Barrier Function and Cytologic Features of the Ocular Surface Epithelium After Autologous Cultivated Oral Mucosal Epithelial Transplantation

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Objective: To determine the barrier function and cytologic features of ocular surface epithelium after autologous cultivated oral mucosal epithelial transplantation in a prospective observational study.

Methods: The status of the epithelium in 4 eyes with limbal stem cell deficiency was studied preoperatively and postoperatively. We used an impression method to determine the cytologic features and anterior fluorophotometry to determine barrier function.

Results: Impression cytology showed nonkeratinized, squamous, polygonal, cohesive cells with a low nuclear to cytoplasmic cell ratio and no goblet cells, corresponding to cultivated oral mucosal epithelium, at up to 16 months after surgery. In some cases, the epithelium displayed a mixture of oral mucosal and conjunctival epithelium, especially in cases with a longer postoperative period. Central epithelial permeability remained persistently high throughout the follow-up period, regardless of the epithelial phenotype.

Conclusions: Cultivated oral mucosal epithelial cells were observed to survive for more than 1 year after transplantation, with gradual replacement by conjunctival epithelium in some cases. Decreased barrier function of the transplanted epithelium may have prognostic implications, suggesting the presence of oral mucosal epithelium long after surgery.

CULTIVATION OF ORAL MUCOSAL EPITHELIAL CELLS

All patients received guidance on oral hygiene and treatment of tooth decay before biopsy. After sterilizing the oral cavity, inferior buccal mucosa was excised using an 8-mm punch biopsy tool (KAI Industries Co, Ltd, Gifu, Japan) while the patient was under local anesthesia. We dissected the specimens and used scissors to remove submucosal connective tissues. The mucosal epithelium was cut into small pieces, and then washed several times to remove blood and adipose tissue in sterile phosphate-buffered saline solution free of calcium and magnesium ions. Specimens were then submerged in a mixture of Dulbecco Modified Eagle Medium and Ham F12 at a ratio of 1:1 (Invitrogen Corporation, Grand Island, New York) with 10% fetal bovine serum, 3-µg/mL gentamicin (Invitrogen Corporation), and 0.25-µg/mL amphotericin B (Sigma-Aldrich Corp, St Louis, Missouri). The basal cells of the oral mucosal epithelial cells were harvested after enzymatic treatment with 0.81U of a grade II neutral protease (Dispase II; Roche Diagnostics, Indianapolis, Indiana) at 4°C for 5 hours, and a solution of 0.05% trypsin and 0.33mM EDTA (Invitrogen Corporation) at room temperature for 10 minutes. The cell suspension was washed and filtered through nylon mesh (BD Biosciences, Bedford, Massachusetts) to remove debris and small pieces of residual material in the Dulbecco Modified Eagle Medium–Ham F12 medium (1:1 mixture) with 10% fetal bovine serum. A single-cell suspension of basal cells from oral mucosal epithelium was resuspended in conditioned medium for oral mucosal epithelium (ArBlast Co, Ltd, Kobe, Japan). The suspension was then seeded (range, 1.0 × 10^5 cells/well) onto human dermed amniotic membrane on the bottom of the culture plate inserts in a 6-well plate (Corning Inc, Corning, New York) containing mitomycin (mitomycin C) (Sigma-Aldrich Corp) treated with 3T3 fibroblasts (2.0 × 10^6 cells/cm^2). The culture was submersed in medium for 2 weeks and exposed to air by lowering the level of the medium at the end of the culture period.15

POSTOPERATIVE MANAGEMENT

Preservative-free 0.1% betamethasone (Rinbeta PF; Nitten, Nagoya, Japan) and levofloxacin (Cravit; Santen Pharmaceutical Co, Osaka, Japan) were instilled 5 times a day postoperatively, and the doses were tapered over several months. Preservative-free artificial tears, 0.1% or 0.3% preservative-free hyaluronate sodium (Hyalomin-Mini; Santen Pharmaceutical Co), and autologous serum eyedrops were used for epithelial management. All patients received systemic betamethasone (Rinderon; Shionogi, Osaka, Japan), 2 mg/d, to reduce postoperative inflammation; the dose was then tapered over the next 2 weeks. No local or systemic immunosuppressants were prescribed during follow-up.

