Cultivated Human Conjunctival Epithelial Transplantation for Total Limbal Stem Cell Deficiency


PURPOSE. To determine the feasibility of cultivated conjunctiva as a viable epithelial sheet for transplantation and corneal resurfacing in eyes with limbal stem cell deficiency (LSCD).

METHODS. Human corneal epithelial (HCE) and human conjunctival epithelial (HCJE) cells were cultivated on human amniotic membrane (AM) to confluence and then air lifted to allow further stratification and differentiation. Denuded AM and cultivated HCE and cultivated HCJE cells were then transplanted into 18 eyes of rabbits with induced LSCD. The cultivated and engrafted epithelia were examined by transmission electron microscopy (TEM) and immunohistochemistry. Two weeks after transplantation, the eyes were examined by slit lamp biomicroscopy and scored on epithelial integrity, corneal haze, and corneal neovascularization.

RESULTS. Both cultivated and engrafted HCJE sheets demonstrated confluent epithelial sheets with five to six layers of well-stratified epithelium. TEM examination of engrafted HCJE revealed numerous microvilli, desmosomes, and hemidesmosomes, identical with in vivo corneal epithelium. Immunohistochemical analysis of both HCJE and HCE cells showed the presence of CK3, CK4, and CK12, with absence of Muc5AC. Clinical outcomes for eyes receiving HCJE transplants and HCE transplants were comparable, with most having transparent, smooth corneas, free of epithelial defects.

CONCLUSIONS. The study showed that microscopically, HCJE cells have features similar to HCE cells, with clinically equivalent outcomes. The ex vivo cultivation of conjunctiva to form transplantable epithelial sheets for corneal replacement is a promising new treatment modality in patients with LSCD.

The ocular surface forms a delicate and dynamic biological system which serves to protect the eye against external insults. Damage to the ocular surface and corneal epithelial stem cells at the limbus from severe cicatricial ocular surface diseases like chemical and thermal burns or Stevens-Johnson Syndrome (SJS), has long posed a major challenge to ophthalmologists. Limbal stem cell deficiency (LSCD) results in poor corneal epithelial healing, replacement of the corneal epithelium with conjunctiva (conjunctivalization), and corneal scarring that contribute to severe visual loss.

In eyes with unilateral LSCD, autologous transplantation of limbal tissue from the contralateral eye can be performed in an attempt to replace the stem cell population of the diseased eye. In bilateral disease in which there is extensive involvement of both eyes, limbal stem cell transplantation with allogeneic tissue is necessary. However, transplantation of allogeneic tissue necessitates postoperative immunosuppression, bringing about the attendant systemic adverse effects associated with its use. Despite immunosuppression, graft outcomes are still unsatisfactory, as rejection and failure frequently occur. Subsequent allogeneic transplantation would be associated with even more dismal results. The use of autologous transplantation therefore has significant advantages, as it reduces the risks of transmission of infection or graft rejection and eliminates the need for long-term immunosuppression.

Recently, transplantation of cultivated autologous oral mucosal epithelium has been used in the treatment of LSCD. However, oral mucosa is nonocular tissue, and it retains the characteristics of the tissue of origin. The use of conjunctiva would therefore have obvious advantages for corneal surface epithelial replacement. We have previously demonstrated the effective use of cultivated conjunctival transplantation for conjunctival epithelial replacement in various ocular surface conditions. The use of cultivated conjunctival transplantation for corneal epithelial replacement would be a novel method for treating severe ocular surface disease.

