Oral Oncology 75 (2017) 81–88

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Plasticity of oral mucosal cell sheets for accelerated and scarless skin wound healing

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A R T I C L E I N F O

Keywords:
Skin
Wound healing
Cell sheet
Oral mucosa
Graft
Plasticity

A B S T R A C T

Objectives: Wound healing is generally faster and associated with less scarring in the oral mucosa than in the skin. Although rarely studied, oral mucosa equivalents may contribute to rapid, scarless cutaneous wound healing. Therefore, we examined the potential utility of our newly developed oral mucosal cell sheet in skin wound healing.

Materials and methods: Oral mucosa and skin samples were obtained from surgical patients and Sprague-Dawley rats. Keratinocytes and fibroblasts were primarily cultured for *in vitro* cell expansion. Mucosa and skin equivalents were produced with a mixture of cultured fibroblasts and autologous fibrin from plasma and seeding keratinocytes. Mucosal and skin cell sheets were transplanted in full-thickness excisional wounds of rat skin with control wounds. Gross, histological, and molecular characteristics of wound healing according to different postsurgical days were compared in control and cell sheet-covered wounds.

Results: Keratinocytes and fibroblasts derived from the oral mucosa were cultured faster than those derived from the skin. The *in vitro*-engineered oral mucosa and skin equivalents were successfully produced using complete autologous mucosa or skin and plasma fibrin, showing similarity to the histological characteristics of the skin or mucosa. In the *in vivo* rat model, the oral mucosal and skin cell sheet promoted wound healing with early wound closure and less scarring. The cell sheet-treated wounds showed lower TGF-β1, α-smooth muscle actin, and fibronectin mRNA expression than the control wounds.

Conclusions: The oral mucosal cell sheet demonstrated *in vivo* tissue plasticity through good adaptation to skin wounds, contributing to accelerated and scarless healing.

Introduction

Skin defects or wounds are caused by burns, traumatic injury, chronic inflammation, ulcers, surgical extirpation of pathological lesions, or irradiation. These defects, if not properly treated, lead to pain, infection, and undesirable sequelae such as scarring and adhesion. This might result in significant cosmetic disfigurement or functional deficits in body motion. Skin grafts or local/regional flaps are currently used to restore the skin surface lining or soft tissue deficits. Skin or tissue harvesting is commonly required to cover surface defects in the same patient, potentially leaving esthetically unacceptable scarring in the donor site. For nearly three decades, the *in vitro*-cultured cell-based regenerative approach has been established as a feasible alternative method to the re-epithelialization of split-thickness wounds [1–3].

Autografts or allografts of epidermal cells have produced epithelial cell sheets for application in the grafting of burn patients [4,5]. Epithelial cell sheets for covering skin defects or burns are commonly produced from the skin. A report has suggested that cell sheets cultured from the mucosa, in addition to cell sheets cultured from epidermal cells, are used for grafting skin defects [6]. Tissue-engineered cell sheets composed of oral mucosal epithelium have also been proposed for the reconstruction of the cornea [7], bladder and urinary tract [8] as well as large intraoral mucosal defects [9]. Cell sheets or equivalents from the oral mucosa can be engineered *in vitro* to be potentially used for the closure of oral wounds [10–12]. Oral mucosa equivalents consist of human lamina propria fibroblasts and oral epithelial cells mimicking the normal oral mucosa, with a similar expression of histological and immunohistochemical markers [13].

Response to injury and wound healing are commonly faster along with less inflammation and angiogenesis in the oral mucosa than in the skin [14,15]. In addition to accelerated wound healing, intraoral wounds display minimal scar formation compared to cutaneous wounds. This may be due to differences in the expression of extracellular matrix components [16]. Therefore, oral mucosa equivalents

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http://dx.doi.org/10.1016/j.oraloncology.2017.10.024
Received 10 July 2017; Received in revised form 27 September 2017; Accepted 28 October 2017
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composed of oral keratinocytes and fibroblasts may contribute to rapid, scarless wound healing in the skin. This has been rarely studied in the literature. We previously developed an *in vitro*-engineered autologous mucosal cell sheet consisting of keratinocytes and plasma fibrin containing mucosal fibroblasts, and prove its efficacy in promoting oral surgical wound healing [17,18]. We further showed that the oral mucosal cell sheets might have the necessary tissue plasticity for promoting rapid and scarless cutaneous wound healing, with *in vivo* effects equivalent to skin-derived cell sheets in a rat skin wound model. This study examined the potential utility of this newly developed oral mucosal cell sheet in skin wounds.

