Reconstruction of a full-thickness collagen-based human oral mucosal equivalent

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ABSTRACT

Tissue engineered human oral mucosa has the potential to be applied to the closure of surgical wounds after tissue deficits due to facial trauma, malignant lesion surgery or preprosthetic procedure. It can also be used to elucidate the biology and pathology of oral mucosa and as a model alternative to animals for safety testing of oral care products. Using the technology previously developed in our laboratory for the production of a skin equivalent, we were able to reconstruct a nonkeratinized full-thickness human oral mucosal equivalent closely mimicking human native oral mucosa. The successive coculture of human lamina propria fibroblasts and human oral epithelial cells isolated from the nonkeratinized region of oral cavity in a porous collagen–glycosaminoglycan (GAG)–chitosan scaffold gave rise to a lamina propria equivalent (LPE) and then to an oral mucosa equivalent (OME). The results of the histology, immunohistology and transmission electron microscopy of this OME demonstrated the presence of a non-keratinized pluristratified and differentiated epithelium as in native nonkeratinized human oral mucosa expressing both K13 and K3/76. This epithelium was firmly anchored to the LPE by a continuous and ultrastructurally well-organized basement membrane. In the LPE, fibroblasts synthesized new extracellular matrix where the average collagen fibre diameter was 28.4 nm, close to that of native oral mucosa. The proliferative capacity of the basal cells was demonstrated by the expression of Ki67.

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1. Introduction

Tissue engineering of oral mucosa holds the promise to use a human oral mucosal equivalent for treatment and closure of surgical wounds, as well as for studies of the biology and pathology of oral mucosa, as a vehicle for gene therapy, and as a model alternative to animals for safety testing of consumer products in an in vitro system [1]. Besides accidents and congenital defects, a great deal of patients require oral tissue regeneration annually following craniofacial cancer surgery [2]. Accordingly, the development of an oral mucosal equivalent would offer the oral and maxillofacial surgeon a material to assist in reconstruction of the oral cavity, predesigned and constructed according to the needs of the patient and surgeon [1].

In skin tissue engineering, the “gold standard” has been the use of split-thickness grafts containing all of the epidermis and a part of the underlying dermis [3]. However, for the mucosal graft, limitation of donor tissue size, which has much smaller surface compared to skin, is a problem [4], and treatment of oral defects with skin grafts has several disadvantages due to physiological differences between skin and mucosa such as hair growth, adnexal structures and pattern of keratinization [5]. The ideal tissue engineered oral mucosa should be the one that mimics the native oral mucosa as closely as possible. Accordingly, it should be composed of two parts: an outer layer of stratified squamous epithelium and an underlying layer of dense connective tissue, i.e. lamina propria, providing support for the epithelium. There have been several attempts to create epithelial sheets for grafting on superficial oral mucosal defects, however these epithelial sheets are not only fragile and difficult to handle but also have low engraftment rates [1]. It was also reported that the presence of a dermis assisted in epithelial graft adherence and epithelial maturation, and minimized wound contraction, while encouraging the formation of a basement membrane [6]. Oral mucosa engineering based on the use of oral mucosal cells is a newly emerging field and there is little work in the literature on the in vitro reconstruction of full-thickness oral mucosa equivalents composed of both an epithelium and a lamina propria, and their in vivo performances are yet to be tested. Promising results have been obtained so far from the culture of oral mucosal cells on various types of scaffolds such as porcine skin [7].
human cadaver dermis [8] and [9], fibrin-based [10] and collagen-based scaffolds [11] and [12]. However, the constructs using porcine skin and cadaver skin do not include fibroblasts. One study of the implantation of oral mucosal substitutes composed of acellular dermis and autologous oral keratinocytes in dogs has failed, probably due to insufficient vascularization after implantation [13]. There is one clinical trial of implantation of a tissue engineered oral mucosa which has resulted in better healing [14].

