Published in final edited form as:

Int J Oral Maxillofac Implants. 2013 ; 28(5): e295–e303.

Intraoral Grafting of Tissue-Engineered Human Oral Mucosa

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Abstract

Purpose—The primary objective of this study was to evaluate the safety of a tissue-engineered human ex vivo–produced oral mucosa equivalent (EVPOME) in intraoral grafting procedures. The secondary objective was to assess the efficacy of the grafted EVPOME in producing a keratinized mucosal surface epithelium.

Materials and Methods—Five patients who met the inclusion criteria of having one mucogingival defect or a lack of keratinized gingiva on a nonmolar tooth, along with radiographic evidence of sufficient interdental bone height, were recruited as subjects to increase the width of keratinized gingiva at the defect site. A punch biopsy specimen of the hard palate was taken to acquire oral keratinocytes, which were expanded, seeded, and cultured on an acellular dermal matrix for fabrication of an EVPOME. EVPOME grafts were applied directly over an intact periosteal bed and secured in place. At baseline (biopsy specimen retrieval) and at 7, 14, 30, 90, and 180 days postsurgery, Plaque Index and Gingival Index were recorded for each subject. In addition, probing depths, keratinized gingival width, and keratinized gingival thickness were recorded at baseline, 30, 90, and 180 days.

Results—No complications or adverse reactions to EVPOME were observed in any subjects during the study. The mean gain in keratinized gingival width was 3 mm (range, 3 to 4 mm). The mean gain in keratinized gingival thickness was 1 mm (range, 1 to 2 mm). No significant changes in probing depths were observed.

Conclusion—Based on these findings, it can be concluded that EVPOME is safe for intraoral use and has the ability to augment keratinized tissue around teeth. Future clinical trials are needed to further explore this potential.

Keywords

clinical trial; Good Manufacturing Practices; keratinized gingiva; keratinocytes; oral mucosa; tissue engineering

Standard surgical replacements for use in reconstruction of the oral cavity, such as split-thickness skin grafts, have the disadvantages of including adnexal structures and a different keratinization pattern.¹ The major disadvantages of harvesting oral mucosa are its limited supply in the oral cavity and marked pain at the palatal donor site.² Thus, there is a real need for tissue-engineered oral mucosa materials for use after major trauma, surgical resections, and maxillofacial preprosthetic surgery, as well as periodontal treatments. In recent decades, human skin equivalents, with anatomical and biochemical similarities to human skin, have become more available for use in the treatment of large full-thickness skin defects.² In contrast, the development of a human oral mucosa equivalent for use in intraoral grafting has lagged behind. However, a few reports have introduced clinical application of several types of tissue-engineered oral mucosa substitutes.^{3–6}

Previous studies have developed a bilayered, tissue-engineered oral mucosa (ex vivo–produced oral mucosa equivalent [EVPOME]) that had similar histologic appearance, handling, and functional properties (ie, secretion of appropriate cytokines) to those of native oral mucosa.^{6–10} The EVPOME was made from primary human oral keratinocytes, which were harvested from palatal keratinized mucosa and expanded in vitro in an environment free of serum, transformed irradiated xenogeneic feeder cells, and pituitary extract in a defined culture medium.⁶ After sufficient oral keratinocytes were produced, they were seeded onto AlloDerm (an acellular dermal matrix; LifeCell). The entire process from harvesting of tissue to production of a full-thickness EVPOME suitable for intraoral grafting took less than a month. This required the manufacturing of the EVPOME under strict current Good Manufacturing Practices (GMP) standards to ensure that the cells utilized in the fabrication were consistent with the guidelines for cell-based products developed by the Center for Biologics Evaluation and Research (CBER) of the United States Food and Drug Administration (FDA). Current GMP regulations require a quality approach to manufacturing to minimize or eliminate instances of contamination and errors; this includes recordkeeping, personnel qualifications, sanitation, cleanliness, equipment verification, process validation, and complaint handling. In addition, a series of tests must be performed to assess sterility (bacteria, mycoplasma) and develop release criteria to ensure a level of quality control.¹¹

The primary objective of this study was to evaluate the safety of the autologous EVPOME in intraoral grafting procedures. The secondary objective was to assess the efficacy of the grafted EVPOME in increasing the amount of keratinized mucosa after grafting around teeth lacking sufficient masticatory epithelium. The clinical trial presented in this paper was done under an investigator-initiated Independent New Drug (IND) application approved by CBER of the FDA.

