium consisting of 7 to 10 layers, along with significant accumulation of inflammatory cells at the ablation site. (c) Two months after laser keratectomy, control corneas revealed less hyperplastic changes and begin to resemble more closely the native cornea.

FIGURE 5. Cell sheet transplantation prevents keratocyte activation and myofibroblast transformation. Both 1 week and 2 months after surgery, transplanted eyes show negative staining for α-smooth muscle actin (a, left) and collagen III (b, left). Whereas at 1 week, control corneas showed similar results to the transplant group, at 2 months after surgery, control eyes demonstrated the presence of myofibroblasts, as evidenced by α-smooth muscle actin staining (a, right), and activated keratocytes with the deposition of collagen III (b, right).
Overall, cell sheet keratoplasty after laser ablation seemingly provides several distinct advantages over the traditional methods of PRK, PTK, and LASIK, by allowing for rapid visual recovery and a reduction in both postoperative eye pain and corneal haze. For clinical applications, avoiding the use of 3T3 feeder layers overcomes an important obstacle by eliminating the use of mouse-derived cells. With the current method, numerous cell sheets can theoretically be created from a single donor eye, thereby allowing for the treatment of a wide range of patients. In the relative simplicity with which cell sheet keratoplasty can be performed also allows for repeated ablations without the use of mitomycin C, which is generally required for the prevention of the postoperative haze and lower postoperative refractive regression that occurs with additional operations.

In summary, our results indicate that cell sheet keratoplasty after excimer laser photoablation enhances postablation wound healing and can significantly reduce postoperative corneal haze. This approach may therefore allow for a novel method in the correction of refractive errors and superficial corneal opacities in cases of ocular surface disorders such as recurrent corneal dystrophy or erosion.

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References