Original

Expression of Cross-Linked Protein on Tissue-Engineered Epithelial Cell Sheets from Rabbit Oral Mucosa

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Abstract: Tissue engineering is a rapidly evolving field involved in the restoration of functions and esthetics of tissues after surgery. Utilizing the oral mucosa as a source of tissue-engineered epithelial cell sheets is beneficial due to the fast healing rate and regenerative capability of the tissue. This study investigated the effect of mesenchymal fibroblasts on epithelial cells taken from rabbit oral mucosa. Rabbit oral epithelial cells were cultured on collagen gels both with oral mucosa fibroblasts (OMFs+) and without (OMFs-). Samples were stained with hematoxylin and eosin and observed under a light microscope. The expressions of cytoskeletal, adhesion, basement membrane and junctional complex as well as cell proliferation protein markers were also determined through immunohistochemistry. Results showed that the OMFs+ group had epithelial cell sheets that were better developed than those of the OMFs- group. Moreover, cytoskeletal, adhesion, basement membrane, junctional complex and cell proliferation markers were more intensely expressed by epithelial cells in the OMFs+ group than in the OMFs- group, and the expression increased over time. Immunohistochemistry demonstrated how stem cells taken from the oral mucosa contributed to the expression of various cytokines indispensable for the growth of the cells. The epithelial-mesenchymal interaction between the keratinocytes and fibroblasts accounts for the architecture of the epithelial cell sheets.

Key words: Cell sheet, Oral mucosa, Keratinocyte, Fibroblast

Introduction

The necessity of autologous graft to improve or replace biological functions brought about advancement in tissue engineering and regenerative medicine. Whether the replacement of tissue defect is to correct gingival recession, chronic inflammation or tumor resection and ablation, the aim is to restore esthetics and function nearly close to normal structures. The skin being the largest organ of the body is an abundant source of autologous graft. Skin grafts taken from the radial forearm or lateral forearm and rectus abdominis have been utilized to restore the shape and function of the oral cavity after surgery¹⁻³⁾. However, because of the shortage of donor, tissue engineering is now becoming a promising technology for a continuous supply of tissue⁴⁾.

The use of oral mucosa in tissue engineering highlights its advantages as a tissue source. Unlike the skin, the oral mucosa heals mainly by regeneration. Tissues can be obtained fairly simple and with less pain and scarring and with a faster healing rate

compared to skin. The use of oral mucosal epithelial cell sheets is gradually being applied in clinical practice in tissue reconstruction following surgical removal of malignant tumors in the esophagus and stomach⁵⁻¹⁰⁾. The oral mucosa has also been used in corneal and limbal surfaces of patients in which improvement of the visual acuity was observed^{11,12)}.

The oral mucosa consists of stratified squamous epithelium and the lamina propria. The stratified epithelium may or may not be keratinized depending on the location. Stratum basale or the basal layer resting on the basement membrane is known to harbor progenitor cells, which then terminally differentiate as they migrate to the superficial layer¹³⁾. It has been reported that mesenchymal stem cells derived from the lamina propria of human oral mucosa are multipotent¹⁴⁾. The basement membrane separates the epithelium from the underlying lamina propria. In the skin, basement membrane proteins perform significant role in the characteristics of the epithelium. Therefore, the communication between the epithelium and the lamina propria affects tissue morphology and function.

Although in *refor*, the study provides a better understanding of the effect of tissue-engineered biomaterial on the surrounding

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Table 1. Primary Antibodies Used

