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Ex vivo produced oral mucosa equivalent preliminary report: a technical note

Gürkan Raşit BAYAR

Aim: The aim of this study was to produce of ex vivo oral mucosal equivalent. The continuous stratified layer of human oral keratinocytes was grown on a cadaveric human dermal matrix in a defined culture medium without a feeder layer.

Materials and methods: Human oral keratinocytes from a keratinized oral mucosa biopsy specimen were enzymatically dissociated and cultured in a serum-free defined culture medium, "EpiLife" (Cascade Biologics, Portland, OR, USA), containing a low calcium concentration of 0.06 mM. Oral keratinocytes were expanded one passage. Once a sufficient population of keratinocytes was reached, they were seeded onto the type IV collagen coated acellular nonimmunogenic cadaveric human dermis (AlloDerm), at cell densities of 1.8×10^5 . To form a continuous epithelial monolayer, and to enhance keratinocyte differentiation, oral keratinocyte-AlloDerm composites were cultured while submerged in a high calcium concentration of 1.2 mM medium for 4 days. After 4 days, AlloDerm-keratinocyte composites were raised to an air-liquid interface to encourage stratification of the epithelial monolayer. After additional 7 days, they were taken for histologic examination at 11 days postseeding of the keratinocytes on the AlloDerms.

Results: Histologically, on day 11, EVPOME's development showed multilayered epithelium comprising basal, suprabasal, and parakeratinized layers.

Conclusion: Histologically, an ex vivo produced oral mucosa equivalent (EVPOME) that consisted of a stratified epidermis on a dermal matrix was successfully developed by the method used.

Key words: Oral, mucosa, substitute, ex vivo, production

Vücut dışında üretilmiş ağız mukozası eşdeğeri ön çalışma raporu: Bir teknik not

Amaç: Bu çalışmanın amacı, vücut dışında insan ağız mukozası eşdeğeri üretmektir. İnsan ağız mukozası keratinositlerinden oluşan ve devamlı, sıralı şekilde katmanlanmış olan canlı bir tabaka, kadavradan elde edilmiş olan hücreli insan deri matrisi üzerinde ve tanımlanmış bir doku kültürü solüsyonu ortamında, hayvan kaynaklı besleyici herhangi bir materyal kullanılmadan üretilmeye çalışıldı.

Yöntem ve gereç: Keratinize ağız mukozası biyopsi örneğinden elde edilen insan ağız mukozası keratinositleri enzimatik olarak ayrıştırıldı ve hayvan kaynaklı herhangi bir besleyici materyal içermeyen, 0,06 mM oranında düşük kalsiyum içeren, tanımlanmış bir kültür ortamında "EpiLife" (Cascade Biologics Portland, OR, USA), bir pasaj çoğaltıldı. Yeterli keratinosit popülasyonuna ulaşıldıktan sonra, keratinositler tip IV kollajen emdirilmiş hücreli, immünojenik olmayan kadavradan elde edilmiş deri matrisi (AlloDerm) üzerine $1,8 \times 10^5$ hücre dansitesi ile ekildi. Devamlı tek tabakalı epiteliyal bir katman elde etmek ve keratinosit farklılaşımını hızlandırmak amacıyla, ağız mukozası keratinositleri ve AlloDerm' den oluşan doku kompoziti, 1,2 mM yüksek kalsiyum konsantrasyonu içeren, tanımlanmış doku kültürü solüsyonu içerisine batacak şekilde 4 gün kültüre edildi. AlloDerm-keratinosit doku kompoziti, epiteliyal tek tabakanın katmanlaşmasını uyarmak amacıyla, 4 gün sonra hava-sıvı etkileşimli ortama aktarıldı. Bu ortamda geçen 7 günden sonra ise, toplam 11 gün sonra, doku kompoziti örnekleri histolojik inceleme için hazırlandı.

Bulgular: Histolojik olarak 11. günde, vücut dışında üretilmiş ağız mukozası eşdeğeri, bazal, suprabazal ve parakeratinize tabakalardan oluşan çok katlı bir epitel gelişimi sergiledi.