Oral mucosa represents the barrier between the mouth and the oral cavity and has a stratified epithelium, however, according to the region in the oral cavity its epithelium may be keratinized (gingival and hard palate), nonkeratinized (all the remaining region, such as cheek, lips, floor of the mouth) or both (dorsum of the tongue, which consists of both keratinized and nonkeratinized regions) since these distinct regions have distinct functions, some requiring more strength, some more elasticity [15]. It may be possible to reconstruct both nonkeratinized or keratinized oral mucosal equivalents by using oral cells isolated from either nonkeratinized or keratinized regions of oral cavity, according to the need.

The aim of the work described here was to create a nonkeratinized oral mucosal equivalent mimicking as much as possible the native nonkeratinized human oral mucosa. The reconstruction of the oral mucosal equivalent was based on technology previously developed in our laboratory for the production of a skin equivalent, using our collagen–glycosaminoglycan (GAG)–chitosan dermal substrate [16]. This skin model was developed to evaluate cutaneous toxicity [17], safety [18], and efficiency of cosmetic products [19] as well as to reconstruct a hemicoxma [20]. The choice for material to be used as a scaffold is a crucial one. There is growing recognition that the physical and the chemical properties of biomaterials can regulate biological responses [21]. The collagen–GAG–chitosan scaffold used in the present study was a good candidate for the reconstruction of an oral mucosal equivalent since it matched the desired properties of an ideal biomaterial, being bioreparable, non-toxic, with the physical properties of strength, compliance and density similar to oral mucosa/skin. In the present study, this porous collagen–GAG–chitosan scaffold was seeded with human oral fibroblasts isolated from the nonkeratinized cheek region of oral cavity and cultured for three weeks forming a lamina propria equivalent. Then, human oral epithelial cells isolated from the same biopsy were seeded on top of this scaffold to give rise to an epithelialized full-thickness oral mucosal equivalent. We characterized this tissue engineered oral mucosa by histology, immunohistochemistry and transmission electron microscopy.

2. Materials and methods

2.1. Origin, isolation, and culture of epithelial cells

Epithelial cells were isolated from human oral mucosal biopsies removed from the nonkeratinized cheek region of the mouth, and obtained with informed consent from patients undergoing oral surgery. The biopsies were first measured, and then cut into small pieces in order to increase the efficacy of the enzymes used. The separation of the epithelium from the lamina propria was performed with dispase (GIBCO), 10 mg/mL for 3 h at 4 °C. After separation, epithelium was treated with trypsin 0.5 g/L–EDTA 0.2 g/L for 20 min to extract the cells, which were collected every 10 min. Epithelial cells were grown at 8000–10 000 cells/cm² on a feeder layer of irradiated human fibroblasts in a specially designed medium as follows: DMEM–Ham-F12 2.7/1 (Sigma), 10% fetal calf serum (Hyclone), 0.4 mg/mL ascorbic acid (Bayer), 0.12 UI/mL insulin (Umuline, Lilly), 50 μg/mL ascorbic acid (Bayer), and antibiotics.

2.2. Origin, isolation, and culture of fibroblasts

Fibroblasts were isolated from the same biopsies as epithelial cells. After epithelium-lamina propria separation, isolation was performed with collagenase A (Roche Diagnostics), 0.1 U/mL for 20 min at 37 °C with continuous stirring. The digest was purified through a 70 μm cell strainer (BD Biosciences). This procedure was repeated 6 times, and then the digest was immediately placed in monolayer culture.

Fibroblasts were seeded at a density of 10,000 cells/cm² and cultured in fibroblast medium composed of DMEM, 10% newborn calf serum (NCS), and antibiotics. The medium was changed every two days until cell confluence was reached. At confluence, cells were resuspended using trypsin 0.5 g/L–EDTA 0.2 g/L, then amplified over three processes (from P0 to P2) and seeded into the matrix at P3.

