Intraoral grafting of an ex vivo produced oral mucosa equivalent: a preliminary report


Abstract. The objective of this study was to assess the efficacy of the use of an ex vivo produced oral mucosa equivalent (EVPOME) for intraoral grafting procedures. Autogenous keratinocytes were harvested from a punch biopsy 4 weeks prior to surgery, placed in a serum-free culture system and seeded onto a human cadaveric dermal equivalent, AlloDerm®. Thirty patients with either a premalignant or cancerous lesion were triaged into two groups, depending on the stage of disease: Group 1: EVPOME or Group 2: AlloDerm®, control without an epithelial layer. Clinically, EVPOME grafts were easy to handle and showed excellent compliance on grafting. Both, EVPOME and AlloDerm® grafts, showed a 100% take rate. At 6 days post-grafting, the EVPOME clinically showed changes indicating vascular ingrowth and had cytologic evidence of the persistence of grafted cultured keratinocytes on the surface. The EVPOME grafts had enhanced maturation of the underlying submucosal layer associated with rapid epithelial coverage when compared to the AlloDerm® grafts at biopsies taken at 28 days post-grafting. In summary, EVPOME appears to be an acceptable oral mucosal substitute for human intraoral grafting procedures and results in a more favorable wound healing response than AlloDerm® alone.

Key words: tissue-engineering; oral mucosa equivalent; AlloDerm®; oral keratinocytes; human clinical trial.

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Introduction

Preprosthetic and reconstructive procedures often result in open wounds in the mouth during intraoral surgery. A free mucosal graft used for reconstruction or closure of the open wounds can prevent microbial infection, excessive fluid loss, and foreign material contamination or relapse secondary to wound contracture. Oral mucosa or skin grafts both require harvesting of tissue from a second surgical site resulting in increased patient morbidity. In addition, oral mucosa is limited in supply while split-thickness skin grafts, though more easily available, contain adnexal structures, and express a different pattern of keratinization. Technological advancements in the field of tissue-engineering have allowed the development of innovative approaches to the fabrication of skin and mucosal substitutes. Over the last decade, two prevalent skin substitute methods have been frequently used in oral and maxillofacial surgery. One is a mucosal epithelial sheet fabricated by the technique developed by GREEN and RHEINWALD. However, several disadvantages of the use of sheets of epithelial cells exist. First is the difficulty in handling of the friable epithelial sheets.

Second is the use of an irradiated xenogeneic 3T3 mouse fibroblast feeder layer that is necessary to enhance growth of cultured keratinocytes. Unfortunately, the feeder layer has the potential to transfer xenogenous components to the co-cultured autologous cells of the grafted patients. A second skin substitute technique uses a bi-layered artificial dermis that consists of collagen-glycosaminoglycan/silastic sheet that is an ‘off-the-shelf’ material that is available at the time of surgery. Its major shortcomings are its difficulty with suturing and its susceptibility to infection.
Our research team has been able to overcome these disadvantages by the development of a human ex vivo produced oral mucosa equivalent (EVPOME) in a serum-free culture system without a feeder layer that has excellent handling characteristics. Our EVPOME is composed of a stratified layer of human autogeneous oral keratinocytes seeded on top of an acellular, human cadaver dermis, AlloDerm® (LifeCell, Branchburg, NJ, USA) that is seeded with autogenous human oral keratinocytes to form an overlying stratified parakeratinized epithelial layer. Briefly, our protocol for the manufacture of the EVPOME is the following. First, in the outpatient clinic, under local anaesthesia, a 5 × 5 mm punch biopsy of keratinized oral mucosa is taken from an uninvolved site in the oral cavity. The biopsy is taken at a time point prior to surgery to allow fabrication of an EVPOME that has appropriate dimensions for the planned surgical reconstruction. Oral keratinocytes are dissociated from the biopsy specimen and expanded in a serum-free defined culture medium, MCDB153 (Sigma, St Louis, MO, USA) containing a calcium concentration of 0.06 mM and porcine pituitary extract (COSMO BIO, Tokyo, Japan). The AlloDerm® is pre-soaked in 5 μg/cm² human type IV collagen (Becton Dickinson Labware, Bedford, MA, USA) for 3 h prior to seeding to enhance adherence of the seeded keratinocytes (1.25 × 10⁵ cells/cm²). Once a sufficient number of oral keratinocytes has been harvested, they are seeded onto the collagen coated AlloDerm® (thickness of 0.007 to 0.020 in). The composites of oral keratinocytes and AlloDerm® are then cultured, submerged, for 4 days to form a continuous epithelial monolayer. At this time, the concentration of calcium in the culture medium is raised to 1.2 mM to enhance keratinocyte differentiation. When the equivalents are raised to an air-liquid interface to encourage stratification of the epithelial monolayer, they are cultured for an additional 7 days resulting in reconstruction. To our knowledge, this is the first reported clinical study using a tissue-engineered ex vivo produced human oral mucosa composed of both an epithelial and dermal component for intraoral grafting procedures with an experimental protocol that includes a parallel control group, AlloDerm®, without epithelium.