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Sonuç: Histolojik olarak kullanılan yöntemle, vücut ortamı dışında hücresiz bir dermal matris üzerinde doğal olarak katmanlanmış epidermis başarıyla üretilmiş oldu.

Anahtar sözcükler: Ağız, mukoza, eşdeğer, vücut dışı, üretim

Introduction

Burns, chronic ulcers, and most reconstructive procedures in oral and maxillofacial surgery require skin and oral mucosa substitutes for enhancing the healing of wounds. Autogenous keratinized oral mucosa and split thickness skin grafts are still being used despite their disadvantages like requiring a second surgical procedure, limiting to supply and unsuitable texture of skin grafts for intraoral grafting. However, the ability to produce ex vivo oral mucosa equivalent from a punch biopsy, composed of an epithelial and dermal component, within 1 to 2 months can assist oral and maxillofacial surgeons. This will make available an unlimited supply of oral tissue that will have similar characteristics to native mucosa (1-3).

In designing a tissue-engineering human oral mucosal equivalent, it should duplicate the tissue's anatomic structures and physiologic functions. In the development of a mucosal equivalent 2 basic components exist: a superficial portion or epidermis that contains keratinocytes, and the deeper portion or dermis (4).

In the past, Rheinwald and Green's technique (5) was used to fabricate cultured oral epithelial sheets. Their protocol was using a feeder layer composed of irradiated 3T3 mouse fibroblasts to grow keratinocytes in vitro. Oral mucosal sheets cultured with an irradiated feeder cell layer were undesirable in elective surgery because of the undetermined risk of introducing a high mouse DNA content onto proliferating human cells (1,6-8).

AlloDerm (LifeCell, Woodlands, TX, USA) was recommended by many investigators as a more suitable dermal matrix for development of an oral mucosal equivalent (1,9). AlloDerm is an acellular nonimmunogenic cadaveric human dermis. It has a polarity by which one side of the material has a basal lamina to grow epithelial cells on, and the other side, an underlying porous dermal matrix, allows ingrowth of fibroblasts and angiogenic cells (1,10).

In 1999, Izumi et al (1) successfully developed an ex vivo composite oral mucosal equivalent that consisted of stratified epidermis on an acellular nonimmunogenic cadaveric human dermis matrix, and they have optimized their method (1,4,11-15). Moreover, after in vivo studies, it was reported that ex vivo produced oral mucosa equivalent (EVPOME) appears to be an acceptable oral mucosal substitute for human intraoral grafting procedures (7,13,14).

In the current study, an optimized version of the original method described by Izumi et al in 1999 (1) was used to obtain a much cleaner keratinocyte culture by using an animal-product-free culture medium (EpiLife Cascade Biologics, Portland, OR, USA) supplemented with human keratinocyte growth factors (EDGS Cascade Biologics, Portland, OR, USA). EVPOME developed by this optimized method can be more suitable for intraoral grafting procedures.

Materials and methods

University of Michigan Internal Review Board approved protocol utilized human oral keratinized tissue for the development of an oral mucosa equivalent.

Dissociating human oral keratinocytes from primary tissue

The basic "culture medium" [chemically defined culture medium (EpiLife Cascade Biologics, Portland, OR, USA) supplemented with human keratinocyte growth factors (EDGS Cascade Biologics, Portland, OR, USA), 125 µg/mL gentamycin, and 1 µg/mL fungizone (Sigma Chemicals Co.)] was prepared with a calcium concentration of 0.06 mM.