2.3. Substrate preparation

Collagen–GAG–chitosan substrates were prepared as previously described [16]. Briefly, types I and III bovine collagens, chitosan (95% deacylated) and chondroitin 4–6 sulfates (LPI, Lyon, France) were dissolved in water and mixed. After mixing, the gel, which contained 72% collagen, 20% chitosan and 8% GAG, was poured into Snapwell inserts (Costar) and frozen overnight at −70 °C. The frozen plates were then lyophilized, submerged in 70% ethanol for 24 h, rinsed and equilibrated in 5 mL of DMEM, and incubated at 37 °C with 5% CO₂ for a minimum of 24 h.

2.4. Preparation of lamina propria equivalents

Lamina propria equivalents consisted of collagen–glycosaminoglycan–chitosan (CGC) foams in which human oral mucosal fibroblasts were cultured. Briefly, lamina propria equivalents were prepared by adding a suspension of 2.5 × 10⁶/cm² on top of the 4 cm² CGC foam. Equivalents were then cultured for 21 days in a medium composed of DMEM, 10% fetal calf serum, 10 ng/mL epidermal growth factor, 50 μg/mL ascorbic acid (Bayer). Culture medium was changed daily until the seeding of epithelial cells.

2.5. Preparation of epithelialized oral mucosa equivalents

Human epithelial cells were plated on lamina propria equivalents at a concentration of 2.5 × 10⁶/cm². Epithelialized oral mucosal substitutes were cultured in epithelial cell medium supplemented with 50 μg/mL ascorbic acid (Bayer) under submerged conditions for 7 days. They were then elevated at the air–liquid interface for the remaining 14 days in another medium with DMEM–Ham-F12 2.2/1 (Sigma), 8 μg/mL bovine serum albumin, 0.4 μg/mL hydrocortisone (Upjohn), 0.12 UI/mL insulin (Umuline, Lilly), 50 μg/mL ascorbic acid (Bayer), and antibiotics.

2.6. Histology

Tissue equivalents were fixed in 4% formaldehyde solution and embedded in paraffin. Sections, 5 μm thick, were cut and stained using hematoxylin–phloxin–saffron (HPS).

2.7. Immunofluorescence

The primary antibodies used in this study to label oral mucosal equivalent, native oral mucosa and native skin were anti-cytokeratin 3 (Progen), anti-cytokeratin 10 (Novocastra), anti-cytokeratin 13 (Chemicon), anti-laminin 5 (Chemicon) and anti-Ki67 (Novocarsta). For the detection of K3, K10, K13 and laminin 5, tissue equivalents were embedded in OCT and frozen at −20 °C. Then, sections of 6 μm thickness were fixed in acetone for 10 min at −20 °C and blocked in phosphate buffered saline containing 4% bovine serum albumin and 5% normal goat serum. All primary antibodies were incubated for 90 min at room temperature. The secondary antibody was AlexaFluor 488 IgG (Invitrogen). For Ki67 antigen detection, same procedure was applied on formalin fixed paraffin embedded tissue with high temperature antigen retrieval and overnight incubation of the primary antibody. Propidium iodide or Hoechst 33,258 stain was used to stain the cell nuclei. In all immunofluorescence stainings, native nonkeratinized human oral mucosa and native human skin were used as either positive or negative controls. Specimens were analyzed with a Zeiss LSM 510 Confocal Laser Scanning Microscope and a Nikon Eclipse Fluorescence Microscope.

2.8. Transmission electron microscopy

Tissue equivalents were fixed with 2% glutaraldehyde−0.1 M NaCacodylate/HCl, pH 7.4 for 2 h and postfixed with 1% osmium tetroxide−0.15 M NaCacodylate/HCl, pH 7.4 for 1 h. After dehydration in a growing gradient of ethanol, the samples were embedded in Epon A + B and finally polymerized at 48 °C for 48 h. The blocks were cut using an ultramicrotome and sections of 60–80 nm thickness were contrasted with uranyl acetate and lead citrate. Observations were performed with a JEM JEOl 1400 transmission electron microscopy. Images were recorded using an Orius Gatan camera and 20 fibrib diameters were measured using a digital micrograph by two observers on three different regions of lamina propria, and average values were calculated.
3. Results

