Establishment of a Cultivated Human Conjunctival Epithelium as an Alternative Tissue Source for Autologous Corneal Epithelial Transplantation


PURPOSE. The corneal epithelium is essential for maintaining corneal transparency, and efforts have been made to develop improved techniques for corneal epithelial transplantation in patients with total limbal failure. We evaluated the suitability of transplanted cultivated human conjunctival epithelium (HCjE) as a corneal epithelium replacement in rabbits with total corneal and limbal deficiency.

METHODS. HCjE cells, cultivated on human amniotic membrane (AM) to confluence and exposed to an air–liquid interface (air-lifted), were transplanted onto denuded rabbit corneas and monitored for 2 weeks. The cultivated HCjE sheet and the engrafted epithelium were analyzed by immunohistochemistry and transmission electron microscopy (TEM).

RESULTS. The transplanted HCjE remained transparent, smooth, and without epithelial defects during the follow-up period. Both the cultivated HCjE cells and the engrafted epithelium manifested five to six layers of stratified squamous epithelium similar in morphology to normal corneal epithelium. The basal cells expressed the putative stem cell markers (ABCG2 and P63) and hemidesmosome and desmosome component proteins. The cytokeratins (CK4, CK13, CK3, and CK12) and MUC4 were found in the engrafted epithelium. However, MUC5AC was not expressed. The results indicate that HCjE cultivated on AM has the potential to be used as an alternative corneal epithelium.

CONCLUSIONS. The transplantation of cultivated HCjE sheets is a promising technique for the treatment of eyes with limbal failure. (Invest Ophthalmol Vis Sci. 2006;47:3820–3827) DOI: 10.1167/iovs.06-0293

The ocular surface is covered by at least two different types of epithelia: corneal and conjunctival. These two epithelial tissues are indispensable in keeping homeostasis of the eye by expressing various specific genes such as cytokeratin 3/12 or secretory mucin and is necessary for ocular surface homeostasis. In patients with severe ocular surface disorders such as Stevens-Johnson syndrome (SJS), ocular cicatricial pemphigoid (OCP), and chemical injuries, the corneal epithelium may be destroyed and replaced by conjunctival epithelium (conjunctivalization). The ocular surface is often inflamed, vascularized, opacified, and keratinized, and vision is severely compromised.

Cultivated corneal stem cells and oral epithelium transplants are a newly developed surgical strategy in which to treat such pathologic conditions. Although these treatments were reported to be effective in applying regenerative medicine, several problems remain. For example, tissue transplantation from allogeneic donors carries the risk of rejection and may require postoperative immunosuppressive therapy that can induce severe systemic and local side effects. The longevity of cultivated corneal and oral mucosal epithelium remains to be investigated.

In addition to corneal and oral mucosal epithelium, conjunctival epithelium is a third epithelial cell source that can be cultivated to be transplanted for ocular surface reconstruction. Among all stratified epithelial tissues in the body, these cells are most akin biologically to corneal epithelial cells. Therefore, conjunctival epithelial cells transplanted onto the corneal surface may serve some of the functions of corneal epithelial cells. As the transplantation of cultivated human conjunctival epithelial cells (HCjE) succeeded in reconstructing the conjunctiva of patients with various ocular surface conditions, e.g., pterygium, we postulated that cultivated HCjE sheets could be transplanted onto the corneal surface.

To test our hypothesis, we cultured HCjE on human amniotic membrane (AM) and transplanted them onto denuded rabbit corneas. The transplanted HCjE were well-maintained and remained clear and smooth during the postoperative period. Histologic and immunohistochemical analyses revealed that the engrafted epithelium shared the morphology and characteristics of corneal epithelium, suggesting that cultivated HCjE may represent a viable alternative to replace damaged corneal epithelium.

METHODS

Human Subjects

This research was approved by the Committee for Ethical Issues on Human Research of Kyoto Prefectural University of Medicine and adhered to the tenets of the Declaration of Helsinki. Normal conjunctival tissues were obtained from patients with conjunctivochalasis. Human AM was harvested at the time of Cesarean section and processed by previously reported methods. The procedures were carefully explained to all donors, and their prior informed consent for use of their tissue was obtained.