We have described the use of cultivated human conjunctival epithelial cells for corneal resurfacing in a rabbit LSCD model. This is the first study in which relative efficacy of cultivated conjunctival epithelial transplantation was compared with cultivated limbal stem cell transplantation for corneal epithelial replacement. We showed that cultivated conjunctival epithelial transplantation is a viable alternative to cultivated limbal stem cell transplantation for corneal resurfacing in eyes with LSCD.
Table 1. Summary of Clinical Findings for Eyes Receiving Cultivated HCjE, HCE, and Denuded AM Transplants

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Epithelial Integrity n (%)</th>
<th>Corneal Haze n (%)</th>
<th>Corneal Neovascularization n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1: HCjE transplant (n = 6)</td>
<td>4 (66.7), no defect</td>
<td>4 (66.7), clear</td>
<td>2 (33.3), not significant</td>
</tr>
<tr>
<td></td>
<td>2 (33.3), grade 1 defect</td>
<td>2 (33.3), grade 1 haze</td>
<td>2 (33.3), grade 1</td>
</tr>
<tr>
<td>Group 2: HCE transplant (n = 6)</td>
<td>4 (66.7), no defect</td>
<td>5 (83.3), clear</td>
<td>3 (50), not significant</td>
</tr>
<tr>
<td></td>
<td>2 (33.3), grade 1 defect</td>
<td>1 (16.7), grade 1 haze</td>
<td>1 (16.7), grade 1</td>
</tr>
<tr>
<td>Group 3: Denuded AM transplant (n = 6)</td>
<td>2 (33.3), no defect</td>
<td>1 (16.7), clear</td>
<td>2 (33.3), grade 2</td>
</tr>
<tr>
<td></td>
<td>2 (33.3), grade 1 defect</td>
<td>5 (83.3), grade 1 haze</td>
<td>2 (33.3), grade 2</td>
</tr>
</tbody>
</table>

Materials and Methods

All experimental procedures performed conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Committee for Ethical Issues on Human Research of Kyoto Prefectural University of Medicine. Normal human conjunctival epithelial (HCjE) tissues were obtained from patients with conjunctivochalasis who had given informed consent, and human amniotic membranes (AMs) were obtained from consenting mothers at the time of cesarean section. Normal human corneal epithelia (HCE) were obtained from human corneoscleral rims from the Northwest Lion Eye Bank (Seattle, WA).

Preparation of Human Amniotic Membranes

In accordance with the tenets of the Declaration of Helsinki, human placentas were obtained from mothers who had undergone cesarean section and whose test results were negative for hepatitis B and C, syphilis, and human immunodeficiency virus. The membranes were washed with sterile phosphate-buffered saline (PBS) to remove the blood clots and the HAM was peeled away from the chorion and flattened, with the stromal side on a sterilized nitrocellulose filter paper (Millipore, Bedford, MA). The paper with adherent HAM was then stored in 50% DMEM and 50% glycerol (Invitrogen-Gibco, Rockville, MD) at −80°C. Immediately before use, the amniotic membrane was thawed, rinsed with sterile PBS, and incubated in 0.02% EDTA solution (Nakalai Tesque, Kyoto, Japan) for 2 hours, followed by gentle scraping to remove any remaining amniotic epithelial cells.

Ex Vivo Expansion of Conjunctival and Corneal Epithelial Cells on HAM

Corneal and conjunctival epithelial cells were cocultured with mitomycin C (MMC)-treated 3T3 fibroblasts (NIH 3T3; American Type Culture Collection, Manassas, VA). Confluent 3T3 fibroblasts were incubated with 4 mg/mL of MMC for 2 hours at 37°C under 5% carbon dioxide. These were then trypsinized and plated onto plastic dishes with a density of 2 × 10^5 cells/cm². Denuded HAM (basement membrane-side up) was placed on the porous support membrane (Millipore Corp., Bedford, MA). The membrane was then introduced into wells of a six-well culture plate containing mitomycin-treated 3T3 feeder cells to achieve a dual-chamber culture.

The corneoscleral rims and the conjunctival epithelia were first incubated at 37°C for 1 hour with 1.2 IU dispase (Roche, Tokyo, Japan). The purpose for using dispase was to facilitate separation of epithelial cells from the underlying stromal tissue, so as to promote the outgrowth of epithelial cells on HAM. The epithelium was then removed from the underlying stroma by mechanical scraping and further dissociation with 0.1% trypsin-EDTA. Epithelial cells from the limbal and peripheral corneal region were separated from the underlying stroma carefully. After cell separation, the human conjunctival and corneal epithelial cells were then seeded onto the AM at a density of 1 × 10^5 cells/cm² in defined keratinocyte-SFM (Invitrogen, Tokyo, Japan) containing 2% FBS.