MATERIALS AND METHODS

Patients

Subjects were recruited for this pilot clinical trial at the University of Michigan. All participants had to sign a University of Michigan Internal Review Board–approved informed consent document. All patients who met the inclusion criteria of having one mucogingival defect on a nonmolar tooth, radiographic evidence of sufficient interdental bone height (≥ 2 mm between crestal bone and cemento-enamel junction), and a need for surgery to increase the width of keratinized gingiva (clinically indicated) were selected as potential subjects. Patients were excluded from the study for any of the following reasons: (1) history of medical complications caused by compromised wound healing; (2) current pregnancy or currently attempting to become pregnant; (3) evidence of clinically significant renal, hepatic, cardiac, endocrine, hematologic, autoimmune, or systemic disease that might make implication/ interpretation of the protocol or results difficult; (4) known or suspected allergy to bovine protein; (5) previously considered to be noncompliant; (6) inability to provide informed consent; or (7) participation in another clinical trial using an investigational new drug or device within 30 days of entrance into this study.

Presurgical Procedures

Each participant received initial periodontal therapy consisting of oral hygiene instruction, scaling and root planing, coronal polishing, and occlusal adjustment as needed prior to entry into the study. Preoperative periapical radiographs and photographs were obtained. Plaque Index (PI), Gingival Index (GI), probing depths (PDs), and keratinized gingival width (KGW) were recorded for the involved teeth at baseline prior to the EVPOME grafting. The KGW (the distance between the gingival margin and the mucogingival line) was measured to the nearest millimeter with a standard University of North Carolina manual probe. Prior to the study, the examiner (RFN) was calibrated to reduce intraexaminer error ($\kappa > 0.75$) to ensure reliability and consistency.

Cell Harvesting

Following administration of local anesthetic, the palatal donor site was prepped with betadine swabs. Then a 5-mm-wide, 2-mm-deep punch biopsy specimen of the hard palate was obtained (Fig 1a). The harvested tissue specimen was immediately placed into transportation solution of phosphate-buffered saline solution supplemented with 6 mmol/L glucose, 125 µg/mL gentamycin, and 1 µg/mL amphotericin B in a 50-mL conical tube, which was labeled appropriately. The tube was then taken to the University of Michigan Human Applications Laboratory, a current GMP/good tissue practice facility, for processing of the tissue specimen.¹² A collagen wound dressing material (CollaTape, Zimmer Dental) was used as needed to establish hemostasis at the punch biopsy site. A cyanoacrylate dressing was used to cover the donor site area.

Cell Culture

Oral mucosal samples were removed from the transportation solution, scraped clean, and washed three times to remove blood. The remaining mucosal tissue was punctured several

times to enhance penetration of a 0.03% trypsin solution prepared in phosphate-buffered saline, in which the tissue was incubated overnight at room temperature to allow separation of the epithelium from the underlying connective tissue at the submucosal-mucosal junction. The trypsin was inactivated the next day by transferring the tissue into a 0.01 25% defined trypsin inhibitor solution (Invitrogen). The epithelial layer was then mechanically separated from the submucosal layer and the interface area was scraped to dissociate the underlying basal cells. The cell suspension was filtered using a 100- μ m cell strainer (Fisher Scientific), counted with a hemacytometer, and plated (up to 7.0×10^6 cells) in 5-mL complete medium (EpiLife containing 0.06 mmol/L Ca^{2+} supplemented with EpiLife Defined Growth Supplement; Invitrogen) containing 25 μ g/mL gentamicin and 0.375 μ g/mL amphotericin B per T-25 flask and incubated at 37°C in 5% CO_2 . The medium was changed 48 hours after the initial plating of cells. Gentamicin and amphotericin B were added to the complete medium only for the first 2 days of cell culturing to assist in preventing contamination of the cultured cells. The cultures were fed every other day with the complete medium. When cells reached 70% to 80% confluence, they were subcultured at 7.0×10^5 cells per T-25 flask.