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Primary Culture of HCJE Cells

The cells were cultured according to a slightly modified, previously reported system.22 Briefly, denuded human AM was placed on a porous support membrane (Millipore Corp., Bedford, MA) with the epithelial basement membrane side up. The membrane was then introduced into wells of a six-well culture plate containing mitomycin-treated feeder cells (NHI 3T3; American Type Culture Collection, Manassas, VA) to achieve a dual-chamber culture. After a 1-hr incubation with 1.2 IU dispase (Roche, Tokyo, Japan), the human conjunctival epithelium (the area of this conjunctival source was ~15 mm²) was removed from the underlying stroma by mechanical scraping and further dissociated by digestion with 0.1% Trypsin-EDTA. The HCJE cells were then seeded on the upper chamber of the culture system and grown according to a three-step culture regimen. Until they reached confluence (6–8 days), the cells were grown in low-calcium medium (Defined Keratinocyte-SFM; Invitrogen, Tokyo, Japan) containing 2% FBS. After reaching confluence, they were grown for 7 days in high-calcium medium (mixture of Defined Keratinocyte-SFM and DMEM/F12/10% FBS at a ratio of 1:1) to promote differentiation. They were then exposed to air by decreasing the volume of the medium (air-lifting) over the course of 1 week to promote epithelial integrity. All cultures were incubated at 37°C in a 5% CO₂/95% air incubator. The medium was changed every 3 days, the ocular surface of the eight transplant recipients was examined and photographed with a slit lamp biomicroscope (SL-1600; Nidek Co., Ltd., Aithi, Japan).

On the day of transplantation and on the 4th and 14th postoperative days, the rabbits were housed and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental procedures were approved by the Committee for Animal Research of Kyoto Prefectural University of Medicine.

Using eight Japanese white rabbits weighing 2.4 to 2.8 kg (OBS, Kyoto, Japan), we performed superficial lamellar keratectomy to remove the entire corneal epithelium. To ensure complete removal of the limbal epithelium, we surgically excised the entire limbal epithelium and surrounding conjunctival tissue up to 2 mm from the limbus from one eye, down to the bare sclera. The cultured HCJE sheets were transplanted onto the denuded ocular surface to completely cover the resected area and were sutured in place with 10-0 nylon (8–12 sutures per sheet). The graft was then covered with a soft contact lens secured with four peripheral anchoring sutures. Finally, tarsorrhaphy was performed with 6-0 nylon sutures (Fig. 1B). After surgery, the rabbits were treated with topical antibiotics (0.3% ofloxacin ointment; Santen Pharmaceutical Co., Ltd, Osaka, Japan), trimcinolone acetoni de (0.2 mL injected subconjunctivally; Bristol-Myers Squibb Co., Tokyo, Japan), and systemic antibiotics (10 mg gentamicin/rabbit, delivered intramuscularly [IM]; Nacalai Tesque Inc. Kyoto, Japan). They also received a daily IM injection of 0.2 mg/kg of the immunosuppressant agent FK506²³ (Astellas Co., Ltd., Tokyo, Japan) to inhibit a possible zonogeneic reaction or nonspecific inflammation.

Conjunctival Epithelium Transplantation onto Rabbit Corneas

At all times, the rabbits were housed and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental procedures were approved by the Committee for Animal Research of Kyoto Prefectural University of Medicine.

