Fabrication and Validation of Autologous Human Oral Mucosal Epithelial Cell Sheets to Prevent Stenosis after Esophageal Endoscopic Submucosal Dissection

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Key Words

Cell culture • Human oral mucosal epithelial cells •
Esophageal squamous cell carcinoma • Flow cytometry

Abstract

Objectives: Human oral mucosal epithelial cells derived from 7 healthy volunteer donors were cultured in a clean room in a cell-processing center (CPC) according to good manufacturing practice guidelines. Cell culture and fabricated transplantable epithelial cell sheets were validated for treating ulcers after endoscopic mucosal dissection. **Methods:** The clonal growth and morphology of the human oral mucosal epithelial cells seeded on temperature-responsive surfaces were observed. During the cultivation, sterilization tests were performed to validate the environment in the CPC. To validate the purity and morphology of fabricated epithelial cell sheets, cell sheets harvested from temperature-responsive surfaces by temperature reduction were examined by histology and flow cytometry. **Results:** Human oral mucosal epithelial cells were successfully cultured and harvested as continuous cell sheets from temperature-responsive culture inserts without any animal-derived materials. During the cultivations, the sterile environment in the

CPC was confirmed. The results of histological and flow cytometry analysis showed the high reproducibility of stratification and the purity of the fabricated human oral mucosal epithelial cell sheets. **Conclusions:** The method for fabricating epithelial cell sheets shown in this study was suitable for the validation for clinical trials and suggested usability of the fabricated cell sheets.

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Introduction

With a progression of the culture method for epidermal keratinocytes, cultured cell grafts are used for the clinical treatment of severe burns [1], ulcers [2] and giant congenital nevi [3]. These cultured keratinocytes are cocultured with a mouse 3T3 feeder layer developed by Rheinwald and Green, and the cultured keratinocytes form stratified squamous epithelium with tonofilaments and desmosomes [4]. Moreover, the 3T3 feeder layer permits cultured human epidermal keratinocytes to subculture more than 150 passages [5]. These perceptions indicate the importance of the 3T3 feeder layer for promoting cell growth and differentiations and, at least in part, main-

taining stem and/or progenitor cells of keratinocytes in vitro. In a similar way to epidermal keratinocytes, human oral mucosal epithelial cells are also used for treating gingival defects [6], skin defects [7] and corneal reconstruction [8], and these previous studies indicate the usability of cultured oral mucosal epithelial cells for clinical therapies.

Aside from the 3T3 feeder layer methods, fetal bovine serum (FBS) is conventionally used as a supplement for culture medium. Although these materials derived from animals are useful for cell culture in laboratory-grade experiments, it would be better to eliminate the materials from cultures as a condition for clinical use to prevent the possibility of unknown pathogen transmission and infection. Additionally, human embryonic stem cells cultured in medium containing FBS show expression of immunogenic nonhuman sialic acid [9]. In our previous studies, a culture method for oral mucosal epithelial cells was developed without using a 3T3 feeder layer and FBS, and the culture epithelial cells formed stratified epithelial cell grafts harvested from temperature-responsive culture surfaces [10–13]. This culture method can be useful to permit the cultured epithelial cells to be used as autologous epithelial cell graft for clinical therapies because there is no animal-derived material involved.

A possibility is indicated of applying fabricated oral mucosal epithelial cell sheets in the treatment of esophageal ulcer surfaces after endoscopic submucosal dissection (ESD), for promoting wound healing and re-epithelialization and preventing esophageal stenosis [14]. Although ESD is a less invasive therapy than open surgery for removing early-stage cancerous lesions from the gastrointestinal tract [15–18], stenosis is a major complication in the esophageal tract after ESD [19]. For this reason, formulae of culture media for epithelial cells were developed by using pharmaceutical-grade ingredients [20], and it was confirmed that human oral mucosal epithelial cell sheets cultured in the medium attached to the esophageal ulcer surface of the animal model [21].

In this study, human oral mucosal epithelial cells derived from 7 healthy volunteers were cultured and harvested from temperature-responsive culture surfaces to fabricate transplantable epithelial cell sheets in a cell-processing center (CPC) according to the culture methods documented in standard operational procedures (SOP). During the operation, the environment of the CPC was validated, and the cultured human epithelial cell sheets were also validated to indicate their adequacy for applying the cultured cell grafts to treat ulcers after ESD.

