Ocular Surface Reconstruction Using Autologous Rabbit Oral Mucosal Epithelial Sheets Fabricated Ex Vivo on a Temperature-Responsive Culture Surface

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PURPOSE. Autologous stem cell transplantation for total limbal stem cell deficiency is immunologically preferable, to avoid allograft rejection. This study was undertaken to investigate the possibility of a novel tissue engineering approach for ocular surface reconstruction, using autologous oral mucosal epithelial stem cells expanded ex vivo on temperature-responsive cell culture surfaces.

METHODS. Rabbit oral mucosal epithelial cells cultured on temperature-responsive culture surfaces with mitomycin-C-treated 3T3 feeder cells for 2 weeks produced confluent epithelial cell sheets. Putative progenitor cell populations were estimated by colony-forming assays. Autologous transplantation of these cell sheets to surgically manipulated eyes was performed, and ocular surface reconstruction and cell phenotypic modulation were examined.

RESULTS. All cultured oral epithelial cells were nonenzymatically harvested as transplantable intact cell sheets by reducing culture temperature to 20°C. Oral epithelial cells were stratified in three to five cell layers more similar to corneal epithelium than to oral mucosal epithelium. Colony-forming assays and immunofluorescence for p63, β1-integrin, and connexin 43 indicated retention of viable stem and/or progenitor cell populations in cell sheets. Autologous transplantation to rabbit corneal surfaces successfully reconstructed the corneal surface, with restoration of transparency. Four weeks after transplantation, epithelial stratification was similar to that in the corneal epithelium, although the keratin expression profile retained characteristics of the oral mucosal epithelium.

CONCLUSIONS. Cell sheet harvest technology enables fabrication of viable, transplantable, tissue-engineered epithelial cell sheets that retain putative progenitor cells from autologous oral mucosal epithelial cells. Promising clinical capabilities for autologous tissue-engineered epithelial cell sheets for ocular surface reconstruction are indicated. (Invest Ophthalmol Vis Sci. 2005;46:1632–1639) DOI:10.1167/iovs.04-08183

The ocular surface is covered by conjunctival, limbal, and corneal epithelium, which are adjacent but stratified squamous and nonkeratinized epithelia with distinct phenotypes.1–5 These cell layers protect the eye from pathogenic microbes, foreign invasion, and dryness while providing optical advantages. The basal layer of the limbal epithelia located at the transitional zone between corneal and bulbar conjunctiva contains corneal epithelial stem cells1–5 that renew the corneal epithelium by generating transient amplifying (TA) cells that migrate outward from the limbal basal layer.6–10 Limbal epithelial-stem cells are often completely lost in patients who have severe trauma or eye disease, such as Stevens-Johnson syndrome, ocular pemphigoid, and thermal and chemical burns, so that adjacent conjunctival tissues cover the cornea, leading to corneal vascularization, opacification, and severe visual loss.11–15 Such pathologic events are considered to represent limbal stem cell deficiencies.

Corneal reconstruction in patients with limbal stem cell deficiencies can be facilitated by transplantation of limbal epithelial stem cells. Transplantation of autologous limbal epithelium has been a promising procedure for treating unilateral limbal stem cell deficiencies,2 but reconstruction of the ocular surface that has been affected bilaterally is a challenging problem. Transplantation of allogeneic limbal epithelium in the form of a keratolimbal allograft from cadaveric tissue10,14–15 or conjunctival limbal allografts from living-related donors16,17 can be performed in patients with bilateral deficiencies. These procedures, however, necessitate postoperative immunosuppression, and even after systemic immunosuppression with oral cyclosporine, the long-term success rate (>1 year) ranges from 20% to 60% (cadaveric donors)10,14–15 and 70% to 80% (living-related donors).16,17 Moreover, success rates tend to decrease gradually with time.