Using eight Japanese white rabbits weighing 2.4 to 2.8 kg (OBS, Kyoto, Japan), we performed superficial lamellar keratectomy to remove the entire corneal epithelium. To ensure complete removal of the limbal epithelium, we surgically excised the entire limbal epithelium and surrounding conjunctival tissue up to 2 mm from the limbus from one eye, down to the bare sclera. The cultured HCJE sheets were transplanted onto the denuded ocular surface to completely cover the resected area and were sutured in place with 10-0 nylon (8–12 sutures per sheet). The graft was then covered with a soft contact lens secured with four peripheral anchoring sutures. Finally, tarsorrhaphy was performed with 6-0 nylon sutures (Fig. 1B). After surgery, the rabbits were treated with topical antibiotics (0.3% ofloxacin ointment; Santen Pharmaceutical Co., Ltd, Osaka, Japan), trimcinolone acetoni de (0.2 mL injected subconjunctivally; Bristol-Myers Squibb Co., Tokyo, Japan), and systemic antibiotics (10 mg gentamicin/rabbit, delivered intramuscularly [IM]; Nacalai Tesque Inc. Kyoto, Japan). They also received a daily IM injection of 0.2 mg/kg of the immunosuppressant agent FK506²³ (Astellas Co., Ltd., Tokyo, Japan) to inhibit a possible zonogeneic reaction or nonspecific inflammation.

Slit Lamp Examination

On the day of transplantation and on the 4th and 14th postoperative days, the ocular surface of the eight transplant recipients was examined and photographed with a slit lamp biomicroscope (SL-1600; Nidek Co., Ltd., Aithi, Japan).

Tissue Preparation

Engrafted tissues were removed from the eyes of eight rabbits killed 14 days after transplantation. In vivo conjunctival tissues, cultivated HCJE cells, and transplanted conjunctival tissues were divided into two portions, one of which was embedded in optimal cutting temperature 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 electron microscopy (EM).

Immunostaining and Light-Microscopic Analysis

Tissue sections (8 μm) were placed on glass slides and subjected to hematoxylin staining or indirect-immunostaining analysis. Briefly, the sections were fixed with Zamboni’s fixative or acetone (4°C, 5 minutes), immersed for 1 hour in blocking solution (1% BSA in 0.01M PBS), and treated with primary antibody solutions (Table 1) and normal goat IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as the negative controls. After a 1-hour incubation, the sections were washed with 0.01 M PBS and mounted with medium containing an anti-photobleaching reagent (3% Dabco; Wako Pure Chemical Industries Ltd., Osaka, Japan). Fluorescent images of the sections were inspected and photographed with a confocal laser scanning microscope (TCS-SP2; Leica, Tokyo, Japan). Unless otherwise stated, all incubations were at room temperature.

Transmission Electron Microscopic Examination

Specimens were fixed in 2.5% glutaraldehyde in 0.1 M PB, washed 3 times in PB, and postfixed for 1 hour in 2% aqueous osmium tetroxide. They were then passed through a graded ethanol series, transferred to propylene oxide, and embedded in Epon-812 (TAAB, Berkshire, England). Ultrathin sections were cut and stained with uranyl acetate and lead citrate before examination under a TEM (H-7000; Hitachi, Tokyo, Japan).
RESULTS

Analysis of HCjE Sheets

HCjE sheets, grown on AM for 3 weeks, manifested five to six layers of well-stratified epithelium (Fig. 2A, 2D) without goblet cells (Fig. 2C). Thus, they were similar to in vivo corneal epithelium (Fig. 2B). The TEM examination revealed many microvilli on the surface of the superficial cells (Fig. 2E), desmosomes at intercellular junctions (Fig. 2F), and hemidesmosomes on the basal side of the basal cells (Fig. 2G).

TABLE 1. Antibodies Used in the Study

<table>
<thead>
<tr>
<th>Group</th>
<th>Antigen</th>
<th>Dilution</th>
<th>Type of Antibody</th>
<th>Immunized Animal</th>
<th>Company*</th>
<th>Annotation</th>
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<tr>
<td>Putative stem cell markers</td>
<td>ABCG2</td>
<td>×40</td>
<td>(Mo)</td>
<td>M</td>
<td>Kamiya</td>
<td>ATP-binding cassette transporter</td>
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<td></td>
<td>p63</td>
<td>×100</td>
<td>(Mo)</td>
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<td>Cymbus</td>
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<td>Desmoplakin</td>
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<td>Nuclei</td>
<td>Human nuclei</td>
<td>×30</td>
<td>(Mo)</td>
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<td>Possible to distinguish human cells from other animal cells</td>
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<td>Cytokeratin</td>
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<td>CK4</td>
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<td>Major cytokeratin in nonkeratinizing mucosal epithelium</td>
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<tr>
<td></td>
<td>MUC5AC</td>
<td>×100</td>
<td>(Mo)</td>
<td>M</td>
<td>Novostra</td>
<td>Secreted mucin/goblet cell mucin</td>
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</tbody>
</table>