Antibody	Dilution	Manufacturer
Keratin 4 (6B10)	1:20	DBS, Pleasanton, CA, USA
Keratin 13 (2D7)	1:10	Progen, Heidelberg, Germany
Laminin (NU-01-LA3)	1:50	Cosmo Bio LTD, Tokyo, Japan
Type IV collagen	1:400	Southern Biotech, Birmingham, AL, USA
Occludin(OC-3F10)	1:50	Invitrogen, Grand Island, NY, USA
ZO-1(ZO-1-1A12)	1:50	Invitrogen, Grand Island, NY, USA
Ki67(M7240)	1:50	DakoCytomation, Heidelberg, Germany
p63 (4A4)	1:50	SantaCruz, CA, USA

tissues, the process is difficult to elucidate due to numerous phenomena that may influence the growth of the tissues and accruing consequence of cell-cell interaction. Moreover, it has been established that epithelial-mesenchymal interaction is a very important process in regulating tissue homeostasis. Analysis of epithelial-mesenchymal cell interaction using mouse organotypic co-culture in vitro is essential because it apparently shows how the cells cooperate with one another¹⁵⁾. The aim of the study was to determine the effects of undifferentiated mesenchymal fibroblasts on the epithelial cells taken from the mucosa of white rabbits. The formation of epithelial cell sheet was observed histologically and its phenotype was determined through the expression of cytoskeletal, adhesion, basement membrane, junctional complex and cell proliferation protein markers.

Materials and Methods

Preparation of rabbit oral tissues

The oral mucosal tissues were taken from female white rabbits (Japan CLEA, Tokyo, Japan) weighing 2.5 kg each. The rabbits were anesthetized with 100 mg/kg pentobarbital sodium (Kyoritsu Seiyaku Co., Tokyo, Japan) and sacrificed using 1 M potassium chloride (Wako, Osaka, Japan). All experimental procedures followed the guidelines approved by the Animal Care and Use Committee of Tokyo Dental College (Approval Number: 250105) and conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Isolation of oral mucosal epithelial cells

The mucosa was carefully separated from the submucosal connective tissue with scissors. The epithelium was cut into small pieces and washed several times using 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Grand Island, NY, USA) and Ham's F12 (Invitrogen, Grand Island, NY, USA) containing mycotic-antibiotic mixture of 5 g/ml gentamycin (Invitrogen, Grand Island, NY, USA) and 0.25 g/ml amphotericin B (Sigma-Aldrich, St. Louis, MO, USA). Epithelial sheets were isolated as previously described by Higa et al¹⁶⁾ using 1.2 U/ml dispase II (Roche, Mannheim, Germany) overnight at 4 °C. Cell suspension was isolated from epithelial sheets using trypsin-EDTA

for 10 min. Suspensions of oral epithelial cells were used for the following co-culture.

Isolation of oral mucosal fibroblasts

Fibroblasts were isolated from the submucosal connective tissue remnants of the oral mucosal tissues. The tissues were treated with 2 mg/ml collagenase. Harvested fibroblasts were cultured in mesenchymal stem cell growth medium (MSCGM, Lonza Walkersville, Inc., Walkersville, MD, USA) incubated at 37 °C until confluency. Single fibroblasts (8.0~10³ cells/ml) were cultured with 0.8 % methylcellulose in advanced-DMEM containing 10 % fetal cow serum (FCS) on low adhesive plates (HydroCell, CellSeed, Tokyo, Japan) to avoid attachment of cells to the bottom of the plate. The cells were incubated for 2 weeks at 37 °C with 5 % CO₂ humidified air. Clusters of cells from the single cells were re-plated on adhesive plates and amplified by explant adhesive culture.

Organotypic co-cultures to make cell sheets

To prepare an equivalent of oral mucosa, a suspension of cultured fibroblasts in DMEM supplemented with 10% FCS was added to collagen solution to make a final concentration of 0.21 % type-1A collagen and a density of 6.25 ~ 10⁴ cells/ml collagen gel. Each well contained 800 l of the gel mixture and allowed to set for 30 min at 37 °C in atmosphere of 5 % CO₂. The isolated epithelial cells were gently added onto the surface of the equilibrated gel and co-cultured with supplemented hormonal epithelial medium (SHEM) with 10 % FCS and 666 KIU/ml aprotinin (Wako, for the inactivation of nonspecific proteases that completely degrades collagen) for 2 weeks at 37 °C in an atmosphere of 5 % CO₂. The changes in the co-culture were observed at day 5, 11 and 15 after culture. Fig. 1A is the schematic summary of co-culture of epithelial cells and fibroblasts.