In the outpatient clinic, under local anesthesia, a 5 × 5 mm keratinized oral mucosa sample was obtained from retromolar pad of a 19-year-old female patient undergoing a dental extraction. The tissue sample was transported in "washing solution" [phosphate-buffered saline (PBS) (Ca⁺⁺ and Mg⁺⁺ free) which was supplemented with 125 µg/mL gentamycin, and 1 µg/mL fungizone (Sigma Chemicals Co.)]. The oral

mucosal sample was placed in a new washing solution, scraped to remove blood, and trimmed of excess tissue. The mucosal tissue in the 0.04% trypsin solution (Sigma Chemicals Co.) was digested overnight at room temperature to allow the separation of the epithelium at the suprabasal layer. The next day, after inactivation of the trypsin by 0.0125% trypsin inhibitor (DTI Cascade Biologics, Portland, OR, USA), the epithelial layer of the mucosal sample was mechanically separated above the basal layer, and the interface area was scraped to dissociate the basal cells from the sub-mucosal layer. The cells in the filtered (by 240 μm nylon mesh filter) cell suspension were counted with a hemacytometer. After it was centrifuged (5 min/1000 rpm/room temperature), and re-suspended by 5 mL culture medium, 2.3×10^6 cells were plated in a T-25 flask (Laboratory Science Co. Corning, NY, USA), and incubated at 37 °C in 5% CO_2 . The medium was changed 2 days after initial plating of the cells. The culture was fed every other day with culture medium containing a low calcium concentration of 0.06 mM. After 10 days, oral mucosa keratinocytes were harvested when they reached 70%-80% confluent. Some of the first passaged keratinocytes were split and used for the fabrication of EVPOME. Others were re-plated into the 3 different T-25 flasks at a density of 2.0×10^4 cells/cm² and used for subsequent cell proliferation assays in monolayer culture.

Preparation of AlloDerm

AlloDerm (LifeCell, Woodlands, TX), the acellular human cadaver dermis, was cut into circular pieces 11.3 mm diameter in order to conform to the area of a 48-well microplate (Corning Inc. Corning, NY, USA). Two circular samples were rehydrated in washing solution. Epidermal and dermal surfaces of the samples were identified and transferred to the 48-well microplate with epidermal surfaces up. They were pre-soaked and kept overnight at 4 °C in 100 μL of PBS and Human Type IV Collagen 5 $\mu\text{g}/\text{cm}^2$ to enhance attachment of seeded keratinocytes.

Production of keratinocytes on AlloDerm

Oral keratinocytes from the first passage of actively dividing cells were used to seed onto AlloDerm samples. They were harvested by adding a solution of trypsin-ethylenediaminetetra-acetic acid

(Trypsin-EDTA, TE Cascade Biologics, Portland, OR, USA) at 37 °C. Trypsin-EDTA activity was inhibited with an equal volume of 0.0125% trypsin inhibitor. Disaggregated cells were collected, counted, centrifuged, and re-suspended. Cells were seeded onto AlloDerm samples in 2 different 48-well microplates at the density of 1.8×10^5 cells. At that time, 1.2 mL of "culture medium" containing a high concentration of calcium (1.2 mM) was gently added to 48-well microplates without disturbing the cells. The oral keratinocyte-AlloDerm composites were cultured and submerged for 4 days in the 48-well culture microplates. Composites were fed daily during this period with culture medium.

After incubating the composites in a submerged environment for 4 days, they were transferred to organotypic tissue culture flasks (Organogenesis Inc. Canton, MA, USA). The medium was changed every other day for 7 more days. The organotypic tissue culture flasks allowed the composites to grow at an air-liquid interface. This culturing technique has been shown to encourage stratification of the epithelial layers (1,16).

Histological examination

EVPOMEs were removed from the culture at 11 days post-seeding of the keratinocytes and fixed in 10% formaldehyde (Figure 1). The fixed EVPOMEs were embedded in paraffin and cut at 5 μm and stained with hematoxylin and eosin.