Methods

In vitro culture of mucosa and skin samples

Small pieces of normal mucosa and the skin were sampled from the oral cavity, pharynx, esophagus, and the neck of patients who underwent transoral and neck surgery. Approximately 10 mL of plasma, along with skin samples, were obtained from the blood of the same patient using a Vacutainer tube (BD Bioscience, Franklin Lakes, NJ, USA) at the time of surgery. This work was approved by the Institutional Review Board of our hospital, and all patients provided written informed consent prior to the procedures. The samples were disinfected with povidone–iodine (Sigma-Aldrich, St. Louise, MO, USA) and washed three times in phosphate-buffered saline solution. All tissues were treated with 1 U/mL dispase (STEMCELL Technologies, Vancouver, Canada) for 1 h at 37 °C, and subsequently, epithelium was separated from subepithelial layer using fine forceps. Both layers were treated with trypsin–ethylenediaminetetraacetic acid (ThermoFisher Scientific, Waltham, MA, USA) for 30 min at 37 °C. The cells were seeded in culture dishes and grown in a culture medium containing a 3:1 ratio of Dulbecco's modified Eagle's minimal essential medium and Ham's F12 (ThermoFisher), with 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 penicillin/streptomycin/amphotericin antibiotic–antimycotic solution (ThermoFisher). Human recombinant epidermal growth factor (10 ng/mL; ThermoFisher) was also added to the medium for mucosal keratinocytes. The medium and supplements were replaced every 3 days.

Generation of in vitro-engineered cell sheets

The source of scaffolds for cell sheets was fibrin glue obtained from the plasma of each patient. The fibrin glue was composed of a mixture of 0.5 mL plasma, 1% calcium chloride, 70 μL tranexamic acid (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and 0.5 mL medium including 5×10^5 fibroblasts. The mixture was solidified in Transwell cell culture inserts with a 0.4 mm pore polyester membrane (Corning, Inc., Corning, NY, USA) at 37 °C for 60 min. The inserts were placed in the plates along with the medium and supplements. Keratinocytes were seeded in the mixture of fibrin glue and fibroblasts and grown in an air–liquid interface culture condition containing the medium, autologous serum, and supplements mentioned above.

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

Histological examinations of cell sheets and tissues

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 sheet or tissue samples were prepared as 5 μm-thick frozen sections for a histological examination. The sections were stained with hematoxylin and eosin (Sigma-Aldrich). The samples were also stained with pancytokeratin AE1/AE3 (1:200 dilution, Dako, Glostrup, Denmark), cytokeratin 14 (1:200, Novus Biologicals, Littleton, CO, USA), p63 (1:200, GeneTex, Inc., Irvine, CA, USA) or vimentin (1:100, Santa Cruz Biotechnology, Dallas, TX, USA) according to the manufacturer's protocol for immunohistochemistry, and examined under fluorescence microscopy (Olympus®).

Cell growth, viability, and colony-forming assays

Cell growth and viability were assessed using the trypan blue exclusion assay. The keratinocytes and subepithelial fibroblasts obtained from the oral mucosa and skin were plated in 10 mm culture dishes with initial cell numbers of 3.5×10^5. The cells were harvested at days 3, 6, 9, 12, and 15. Trypan blue exclusion was performed by 0.4% trypan blue staining, and counting was performed using a hemocytometer. The colony-forming ability of keratinocytes was assessed using a clonogenic assay. A total of 1000 cells were plated and cultured for 2–3 weeks. The plate was stained with 0.5% crystal violet solution, and the number of colonies (> 50 cells) was counted. Cell number and viability were compared between oral and skin keratinocytes. The colony-forming ability was also compared among oral, pharyngeal, and esophageal mucosae and the skin and between different culture conditions of oral keratinocytes with and without fibrin-coated dishes.

Skin wound model and in vivo testing of cell sheets

All animal study procedure protocols were approved by the Institutional Animal Care and Use Committee of our institution. The male SD rats, weighing 180–220 g, were purchased from Central Lab Animal Inc. (Seoul, Republic of Korea). The animals were anesthetized by an intramuscular injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The skin of each SD rat was removed with iris scissors to create a 175 mm^2-sized full-thickness wound (Fig. 1). The autologous oral mucosal and skin cell sheets were detached from culture dishes and placed on the skin defect. The cell sheet was fixed on the wound with 5–0 nylon sutures and subsequently overlaid with a thin transparent silastic sheet (0.010″ thickness; Bentec Medical, Woodland, CA, USA). All silastic sheets were removed 3 days after grafting. The experiment included a total of 40 rats. Each rat had three skin excisional wounds including the control wound and treatment wounds using the oral mucosal and skin cell sheets.

Gross and microscopic examinations of postsurgical wounds

Gross photographs of each wound were regularly captured at 1 day and twice a week post surgery, and the wound size was measured. The largest dimensions were measured across the diameter of the wound and multiplied by the wound area. Five rats were sacrificed at 3, 7, 10, and 14 days post surgery, and 10 rats were sacrificed at 21 and 28 days post surgery. The tissues from previous wounds were harvested and snap-frozen at −80 °C. Thereafter, 5 μm-thick sections were stained with hematoxylin–eosin and Masson's trichrome (Sigma-Aldrich) and observed under a microscope (Nikon Co., Tokyo, Japan). Collagen density was blindly measured in 10 randomly selected fields, using the ImageJ processing program (National Institutes of Health, Bethesda, MD).

Quantitative real-time reverse transcription polymerase chain reaction

Tissues were obtained from skin wounds at different days after wounding. Cells were prepared using the QIAzol lysis reagent and RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was generated from RNA using a QuantiTect Reverse Transcriptional Kit (Qiagen) according

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