On confluence (6–8 days), the epithelial cells were exposed to 7 days of high-calcium conditions (1:1 mixture of defined keratinocyte-SFM containing 2% FBS and DMEM/F12 containing 10% FBS) to promote differentiation and stratification. These conjunctival and corneal equivalents were air-lifted for 7 days, to allow further stratification and differentiation of the epithelial cells. Air-lifting was performed by lowering the level of medium to the level of the membrane. Close monitoring of the fluid level was performed twice daily to ensure that the desired level of medium was maintained.

All cultures were incubated at 37°C in a 5% CO₂–95% air incubator, with medium changed every 1 to 2 days. The cultures were monitored under an inverted phase-contrast microscope (Axiovert; Carl Zeiss Meditec, Oberkochen, Germany).

Transplantation of Cultivated Conjunctival and Corneal Epithelial Cells

Total LSCD was created in 18 Japanese white rabbit eyes by surgically removing the entire corneal epithelium by superficial keratectomy. To ensure complete removal of the limbal epithelium, we surgically excised the entire limbal epithelium and surrounding conjunctival tissue up to 2 mm beyond the limbus from one eye, down to the bare sclera.

The rabbits were then divided into three treatment groups (Table 1): group 1, cultivated human conjunctival epithelial (HCjE) transplantation (n = 6); group 2, cultivated human corneal epithelial (HCE) transplantation (n = 6); and group 3, denuded human amniotic membrane transplantation (n = 6).

The epithelial sheets and HAM were transplanted onto the denuded corneal surface to completely cover the resected area and sutured with 10-0 nylon sutures. The graft was covered with a therapeutic soft contact lens and secured with four peripheral anchoring sutures. Total tarsorrhaphy was performed with 60 nylon sutures.

After surgery, the rabbits were treated with topical antibiotics of 0.3% oxofloxacin ointment (Santen Pharmaceutical Co., Ltd, Osaka, Japan), trimethoprim acetic acid 0.2 mL injected subconjunctivally (Bristol Myers Squibb Co., Tokyo, Japan), and systemic antibiotics (10 mg gentamicin/rabbit, delivered intramuscularly; Nacalai Tesque, Inc.). They also received a daily IM injection of 0.2 mg/kg of the immunosuppressant agent FK506 (Astellas Co., Ltd., Tokyo, Japan) to inhibit a possible xenogenic reaction or nonspecific inflammation.

Slit Lamp Examination

The ocular surfaces of the rabbits were examined, stained with fluorescein, and photographed with a slit lamp biomicroscope (SL-1600; Nidek Co., Ltd., Aithi, Japan) on the day of transplantation and on the 14th postoperative day. The main outcome measures were graded as follows.

Epithelial Integrity. Grade 0, no epithelial defect; 1, less than or equal to one fourth of the corneal surface; 2, from one fourth to one
half of the corneal surface; 3, from one half to three fourths of the corneal surface; 4, more than three fourths of the corneal surface.

**Corneal Haze.** Grade 0, no haze; 1, trace haze; 2, mild haze easily visible on slit-beam illumination; 3, moderate haze partially obscuring iris details; and 4, marked haze that obscures iris details.

**Corneal Neovascularization.** Grade 0, no neovascularization and 1, up to 3 clock hours; 2, 3 to 6 clock hours; 3, 6 to 9 clock hours; and 4, >9 clock hours of corneal neovascularization.

### Immunostaining and Light-Microscopic Analysis

Two weeks after transplantation, the corneas were excised and further analyzed by histology, immunohistochemistry, and transmission electron microscopy (TEM). Engrafted tissues were removed from the eyes of the 18 rabbits. Normal conjunctival and corneal tissues were also examined for the purpose of comparison. In vivo tissues, cultivated HCjE and HCE cells, and transplanted tissues were divided into two portions, one portion of each of which was embedded in OCT compound (Tissue-Tek; Sakura Fine Technical Co., Ltd.; Tokyo, Japan) and snap frozen with liquid nitrogen for immunostaining analysis. The other portion was processed for TEM.