Results

After the enzymatic dissociation of keratinized oral mucosal tissue samples with 0.04% trypsin overnight, keratinocyte proliferation was enhanced and shown to be in a hyperproliferative state without evidence of fibroblastic contamination when they were cultured in a chemically defined serum-free culture medium (Figure 2). Histologically, on day 11, EVPOMEs development showed multilayered epithelium comprising basal, suprabasal, and parakeratinized layers (Figure 3). The epithelial architecture of EVPOMEs resembled that of normal mucosa (16) (Figure 4). Highly stratified EVPOMEs showed evidence of parakeratosis. Keratinocytes of the basal layer were cuboid or circular, and they were aligned along the AlloDerm surface. Under the basal

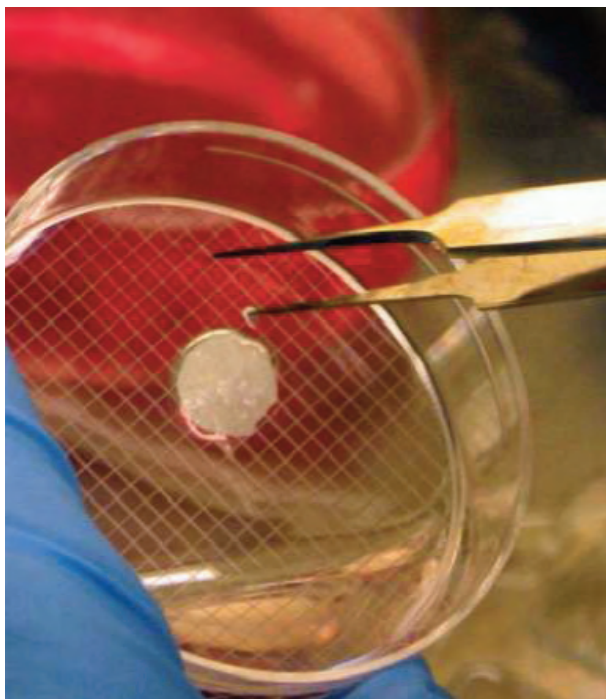


Figure 1. Macroscopic appearance of EVPOME sample before histologic examination.

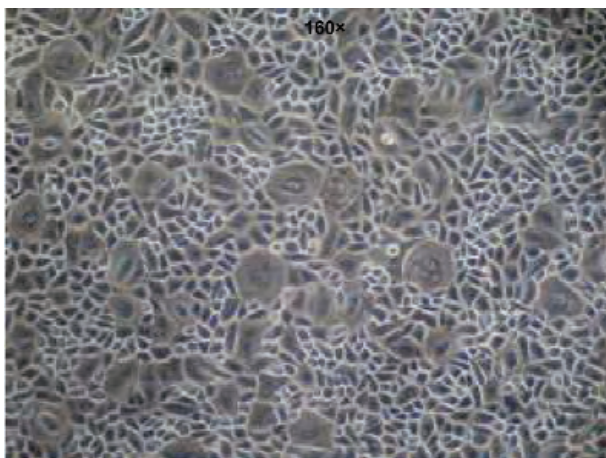


Figure 2. Hyperproliferative state of keratinocytes in a chemically defined serum-free culture medium (160x).

layer of EVPOMEs AlloDerm showed no evidence of any cellular components and consisted of interlacing dense collagen bundles of varying sizes, indicating that the structural integrity of the extracellular matrix was intact (Figure 2) (1,3).

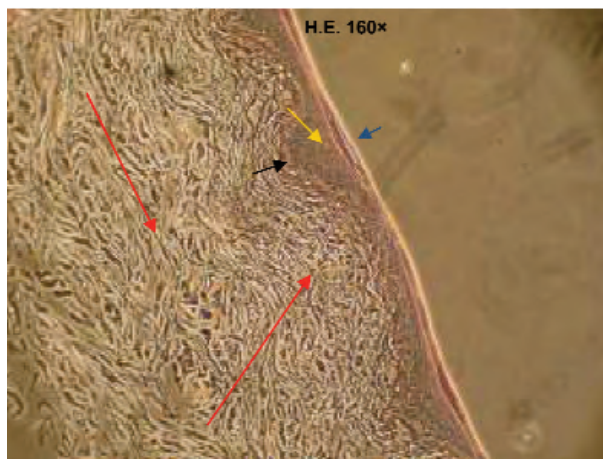


Figure 3. Histological appearance of EVPOME sample stained by hematoxylin and eosin showed that multilayered epithelial layer comprised a basal layer (black arrow), suprabasal layer (yellow arrow), and a parakeratinized layer (blue arrow). Under the basal layer of EVPOMEs. AlloDerm shows no evidence of any cellular components and consisted of interlacing dense collagen bundles of varying sizes, indicating that the structural integrity of the extracellular matrix was intact (red arrows) (H.E. 160x).