Mo, monoclonal; Po, polyclonal; M, mouse; G, goat.

* Kamiya: Kamiya Biomedical Company, Seattle, WA; Santa Cruz: Santa Cruz Biotechnology Inc., Santa Cruz, CA; Chemicon: CHEMICON International Inc., Temecula, CA; Symbus: Symbus Biotechnology LTD, Hampshire, UK; Progen: PROGEN Biotechnik GmbH, Heidelberg, Germany; Novostra: Novocastra Laboratories Ltd, Newcastle, UK; Zymed: ZYMED Laboratories Inc., South San Francisco, CA.

Figure 2. Histologic examination of HCjE cells grown on human amniotic membrane. Cultivated human conjunctival epithelium and in vivo corneal- and conjunctival epithelium were examined by light microscopy (A–C: semithin section stained with toluidine blue) or transmission electron microscopy (D–G). The cultivated epithelium was five to six layers thick (A, D) and exhibited typical microvilli (E), and desmosome (F) and hemidesmosome (G) formation.
Frozen sections of in vivo ocular tissues and cultivated HCjE were subjected to indirect immunostaining analysis. The basal cells of the cultivated HCjE sheets expressed the putative stem cell markers ABCG2 and p63 (Fig. 3Aa–Af); their expression patterns were almost identical with those of in vivo limbal epithelium. The hemidesmosome component proteins laminin5 and integrin α6β4 were restricted to the interface between the basal cells and the AM. Desmoplakin, a desmosome-associated protein, was expressed at cell–cell borders. These expression patterns were almost identical with those of in vivo HCjE (Fig. 3Bg–Bn).

Transplantation of Cultivated HCjE Sheets
Cultivated HCjE sheets were successfully transplanted onto the cornea of all eight rabbits. The transplanted conjunctival epithelium completely covered all corneas and remained transparent, smooth, and devoid of epithelial defects during the 2-week postoperative observation period (Fig. 4). The transplanted HCjE was well-maintained on the recipients’ corneal surface; there were no instances of graft retraction or dislodgement. The engrafted epithelium manifested five to six layers of stratified squamous epithelium, rendering it morphologically similar to normal corneal epithelium (Figs. 5A–D). We observed no goblet cells in the engrafted epithelium. As the grafts stained positive for the anti-human nuclei antibody that specifically reacts with human tissue,24,25 we were able to confirm that the epithelial cells on the rabbit corneas were of human origin (Fig. 5E).

Histologic and EM Appearance of the Engrafted Conjunctival Epithelium
The engrafted epithelium consisted of five to six well-stratified layers harboring cuboidal or columnar basal cells, winged suprabasal cells, and flattened squamous superficial cells (Fig. 6A). There were many microvilli on the surface of the superficial cells. Tight junction-like structures were present at the cell–cell border of the superficial cells (Fig. 6B), and desmosomes were at the intercellular regions of the epithelial cells (Fig. 6C). Hemidesmosomes were seen at the basal cell-AM substrate junction zone (Fig. 6D).
Immunohistochemistry

Although MUC4 and MUC5AC were expressed by HCjE in vivo (Figs. 7A, 7D), neither cultivated nor engrafted HCjE cells stained positive for MUC5AC (Figs. 7E, 7F). In vitro cultivated HCjEs did not express MUC4, but engrafted HCjE was found to express MUC4 (Figs. 7B, 7C). CK4/13, normally expressed in conjunctival epithelium, was present in the cultivated HCjE sheets (Figs. 7G–L). In vivo conjunctival epithelium contained a few CK3/12-positive cells, as did cultivated and engrafted HCjE (Figs. 7M–R).