EVPOME Fabrication

Oral keratinocytes from the second passage of cells were used to seed onto AlloDerm. The oral keratinocytes were harvested by a solution of recombinant trypsin–ethylenediaminetetraacetic acid (0.025%/0.01%) (Invitrogen) at 37°C. Trypsin activity was inhibited with an equal volume of 0.0125% defined trypsin inhibitor. Disaggregated cells were collected, counted, spun, and resuspended in the complete medium, which contained a high concentration of calcium (1.2 mmol/L). A density of 1.25×10^5 cells/200 μ L/cm² was placed onto the AlloDerm, and 1 mL of a high-calcium complete medium was gently added to the microwell plates and then allowed to remain undisturbed for 4 hours. The oral keratinocyte/AlloDerm composites were then cultured, submerged, for 4 days in the microwell plate. Composites were fed daily during this time period with a high-calcium medium. At day 4, they were raised to an air-liquid interface and then cultured for another 7 days. They were fed a total of three times during these 7 days in an air-liquid interface. At 4 weeks after biopsy retrieval, several circular pieces of EVPOME, 20 mm in diameter, were ready for grafting (Fig 1b). For quality assurance/ control of the EVPOME, under the regulation of current GMP/good tissue practice standards, the culture medium supernatants of EVPOME grafts were sampled on all feeding days during the fabrication of EVPOME at the Human Applications Laboratory. On the day of grafting, prior to product release, the culture supernatant was used to assess microbial contamination and the level of endotoxin for the sterility test. Trimmed pieces of EVPOME to be grafted were examined for the presence of mycoplasma. To assess cell viability on the EVPOME, the amount of glucose consumed in the culture supernatant in the last 2 days of culturing was measured and had to exceed a preestablished minimum to be considered acceptable; this was the release criterion to allow the EVPOME to be used for intraoral grafting as required by the FDA. Figure 2a shows a representative graph of glucose consumption of six EVPOMEs cultured from the same patient under the same culturing conditions. The EVPOMEs that showed the highest glucose consumption were always selected for intraoral grafting as long as they exceeded the minimum required level of consumption (ie, the release criterion). Figure 2b shows an EVPOME that had high glucose consumption in comparison to Fig 2c, which had lower

glucose consumption (lower cell viability). The histologic image shown in Fig 2b reveals a well-organized stratified epithelial layer; in contrast, the substandard EVPOME shown in Fig 2c, which had low glucose consumption, displays a much thinner stratified epithelial layer. The number of basophilic cell nuclei was counted in the hematoxylin-eosin-stained sections. A randomly chosen, 100- μ m-wide epithelial layer excluding the eosinophilic superficial layer contained 15.8 ± 3.3 cells in the standard high-glucose-consumption EVPOME specimen; in contrast, 5.2 ± 0.4 cells were present in the substandard low-glucose-consumption EVPOME.

Surgical Protocol and Follow-up

The same surgeon (RFN) performed all surgeries, including biopsy specimen retrieval. Examples of treated patients are shown in Figs 3 and 4. For the grafting procedure, after profound local anesthesia had been achieved, partial-thickness supraperiosteal flaps were elevated (Fig 3b). EVPOME grafts were applied directly over the intact periosteal bed (Fig 3c) and secured to the surrounding gingiva and underlying periosteum with interrupted resorbable sutures. A periodontal dressing (Coe-Pak, GC America) was used to cover the EVPOME-treated sites for 7 days to ensure that the grafts were firmly adapted to the recipient site to prevent underlying hematoma formation. After surgery, routine written and oral postoperative care instructions were given to the patients. For analgesia, a nonsteroidal anti-inflammatory drug was prescribed (ibuprofen, 800 mg four times daily for 5 days). Postoperative home care instructions included refraining from any mechanical oral hygiene procedures at the surgical areas for 2 weeks. In addition, patients were instructed to rinse two times daily with a 0.12% chlorhexidine gluconate solution for 1 minute for 2 weeks.