To improve the surgical outcome of ocular surface reconstruction, recent attempts have been made to fabricate corneal epithelial graft constructs ex vivo from expanded autologous limbal stem cells. Pellegrini et al.18 initially reported the clinical use of autologous cultivated corneal epithelium. Their work was followed by that of several groups who used different carriers, such as collagen,19 amniotic membranes,20–23 fibrin gel,24 and cross-linked gels of fibropectin and fibrin.25 We have recently obtained and clinically applied a new, carrier-free transplantable cell construct harvested from novel temperature-responsive cell culture surfaces, producing intact, transplantable, multilayered epithelial sheets.26,27 A temperature-responsive polymer, poly(N-isopropylacrylamide; PIPAAm), when covalently immobilized to surfaces of commercial cell culture dishes, facilitates cell adhesion, spreading, and growth in culture conditions at 37°C above the polymer's lower critical solution temperature of 32°C. When the culture temperature is reduced below that temperature, the surface rapidly

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al.30 have reported that the amniotic membrane remains intact of the amniotic membranes after grafting. Indeed, Cooper et

traction. The drawback is that little is known about the integration
membranes give the constructs easy handling for transplanta-
plantation) study in a rabbit model similarly using autologous
oral mucosal epithelial cells cultured on amniotic membranes
received these autografts have fully restored eyesight. It is im-
portant to note that transplanted cell sheets become increas-
hydrates and swells, prompting rapid and complete detach-
ment of adherent cells without the need for typical proteolytic
enzymes or EDTA treatments. Using this approach, we have
reported treatment of damaged cornea by transplantation of
these viable, bioengineered, corneal epithelial sheets ex-
for 1 year after cultivated corneal epithelial transplantation
using an amniotic membrane carrier.

In the present study, we transplanted autologous tissue-
engineered epithelial cell sheets fabricated from harvested oral
mucosal epithelium in a rabbit model. Keratin expression pro-
files were examined in the tissue-engineered cell sheets just
before and 4 weeks after transplantation and were compared
with those from native corneal and oral mucosal epithelia. Data
support the occurrence of phenotypic modulation of oral mu-
cosal epithelial cells.

**Materials and Methods**

**Preparation of Temperature-Responsive Cell
Culture Surfaces**

Specific procedures for the preparation of PIPAAm-grafted cell culture surfaces have been described. Briefly, N-isopropylacrylamide monomer (PIPAAm, kindly provided by Kohjin Co., Ltd, Tokyo, Japan) in 2-propanol solution was spread onto commercial cell culture inserts (Falcon 3090; BD Labware, Bedford, MA) and subjected to irradiation with a 0.3-MGy electron beam generated by an area beam electron processing system (Nissin High Voltage, Kyoto, Japan). After the PIPAAm-immobilized inserts were rinsed with cold distilled water to remove nonimmobilized PIPAAm, they were sterilized with ethylene oxide gas.

**Primary Culture of Oral Mucosal Epithelial Cells**

Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the experimental procedure approved by the Committee for Animal Research of Osaka University Medical School. Oral mucosal biopsy specimens (3 mm radius) were taken from New Zealand White rabbits (2.0 kg) under deep anesthesia induced by intramuscular injection of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (40 mg/kg). The biopsy specimens were washed three times with Dulbecco's

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**FIGURE 1.** Ocular surface reconstruction by autologous transplantation of tissue-engineered cell sheets fabricated from oral mucosal epithelial cells on temperature-responsive cell culture inserts. Oral mucosal tissues containing whole epithelial cell layers were excised from rabbit oral cavities and treated with dispase and trypsin-EDTA to obtain single epithelial cell suspensions. Cells were then seeded onto temperature-responsive culture inserts preseeded with mitomycin-C (MMC)-treated 3T3 cell feeder layers. Cultured cells were harvested as a cell sheet by reducing culture temperature and transplanted onto keratectomized corneal surfaces.