Immunohistochemistry (IHC)

The specimens were mounted with optimum cutting temperature (OCT) compound and sectioned into 5-mm frozen sections for hematoxylin and eosin (H&E) staining and IHC. Frozen sections were fixed in 2 % paraformaldehyde (Wako) or acetone (Wako) for 10 min. Then after, sections were incubated with 10 % normal donkey serum (Chemicon International Inc.) and 1 % bovine serum

Figure 1A: Schematic presentation of co-culture of epithelial cells and fibroblasts harvested from rabbit oral mucosa.

1B: Hematoxylin and eosin sections of OMFs- and OMFs+ groups. An increased in the stratification of the epithelium is evident in OMFs+ group compared to OMFs- group. Likewise, more fibroblasts proliferated underneath the epithelium in OMFs+ group compared to OMFs- group.

albumin (Sigma-Aldrich) for 1 h at room temperature. Sections were then incubated with primary antibody for 90 min at room temperature. Table 1 shows the list of primary antibodies used in the experiment. This was followed by incubation with fluorescein isothiocyanate- (FITC), rhodamine- (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or Cy3-conjugated secondary antibodies (Chemicon International Inc., Temecula, CA, USA). Then after, sections were washed three times with PBS, incubated with 1 mg/ml 4', 6-diamidino-2-phenylindole (DAPI, Dojindo Laboratories, Tokyo, Japan) for 5 min at room temperature. After washing twice with PBS, slides were mounted using aqueous mounting medium containing anti-fading agent (Fluoromount/Plus, Diagnostics Biosystems, Pleasanton, CA, USA). Images were viewed under fluorescence microscope (Axioplan2 Imaging, Carl Zeiss Inc., Thornwood, NY, USA).

Results

Generation of epithelial cell sheet

Fig. 1B shows the H&E sections of the control (OMFs-) and co-cultured with oral mucosa fibroblasts (OMFs+) groups. At 5 d, a thin layer of epithelium is present on both OMFs- and OMFs+ groups with no difference observed between the two groups. Few fibroblasts also started to proliferate in OMFs+ group. At 11 d, the epithelial stratification increased in OMFs+ group compared

to OMFs- group. Also, more fibroblast proliferation can be seen in OMFs+ group. At 15 d, epithelial sheet and the underlying connective tissue are more evident in OMFs+ group compared to OMFs- group.

Expression of epithelial differentiation markers

Fig. 2 shows keratin 4 and 13 immunofluorescence sections of OMFs- and OMFs+ groups. Both proteins were expressed in the epithelium. However, in OMFs+ group, more cells expressed both proteins compared to those in OMFs- group. The number of cells, which expressed both proteins increased over time.

Expression of basement membrane markers

Fig. 3 shows the type IV collagen and laminin immunofluorescence sections of OMFs- and OMFs+ groups. Type IV collagen was expressed some fibroblasts especially in OMFs+ group. The number of cells expressing type IV collagen increased over time. On the other hand, laminin was expressed by mostly basement membrane as well as by some fibroblasts. Laminin expression also increased over time.

Expression of junctional complex markers

Fig. 4 shows occludin and ZO-1 immunofluorescence sections of OMFs- and OMFs+ groups. Both proteins in both groups were prominent among superficial layer of epithelium and the expressions increased with the stratification of epithelium over time (Arrowheads).

Expression of cell proliferation markers

Fig. 5 shows Ki67 and p63 immunofluorescence sections of OMFs- and OMFs+ groups. Ki67 was expressed by dividing cells in both groups. At 11 d and 15 d, Ki67 was localized in few basal cells in OMFs+ groups. p63 was expressed by proliferating cells in both groups. The difference of p63 expression was observed between the two groups. The p63 expression was shown a propensity to be higher in OMFs+ groups than OMFs- groups at all the times.