Immunohistochemistry studies of several tissue-specific cytokeratins in cultivated and transplanted conjunctival and corneal epithelial sheets were performed by previously described methods.18,20 Tissue sections (8 μm) were placed on silanized slides (Dako Japan, Kyoto, Japan), air dried, and subjected to hematoxylin staining or indirect-immunostaining analysis. The sections were fixed with Zamboni’s fixative or acetone (4°C, 5 minutes), incubated for 1 hour in blocking solution (1% BSA in 0.01 M PBS). The sections were incubated at room temperature for 1 hour with primary antibody solutions (Table 2) and normal mouse IgG1, IgG2a, and IgG2b (Dako Japan), and goat IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as negative controls. After they were stained with primary antibodies, the sections were washed with 0.01 M PBS and then treated for 1 hour with the appropriate secondary antibodies: Alexa-488–labeled donkey anti-mouse IgG and Alexa-594–labeled donkey anti-goat IgG (Molecular Probes, Eugene, OR). Subsequently, the sections were washed with 0.01 M PBS and mounted with medium containing an anti-photo-bleaching reagent (3% DABCO; Wako Pure Chemical Industries Ltd., Osaka, Japan). Fluorescent images of the sections were examined by confocal laser microscopy (TCS-SP2; Leica, Tokyo, Japan).

### TEM Examination

The specimens were fixed in 2.5% glutaraldehyde in 0.1 M PB, washed three times in PB, and postfixed for 1 hour in 2% aqueous osmium tetroxide. The samples were dehydrated by being passed through a graded ethanol series, transferred to propylene oxide, and embedded in epoxy resin (Epon-812; Shell Chemical, San Francisco, CA). Ultrathin (70 nm) sections were cut and stained with uranyl acetate and counterstained with lead citrate before examination under a TEM (H-7000; Hitachi, Tokyo, Japan).

### Table 2. Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Type of Antibody</th>
<th>Immunized Animal</th>
<th>Company*</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK3</td>
<td>×50</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>PROGEN</td>
<td>Major cytokeratin in corneal epithelium</td>
</tr>
<tr>
<td>CK4</td>
<td>×100</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>Novocastra</td>
<td>Major cytokeratin in non-keratinizing mucosal epithelium</td>
</tr>
<tr>
<td>CK12</td>
<td>×100</td>
<td>Polyclonal</td>
<td>Goat</td>
<td>Santa Cruz</td>
<td>Major cytokeratin in corneal epithelium</td>
</tr>
<tr>
<td>Muc5AC</td>
<td>×100</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>Novocastra</td>
<td>Secreted mucin/goblet cell mucin</td>
</tr>
</tbody>
</table>

* PROGEN: Biotechnik GmbH, Heidelberg, Germany; Novocastra: Novocastra Laboratories Ltd., Newcastle, UK; Santa Cruz: Santa Cruz Biotechnology Inc., Santa Cruz, CA.

### RESULTS

#### Histologic Analysis of HCjE and HCE Sheets

Human conjunctival and corneal epithelial cells formed confluent epithelial sheets over the amniotic membranes after 21 days, consisting of five to six layers of well-stratified epithelium, with cuboidal basal cells, and progressive flattening of the cells toward the surface (Fig. 1). TEM examination of the epithelial culture sheet showed that the cells had differentiated into basal columnar cells, suprabasal cuboidal wing cells, and flat squamous superficial cells (Figs. 2A, 2E). Morphologic patterns were similar between cultured conjunctival and corneal epithelia. Numerous microvilli were evident on the surface of the superficial cells (Figs. 2B, 2F), which was almost identical with that found in vivo corneal epithelium. The basal epithelial cells adhered well to the AM substrate with hemidesmosome attachments (Figs. 2D, 2H), and numerous desmosomal junctions were evident between the epithelial cells in all cell layers (Figs. 2C, 2G).