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**FIGURE 2.** Transplantable carrier-free epithelial cell sheets. Oral mucosal epithelial cells grew to confluence within 1 to 2 weeks (a). After one additional week of culturing, all cells were harvested as intact cell sheets having three to five cell layers (b). These harvested oral mucosal epithelial cell sheets were similar in morphologic appearance to corneal epithelial cell sheets fabricated from limbal epithelial cells (c). From colony-forming assays of both primary (d) and secondary cells (e), the number of putative progenitor cells was determined (f). Scale bar, 50 μm.
phosphate-buffered saline (PBS) containing antibiotics and antimycotics. These specimens were then incubated at 4°C for 4 hours with Dispase II (Roche Diagnostics GmbH, Mannheim, Germany) and treated with trypsin-EDTA solution for 20 minutes at room temperature, to scatter the epithelial cells. The suspended cells were cultured on temperature-responsive culture inserts (CellSeed, Inc., Tokyo, Japan) at an initial cell density of $1.7 \times 10^5$ cells/23-mm insert with mitomycin C (MMC)-treated NIH/3T3 cells$^{32}$ separated by cell culture inserts (Fig. 1). One week later, oral epithelial cells reached confluence, and after an additional week of culturing, the resultant cell sheets were harvested by reducing the culture temperature to 20°C for 30 minutes.

Colony-Forming Assay
To evaluate putative progenitor cell populations in the biopsied and cultured cells, colony-forming assays were performed. Primary cells isolated from rabbit oral epithelial tissues were divided and seeded on normal commercial cell culture dishes (60 mm, Falcon 3002; BD Labware) for colony-forming assays (CFAs) and onto temperature-responsive culture inserts, to prepare and the harvest cell sheets. Secondary cells isolated from tissue-engineered cell sheets by trypsin digestion were also subjected to CFA. Culture conditions for both primary and secondary CFA were the same as that for cell sheet preparation except for the seeded cell density of $1 \times 10^4$ and $3 \times 10^4$ cells/dish (60-mm diameter), respectively. After 10 to 12 days in culture, cells were fixed and stained with rhodamine B. Colony formation was screened over the entire dish under a dissecting microscope. The primary colony-forming efficiency (CFE) was calculated by dividing the number of colonies per dish by the total number of cells initially seeded onto the dishes. The CFE for fabricated cell sheets (secondary cell CFE) was calculated by dividing the number of colonies per dish originating from cells dissociated from harvested cell sheets by the number of seeded cells ($n = 6$ duplicates of each sample). The number of putative progenitor cells within the cell population initially seeded onto each temperature-responsive culture insert to prepare cell sheets was obtained by multiplying the primary CFE by the number of cells seeded onto each temperature-responsive culture insert. Similarly, the number of putative progenitor cells within a harvested cell sheet was obtained by multiplying the secondary CFE by the total number of cells in each cell sheet harvested by reducing the culturing temperature, followed by trypsin digestion and counting.

Cultured Oral Epithelial Cell Sheet Transplantation
Tissue-engineered cell sheets fabricated from oral mucosal epithelial cells were autologously transplanted onto keratectomized ocular surfaces of the rabbit stem cell deficiency model$^{33}$ surgically prepared 3 weeks before transplantation. The rabbits were chosen randomly for epithelial cell transplantation or the sham surgery. Keratectomy was used to excise the entire corneal surface, including the limbus and the conjunctival tissue within 5 mm of the limbus, completely removing the corneal and limbal epithelium and exposing the stroma. Three weeks after keratectomy, conjunctival scar tissue with some neovascularization covered the entire corneal stromal surface, inducing severe corneal opacity. Before cell sheet transplantation, the conjunctivalized ocular surface was surgically removed to reexpose the native transparent corneal stroma. Then, tissue-engineered cultured cell sheets, fabricated ex vivo from autologous oral mucosal epithelial cells, were harvested by temperature reduction and transferred to a poly(vinylidene difluoride) (PVDF) support membrane (outer diameter, 25 mm, with a 16-mm hole in the center) and placed over the transparent stromal bed immediately (Fig. 1). Within 5 minutes, the cell sheets spontaneously produced stable attachment to the stroma, and the PVDF membranes were removed with scissors. For healing protection, the corneal surface was finally covered with a soft contact lens, and a tarsorrhaphy was performed. Antibiotics (0.3% ofloxacin) and steroids (0.1% betamethasone) were topically applied three times daily after transplantation. The eyes were carefully observed with a slit lamp biomicroscope and recorded with serial photographs of the rabbit anterior ocular segment for 4 weeks. Control (sham) rabbits underwent all procedures but did not undergo transplantation. Four weeks after surgery, rabbits were killed with an overdose of anesthetic agent (pentobarbital) and eyes were enucleated for histology. Three ophthalmologists carefully examined the histologic slides in a masked fashion. All found corneal epithelial regeneration in each eye of the transplan-