3.1. Histology

Histological analysis showed that the fibroblasts seeded into the collagen–GAG–chitosan foam were able to proliferate, migrate within the thickness of the substrate, and synthesize new extracellular matrix filling the pores of the substrate, giving rise to a lamina propria equivalent (LPE) (Fig. 1a). At the top of this LPE, epithelial cells proliferated during 7 days of culture under submerged conditions and 14 days of culture at an air–liquid interface, forming a nonkeratinized, multilayered (9–10 layers thick) epithelium and giving rise to a full-thickness oral mucosal equivalent (OME). The epithelial cells in the superficial layer were seen to retain their nuclei and stratum corneum was absent as in native nonkeratinized oral mucosa (Fig. 1c), whereas the skin equivalent cultured under the same conditions (our control of differentiation) is completely differentiated with a basal layer, a stratum spinosum, a stratum granulosum and a well-developed stratum corneum (Fig. 1b), as in native skin (Fig. 1d).

3.2. Immunohistochemistry

Keratin 3/76 (K3/76, clone AE5), which recognizes both K3 in cornea and K76 in oral mucosa, was strongly expressed in the OME and the native human OM, especially in suprabasal layers (Fig. 2a and b). It was, however, found to be completely absent in native human skin (Fig. 2c) (Table 1).

Keratin 10 (K10), marker of keratinized epithelium, was found to be completely absent in both our OME and native oral mucosa (Fig. 2d and e), but expressed in native human skin (Fig. 2f) (Table 1).

Keratin 13 (K13), the major differentiation-associated marker of nonkeratinized oral mucosa, was very strongly expressed in both OME and native OM (Fig. 2g and h) but not in skin (Fig. 2i). Its expression was restricted to suprabasal layers of our model (basal cells did not express it) (Fig. 2g) (Table 1).

The basement membrane protein laminin 5, the major component of anchoring filaments, was strongly expressed all along the interface between lamina propria and epithelium in the reconstructed oral mucosa, as well as in native oral mucosa and skin (Fig. 2j–l; Table 1).

Ki67 antigen, a marker of proliferative cells, was detected in basal cells of the OME as in native oral mucosa and skin. The number of proliferating cells labelled using anti-Ki67 was highest in native oral mucosa, followed by native skin and OME (Fig. 2m–o; Table 1).

3.3. Transmission electron microscopy

Transmission electron microscopy analysis of the reconstructed oral mucosa showed the ultrastructural organization of the
Fig. 2. Immunofluorescence labelling of keratins 3, 10 and 13; basement membrane protein laminin 5; and proliferating cell antigen Ki67 in the oral mucosal equivalent, native oral mucosa and native skin. Immunolabelling is shown in green, cell nuclei are shown either in blue (K3) or in red (K10, K13, laminin 5) (for K3: OME, OM, skin: ×100; for K10, K13, laminin 5: OME, skin: ×200, OM: ×100; for Ki67: OME, OM, skin: ×200).
epithelium, lamina propria and basement membrane (Fig. 3). In the epithelium, the size and shape of the cells were changing, the cells became larger and flattened as they moved and differentiated towards the oral surface (Fig. 3a). Encouragingly, numerous desmosomes were observed between adjacent epithelial cells (Fig. 3e and f). No stratum corneum and stratum granulosum were observed, as in the case for nonkeratinized native oral mucosa. A continuous and ultrastructurally well-organized basement membrane was formed on the lamina propria equivalent all along the interface between the epithelium and the lamina propria (Fig. 3c and d). In the subepithelial layer and in the deep layer of lamina propria of the reconstructed oral mucosa, high amount of newly synthesized collagen was detectable by

Table 1
Results of the immunohistochemical staining of the human oral mucosal equivalent, native human oral mucosa and native human skin.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Human oral mucosal equivalent</th>
<th>Native human oral mucosa</th>
<th>Native human skin</th>
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<tr>
<td>K3</td>
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<td>Ki67</td>
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* +++: very strong, ++: strong, +: moderate, –: no expression.