**Experimental design and methodology**

**Fabrication of Ex vivo Produced Oral Mucosa Equivalent (EVPOME)**

The development and characteristics of EVPOME have been previously described. EVPOME is a composite human oral mucosa equivalent consisting of a dermal component composed of a human cadaver dermis, AlloDerm® (LifeCell, Branchburg, NJ, USA) that is seeded with autogenous human oral keratinocytes to form an overlying stratified parakeratinized epithelial layer. Briefly, our protocol for the manufacture of the EVPOME is the following. First, in the outpatient clinic, under local anaesthesia, a 5 × 5 mm punch biopsy of keratinized oral mucosa is taken from an uninvolved site in the oral cavity. The biopsy is taken at a time point prior to surgery to allow fabrication of an EVPOME that has appropriate dimensions for the planned surgical reconstruction. Oral keratinocytes are dissociated from the biopsy specimen and expanded in a serum-free defined culture medium, MCDB153 (Sigma, St Louis, MO, USA) containing a calcium concentration of 0.06 mM and porcine pituitary extract (COSMO BIO, Tokyo, Japan). The AlloDerm® is pre-soaked in 5 μg/cm² human type IV collagen (Becton Dickinson Labware, Bedford, MA, USA) for 3 h prior to seeding to enhance adherence of the seeded keratinocytes (1.25 × 10⁵ cells/cm²). Once a sufficient number of oral keratinocytes has been harvested, they are seeded onto the collagen coated AlloDerm® (thickness of 0.007 to 0.020 in). The composites of oral keratinocytes and AlloDerm® are then cultured, submerged, for 4 days to form a continuous epithelial monolayer. At this time, the concentration of calcium in the culture medium is raised to 1.2 mM to enhance keratinocyte differentiation. When the equivalents are raised to an air-liquid interface to encourage stratification of the epithelial monolayer, they are cultured for an additional 7 days resulting in reconstruction.

**Our research team has been able to overcome these disadvantages by the development of a human ex vivo produced oral mucosa equivalent (EVPOME) in a serum-free culture system without a feeder layer that has excellent handling characteristics.**

Our EVPOME is composed of a stratified layer of human autogeneous oral keratinocytes seeded on top of an acellular, human cadaver dermis, AlloDerm®. It is the use of AlloDerm® as the dermal base that confers enhanced handling characteristics and assists in compliance of the graft.

The elective nature of maxillofacial surgical procedures allows the flexibility and timing to coordinate the manufacture of a human ex vivo produced engineered-tissue of adequate dimensions within sufficient time to allow its use in the reconstruction of mucosal tissues within the oral cavity.

The objective of this study was to evaluate the efficacy and clinical handling of EVPOME for intraoral reconstruction. To our knowledge, this is the first reported clinical study using a tissue-engineered ex vivo produced human oral mucosa composed of both an epithelial and dermal component for intraoral grafting procedures with an experimental protocol that includes a parallel control group, AlloDerm®, without epithelium.
in the formation of a parakeratinized layer in preparation for intraoral grafting of the EVPOME (Fig. 1A).

This optimal fabrication protocol for the EVPOME, i.e., the number of days in culture, was determined by experimental studies that involved the grafting of different stages of epithelial development of the EVPOME into SCID mice. Grafted EVPOMEs were then evaluated for microvessel ingrowth within the underlying AlloDerm® layer, since re-vascularization is an important factor in free graft survival. At this stage of development the EVPOME possessed excellent handling characteristics that allowed it to be easily transferred from the culture flask to the oral cavity (Fig. 1B). These EVPOME grafts have been referred to as day 11 grafts, and day 10 to 14 grafts are the ones that were used in this reported study.

