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Promotion of oral surgical wound healing using autologous mucosal cell sheets

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ABSTRACT

Objectives: Severe oral mucosal and tissue defects can lead to pain, infection, and later undesirable healing of scarring and adhesion, resulting in a poor quality of life. *In vitro*-engineered oral mucosal equivalents for covering such defects are an alternative to avoiding the donor site morbidity of conventional skin or tissue grafts. We examined the efficacy of our newly developed three-dimensional mucosal cell sheets in an *in vivo* tongue wound model mimicking the surgical extirpation of tongue cancer.

Materials and methods: Small oral mucosal and autologous fibrin samples were obtained from surgical patients and Sprague-Dawley rats. The fibrin was mixed with fibroblasts and seeded with keratinocytes that had been primarily cultured for *in vitro* cell expansion. The three-dimensional autologous cell sheets, cultured in air-lift interface inserts, were transplanted into deep wounds of the rat ventral tongue. Gross and microscopic findings of the postsurgical wounds were compared between wound control and cell sheet groups.

Results: The cell sheets were flexible, expandable, and easy to transfer, and had histological characteristics similar to that of the normal oral mucosa, with high p63 positivity. They promoted oral wound healing with earlier re-epithelialization and less fibrosis than that in the wound control. The cell sheet-healed tongue had similar histology to that of a normal tongue.

Conclusions: Our engineered cell sheets have potential applicability for the rapid healing of oral mucosal and soft tissue defects, without scarring, adhesion, and functional deficits.

Condensed abstract: The efficacy of *in vitro*-engineered mucosal equivalents, using completely autologous mucosa and plasma, was examined. Transplantation of the autologous cell sheets into deep wounds of the rat ventral tongue promoted oral wound healing with earlier re-epithelialization and less fibrosis than that in controls. Healed and normal tongues showed similar histology.

Introduction

Oral mucosal and soft tissue defects can be caused by surgical extirpation of intraoral pathological lesions, trauma, recurrent ulcers, and irradiation. If not properly treated, the defects lead to pain, infection, and later undesirable healing, such as scarring and adhesion to adjacent tissues. This might cause significant deficits of functions that are essential for daily life, such as deglutition, articulation, and speech, as well as respiration. To prevent undesirable discomfort and sequelae, a skin graft, local or regional flap, or microvascular free flap is currently used to restore the mucosal lining or soft tissue defects in the oral cavity. The procedures commonly require harvesting skin or tissues from the same patients, which might result in morbidity and leave aesthetically unacceptable scarring in the donor sites. The current surgical approach for filling a large soft tissue defect is microvascular free flap transfer, which commonly requires a considerable operation time and experienced surgical hands. A superficial intraoral defect without considerable soft tissue deficits can be covered with a split thickness skin graft. *In vitro*-cultured cell-based regenerative approaches have been introduced as feasible alternative methods to restoring the mucosal lining and tissue defects [1–3].

Epithelial cell sheets prepared from the autografts or allografts of epidermal cells have been applied to the grafting of burns [4–6]. In addition, it has been reported in the literature that cell sheets or equivalents from human mucosa can be *in vitro*-engineered for potential application to the closure of oral wounds [7–9]. Oral mucosal equivalents consisting of human lamina propria fibroblasts and oral epithelial cells have been shown to mimic the normal

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oral mucosa, with similar histological and immunohistochemical marker expression levels [8]. Furthermore, *in vitro*-produced oral mucosal equivalents were introduced for intraoral grafting after their production using scaffolds of acellular dermis [7], amniotic membrane [10], and collagen glue [11], etc. These were not autologous, but obtained from animals or cadavers and required pretreatments for eventual immunorejection of the equivalents.

Human plasma is an abundant source of autologous fibrin that might be an optimal scaffold for producing skin and mucosal epithelial equivalents [12,13]. Lamina propria fibroblasts can be easily cultured *in vitro*, mixed in the autologous fibrin glue, and then used to facilitate the growth of keratinocytes seeded on the mixture, without potential immunorejection [13]. Therefore, we developed an *in vitro*-engineered autologous mucosal cell sheet consisting of keratinocytes and of plasma fibrin containing lamina propria fibroblasts. The keratinocytes and fibroblasts were obtained by *in vitro* culture using animal-product-free and completely autologous materials. Herein, this study examined the potential efficacy of our newly developed three-dimensional oral mucosal cell sheet in an *in vivo* tongue wound model mimicking the clinical setting of surgical extirpation of tongue cancer.