Materials and Methods

Validation Test of the CPC

The preparation of human oral mucosal epithelial cells and the fabrication of the epithelial cell sheets were performed in a good manufacturing practice-grade CPC (fig. 1). The cleanliness of the CPC was continuously monitored with an aerosol particle counter, and the number of aerosol particles was controlled by air-conditioning systems using HEPA filters. Atmospheric pressures were designed and controlled for each room in the CPC. Sterilization tests were performed 16 times over 8 months during the preclinical study as a validation to confirm that the CPC maintained an aseptic environment. Floor and equipment wipes in the CPC were inoculated on agar media for detecting adherent bacteria and fungi.

Preparation of Culture Medium

Keratinocyte culture medium (KCM) was prepared to culture human oral mucosal epithelial cells. KCM was composed of a basal mixture of 3 parts Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, Mo., USA) and 1 part nutrient mixture Ham's F-12 medium (Sigma), and supplemented with 2 nM triiodothyronine (Wako Pure Chemicals, Osaka, Japan), 5 µg/ml insulin (Eli Lilly, Indianapolis, Ind., USA), 10 ng/ml epidermal growth factor (Higeta Shoyu, Chiba, Japan), 0.4 µg/ml hydrocortisone (Kowa Pharmaceuticals, Tokyo, Japan), 1 nM cholera toxin (List Biological Laboratories, Campbell, Calif., USA), 0.25 mg/ml amphotericin B (Bristol-Myers Squibb, New York, N.Y., USA), 4 or 40 µg/ml gentamicin (Schering-Plough, Kenilworth, N.J., USA), and 5% FBS (Moregate BioTech, Bulimba, Qld., Australia) or autologous human serum (AHS).

Isolation of Oral Mucosal Tissues

All animals were treated in accordance with experimental procedures approved by the Committee for Animal Research of the Tokyo Women's Medical University. Swine oral mucosal tissues were taken, under deep anesthesia, from the buccal cavities of 6-month-old male pigs weighing approximately 20 kg. The animals received intramuscular injections of ketamine hydrochloride (5 mg/kg) and medetomidine hydrochloride (0.2 mg/kg), and an intravenous injection of propofol (1 mg/kg). An endotracheal tube was then inserted, and anesthesia was maintained using sevoflurane and nitrous oxide inhalation. The use of human oral mucosal tissues from volunteer donors was approved by the Institutional Review Board of the Tokyo Women's Medical University. Oral and written informed consents were obtained from healthy volunteers. Four volunteers (ID 0001, 0002, 0003 and 0004) kindly donated mucosal tissue twice. After a volunteer's oral cavity had been sterilized with topical povidone-iodine under local anesthesia with xylocaine (AstraZeneca, Osaka, Japan), 5- or 6-mm (i.d.) punch biopsies of oral mucosa were surgically excised from the interior buccal mucosal epithelium, and the wounds were then sutured.

Preparation of Oral Mucosal Epithelial Cells

Instruments for preparing oral mucosal epithelial cells are shown in figure 2a. Biopsy specimens were sterilized with povidone-iodine and 3 times with DMEM containing 57 µg/ml ampicillin and 100 µg/ml streptomycin (fig. 2b). This operation was repeated twice. Sterilized oral mucosal tissues (fig. 2c) were divided into quarters (fig. 2d) and treated with 1,000 U/ml of dispase (Godo Shusei, Tokyo, Japan) for 2 h at 37°C. The oral muco-

Fig. 1. The design of the CPC. Atmospheric pressure (Pa) and cleanliness classes of each room in the CPC are shown. Numbers of aerosol particles in class 100, 10⁴ and 10⁵ areas were kept less than 100, 10⁴ and 10⁵ particles/ft³, respectively.

Fig. 2. Preparation of oral mucosal epithelial cells (swine oral mucosal tissues were used). **a** Instruments for preparing epithelial cells. **b** Petri dishes for sterilization and washing of oral mucosal tissues. Povidone-iodine (left dish) was used for sterilization, and DMEM, containing ampicillin and streptomycin, was used for washing the tissues. **c** Swine oral mucosal tissues taken by punch

biopsy after sterilization and washing. **d** The mucosal tissues were divided into quarters before treatment with 1,000 U/ml of dispase. **e** Swine oral mucosal epithelia were separated from the substantia propria with surgical forceps after treating with dispase. **f** Oral mucosal epithelia minced by surgical microscissors before treatment with 0.25% trypsin-0.1% EDTA.