IMPRESSION CYTOLOGY

Impression cytology was performed after administration of topical anesthesia with 0.4% benoxinate hydrochloride (oxybuprocaïne). Strips of cellulose acetate filter paper (Millipore Corp, Bedford, Massachusetts) were placed on the central cornea and/or the periphery of the transplanted epithelial sheet with a glass rod. The specimens were then fixed with 10% formaldehyde. Specimens were stained with periodic acid–Schiff–hematoxylin agent (Muto Pure Chemicals Co, Ltd, Tokyo, Japan) that had been dehydrated in ascending grades of ethanol and then xylol, immersed in a 1% alcoholic osmic acid (Wako Chemicals, Osaka, Japan), and postfixed in 1% osmium tetroxide solution (Wako Chemicals). The same researcher (M.D.), masked to clinical information, examined the specimens for the presence of goblet cells, keratinization of epithelium, and mucin pickup.19 Impression cytology was also performed on the inferior buccal mucosa from a healthy donor. The specimens were then fixed with 10% formaldehyde solution of 0.05% trypsin and 0.53mM EDTA (Invitrogen Corporation, Grand Island, New York) at 4°C for 5 hours, and a solution of 0.05% trypsin and 0.33mM EDTA (Invitrogen Corporation) at room temperature for 10 minutes. The cell suspension was washed and filtered through nylon mesh (BD Biosciences, Bedford, Massachusetts) to remove debris and small pieces of residual material in the Dulbecco Modified Eagle Medium–Ham F12 medium (1:1 mixture) with 10% fetal bovine serum. A single-cell suspension of basal cells from oral mucosal epithelium was resuspended in conditioned medium for oral mucosal epithelium (ArBlast Co, Ltd, Kobe, Japan). The suspension was then seeded (range, 1.0 × 10^5 cells/well) onto human dermed amniotic membrane on the bottom of the culture plate inserts in a 6-well plate (Corning Inc, Corning, New York) containing mitomycin (mitomycin C) (Sigma-Aldrich Corp) treated with 3T3 fibroblasts (2.0 × 10^6 cells/cm^2). The culture was submersed in medium for 2 weeks and exposed to air by lowering the level of the medium at the end of the culture period.15

MEASUREMENT OF PERMEABILITY TO FLUORESCIN

Fluorescein permeability was measured to determine the barrier function of the transplanted epithelium by using a slitlamp.
fluorophotometer (Anterior Fluorophotometer FL-500; Kowa Co Ltd, Tokyo) as described previously.20 This procedure was performed before surgery, and again during the 1- to 3-month, 6- to 9-month, and 12- to 16-month follow-up periods after surgery. First, intensity of background fluorescence (autofluorescence) was measured 10 times at the central area (0.3/0.5 mm) of the grafted cornea, and the average was calculated. We applied 3 µL of 0.5% fluorescein in sterile isotonic sodium chloride solution (5 mg/mL) to the lower conjunctival sac, making sure that no physical contact occurred during the procedure. Ten minutes later, the ocular surface, including the lower tarsal conjunctiva, conjunctival sac, cornea, bulbar conjunctiva, and upper tarsal conjunctiva, were washed with 20 mL of sterile saline solution. After another 20 minutes, intensity of fluorescence at the same central area was measured, and the average intensity was calculated. The mean background value was subtracted from this mean value. The counts obtained were converted into fluorescein concentrations using calibration lines (range, 0-5000 ng/mL) incorporated into the software.

**PERMEABILITY OF EPITHELIAL SHEET TO HORSE RADISH PEROXIDASE**

Cultivated oral mucosal epithelial sheets were rinsed with phosphate-buffered saline solution, and then incubated in phosphate-buffered saline solution with 100-mg/mL horseradish peroxidase (HRP) (Wako Pure Chemical Industries, Ltd, Osaka, Japan) for 45 minutes at room temperature. After rinsing twice again with phosphate-buffered saline solution, the epithelial sheets were fixed in 2.5% glutaraldehyde solution (Tabb Laboratory Equipment Ltd, Berkshire, England) overnight. After fixation, the epithelial sheets were rinsed and visualized with 3,3’-diaminobenzidine tetrahydrochloride (Vector Laboratories, Burlingame, California) as a substrate for 10 minutes. Finally, they were rinsed with deionized water, and paraffin sections were prepared for histochemical analysis.