#### Transplantation of Cultivated HCjE and Cultivated HCE Sheets

Cultivated HCjE sheets were successfully transplanted onto the corneas of the six rabbits. Complete epithelialization was confirmed by fluorescein staining at the end of surgery. The clinical results of the transplants are summarized in Table 1.

In the group that underwent denuded AM transplantation, four (66.7%) eyes had epithelial defects, and only one (16.7%) eye had a clear cornea. In contrast, four eyes in the HCjE transplantation group had no epithelial defects, and four eyes had clear corneas. These results were comparable to the HCE-transplanted eyes, where similarly, four eyes had no epithelial defects, and five (83.3%) eyes had clear corneas (Fig. 3). For both the HCjE and HCE transplant groups, the epithelial defects were found in eyes in which the contact lens had dislodged or had folded on itself and was abrading the cornea.

Denuded amniotic membrane transplants were associated with significant peripheral corneal neovascularization, with five (83.3%) eyes having grade 4 neovascularization. Cultivated HCjE and HCE transplants were associated with significantly less neovascularization, with only two (33.3%) eyes having grade 2 or 3 neovascularization in each group, and no eyes with grade 4 neovascularization. The majority of corneas that underwent cultivated HCjE transplantation were transparent, smooth, and devoid of epithelial defects 2 weeks after transplantation (66.7%). There were no instances of graft retraction or dislodgement.

### Immunohistochemistry

The engrafted HCjE and HCE tissues consisted of five to six layers of stratified epithelial cells, with cuboidal or columnar basal cells and flattened squamous superficial cells. The grafts stained positively for the antihuman nuclei antibody, confirm-
ing that the epithelial cells on the rabbit corneas were of human origin. The engrafted cultivated conjunctival epithelium expressed the conjunctiva-specific keratin CK4 which was consistent with engrafted corneal epithelium and also normal in vivo conjunctiva (Figs. 4B, 4E, 4H). Cornea-specific CK12 was seen in both in vivo cornea and engrafted HCE and although it was present only minimally in in vivo conjunctiva, it became more obvious in transplanted conjunctiva. In cultivated (Fig. 4D) and engrafted (Fig. 4G) HCE, specimens stained more heavily for cornea-specific keratin 3 than did in vivo conjunctiva (Fig. 4A). This staining pattern was similar to both in vivo and engrafted HCE (Figs. 5A, 5G). Muc5AC staining for goblet cells was absent in both the cultivated HCE sheet and the engrafted conjunctival epithelium (Figs. 5F, 5I). This result was morphologically similar to that of in vivo corneal epithelium (Fig. 5C).

**DISCUSSION**

Limbal stem cell transplantation has for the past two decades been the standard treatment for LSCD. However, many patients with severe ocular surface disorders have bilateral disease...
requiring allogeneic transplantation and long-term immunosuppression, with the attendant systemic risks. In recent years, the use of bioengineered epithelial transplantation has revolutionized the treatment of patients with severe ocular surface disease. The novel use of cultivated autologous conjunctival epithelial transplantation for corneal resurfacing is a promising treatment modality with the advantages that using autologous tissue is safer, has a reduced risk of graft rejection and transmission of infection, and reduces the need for long-term treatment with steroids or immunosuppression. This study demonstrates the effective use of cultivated conjunctival transplantation for corneal epithelial reconstruction and suggests that it may be a viable alternative to cultivated or conventional limbal stem cell transplantation for severe ocular surface disease and severe LSCD.

We recently demonstrated that cultivated conjunctival transplantation may be used for corneal epithelial replacement.18 The study was focused on morphologic and immuno-histochemical characteristics of the cultivated epithelial sheet and showed that cultivated HCjE and the engrafted epithelium manifested five to six layers of stratified squamous epithelium similar in morphology to normal corneal epithelium. The basal cells expressed putative stem cell markers (ABCG2 and P63) and hemidesmosome- and desmosome-component proteins. The cytokeratins CK4, CK13, CK3, and CK12 and the mucin MUC4 were found in the engrafted epithelium.