Table 1. Validation of the CPC

Tested area	Total colony		Average
Gowning room 1			
Floor (near entrance)	bacteria	43	2.7 ± 2.6
	fungi	0	
Floor (near exit)	bacteria	14	0.88 ± 1.7
	fungi	0	
Gowning room 2			
Floor (near entrance)	bacteria	10	0.63 ± 1.1
	fungi	0	
Floor (near exit)	bacteria	3	0.19 ± 0.75
	fungi	0	
Culture room			
Safety cabinet (inside table)	bacteria	0	0
	fungi	0	
Safety cabinet (outside lug)	bacteria	0	0
	fungi	0	
Floor (in front of desk)	bacteria	0	0
	fungi	0	
Microscope (eyepiece lens)	bacteria	0	0
	fungi	0	
Floor (near exit)	bacteria	0	0
	fungi	0	

Sterilization tests were performed from June 6, 2007, to February 21, 2008 (n = 16).

sal epithelia were separated from the underlying substantia propria using surgical forceps (fig. 2e). The epithelia were minced by surgical microscissors (fig. 2f) and treated with 0.25% trypsin-0.1% EDTA for 20 min at 37°C for preparing the epithelial cell suspensions. Disaggregated human oral mucosal epithelial cells were filtered through a cell strainer (40 µm, BD Biosciences, Franklin Lakes, N.J., USA) and subjected to epithelial cell culture.

Colony-Forming Assay

Primary human oral mucosal epithelial cells in KCM including 5% FBS or AHS were seeded at a density of 68 cells/cm² and cocultured with NIH-3T3 treated with mitomycin C for detecting clonal growth of seeded epithelial cells. Colony-forming efficiency was calculated by dividing the observed number in the colony with the initial cell number.

Fabrication of Human Oral Mucosal Epithelial Cell Sheets

Primary human oral mucosal epithelial cells in KCM including 5% FBS or AHS were seeded on temperature-responsive cell culture inserts (CellSeed, Tokyo, Japan) at a density of $4-8 \times 10^4$ cells/cm², and cultured for 2 weeks at 37°C in a humidified atmosphere containing 5% CO₂. The morphology of cultured mucosal epithelial cells was observed under a phase-contrast microscope (TE300, Nikon, Tokyo). After the cultivation, cultured human oral mucosal epithelial cells were harvested from temperature-responsive cell culture inserts by reducing the temperature to 20°C for 30 min and using surgical forceps (fig. 3a).

Validation Tests of Fabricated Human Oral Mucosal Epithelial Cell Sheets

Harvested human oral mucosal epithelial cell sheets (fig. 3b) were divided into 4 parts for validation tests (fig. 3c, d). One portion of divided cell sheets was fixed with 10% neutral-buffered formalin and routinely processed into 3-µm-thick paraffin-wax-embedded sections. Hematoxylin and eosin staining was performed by conventional methods. The other portion of divided cell sheets was treated with 0.25% trypsin-0.1% EDTA for 20 min at 37°C and filtered through a 40-µm cell strainer. Suspended cultured oral mucosal epithelial cells were fixed and permeabilized with a BD Cytotfix/Cytoperm kit (BD Biosciences) in accordance with the manufacturer's protocol provided with this reagent for treatment with FITC-conjugated anti-pan-cytokeratin antibody (Progen, Heidelberg, Germany) for 1 h at room temperature. Before flow cytometry analysis, treated epithelial cells were washed with BD Perm/Wash buffer (BD Biosciences) in accordance with the manufacturer's protocol, along with the BD Cytotfix/Cytoperm Kit. Flow cytometry analysis was performed with EPICS XL EXPO32 (Beckman Coulter, Palo Alto, Calif., USA).

Results

Validation of CPC

During this preclinical study (8 months), sterilization tests had been performed in gowning room 1, gowning room 2, and the cell culture room of the CPC 16 times (fig. 1). The results are shown in table 1. Adherent bacteria were detected on the floors of gowning rooms 1 and 2, but there was no detection of bacteria or fungi in the cell culture room.

Preparation of Human Oral Mucosal Epithelial Cells

Human oral mucosal tissues were taken from 7 healthy volunteers and used for fabricating human oral mucosal epithelial cell sheets similar to figure 2 shown by swine tissues. To confirm reproducibility, the total numbers of isolated epithelial cells were counted and divided by the total area of oral mucosal tissues taken by punch biopsy (table 2). The average of collected cells per size of tissue (in cm²) was $(12 \pm 3.3) \times 10^5$ cells/cm².

Cultivation of Human Oral Mucosal Epithelial Cells

A colony-forming assay of primary human oral mucosal epithelial cells was performed to confirm the efficiency of AHS informing colonies of epithelial cells. FBS was used as a positive control serum. Human oral mucosal epithelial cells cocultured with the 3T3 feeder layer were able to form colonies regardless of the serum source (fig. 4a, b), and there was no significant difference between FBS and AHS for promoting the colony formation of human oral mucosal epithelial cells (n = 7) (fig. 4c).