At 7, 14, 30, 90, and 180 days after grafting, the following clinical measurements were recorded for each subject: PI, GI, PD, KGW, and keratinized gingival thickness (KGT). Bone sounding was performed using a periodontal probe at 30, 90, and 180 days. A visual analog scale for self-reporting of pain (scores ranging from 0 to 5) was also completed at every visit.

RESULTS

Five subjects participated in the trial. All were female and presented with a mean age of 55.4 years (range, 49 to 64 years). All subjects were Caucasian with noncontributory medical and dental histories.

The biopsied palatal wound healed without complications with minimal patient discomfort, as reported with the visual analog scale. Seven days after grafting with the EVPOME and removal of the periodontal dressing, the tissue-engineered graft appeared to be firmly adhered to the underlying recipient site with a red color indicative of revascularization. No complications or adverse reactions to EVPOME were observed on any subjects during the study. Treatment sites followed a healing pattern similar to that seen with free gingival grafts (FGGs) (Figs 3 and 4). Consistent low levels of plaque accumulation and gingival inflammation were observed throughout the study (Table 1). KGW at treatment sites ranged from 1 to 2 mm (mean, 1.2 mm) at baseline and from 4 to 5 mm at 180 days (mean, 4.2 mm), for an average KGW gain of 3 mm (range, 3 to 4 mm). Surgical procedures involved

an average of 5.2 teeth (range, 2 to 9 teeth). The mean KGT gain was 1 mm (range, 1 to 2 mm). No significant changes in PD were observed. EVPOME graft hue (color) and thickness appeared to be more natural than those of conventional FGGs. Clinically, the EVPOME grafts conferred a healthy periodontal environment with an increase of keratinized gingiva as well as providing a more esthetically appearing gingival graft in both hue (color) and thickness. In the clinician's opinion, no specific skills were required during the surgical procedure because of the easy handling characteristics of EVPOME grafts. A summary of all clinical parameters is provided in Table 1.

DISCUSSION

This study demonstrated the safety and efficacy of a tissue-engineered, autologous oral mucosa substitute, the EVPOME, to increase keratinized gingival tissue without any adverse events or local complications, thus achieving the main objectives for this prospective clinical trial. For the phase I clinical trial, presented in this paper, the authors had not yet received approval to set aside a control group (ie, AlloDerm without cultured primary oral keratinocytes), since the study was done to determine safety and not efficacy. Thus, no postoperative biopsy specimens were obtained to assess vascularization of the tissue or persistence of the grafted cultured cells. Previous studies^{8,9} showed that the grafted EVPOMEs had a deeper red hue than AlloDerm grafts at postoperative day 6, and there was histologic and immunohistochemical documentation of microvessel ingrowth into the underlying dermal component, AlloDerm, of the EVPOME in a study of severe combined immunodeficient mice, respectively. It can therefore be stated, with reasonable certainty, that the grafted EVPOME used in the present study had undergone revascularization by postoperative days 7 to 14.

The maintenance of an adequate band of attached gingiva, consisting of at least 2 mm of keratinized gingiva, has been shown to be important for maintenance of periodontal health.^{13,14} Traditionally, FGGs and connective tissue grafts have been used to increase and/or create an adequate band of keratinized gingiva.¹⁵ Clinical applications of tissue-engineered autologous/allogeneic products were recently reported to overcome the major drawbacks of FGGs, ie, donor site morbidity and the limited amount of tissue for grafting.^{16,17} McGuire et al¹⁶ and Nevins¹⁷ reported the use of bilayered cell therapy for mucogingival treatment and showed that sufficient keratinized tissue was generated, but the volume was not superior to that obtained with FGGs. Both studies claimed to have a better esthetic (color and texture) outcome compared with FGGs. However, since the allogeneic cells in those products never "take" on the wound bed, the effect of this therapy would be as a novel "biologic dressing" to stimulate surrounding host cells. Another viable product was a biodegradable scaffold with cultured autologous gingival fibroblasts.^{18,19} This augmentation technique was also shown to be effective. However, that culture system contained fetal bovine serum, which was not used in the present culture technique and would delay FDA approval. In addition, a lack of keratinocytes in that cell-based product would necessitate a graft of an epithelial component for a larger wound; otherwise, the wound bed would heal by secondary intention. An earlier clinical study showed that AlloDerm grafts placed without cells undergo prolonged inflammation and take longer to be epithelial-ized over the graft surface.⁸ One would postulate that AlloDerm grafts without cells would take