Fig. 3. Ultrastructural analysis of the oral mucosal equivalent by transmission electron microscopy a) different cell layers and differentiation in the epithelium, epithelial cells become flattened as they move from the basal up to superficial layer (arrow) (bar = 5 μm) b) two adjacent basal epithelial cells (ECs) residing on the lamina propria equivalent (LPE) (bar = 2 μm) c) basement membrane (BM) formed between the LPE and the epithelium (bar = 2 μm) d) the continuous and well-organized BM at higher magnification anchoring the epithelium firmly to the LPE (bar = 0.5 μm) e) numerous desmosomes were detected between adjacent epithelial cells (bar = 0.5 μm) f) a desmosome at higher magnification (bar = 200 nm) g) newly synthesized collagen I fibrils in the LPE (bar = 0.5 μm) h) collagen fibril striations visible at higher magnification (bar = 100 nm).
transmission electron microscopy (Fig. 3g), striations of the collagen fibrils becoming visible at higher magnifications (Fig. 3h). The connective tissue was represented by fibrils of collagen straight in some regions and wavy in others, and some of them oriented parallel to the oral surface, some not (Fig. 3g). The average collagen fibril diameter in the reconstructed oral mucosa was measured as 28.4 nm.

4. Discussion

Our 3-D model based on collagen–GAG–chitosan allowed us to reproduce an OME comprising a pluristratified epithelium firmly anchored to an LPE through an ultrastructurally well-organized epithelial/LP junction mimicking native nonkeratinized human oral mucosa. To our knowledge, we are the first to obtain a nonkeratinized epithelium under optimal conditions for differentiation with complete exposure of the equivalent’s surface to air at the air–liquid interface during 2 weeks in a medium poor in growth factors and serum. Previous studies on tissue engineered oral mucosa have mostly focused on keratinized OMEs. There is only one study where a nonkeratinized OME was reconstructed using gingival cells and adding medium at the top of the epithelium to mimic oral cavity conditions. But the humidity is known to inhibit differentiation [22]. The fact that the epithelial cells are nonkeratinized may be either due to their interaction with fibroblasts of lamina propria, or to their genetic programming. To elucidate this, we plan to compare the differentiation of epithelial cells on dermal, stromal and lamina propria equivalents.

In the epithelium of our model, epithelial cells retained their nuclei and stratum corneum was absent, as revealed by HPS staining (Fig. 1a). The very strong expression of K13, which is the major differentiation-associated marker of nonkeratinized epithelium in oral mucosa, and the absence of keratin 10, which is expressed only by keratinizing epithelial cells, supported the suggestion (Fig. 2n and o). Ki67 antigen, a marker of proliferative cells, was detected in the basal cells of the OME as in native oral mucosa and skin, indicating that the OME was capable of self-renewal (Fig. 2m). However, although the spatial distribution of proliferating cells in the OME was very similar to native tissues, their number was found to be higher in native tissues. The progressive decrease in the number of cells expressing Ki67 has already been shown in the course of kinetics of proliferation in an oral mucosa model [22], passing from a maximum proliferative state at the first week to a few cells at the end of third week, as we have. This is due to the fact that the highest proliferation rate in a developing oral mucosa is at the early stage of epithelial formation (1 week after epithelial cell seeding) and it decreases progressively as time passes [22]. In our model, Ki67 antigen detection was performed at the end of epithelial formation (3 weeks after epithelial cell seeding), resulting in less number of cells retaining proliferative capacity, because what we wanted to show was that even after such a longtime, the cells were still able to proliferate, so that if we transplant this oral mucosal equivalent in vivo, the epithelium will still be able to self-renew.