Methods

In vitro culture of mucosal samples

A small piece of mucosa was sampled from the normal mucosa adjacent to the resection margins in patients who underwent transoral surgery. This work was approved by the Institutional Review Board of our hospital, and informed consent was obtained from each patient. Plasma was also obtained from the 25 mL blood samples drawn from the same patients using a vacutainer tube (BD Bioscience, Franklin Lakes, NJ, USA) at the time of surgery. The mucosae were sterilized with povidone-iodine solution (Sigma-Aldrich, St. Louise, MO, USA) and washed 3 times in phosphate-buffered saline solution. All tissues were treated with 1 U/mL dispase (STEMCELL Technologies, Vancouver, BC, Canada) for 2 h at 37 °C, and the epithelial and subepithelial layers were then separated. Both layers were separately treated with trypsin-ethylene diaminetetraacetic acid (ThermoFisher Scientific, Waltham, MA, USA) for 15 min at 37 °C. The cells were separately seeded in culture dishes and grown in the culture medium, which was a 3:1 mixture of Dulbecco's modified Eagle's minimal essential medium and Ham's F12 (ThermoFisher), containing 10% heat-inactivated autologous serum, human recombinant insulin (5 μg/mL), triiodothyronine (1.3 ng/mL), adenine (24 μg/mL), hydrocortisone (0.4 μg/mL), and cholera toxin (8 ng/mL) (all purchased from Sigma-Aldrich), and supplemented with a penicillin-streptomycin-amphotericin antibiotic-antimycotic solution (ThermoFisher). Human recombinant epidermal growth factor (10 ng/mL; ThermoFisher) was also added to the medium for culturing mucosal keratinocytes. The medium and supplements were replaced at every 3 days.

Generation of in vitro-engineered mucosal cell sheet

The plasma obtained from each patient's blood was used to make fibrin glue as the source of the scaffolds. The fibrin glue was composed of a mixture of 0.5 mL of plasma, 1% calcium chloride, 70 μL of tranexamic acid (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and 0.5 mL of medium with 5×10^5 fibroblasts. The mixture was allowed to solidify in transwell cell culture inserts with a 0.4-mm pore-size polyester membrane (Corning, Inc., Corning, NY, USA) at 37 °C for 60 min. The inserts were placed in the plates with the medium and supplements. Keratinocytes were seeded on the mixture of fibrin glue and fibroblasts, and grown under air-liquid interface culture conditions, with the same medium, autologous serum, and supplements as mentioned above.

The *in vitro* culturing of keratinocytes and fibroblasts was also performed using biopsy samples of the oral buccal mucosa from Sprague-Dawley (SD) rats. Blood was also obtained from each rat and used as a source of autologous fibrin glue. The autologous mucosal cell sheet was produced in the same way as described above.

About 10–14 days after cell culturing, the fibrin glue and submucosal fibroblasts were mixed, poured, and solidified in the insert well, and keratinocytes were then seeded onto the mixture. Five to 7 days later, the mucosal cell sheets were constructed and prepared for transplantation.

Histological examinations of the mucosal cell sheets

The *in vitro*-cultured mucosal cell sheet or tissue samples were harvested, embedded in optimal cutting temperature compound (Sakura Fineteck USA Inc., Torrance, CA, USA), immediately snap-frozen in liquid nitrogen, and stored at −80 °C for subsequent use. The stored sheets or tissue samples were prepared as 5-μm-thick frozen sections for histological examination. The sections were stained with hematoxylin and eosin (Sigma-Aldrich). The samples were also examined immunohistochemically for the staining of p63 (1:200; GeneTex, Inc., Irvine, CA, USA), pancytokeratin AE1/AE3 (1:200 dilution, Dako, Glostrup, Denmark), cytokeratin 5/6 (1:200, Dako), and ki-67 (1:200, GeneTex) according to established protocols and examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Intraoral grafting of the mucosal cell sheet into a surgically wounded rat tongue

All animal study procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of our institution. Male SD rats, weighing 180–220 g, were purchased from Central Lab Animal Inc. (OO). The animals were anesthetized by intramuscular injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Iris scissors were used to remove the mucosa and submucosal soft tissues of the ventral tongue in each SD rat to create a 50-mm^2 deep wound (Fig. 1). The autologous mucosal cell sheet was detached from the culture dish and trimmed to a similar shape and size as the surgical defect. The cell sheet was then overlaid with a silastic sheet (0.010″ thickness; Bentec Medical, Woodland, CA, USA) and grafted onto the defect by use of 5-0 absorbable vicryl sutures. For the wound control group, a silastic sheet without the cultured mucosal sheet was attached to the wound site. All silastic sheets were removed at 3 days after grafting. Each experimental group included 25 rats. Normal tongues were also obtained from 5 unwounded rats. The body weight and food intake of each group were measured at every 3 days.

Gross and microscopic examinations of the postsurgical wounds

Gross photographs of each rat wound were taken regularly. The wound size at each postoperative day was also measured and compared with that of the original wound in each rat, and comparisons were also made between the wound control and cell sheet groups. Five rats of each group were sacrificed at postsurgical days 3, 7, 14, 21, and 28, and the tissues from the previous oral wounds were harvested and quickly frozen at −80 °C. Thereafter, 5-μm-thick sections were stained with hematoxylin and eosin and Masson's trichrome stains (Sigma-Aldrich) and observed under a microscope (Nikon Co., Tokyo, Japan). The epithelial and subepithelial thicknesses and collagen density were measured in a blind manner in