longer to heal, as there are no cellular components (oral keratinocytes) present that have the capability of secreting proangiogenic cytokines, such as vascular endothelial growth factor.^{7,20} For smaller wounds this might not be critical, but with the larger defects seen in oral and maxillofacial reconstruction cases, the EVPOME would be more efficacious to use. More recently, Jhaveri et al reported favorable results with AlloDerm seeded with autologous gingival fibroblasts, as compared to conventional connective tissue grafting for the treatment of Miller Class I and II gingival recession.²¹

It appears from the present study that the EVPOME graft has the potential to be used successfully in periodontal therapy. Using the oral keratinocytes obtained at the second passage, a dentist could produce an EVPOME graft larger than the size of a US\$1 bill, more than would be necessary for any periodontal procedure. For the potential use of periodontal therapy in which a smaller graft is sufficient, the first-passage cells could be seeded onto the AlloDerm to reduce the days in culture and lower the cost and risk of contamination.

This clinical trial was distinctive from other case studies using tissue-engineered products because it was conducted under a CBER/FDA-approved, investigator-initiated IND. In any standard FDA-approved IND protocol, on the day of grafting prior to the product release, specific and rigorous cell testing and monitoring are required.²² Glucose consumption was selected as the dose test in this study because it was considered a good indicator of the number of viable cells present on the EVPOME. Glucose consumption was able to detect cellular variations within EVPOME grafts, even in material from the same individual, showing the acceptability of this test for the necessary quality assurance/control and the release criterion mandated by CBER/FDA. Overall, the cell culture protocols used in this clinical trial proved to be acceptable, since all EVPOMEs were successfully grafted into the subjects with no signs of morbidity and/or contamination. The release criterion of using glucose consumption correlated well with histologic examination of the EVPOMEs, which showed a direct correlation between glucose consumption and the presence of a well-stratified oral mucosa epithelial layer (Fig 2).

It is important to note that, while useful, the test of glucose metabolism by cells as the “standard of function” in vitro is not practical for in situ assessment (ie, to follow EVPOME grafts after transplantation). Thus, the development of sensitive optical spectroscopic and functional assays that allow real-time (under 2 hours) noninvasive study of processes on the subcellular, cellular, and tissue levels is necessary to give a more complete picture of cell behavior, both in vitro and in situ. The development of quantitative noninvasive tools to monitor the structure, composition, and function of engineered tissues in real time using several optical spectroscopic and functional assays is presently being explored in the authors’ laboratory.

Autologous systems require a biopsy specimen— from the palate in this study—prior to the surgical procedure, which may be considered as “donor site morbidity.” Although off-the-shelf bioengineered products, such as those containing allogeneic cells, are beneficial to patients because preoperative biopsy specimens need not be obtained, the use of allogeneic cells may carry a risk of transmitted diseases and may cause an inflammatory response leading to rejection²³; in addition, allogeneic cells are eventually exfoliated.^{24,25} Thus, a

main advantage for the use of direct autologous cells is their ability to survive in situ longer after grafting.²³

This issue raises the question of how long the autogenous cells survive or persist in oral mucosa substitutes. This is pertinent not for only keratinocytes on the EVPOME graft but also for any cell-based engineered construct: what role does the addition of cells to a device impart on the efficacy of the construct? The turnover time of oral mucosa in rodents was reported to be three times shorter than that of skin (approximately 5 to 6 days versus 15 to 18 days).^{26,27} This would imply that keratinocytes from the EVPOME grafts would be sloughed off in the oral cavity in a relatively short period of time. In defense of the use of a cell-based product, a recent study done in immunodeficient mice showed that the cultured EVPOME keratinocytes released numerous cytokines, which not only promoted initial vascularization but also increased the migration of keratinocytes from the wound edge after grafting. The study concluded that the EVPOME epithelium played an important role as a promoter of wound healing and of reepithelialization of oral mucosa.²⁸