As an extension of that study, we proceeded to compare the clinical efficacy of cultivated conjunctival transplantation with cultivated corneal and denuded AM transplantation. We showed that transplanted cultivated conjunctiva had clinical results equivalent to that of transplanted cultivated corneal epithelium. The epithelial surface was completely epithelialized, and the corneas remained clear in most of the eyes in both the cultivated conjunctival and cultivated corneal epithelial transplant groups. This result was in contrast to that obtained with denuded amniotic membrane where the majority (66.7%) of eyes had residual epithelial defects with some having large defects and only 16.7% remaining clear. TEM demonstrated the presence of hemidesmosomal attachments of the basal cells and a basal lamina, which are necessary for ensuring graft attachment and integrity after transplantation. The in vitro cultured conjunctival epithelium and engrafted epithelium contained cells that expressed the cornea-specific markers CK3 and CK12. We hypothesize that this was probably derived from clusters of CK3/CK12-positive cells that were present in the in vivo conjunctival tissue. These findings are consistent with our previous report that demonstrated the presence of ectopic clusters of CK3/CK12-positive cells in the conjunctiva.21

Our study is the first to compare objectively cultivated human conjunctival transplantation with the current preferred method of cultivated limbal stem cell transplantation. In a
recent study, Ono et al.\textsuperscript{22} compared the use of cultivated rabbit conjunctival epithelial transplantation with denuded amniotic membrane transplantation and eyes without any treatment in a limbal-deficiency model. In that study, there was no comparison with cultivated limbal stem cell transplantation, and rabbit tissue was used. As our purpose was to evaluate the clinical feasibility of using cultivated human conjunctival tissue in patients, the use of human tissue would allow us to draw a more direct correlation with the expected clinical results when applied to humans. In addition, using human tissue allowed us to better discriminate between transplanted and migrated host-derived cells, thereby giving us a more objective picture of the true effect of conjunctival transplantation. Our demonstration of the comparable results between cultivated conjunctival and cultivated limbal stem cell transplantation validates the use of conjunctival epithelial transplants as an alternative method of corneal epithelial replacement, while achieving similar good results in terms of ocular surface stability, corneal clarity, and reduction in corneal neovascularization.

In 1989, Kenyon and Tseng\textsuperscript{23} described autologous limbal transplantation from the healthy fellow eye as a therapeutic technique for unilateral ocular surface disease. A drawback to this technique was the potential risk of inducing iatrogenic limbal deficiency in the donor eye when too much limbal tissue was removed, as had been previously demonstrated in animal models.\textsuperscript{24,25} The potential stem cell depletion in the donor eye secondary to inflammation or subclinical disease has also been reported.\textsuperscript{26,27} In cases of bilateral LSCD, treatment with allogeneic limbal epithelium requires the long-term use of steroids and immunosuppression and is associated with systemic complications.\textsuperscript{28,29} With the ex vivo expansion technique described herein, only a small amount of autologous conjunctival tissue is required. Even in cases of bilateral involvement, as long as a small amount of healthy conjunctiva was available (1 × 2 mm), we were still able to cultivate an epithelial sheet of sufficient size ex vivo to resurface the corneal defect. With numerous potential sites of harvest\textsuperscript{30–34} there are no significant additional risks should repeat transplantation be required, because of the small amount of tissue needed. In addition, because there is no prior allosensitization, subsequent allografts are not negatively affected (e.g., penetrating keratoplasty).