Discussion

The oral cavity is protected by the oral mucosa consisting of stratified squamous epithelium and underlying lamina propria. The number of stratification and the presence of keratin on the top layer vary with location. The co-culture of epithelial cells and fibroblasts resemble the architecture of a reconstructed mucosa formed by the paracrine interaction between the keratinocytes and fibroblasts. It is known that the oral epithelium contains progenitor cells responsible for the maintenance and renewal of epithelial cells¹³⁾. Although both OMFs- and OMFs+ group showed the presence of stratified squamous epithelium, the OMFs+ group showed advanced stratification of the epithelium. Moreover, the underlying connective tissue in OMFs+ group is more definite

Figure 2. Immunofluorescence sections of K4 (A) and K13 (B). Both proteins are expressed by the mucosal epithelium. Both groups showed increased in K4 and K13 on 11d. However, the difference in both proteins between groups is evident on 15 d.

Figure 3. Immunofluorescence sections of collagen type IV and laminin. Both proteins were expressed on the basement membrane in both groups. However, in OMFs+ group, some fibroblasts also expressed both proteins.

Figure 4. Immunofluorescence sections of occludin and ZO-1. Both proteins were localized in the superficial epithelial cells with increasing expression over time (arrowheads).

Figure 5. Immunofluorescence sections of Ki67 and p63. Both Ki67 and p63 are localized at the basal cells of the epithelium

compared to the OMFs- group. The maturation of the epithelium is controlled by keratinocyte growth and differentiation. The fibroblasts in the underlying connective tissue generally produce cytokines that can regulate the differentiation of keratinocyte¹⁷⁾. Moreover, fibroblasts are essential cells providing support to most organs. Taken together, the presence of more mesenchymal cells and abundant fibroblasts in the connective tissue account for the growth of the epithelial cell sheet. Thus, the interaction between

the epithelial cells and the fibroblasts encourage the morphogenesis of epithelial cell sheet.

The epithelial cells expressed cytoskeletal proteins K4 and K13. Both proteins are usually found above the basal layers of non-keratinized stratified epithelium and are considered dominant pair of differentiation-related keratins in oral keratinocytes¹⁸⁾. In the study, K4 and K13 were observed in both groups but both proteins were more intense in OMFs+ group as observed on 11d

suggesting that the epithelial cells are in advanced differentiation.

The epithelium rests on the basement membrane consisting mainly of type IV collagen and laminin. Type IV collagen is a significant basement membrane protein needed to support the basal cells for their continuous growth and survival as well as to enhance epithelial phenotype^{19,20}. The study demonstrates the presence of both type IV collagen and laminin suggesting that the proteins could have guided the fate of the keratinocyte. Thus epithelial-mesenchymal interaction initiated the growth of the cells. Moreover, the presence of basement membrane proteins may anchor the epithelium from the underlying connective tissue.

One characteristic of the epithelium is the very little intercellular space in between the cells. Tight junctions hold keratinocytes together and function also as barrier and maintenance of osmotic balance. Occludin and zonula occludens-1 (ZO-1) are known proteins expressed in tight junctions^{21,22}. These proteins particularly ZO-1 was intensely expressed by the epithelial cells indicating the physiological state of the cells.

Ki67 is a measure of growth of cells undergoing cell division²³⁻²⁵. Keratinocytes undergoing physiological cell division are usually found at the basal layer. Although very few cells expressed Ki67, its expression was limited to the basal cells implying normal cell division. The protein p63 is a member of the p53 family required for epidermal development. Its expression has been shown in the proliferation and differentiation of mature keratinocytes²⁶. The expression of p63 observed in the basement membrane of the epithelium indicates the presence of undifferentiated cells, which are the sources of epithelial cells.

In summary, the H&E sections showed the morphology of the epithelial cell sheet formed by co-culture of epithelial cells and fibroblasts harvested from rabbit oral mucosa. The immunohistochemical sections showed how stem cells of mesenchymal origin taken from the oral mucosal epithelium of rabbits contributed directly to the expression of cytoskeletal and adhesion proteins of the epithelium in the absence of feeder cells. However, it cannot be affirmed whether the stem cells are capable of differentiating into osteoblasts, adipocytes, chondrocytes or undifferentiated fibroblasts. The architecture of the reconstructed mucosa might have been formed by the interaction between the keratinocytes and fibroblasts influenced by the direct and / or indirect factors.

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