Keratin 3/76 is the most commonly used antibody prepared from AES clone (K3/76, AES). It was expressed in native oral mucosa and in our OME. Our results are in accordance with a previous study where the expression of keratin 3 has been shown in oral mucosal epithelial cell sheets [27], suggesting that keratin 3, which is known as a specific marker of corneal epithelia cells, is also a marker of oral mucosal epithelial cells cultured under same conditions on a) a lamina propria equivalent forming a thick epithelium (>100) b) a stromal equivalent forming a thin epithelium (<100); however, expressing K13 strongly in both cases. Immunolabeling for K13 is shown in green and cell nuclei are stained in red with PI.

![Fig. 4. Same oral epithelial cells cultured under same conditions on a lamina propria equivalent forming a thick epithelium (>100) b) a stromal equivalent forming a thin epithelium (<100).](image131x600 to 475x727)

**Fig. 4.** Same oral epithelial cells cultured under same conditions on a) a lamina propria equivalent forming a thick epithelium (>100) b) a stromal equivalent forming a thin epithelium (<100); however, expressing K13 strongly in both cases. Immunolabeling for K13 is shown in green and cell nuclei are stained in red with PI.
mucosa. Here, we show for the first time the expression of K3/K76 in an oral mucosal equivalent (Fig. 2a). In fact, it was recently reported that this antibody from the AE5 clone detects not only the expression of K3, but also that of the related K76 (formerly K2p), which is specifically expressed in suprabasal layers of oral masticatory epithelium [28]. So, it is possible that the labelling observed in our OME was due to the reaction of the antibody with K76 and not with K3.

On the other hand, using TEM we could observe at the epithelium level several desmosomes (Fig. 3e), these intercellular junctions are crucial for epithelial adhesion and barrier function in stratifying epithelia [29].

The basement membrane formed in our model was continuous and ultrastructurally well-organized all along the interface between epithelium and the LPE, as shown by both immunolabeling for the classical basement membrane marker laminin 5 (Fig. 2j), and TEM (Fig. 3d). This ultrastructural organization was previously shown to be the result of the interaction between fibroblasts and keratinocytes [30]. Only models allowing contact between fibroblasts of extracellular matrix and keratinocytes present this organization. So, our results indicate that our scaffold permitted a dialogue to occur between epithelial cells and fibroblasts, as would be the case in vivo. The basement membrane is an important feature a reconstructed oral mucosa should possess, it is the attachment zone necessary to withstand shear stress in oral mucosa [1], and also has an important role in wound healing and disease [25].

Deeper, in the LPE, abundant amount of newly synthesized collagen was detected by TEM (Fig. 3g). Some of the collagen fibrils were straight and parallel to the oral surface, and some were wavy and less organized (Fig. 3g). In fact, the collagen fibres in the lamina propria of the nonkeratinized oral mucosa were reported to be loosely and less regularly organized compared to keratinized mucosa where they are arranged in bundles [25]. The average collagen fibril diameter in the reconstructed oral mucosa (28.4 nm) was similar to native human oral mucosa, for which the collagen fibril diameter range has been reported as 20–40 nm. This 20 nm range is due to the fact that the average fibril diameter varies with the region in the mouth, or for the same region with their location in the lamina propria [31] and [32]. Collagen fibrils in native human skin are of larger diameter (53–82 nm) compared to oral mucosa [33].

Because this oral mucosa 3-D model is very close to human tissue, we propose the use of this OME as a model to test the efficiency or toxicity of oral care products, or it may be further tested in vivo for potential clinical applications. The main challenge for success in vivo is to secure the attachment and survival of cultured cells on the wound beds. For this purpose, future strategies to accelerate vascularization, such as reconstruction of endothelialized oral mucosal substitutes by incorporation of endothelial cells and vascular endothelial growth factor (VEGF) are needed.

5. Conclusion

We demonstrated the possibility to reproduce a nonkeratinized oral mucosa with a thick epithelium under culture conditions which favour differentiation. This tissue engineered oral mucosa results from the interactions between fibroblasts of lamina propria and epithelial cells of oral mucosa.

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Appendix

Figures with essential colour discrimination. Most of the figures in this article have parts that are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.08.010.

References