**RESULTS**

**CLINICAL FINDINGS**

Before surgery, all 4 patients had chronic bilateral total LSCD, accompanied by highly vascularized conjunctival tissue on the cornea, stromal opacity, or symblepharon (Table 1 and Figure 1A). After COMET, all 4 patients showed a stable ocular surface, with no epithelial defects, decreased neovascularization and fibrotic tissues on the cornea, and no symblepharon (Table 2 and Figure 1B and C). No complications such as persistent epithelial defects, recurrence of symblepharon, or infection were observed, except in the patient in case 1, in whom increased intraocular pressure was successfully managed by antiglaucoma medication. No adverse effects of postoperative medication were recognized during follow-up. Postoperative visual acuities did not improve dramatically after COMET because of residual stromal opacity. Patients 1 and 3 underwent keratoplasty at 19 and 6 months, respectively, after COMET, resulting in marked improvement in visual acuity (20/40 and 20/30, respectively). No epithelial problem or immunological rejection was observed.

**IMPRESSION CYTOLOGY**

Cytologic evaluation of the superficial oral mucosal layer showed nonkeratinized squamous, polygonal, cohesive cells...
with a low nuclear to cytoplasmic cell ratio, and no goblet or inflammatory cells. Mucosal epithelial cells were larger than those of the conjunctival epithelium (Figure 2A). The normal conjunctival epithelial cells were small, round, and compact, with scanty, eosinophilic-staining cytoplasm, and their large nuclei yielded a nuclear to cytoplasmic cell ratio of 1:1 to approximately 1:2. Goblet cells were abundant (Figure 2B). The superficial layer of the cultivated oral mucosal epithelial sheet showed larger nuclei, resulting in a decrease in the nuclear to cytoplasmic cell ratio compared with normal conjunctival epithelium (Figure 2C).

Representative results of impression cytology on peripheral transplanted tissue showed oral mucosal epithelium and conjunctival epithelium in the same area (Figure 3B). Oral mucosal epithelium transplanted onto the ocular surface shared the same characteristics as the cultivated oral mucosal epithelial sheet. The conjunctival epithelium at the transplantation site was also similar in appearance to normal conjunctival epithelium. These cells were small, round, and compact in the presence of goblet cells (Figure 2B and C and Figure 3B).

Longitudinal changes in the cytologic features of case 1 are shown in Figure 3. Before surgery, the epithelium showed a total loss of goblet cells with mild keratinization. B. Peripheral region of the transplantation site 4.5 months after surgery shows a mixture of oral mucosal epithelium (*) and conjunctival epithelium with goblet cells (arrows). C. Center of the transplantation site at 8 months after surgery shows only the cytologic features of oral mucosal epithelium. D and E. Center of the transplantation site 12 months after surgery shows cytologic features of oral mucosal epithelium (D) and conjunctival epithelium with abundant goblet cells (E) (periodic acid-Schiff-hematoxylin, original magnification ×200).

Barrier function after COMET

Preoperative values for permeability to fluorescein were high in each case, ranging from 700 to 2700 ng/mL. Postoperative values were persistently higher (Table 4). No correlation was seen between permeability to fluorescein and the results of cytologic analysis (Tables 3 and 4). We also assessed the permeability of the cultivated
oral mucosal epithelial sheet to HRP, which has a larger molecule size (40 kDa) than does fluorescein dye (344 Da). Horseradish peroxidase proteins were detected at the apical surface of the superficial layer of the epithelial sheet but not inside the epithelial layer (Figure 4).

**COMMENT**

Autologous cultivated oral mucosal epithelial transplantation has been reported as a new and useful reconstruction procedure for the ocular surface. To maintain a stable ocular surface after surgery, it is important to know how long the transplanted epithelium will survive. In this study, we confirmed the presence of transplanted oral mucosal epithelium on the cornea after COMET. Furthermore, the transplanted cells were observed more than 1 year after surgery. We used impression cytology to assess the cytologic features of the normal oral mucosa, cultivated oral mucosal epithelium, and transplanted epithelium. These samples shared mutual cytologic features, including nonkeratinized, squamous, polygonal, cohesive cells with a low nuclear to cytoplasmic cell ratio and no goblet cells, as described by Aguilar et al. Because of these cytologic characteristics, it was not difficult to differentiate transplanted oral mucosal epithelium from conjunctival epithelium.

A fundamental function of epithelium is to act as a barrier between the external environment and ocular tissue. We used anterior fluorophotometry to determine barrier function to fluorescein dye in the corneas after COMET. Permeability after COMET was very high (range, 2300 to >5000 ng/mL) during the follow-up period compared with that of conjunctival epithelium (700 to approximately 1400 ng/mL) or that of eyes with severe punctate corneal staining (Table 4). The values were persistently high, despite longitudinal changes in cytologic features.