Discussion

Tissue-engineered oral mucosal equivalents have been developed for clinical applications and an in vitro test model for wound healing, mucotoxicity, and biocompatibility studies. In recent years, research has concentrated on the development and characterization of human oral mucosal equivalents by introducing new dermal scaffolds and epithelial cell culture methods (17).

The protocol developed by Izumi et al. (1) to produce ex vivo oral mucosa equivalent for intraoral grafting procedures has several advantages over previously published techniques (1,3). Firstly, they could propagate and expand oral keratinocytes in vitro, in a defined culture medium devoid of the use of an irradiated 3T3 mouse feeder layer. The combination of human cells and 3T3 mouse fibroblasts may contribute to transfection of the mutational or xenogenic DNA to human keratinocytes. That is why it is suggested that the defined culture medium would be more acceptable for human use. The second modification of the Izumi et al.'s protocol is the use of trypsin to dissociate basal cells from the dermal layer in association with the

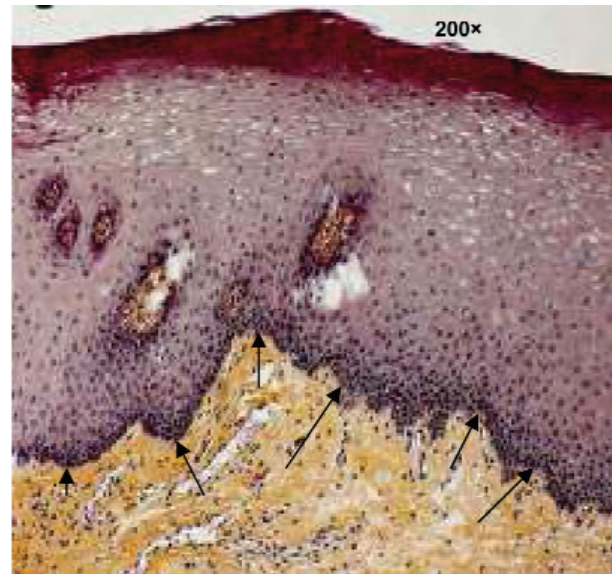
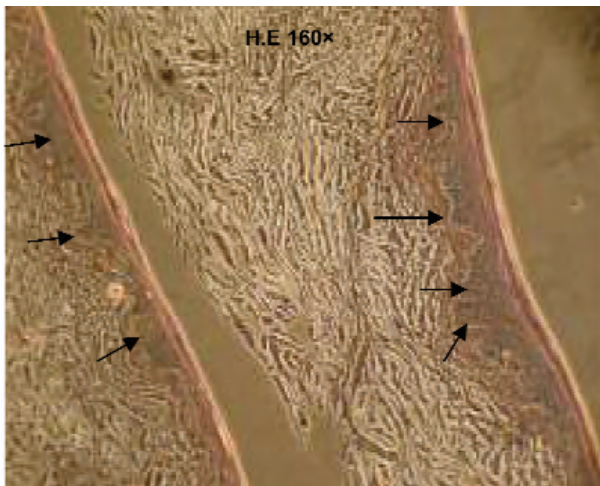


Figure 4. Histologically, the epithelial architecture of EVPOMEs (H.E. 160 \times) (a) resemble that of a normal oral mucosa (H.E. 200 \times) (b) (15). Black arrows show cuboid or circular keratinocytes of basal layer in normal oral mucosa and EVPOME samples

primary cultivation in a defined culture medium. It has allowed us to successfully culture, amplify, and harvest a pure population of keratinocytes without contamination of fibroblasts (1,8,18).