DISCUSSION

We established a method for the culture of well-stratified conjunctival epithelium on human AM. The epithelial sheets we obtained exhibited high physical integrity, were well maintained after transplantation onto denuded rabbit corneas, and contributed to corneal transparency. Our results suggest that it may be possible to use these epithelial sheets for corneal epithelial replacement in patients with various ocular surface disorders.

It was initially intended in this study to culture rabbit conjunctival epithelial cells for transplantation onto rabbit corneas because this procedure is apparently free of undesirable xenogeneic rejection. However, the decision was made to transplant the cultivated HCjE sheets onto rabbit corneas for the following reasons. First, the optimal culture conditions for rabbit and human cells are reportedly different.15,26,27 Consider-
MUC4, one of the mucin core proteins secreted from the surface of in vivo conjunctival epithelium, was not expressed in the cultivated HGjE cells, although it was expressed in the engrafted HGjE. In rats fed a retinoic acid-depleted diet, the expression of mucin genes by the ocular surface epithelium was decreased. Therefore, it is possible that the cultivated HGjE failed to express MUC4 because the culture medium lacked this solute factor. Alternatively, retinoic acid present in rabbit tears may have led to the recovery of MUC4 expression in the engrafted HGjE.

MUC5AC was not found to be expressed in the goblet cells of conjunctival epithelium in either cultivated- or engrafted HGjE, although a series of contiguous sections were inspected. Considering the previous report that approximately 500 goblet cells exist in a 1-mm² section of conjunctival epithelium, 7500 goblet cells may exist in the initial period of cultivation. However, no goblet cells were identified, both in cultivated HGjE at the end stage of the culture and engrafted HGjE at 2 weeks after surgery. This suggests that our culture conditions did not support goblet cell differentiation in culture or after transplantation.

We recently reported that similar to corneal epithelial cells, many as 1% of conjunctival epithelial cells are CK3/12 positive. We postulate that the CK3/12-positive cells in the engrafted HGjE derived from the resected conjunctiva and were maintained in our culture system. We documented elsewhere that the expression of thrombospondin-1, an inhibitor of vascularization, was much higher in corneal than conjunctival epithelium. As the expression level of this gene by CK3/12-positive cells in the engrafted HGjE was similar to the level seen in corneal epithelium, it may contribute to the inhibition of corneal neovascularization.

In patients with unilateral chemical or thermal injury, the conventional repair by limbal autografts from the contralateral eye requires 3 to 6 hours, and this may inflict iatrogenic limbal stem cell deficiency on the donor eye. The transplantation of autologous cultivated limbal stem cells has yielded promising results and requires the harvest of much less tissue, thereby reducing the risk of iatrogenic injury to the donor eye. To treat bilateral ocular surface disorders such as SJS, our group has reported allogeneic transplantation more recently, autologous cultivated oral epithelial transplantation, as promising treatment options. We now add cultivated autologous conjunctival epithelial transplantation for corneal epithelial replacement as a promising new modality to treat severe ocular surface disorders. It may be safer than the conventional methods currently used, and immunologically, it is superior to allogeneic transplantation. From a cytological point of view, autologous conjunctival epithelium represents a better alternative than oral mucosal epithelium for corneal epithelial replacement.
ment. Because we were dealing with xenotransplantation, one of the limitations of this study is the short follow-up period of 14 days. With more prolonged follow-up, it may be that some conjunctival cells would differentiate into goblet cells and that progressive conjunctivalization and neovascularization would occur. More long-term studies are needed to investigate some of these questions.

In summary, ours is the first report that clearly demonstrates the potential of cultivated HCjE as an alternative tissue source for replacement of the corneal epithelium. Our animal study is a step toward the eventual transplantation of autologous cultivated HCjE to treat patients with ocular surface disorders, and studies are ongoing to resolve outstanding issues.

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