In another study, autologous oral mucosa epithelium was transplanted to the cornea²⁹; the authors demonstrated the long-term (10 to 22 months) existence of the cultured oral keratinocytes, which expressed progenitor/stem cell markers. Studies are presently underway in the authors' lab employing physical and pharmacologic methodologies consistent with the GMP conditions to develop techniques that will lead the cultured primary oral keratinocytes to increase the progenitor/stem cell population; these studies will allow this technology to be brought into clinical use more easily for the development of a more robust EVPOME for intraoral grafting procedures.^{30–31}

Because oral mucosa keratinocytes are easily obtainable and expand faster in vitro than skin keratinocytes,^{32–34} they may be more efficacious for use in future clinical applications in regenerative medicine. This platform technique may have other potential extraoral uses, such as repair of facial skin,³² reconstruction of eyelids and nose, or in situ mucosal substitutes for urethra and conjunctiva.³⁵

CONCLUSION

The first objective of this clinical trial, to evaluate the safety of an ex vivo–produced oral mucosa equivalent (EVPOME) for intraoral applications, was successfully demonstrated. No surgical complications or adverse reactions were observed during the study, confirming the safety of the EVPOME for intraoral grafting procedures. The second objective, to assess the efficacy of the EVPOME in increasing keratinized gingiva width, was also successfully confirmed by a mean gain of 3 mm of keratinized gingiva at the treated sites. The success of this study would warrant the development of a larger-scale clinical trial with larger surgical defects to assess the usefulness of EVPOME for oral and maxillofacial reconstruction.

Acknowledgments

The authors wish to acknowledge the continuous support and assistance of Blake Roessler, MD, and Cynthia L. Marcelo, PhD, University of Michigan. This study was supported by a grant to Stephen E. Feinberg (DE 13417) and grant M01 RR00042 from the University of Michigan General Clinical Research Center.

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a **b****Fig 1.**

a A 5-mm punch biopsy specimen was harvested from the palatal keratinized mucosa.

b The EVPOME construct immediately before grafting.

Fig 2.

a A representative chart showing the changes in glucose consumption during EVPOME fabrication. All six pieces showed a similar pattern; however, on the day of grafting, a marked decrease was noted in one piece (*black arrow*), which was not used.

b Histologic view of EVPOME used for grafting (hematoxylineosin).

c Histologic view of EVPOME not released from the laboratory (hematoxylineosin).

Fig 3. Clinical views of a patient in the study

- a** The treatment site at baseline demonstrates a limited band of keratinized tissue.
- b** Split-thickness dissection was performed to create a recipient bed for the EVPOME.
- c** The EVPOME was grafted onto the intact periosteum, secured with interrupted sutures, and subsequently covered by a periodontal dressing.
- d** Clinical view showing typical healing at 7 days postoperative.
- e** Healing at 14 days.
- f** Clinical appearance at 30 days.

g Appearance at 90 days.

h Treatment site at 180 days postoperative, demonstrating a significant gain in keratinized tissue. Compare with Fig 3a.

Fig 4. Clinical views of a second patient in the study

a Preoperative appearance.

b Suprapariosteal recipient site.

c EVPOME grafted in place.

d Seven days postoperative.

e Appearance at 14 days.

f Appearance at 30 days.

g View at 90 days.

h View at 180 days.

Table 1

Clinical Parameters (Means and Ranges)

Parameter	Baseline	7 d	14 d	30 d	90 d	180 d
PI	0.6 (0–1)	1 (0–2)	1.4 (0–2)	1 (0–2)	0.5 (0–1)	0.75 (0–1)
GI	0.8 (0–1)	1.2 (0–2)	1.6 (0–2)	0.25 (0–2)	0.25 (0–1)	0.25 (0–1)
PD	2.2 (1–3)	N/A	N/A	2.2 (2–3)	2 (2)	1.7 (1–2)
KGW	1.2 (1–2)	N/A	N/A	4.7 (4–5)	4.2 (4–5)	4.2 (4–5)
KGT	1.2 (1–2)	N/A	N/A	1.6 (1–2)	1.8 (1.5–2)	2.2 (2–3)
Pain	2.8 (0–4)	2.2 (0–4)	0.4 (0–2)	0	0	0

Baseline = punch biopsy specimen retrieval.