Experimental design

All patients enrolled in the study, at Niigata University, signed a consent form in which they agreed to be part of the investigation to evaluate EVPOMEs for intraoral grafting procedures. Study subjects were selected from patients diagnosed with premalignant lesions or oral cancer who were to undergo surgical excision of the lesion followed by reconstruction. Patients were triaged to Group 1, EVPOMEs, or Group 2, AlloDerm®, control group without an epithelial layer, depending on the urgency of surgery, since the EVPOME required additional time to fabricate. The use of a control group, AlloDerm®, without an epithelial layer, allowed us to assess the efficacy of the use of a stratified layer of oral epithelial keratinocytes on the dermal equivalent.

Inclusion and exclusion criteria

Inclusion criteria were: (1) over 18 years of age and (2) diagnosed as oral squamous cell carcinoma or oral premalignant lesion such as dysplasia or carcinoma in situ. Exclusion criteria were: (1) serologically positive for syphilis, HIV or Hepatitis B or C virus, (2) uncontrolled diseases such as diabetes, pregnancy, and not medically able to withstand a general anaesthetic (ASA class III or higher), (3) immunodeficiency, (4) patients receiving radiation therapy, and (5) cases where the surgical defect could be repaired, primarily, without tension.

Study population

Fifteen patients were recruited for each group, for a total of 30 patients. Patients were triaged to each group depending on the urgency for need of the surgical excision and physical condition of subject so as not to compromise patient care.

Demographics of Group 1 (grafted with EVPOME) were five males, and ten females. The ages of patients ranged from 49 to 84 years with a mean age of 64.2 ± 2.7 years. Lesions were located in the tongue in 13 cases and alveolar gingiva in five cases (locations in three cases of oral leukoplakia were multiple). The lesions were histologically diagnosed as squamous cell carcinoma in six cases (T stage: T3=1, T2=1, T1=4), epithelial dysplasia in eight and oral lichen planus in one. Eight patients were non-smokers, three had a positive smoking history but did not smoke at the time of surgery and four were current smokers in which one patient was classified as a heavy smoker (An index calculated by ‘number of currently smoked cigarettes per day’ × ‘years of smoking at the time of excision’ is 1000 or higher). In contrast, Group 2 (AlloDerm® without seeded oral keratinocytes) contained ten males, and five females. The ages of patients ranged from 41 to 84 years with a mean age of 68.0 ± 3.0 years. Lesions were located in the tongue in 11 cases (T stage: T2=4, T1=7) as well as the buccal mucosa, floor of mouth, oral gingiva, and alveolar gingiva. Histological diagnoses of the lesions were: 11 cases of squamous cell carcinomas, four cases of epithelial dysplasias with two of these cases presenting with melanotic pigmentation. Eleven patients were non-smokers and four were current smokers with one patient classified as a heavy smoker (Table 1).

<p>| Table 1. Research subjects of the EVPOME (Group 1) and AlloDerm® alone (Group 2). |
|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th>Mean age (range)</th>
<th>Male/female ratio</th>
<th>Site</th>
<th>Diagnoses</th>
<th>Smoking history</th>
<th>Surface area (cm²) to be covered</th>
<th>Follow-up (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 EVPOME (15 pts)</td>
<td>64.2 ± 2.7 (49 to 84)</td>
<td>5/10</td>
<td>Tongue (13)*</td>
<td>Non-smoker (8)</td>
<td>12.41 ± 1.70</td>
<td>5–17 months</td>
</tr>
<tr>
<td>Group 2 AlloDerm® alone (15 pts)</td>
<td>68.0 ± 3.0 (41 to 84)</td>
<td>10/5</td>
<td>Tongue (11)</td>
<td>Non-smoker (11)</td>
<td>11.28 ± 1.83</td>
<td>4–18 months</td>
</tr>
</tbody>
</table>

* Several patients had multiple sites thus number of sites is greater than 15.† Previous history of smoking; presently a non-smoker.‡ One smoker is this group of four was classified as a ‘heavy smoker’ (an index showed 1000 or higher).
Intraoral grafting of an ex vivo produced oral mucosa equivalent

confirmed, by a postoperative histological biopsy. The ethical committee in Niigata University Dental Hospital permitted only a single postoperative biopsy to evaluate the grafts. Therefore, in lieu of multiple biopsies from the same subject, we altered the timing of the one biopsy in every ten cases. In fifteen cases, a biopsy was performed at 4 weeks postoperatively, while the last five were done at 2 weeks. The postoperative biopsy was taken from the periphery of the grafts in which both the EVPOME and AlloDerm® clinically appeared, to possess an epithelial layer.