Fig. 3. Validation tests of cultured human oral mucosal epithelial cell sheets fabricated in conformity with good manufacturing practice. **a** Human oral mucosal epithelial cells cultured on temperature-responsive culture inserts were harvested as epithelial cell sheets with surgical forceps after incubation at 20°C for 30 min. **b** A harvested human oral mucosal epithelial cell sheet. **c** Harvested cell sheets were divided for validation tests. **d** A divided cell sheet used for histological analysis and flow cytometry. **e** The result of histological analysis, which confirmed that the fabricated epithelial cells formed a stratified epithelial layer. **f** The result of flow cytometry for the measurement of cytokeratin-positive cells in a fabricated cell sheet.

For the fabrication of transplantable epithelial cell sheets, the 3T3 feeder layer was eliminated by utilizing temperature-responsive culture inserts with submicron pores. The growth of human oral mucosal epithelial cells cultured in the KCM with AHS (fig. 5b, d, f, h) was similar to the growth in the KCM with FBS (fig. 5a, c, e, g). Table 3 shows the results of the fabrication of human oral mucosal epithelial cell sheets. Four volunteers (ID 0001,

0002, 0003 and 0004) kindly donated mucosal tissue twice. In the first trials of ID 0001, 0002 and 0003, the culture medium included 4 µg/ml gentamicin, and cultured mucosal cells derived from 0002 and 0003 were contaminated with bacteria. The concentration of gentamicin was thus increased from 4 to 40 µg/ml in the other trials. Moreover, ampicillin and streptomycin were added to DMEM for transporting the mucosal tissues to the

Fig. 4. Colony-forming assay of human oral mucosal epithelial cells. Human oral mucosal epithelial cells cocultured with the 3T3 feeder layer showed clonal growth and formed colonies in KCM containing FBS (**a**) or AHS (**b**). **c** There was no significant difference between FBS and AHS in colony-forming efficiency (CFE).

Table 2. Isolated cell numbers

Volunteer ID	Age years	Gender	Size of tissue, cm ²	Collected cells × 10 ⁵ cells	Cells/area × 10 ⁵ cells/cm ²
0001	29	M	0.59	5.3	9.0
			1.2	12	10
0002	22	M	0.98	12	12
			1.2	12	11
0003	42	M	0.79	8.9	11
			1.1	22	19
0004	39	F	0.59	5.5	9.3
			1.1	7.4	6.5
0005	24	M	0.79	8.8	11
0006	40	M	1.1	16	14
0007	50	M	1.1	15	13
Average	–	–	–	–	12 ± 3.3

Table 3. Cell sheet fabrication results

Volunteer ID	Serum	Seeding density of cells × 10 ⁴ cells/cm ²	Culture term days	Cell sheet
0001	FBS	4.0	17	harvested
	AHS	4.0	17	harvested
	AHS	6.0	15	harvested
0002	FBS	6.0	–	failed
	AHS	6.0	19	harvested
0003	FBS	6.0	16	harvested
	AHS	6.0	16	harvested
0004	AHS	4.0	18	harvested
	AHS	8.0	15	harvested
0005	FBS	4.8	14	harvested
	AHS	4.8	14	harvested
0006	AHS	6.0	15	harvested
0007	AHS	4.0	15	harvested

CPC and DMEM used for treatment with dispase. After these modifications, there was no contamination. Human oral mucosal epithelial cells cultured in KCM containing AHS were successfully harvested (fig. 3a, b) from temperature-responsive culture inserts except for the first trial of 0004. Incidentally, in the case of volunteers 0002 and 0004, although the oral mucosal epithelial cell sheets were harvested from temperature-responsive culture inserts in KCM containing AHS, the epithelial cells cultured in FBS-containing KCM did not reach confluence (table 3).

Validation Tests for Cultured Human Oral Mucosal Epithelial Cell Sheets

In this preclinical study, fabricated human oral mucosal epithelial cell sheets were divided into 2 parts (fig. 3c, d) for the validation tests by histological analysis and flow cytometry. Histological analysis was performed to confirm that the fabricated human oral mucosal epithelial cell sheets were able to stratify on temperature-responsive culture inserts. In consequence, all fabricated epithelial cell sheets were confirmed to be stratified epithelium (fig. 3e). Moreover, flow cytometry analysis indicated that the fabricated cell sheets consisted entirely of cyto-keratin-positive cells (fig. 3f).