**Figure 3.** Expression of progenitor cell markers in transplantable carrier-free epithelial cell sheets. Oral mucosal epithelial cell sheets were harvested from temperature-responsive culture dishes by reducing the temperature and then were subjected to immunofluorescence with anti-p63 (a), anti-β1 integrin (b), and anti-cx43 (c) antibodies. Every panel consists of three microphotographs: phase-contrast (top), immunofluorescence (middle), and the two images superimposed (bottom). Scale bar, 50 μm.
tation group, but did not observe such regeneration in the sham-operation group. We used 10 rabbits in each group.

Immunofluorescence

Native rabbit corneas, rabbit oral mucosa, tissue-engineered epithelial cell sheets harvested from dishes, and reconstructed ocular surfaces (4 weeks after grafting) were all examined by immunofluorescence. Cryosections (12-μm thick) were treated with 5% bovine serum albumin (BSA) in 50 mM Tris-buffered saline (TBS; pH 7.2) containing 0.4% Triton X-100 for 60 minutes at room temperature. Sections were then incubated overnight at 4°C with primary antibodies diluted with 1% BSA in TBS containing 0.4% Triton X-100. Primary antibodies included a rabbit polyclonal anti-cytokeratin 12 (K12; the kind gift of Winston W. Kao, University of Cincinnati, OH),34 mouse monoclonal anti-cytokeratin 3 (K3; AE5l Progen, Heidelberg, Germany), anti-cytokeratin 4 (K4; MP Biomedicals Inc. [formerly ICN], Irvine, CA), anti-cytokeratin 13 (K13; American Research Products, Inc., Belmont, MA), anti-cytokeratin 1 (K1; YLEM, Roma, Italy), anti-cytokeratin 10 (K10; Biomedica Corp., Foster City, CA), anti-connexin 43 (Chemicon, Temecula, CA), anti-β1 integrin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-p63 (4A4; Santa Cruz Biotechnology, Inc.). 4A4 was shown to recognize both p63 and ΔNp63.35 Negative control sections were incubated identically with normal mouse IgG or rabbit IgG. Rabbit limbal and corneal epithelia were used as positive control tissues. FITC-labeled secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) were used. Sections were costained with propidium iodide (Sigma-Aldrich, St. Louis, MO) and observed by confocal laser scanning microscopy (LSM-410; Carl Zeiss Meditec, Jena, Germany).

RESULTS

Fabrication of Tissue-Engineered Cell Sheets from Expanded Oral Mucosal Epithelial Cells

Mucosal epithelial cells isolated from the oral cavity were seeded on temperature-responsive culture surfaces. Cell colonies grew to confluence within a week (Fig. 2a). After an additional week of culture, tissue-engineered cell sheets were harvested by incubation at 20°C for 30 minutes, comprising three to five stratified, well-differentiated cell layers (Fig. 2b), showing a morphology similar to that of native corneal epithelium and corneal epithelial sheets fabricated from limbal stem cells and harvested by the same technique (Fig. 2c).