Eyes with LSCD have persistent epithelial defects and chronic inflammation, and these chronically sick eyes eventually develop conjunctivalization and pannus formation that is usually associated with significant scarring and neovascularization. Denuded amniotic membrane transplantation has also been used for treating these eyes, but as clearly demonstrated in this study, the lack of an intact epithelium results in delayed healing, more inflammation, and more neovascularization compared with an intact epithelium that is simultaneously transplanted. Transplantation of an intact autologous cultured conjunctival epithelium over an amniotic membrane substrate has significant advantages. In contradistinction to the conjunctivalized, vascularized pannus that encroaches on the cornea in LSCD-affected eyes, cultured conjunctival epithelial transplantation involves merely transplanting the conjunctival epithelium without any associated fibrous or vascular tissue. The advantages of having a completely epithelialized surface immediately after transplantation is clearly evident from our study, as it promotes faster recovery, corneal surface stability, and there is less stimulus for conjunctival advancement with accompanying fibrovascular pannus formation. In addition, amniotic membrane transplantation further helps to promote recovery by reducing ocular inflammation, neovascularization, and scarring.

Cultivated oral epithelium has recently been used as an alternative tissue source in the treatment of bilateral severe ocular surface disease. Short- to mid-term results have been encouraging thus far\textsuperscript{9–11} but long-term outcomes are still unknown. Although this method makes use of autologous tissue, oral tissue is of nonocular origin. The use of conjunctiva has several advantages. The cytology and morphology of conjunctival epithelium is more similar to corneal epithelium than that of oral mucosa, thus making it a more favorable tissue source. Significant peripheral neovascularization of oral mucosa-derived grafts has been reported. In certain autoimmune diseases such as ocular cicatricial pemphigoid, oral mucosa theoretically can secrete a common basement membrane target antigen, possibly making it a less desirable tissue source in these groups of patients.\textsuperscript{32} Last, the use of endogenous tissue from the eye itself is always preferred over nonocular foreign tissue. In our study, only a minority of eyes that underwent HCE and HCE transplantation had three or more clock hours of neovascularization. This finding is particularly important in reducing inflammation and scarring, as well as graft rejection or failure in eyes that may subsequently undergo future corneal transplantation. One limitation of our study was the relatively short follow-up duration (14 days) stemming from the use of human grafts in the rabbits, for the reasons we have described. As such, studies with longer observation periods would be necessary, to provide more information on the long-term efficacy of this treatment modality.

In our study, goblet cells were not found in the in vitro and transplanted conjunctival epithelium. The issue of goblet cell differentiation in culture has been a contentious one. Investigators in other studies have demonstrated variable results, with some reports showing the presence\textsuperscript{56–58} or absence\textsuperscript{59,60} of goblet cells in cultured conjunctival epithelial cells. In many of these studies, rabbit conjunctival epithelium was used. The variability in results may reflect the difference in goblet cell proliferation under various cell culture conditions, such as, if 3T3 cells were in direct contact with cells\textsuperscript{59–57} or were in a separate compartment of the culture system\textsuperscript{38–40} if cells were in a submerged condition throughout\textsuperscript{56–58} or subjected to air-lifting.\textsuperscript{59,60} Goblet cell differentiation is not universal in all studies, and even if present, were usually found to be very scattered and few. In each of these papers, rabbit conjunctival epithelial cells were used. In the present study, we used human conjunctiva, and we exposed cells to air-lifting for 7 days. Under our culture conditions, no distinct Muc5AC-positive cells were present. This variability in results may reflect the relative difficulty in propagating human goblet cells in vitro and may be related to differences in cell culture conditions.

In conclusion, we demonstrated the effective use of cultivated conjunctival transplantation for corneal epithelial replacement in the treatment of total LSCD. This study demonstrated clinically that cultivated conjunctival epithelial transplantation may be a viable alternative to cultivated limbal stem cell transplantation or cultivated oral epithelial transplantation for the treatment of eyes with severe ocular surface disease and limbal deficiency, with the obvious advantages of using an autologous eye tissue source. This finding increases the spectrum of severe ocular surface disorders that can be treated more safely with autologous tissue, as even patients with bilateral disease are likely to have some areas of healthy conjunctival tissue that can be harvested and expanded ex vivo to form a confluent conjunctival sheet. Future studies are needed to assess the long-term efficacy of this procedure.

References