Fig. 5. Observation of human oral mucosal epithelial cells cultured on temperature-responsive cell culture inserts without a 3T3 feeder layer by a phase-contrast microscope. The epithelial cells were seeded at a density of 4×10^4 cells/cm² and cultured in KCM containing FBS or AHS. Cultured cells were observed on days 5 (**a, b**), 8 (**c, d**), 10 (**e, f**) and 16 (**g, h**) after the cell seeding.

FBS

AHS

5

Discussion

As SOP, 75 documents were drawn up to fabricate human oral mucosal epithelial cell sheets for use in a clinical study to treat esophageal ulcers after ESD. The SOP included manuals for preparing human oral mucosal epithelial cells. If human oral mucosal epithelial cell sheets are to be utilized for esophageal ulcers after ESD, the number of epithelial cells for fabricating cell sheets depends on the size of the cancerous area to be removed from the esophagus. Therefore, information about the collection ratio of epithelial cells from human oral mucosal tissues is very important and useful for planning the fabrication of the epithelial cell sheets. We calculated the reproducibility of the collection ratio from the results of the preparation of human oral mucosal epithelial cells.

The SOP documented in this study included manuals and inspection of equipment in the CPC, manuals for preparing reagents, inspections of fabricated epithelial cells and information on how to ensure a sterilized environment. When operators entered the CPC for fabricating human oral mucosal epithelial cell sheets, they had to wear a surgical cap, mask and gloves in the entrance room. In gowning room 1, they had to wear a sterilized gown and pants over their clothes and change into clean shoes. Moreover, the operators had to wear a sterilized coverall, cowl and shoe covers, and had to change their sterilized surgical gloves in gowning room 2 to enter the cell culture room. Additionally, every monitored area in the CPC was cleaned weekly with disinfectant. As a result, although adherent bacteria were detected in gowning rooms 1 and 2, the cell culture room environment had successfully been kept aseptic during preclinical study. Additionally, there was no detection of bacteria or mycoplasma in the culture medium used for the epithelial cells derived from the volunteers. Other aspects of validation and the morphology and purity of the epithelial cells, including the fabricated human oral mucosal epithelial cell sheets, had to be confirmed. In a previous study, a culture method using temperature-responsive culture inserts and AHS is useful for fabricating stratified human oral mucosal epithelial cell sheets consisting of cytokeratin-positive cells [11]. In this study, histological analysis confirmed that all fabricated cell sheets derived from the 7 volunteers formed stratified epithelium. Additionally, the percentage of cytokeratin-positive cells was quantitatively analyzed by flow cytometry and the results showed that the purity of epithelial cells in the cultured cell

sheets was higher than 90%, even though the epithelial cells had been cultured without the 3T3 feeder layer. Moreover, the anchorage dependence of cultured human oral mucosal epithelial cells constructing the cell sheets was demonstrated by culture in soft agar medium (data not shown). These results ensured the safety and efficacy of fabricated human oral mucosal epithelial cell sheets for treating ulcers after endoscopic gastrointestinal cancer surgery.

The efficacy of AHS for cell growth is important for fabricating autologous cell grafts. In this study, the colony-forming efficiency of AHS was compared with that of FBS in the culture of human oral mucosal epithelial cells, and there was no significant difference. Moreover, the epithelial cell sheets were successfully fabricated in the medium containing AHS except for 1 case, where the oral mucosal cells derived from 0004 were seeded at a density of 4×10^4 cells/cm². Although these cells could not fabricate cell sheets in the first trial, when seeded at the same density or 8×10^4 cells/cm² in the second trial, they did proliferate and form stratified epithelial cell sheets. Additionally, in the first trial of 0004, the epithelial cells cocultured with the 3T3 feeder layer showed clonal growth and formed colonies of epithelial cells. These conflicting results of the 0004-derived epithelial cells may include important information for determining the seeding density of human oral mucosal epithelial cells on temperature-responsive culture inserts without the use of FBS or the 3T3 feeder layer for fabricating clinical grade epithelial cell grafts.

Cultured oral mucosal epithelial cell sheets indicate the feasibility of using epithelial cell grafts to treat esophageal ulcers after ESD in animal models [14, 21]. In this preclinical study, the results of the validation tests indicated the ability of the methods documented in the SOP for ensuring the cleanliness of the fabricated autologous human oral mucosal epithelial cell sheets and the CPC environments. Moreover, by culture in soft agar medium, the safety of the epithelial cells in the fabricated cell sheets was confirmed, and their purity was confirmed by flow cytometry. For these reasons, the methods shown in this study may contribute to the successful in vitro fabrication of human oral mucosal epithelial cell sheets for clinical applications.

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