Colony-forming assays were used to estimate the number of putative progenitor cells within cell populations of both primary cultured cells used for cell sheet preparation and harvested cell sheets. From these sources, 3.38 ± 0.61 × 10³ and 1.31 ± 0.31 × 10³ cells (mean ± SE, n = 6) were determined, respectively (Figs. 2d–f). Harvested cell sheets were also examined by immunofluorescence. The putative marker of corneal epithelial and epidermal stem cells p63 and/or ΔNp63 was detected in the nuclei of the basal cell layers (Fig. 3a). β1 Integrin, suggested as a putative marker for stem cells as well as TA cells,37–39 was also expressed in basal layers of the cell sheets (Fig. 3b). Together, these results suggest that harvested cell sheets contain putative progenitor cells. Connexin 43 comprising gap junctions is expressed in epidermal basal cell layers, but is absent from limbal epithelial basal cells,5,11,40–42 and serves as a negative marker for epithelial stem cells.43 It was absent from some portions of basal cell
layers in cell sheets (Fig. 3c), implying that basal cells within harvested cell sheets contain putative progenitor cells.

**Autotransplantation of Tissue-Engineered Cell Sheets**

Autologous tissue-engineered cell sheets were successfully transplanted onto ocular surfaces in a rabbit corneal stem-cell-deficiency model, as previously reported for tissue-engineered corneal epithelial cell sheets fabricated from limbal stem cells, as well as tissue-engineered human epithelial cell sheets fabricated from autologous oral mucosal epithelial cells. Transplanted oral mucosal epithelial cell sheets readily resisted displacement under tension with forceps, implying stable adhesion to the corneal stroma. Damaged ocular surfaces in the model failed to repel fluorescein penetration into the stroma before cell sheet transplantation. However, corneal surfaces were completely protected from fluorescein penetration by grafted cell sheets immediately after transplantation (Fig. 4). During postsurgery healing, corneal transparency gradually improved, with corneal surfaces appearing fairly clear and smooth under slit lamp microscopy 7 days after transplantation (Fig. 4a). Within 4 weeks after transplantation, ocular surfaces were completely reconstructed with clarity and smoothness comparable to those of normal cornea, with faint or no observable defects. Control rabbits not receiving cell sheet transplantation showed no signs of corneal epithelialization at postoperative day 7. However, complete conjunctivalization was observed 2 weeks after surgery under a slit lamp (Fig. 4b). Rabbits were then killed, and the eyes were histologically evaluated 4 weeks after transplantation. Gaps between regenerated epithelium and stroma, underlying stromal vascularization, inflammatory cells, and goblet cells were not observed in corneas receiving cell sheet transplants (Fig. 5a). Reconstructed ocular surfaces resembled native corneas morphologically (Fig. 5b), significantly distinct from oral mucosa in epithelial thickness and surface smoothness (Fig. 5d). However, we observed heterogeneity in basal cell morphology in regenerated epithelium, because most of the basal epithelial cells displayed the characteristic cuboidal cell shape, whereas others were flat. In contrast, corneal surfaces in sham control rabbits that did not undergo cell sheet transplantation were covered with conjunctival cells containing numerous goblet cells, with vascularized stromal layers (Fig. 5c).

**Keratin Expression Profiles**

For analysis of cell phenotypic modulation in grafted oral mucosal epithelial cells after transplantation onto corneal stroma, we used keratin expression profiles compared among tissue-engineered cell sheets, reconstructed ocular surfaces (4 weeks after transplantation), normal oral mucosa, and normal cornea (Fig. 6; Table 1). K3, often used as a specific marker of corneal epithelial cells, was expressed in all epithelial layers in all four tissues. Another corneal-epithelium-specific marker, K12, was expressed only in normal corneal epithelia. K4 and K13 were expressed in all oral mucosal epithelial layers except basal cells. K4, however, was expressed only in superficial cells, and K13 expression was not detectable in normal cornea. Harvested cell sheets expressed K4 only in superficial cells, and expression was retained at 4 weeks after surgery, implying cell phenotypic modulation within the culture. In contrast, K13 was detectable in superficial cells, but undetectable 4 weeks after surgery, suggesting cell phenotypic modulation after cell sheet transplantation. Neither K1 nor K10, both classified as keratinized species, was detected in any epithelial layers.