In general, the barrier function of the epithelium reflects the structural integrity of the tissue. Oral mucosa does not have uniform thickness but shows regional variation and consists of keratinized and nonkeratinized squamous epithelium. Nonkeratinized epithelium in the oral cavity is a highly permeable tissue, and its barrier function depends on its thickness. Areas of single-layer epithelium in the oral cavity, such as the sublingual area, are capable of absorbing drugs. In this study, oral mucosal tissue was harvested from a nonkeratinized region, and only 4 to 6 layers of the epithelium were reconstructed on the amniotic membrane (data not shown). It is possible that the barrier function of cultivated oral mucosal sheets cannot be maintained at this thickness. Highly permeable transplanted epithelium may, therefore, suggest the presence of oral mucosal epithelium.

Low barrier function may indicate susceptibility to infection. However, infection after COMET was outside the scope of this study. We found that HRP, which has a molecular weight of 40 kDa, did not penetrate the superficial layer of the cultivated oral mucosal epithelium (Figure 4). This suggests that cultivated oral mucosal epithelium has sufficient barrier function to prevent invasion by pathological organisms. Avoidance of long-term use of immunosuppressants may be a more important factor in reducing the risk of postoperative infection. Nonetheless, decreased barrier function after COMET may influence the penetration rates of small molecules such as those contained in eyedrops.

Segmental ingrowth of conjunctival epithelium with goblet cells was observed in some cases (Figure 3B). The regenerating conjunctival epithelium showed decreased squamous metaplasia and increased goblet cell density compared with the preoperative findings (Figure 3A and E). Because this regenerating conjunctiva was associated with

**Table 3. Evaluation of Transplantation Site by Impression Cytology**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>After Transplantation, mo</th>
<th>3-5</th>
<th>6-9</th>
<th>12-16</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oral mucosal epithelium</td>
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<td>Oral mucosal epithelium/ conjunctival epithelium</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Conjunctival epithelium</td>
</tr>
<tr>
<td>3</td>
<td>Oral mucosal epithelium/ conjunctival epithelium</td>
<td>Oral mucosal epithelium</td>
<td>NA b</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Oral mucosal epithelium</td>
<td>Oral mucosal epithelium</td>
<td>Oral mucosal epithelium</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: NA, not applicable.

a Before autologous oral mucosal epithelial transplantation, the patients in cases 1 through 3 exhibited conjunctival epithelium cells in the central cornea; the patient in case 4 had conjunctival epithelium cells in the central cornea before autologous cultivated limbal epithelium transplantation.

b Penetrating keratoplasty was performed 6 months after transplantation.

**Table 4. Fluorescein Permeability of Transplanted Cornea**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Before Transplantation</th>
<th>After Transplantation, mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1-3</td>
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<td>2</td>
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<tr>
<td>4</td>
<td>2715.5</td>
<td>4905.8</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not applicable.

a Data are expressed as fluorescein units (nanograms per milliliter).

b Indicates beyond the limitation of measurement.

Penetrating keratoplasty was performed 6 months after transplantation.
low fibrosis and vascularization, the ocular surface remained stable with some transparency. The cytologic improvement of the conjunctival epithelium seen in our study may have been due to reduced inflammation or presence of a suitable substrate amniotic membrane, as previously reported. In addition, possible mucin expression changes after COMET may contribute to the improvement of the ocular surface status.

There were some limitations in the present study arising from factors such as variation in the original disease, tear function, lid abnormality, trichiasis, and meibomian gland function. This variation may have influenced the survival and phenotypic changes in the transplanted epithelium after COMET. In addition, the follow-up periods were relatively short: 2 eyes underwent corneal transplantation for visual recovery at 16 and 9 months after COMET (Table 1). Therefore, further studies with a larger number of cases and a longer follow-up period are necessary to clarify these factors.

In conclusion, impression cytology findings confirmed that transplanted oral mucosal epithelium survived longer than 1 year after COMET. Although increased permeability to fluorescein dye was demonstrated by anterior fluorophotometry, the transplanted epithelium appeared to retain barrier function against large molecules.

Submitted for Publication: May 2, 2007; final revision received June 13, 2007; accepted June 14, 2007.

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Financial Disclosure: None reported.

Funding/Sponsor: This study was supported by Grant-in-Aid for Young Scientists (B) KAKENHI 17791259 from the Japan Society for the Promotion of Science and by a grant from the Advanced and Innovative Research Program in Life Sciences of the Ministry of Education, Culture, Sports, Science and Technology.

Additional Contributions: Morio Tonogi, DDS, Kazunari Higa, PhD, and Fumito Morito provided the oral mucosal tissue samples and cultivation of the oral mucosal epithelium in this study. Jeremy Williams, of Tokyo Den-