In 1975, Rheinwald and Green (5) introduced a method to grow human keratinocytes in serial culture *in vitro* and produce single-layer epithelial sheets. Monolayer cultures have been extremely helpful in the study of the basic biology, and responses to stimuli, of both oral and skin keratinocytes, and many studies have used them. However, the oral epithelium and epidermis are complex multilayer structures, with cells undergoing terminal differentiation, and monolayer cultures may not be a good model of what is happening *in vivo*. The culture of keratinocytes on permeable cell culture membranes at the air/liquid interface facilitated the construction of multilayer sheets of epithelium, which resemble native epithelium and show signs of differentiation, such as basement membrane formation, different cytokeratin expression, and keratinization if the origin of the keratinocytes is keratinized mucosa (17). In this preliminary study, histological samples of EVPOME stained by hematoxylin and eosin showed multilayered epithelium comprising basal, suprabasal, and parakeratinized layers.

Another important element in oral mucosa and skin reconstruction is the scaffold that supports the cells. Choosing the right scaffold with ideal biocompatibility, porosity, biostability, and mechanical properties is a crucial step in tissue engineering. In this preliminary study, acellular cadaveric human dermis (AlloDerm) was used as a scaffold for the tissue engineering of oral mucosa. AlloDerm has a polarity by which one side has a basal lamina suitable for epithelial cells, and the other side has intact vessel channels suitable for fibroblast infiltration. It is a durable acellular dermis that retains extracellular matrix proteins and intact basement membrane structure, while being surgically manageable (1,17). Although several investigations had been reported on the use of either AlloDerm or deepidermized human dermis in the development of reconstituted skin and oral mucosa (13,19,20), the first *ex vivo* produced oral mucosa equivalent utilizing AlloDerm in a serum-free defined culture medium without an irradiated xenogenic feeder layer was reported by Izumi et al. in 1999 (1). In their *in vivo* animal studies, the AlloDerm portion of the EVPOME showed no evidence of an adverse inflammatory reaction or rapid remodeling reaction in the early stages after grafting but rather appeared to

act as a biomimetic template that promoted the intradermal migration and infiltration of fibroblasts and host cells. Furthermore, AlloDerm with epithelium of EVPOME showed marked remodeling of the collagenous bundles in comparison with AlloDerm without epithelium. This eventually progressed to a point of contracture of the EVPOME 21-day postgrafting (13).

When clinical transplantation of EVPOME was compared with that of AlloDerm alone or of artificial dermis, it resulted in: 1) earlier initiation of epithelization, 2) a shorter period until complete healing, and 3) negligible scar contracture (14). Hotta et al. (14) suggested that the EVPOME graft may be a more acceptable oral mucosa substitute for human intraoral grafting procedures, resulting in a more favorable wound healing response. The advantage of the EVPOME over the AlloDerm may have been related to the persistence of a transplanted epithelial basal cell layer resulting in the development of a more rapid and mature epithelium. In the recent literature, it has been stated that the persistent basal layer of grafted keratinocytes and/or the initial presence of an intact epithelial layer of EVPOME may release cytokines and growth factors that are known to be secreted by keratinocytes (12). Furthermore, it has

been shown by in vivo studies that the vascular endothelial growth factor (VEGF) is secreted by EVPOME grafts and it may contribute to early vascularization after grafting (3,12,13).

It was reported that the absence of adequate keratinized mucosa around implants supporting overdentures was associated with higher plaque accumulation, gingival inflammation, bleeding on probing, and mucosal recession (21). From a 4×4 mm² punch biopsy of the palate, it would take approximately 40 days to fabricate an EVPOME the size of approximately 65×150 mm². This size of EVPOME should be large enough to cover most mucosal defects (2). This method may be used during implant treatment. The procedure may be initialized at the implant insertion phase, and subsequently the gathered graft might be used during the second surgery for submerged implants.

Recent and future studies entail shortening the time for fabrication of the EVPOMEs necessary for reconstructive surgery, as well as isolating a long-live transplanted cell that would be able to continue secretion of a transfected recombinant product, such as a cytokine or growth factor, over a sustained period of time. This will require the identification and isolation of a putative progenitor/stem cell (4,15).

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