**DISCUSSION**

We demonstrated that autologous tissue-engineered cell sheets fabricated from oral mucosal epithelium are effective substitutes for allogeneic limbal tissues in ocular surface reconstruction. The choice of the oral mucosal epithelium as a cell source...
for ocular surface reconstruction is based primarily on several considerations. First, oral mucosal epithelium in vivo expresses K3, also expressed by the corneal epithelium but not by epidermis.4,44 Cultured oral mucosal cells showed no obvious loss of nuclei or keratinization, whereas cultured epidermal cells showed extensive keratinization.45,46 The absence of both keratinization and a loss of nuclei in oral mucosal epithelial cell sheets encouraged us to use them to reconstruct corneal epi-

**TABLE 1. Results of Immunohistochemical Staining**

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Staining intensity: +, positive; -, negative. Location: B, basal; IM, intermediate; S, superficial.
epithelial cell sheets originating from limbal stem cells.27

Recently, Nakamura et al.29 reported a short-term (10 days after transplantation) study in a similar rabbit model using autologous oral mucosal epithelial cells cultured on amniotic membranes as a carrier to replace damaged corneal epithelium. They also examined keratin expression profiles, but lack immunofluorescence data for grafted epithelia and any consideration of progenitor cell populations. In the present study, we show evidence of the existence of putative progenitor cells in both oral mucosal epithelia and harvested cell sheets. Colony-forming efficiency for the primary oral epithelial cells is approximately 15% of that of rabbit limbal epithelium.27 Therefore, we seeded oral mucosal epithelial cells at five times higher cell density than limbal epithelial cells, to prepare transplantable epithelial cell sheets. Furthermore, autologous oral mucosal epithelial cells exhibit phenotypic modulation in culture before transplantation and after transplantation onto corneal stroma (Fig. 6, Table 1). Native oral mucosal epithelium on a substantia propria rich in blood vessels was much thicker than native corneal epithelium, and its surface was more irregular than the corneal surface (Figs. 5b, 5d). However, the reconstructed corneal surfaces showed a thin, smooth epithelium (Fig. 5a). Correlating with this histologic finding, keratin expression profiles for harvested oral mucosal epithelial cells were altered during the 4 weeks after transplantation onto corneal stroma (Fig. 6, Table 1). It was presumed that the grafted oral mucosal epithelial phenotype was modulated by the underlying stromal keratocytes, although further investigation is needed to elucidate the role. If so, temperature-responsive cell sheet harvest may be effective in enabling epithelial phenotypic modulation, since these cell sheets are directly transplantable onto corneal stroma in a carrier-free manner, and grafted oral mucosal epithelial cells immediately and directly interact with corneal stromal keratocytes without carrier interference. Hence, clinical outcome, including postoperative corneal transparency and graft survival after autologous transplantation of tissue-engineered oral mucosal epithelial cell constructs may be influenced by the choice of carrier.

In summary, we have demonstrated that tissue-engineered epithelial cell sheet grafts fabricated ex vivo from autologous oral mucosal epithelium can alter their phenotype, depending on their transplanted context. Our cell sheet fabrication approach, exploiting the use of new temperature-responsive culture surfaces, produces robust, viable, multilayered epithelial cell sheets without enzymatic processing, support substrates, or carriers, and promotes strong, rapid adhesion of transplanted sheets onto corneal stroma in vivo without the need for suturing. The transplantation of these epithelial sheets without any accompanying stromal components, as shown in this study, may provide a distinct therapeutic advantage. These results indicate a strong potential for the application of cultured oral mucosal epithelial cells in the reconstruction of ocular surfaces in bilateral diseases such as Stevens-Johnson syndrome and ocular pemphigoid, typically refractory to allograft transplantation.

References