After discharge, all patients were evaluated weekly for the first month, bi-weekly for the next 2 months and then monthly up to 12 months. At each postoperative visit, direct clinical observation and photographs were used to evaluate and document wound healing. The time period required to complete epithelial coverage was determined by the number of days between the day of excision and the day full epithelial coverage was achieved. Adverse events were evaluated by daily reports during hospitalization and at each visit after discharge, as well as by a serum chemistry profile and a complete blood cell count at day 7, 14, 28 days, 3, 6 months, postoperatively.

Statistical analysis

Mann–Whitney U test was used to determine the statistical significance between the two groups: EVPOME and AlloDerm®. Values of \( P<0.05 \) were considered to be statistically significant. Data was expressed as the mean ± SEM.

Results

There were a few demographic differences between, Group 1, EVPOME and Group 2, AlloDerm®. In Group 1, the mean interval between punch biopsy and excision, equal to days it took to fabricate the EVPOME prior to surgery, was 27.7 ± 1.4 days whereas the mean interval between the first visit and excision for Group 2, AlloDerm®, was 13.7 ± 1.5 days. This difference was statistically significant (\( P<0.01 \)). This occurred because our treatment protocol triaged patients who had a clinical diagnosis of carcinoma, T1 or T2 into a group that had the lesion excised as soon as possible while the EVPOME cases were scheduled electively. Our triage treatment policy resulted in a larger sample of malignant lesions and males in Group 2.

grafted keratinocytes. Grafted sites of all patients were evaluated by clinical observation and photographs every day until discharge. Three variables were evaluated to assess patient and graft acceptance. First, postoperative pain was assessed by a visual analog scale (VAS) \((-=0, + =1–24, ++ =25–49, +++ =50–100). Second, ‘pull off strength’ was used to evaluate graft adhesiveness to the underlying layer at the periphery of the wound margin at day 7 postoperatively \((- = non-adherent, + =adherent but easily removed, ++ =adherent difficult to remove, +++ =adherent unable to remove). Third, the percent of epithelial coverage of the graft, clinically evident at 4 weeks after transplantation, was calculated by the formula of A-B/A (\( % \)). ‘A’ was the wound area transplanted with EVPOME or AlloDerm® and ‘B’ was the area with exposed AlloDerm® (lack of epithelial coverage). Areas of the grafts that had epithelial coverage appeared red, while white or opaque regions at the grafted site were seen with AlloDerm® which lacked an epithelial layer \((- =0–50\%, + =51–75\%, ++ =76–99\%, +++ =100\%). This clinical assessment was subsequently

Table 2. Postoperative outcomes of the EVPOME (Group 1) and AlloDerm® alone (Group 2).

<table>
<thead>
<tr>
<th>Postoperative pain</th>
<th>EVPOME (15 pts)</th>
<th>AlloDerm® alone (15 pts)</th>
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<tbody>
<tr>
<td>Adhesiveness</td>
<td>++ (15)</td>
<td>+++ (13)</td>
</tr>
<tr>
<td>Epithelial coverage</td>
<td>+++ (13)</td>
<td>++ (0)</td>
</tr>
<tr>
<td>Required days for epithelialization</td>
<td>27.4 ± 1.2</td>
<td>46.0 ± 2.8</td>
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</table>

Postoperative pain: + and − units used for Visual Analog Scale (VAS) values ranging as follows: − = 0, + =1–24, ++ =25–49, +++ =50–100. Adhesive: + and − units represent ‘Pull off strength’; − =non-adherent, + =adherent but easily removed, ++ =adherent difficult to remove, +++ =adherent unable to remove. Epithelial coverage: + and − unit for % of epithelial coverage of grafted area at four weeks post-grafting: − =0–50%, + =51–75%, ++ =76–99%, +++ =100%. Required days for epithelialization: Values were expressed as the mean ± SEM. \( P \) value of <0.05 indicates statistical significance.
AlloDerm®. In contrast, patient’s with a positive smoking history, which plays a role in etiology of malignant lesions and can affect graft success rate, was equal in both groups. The average surface area surgically covered by the grafted EVPOME was $12.41 \pm 1.70 \text{ cm}^2$, while the mean area of coverage of AlloDerm® was $11.28 \pm 1.83 \text{ cm}^2$. There was no significant difference between the two groups.

Postoperative course for both groups of patients was uneventful. There were no significant differences between both groups in regard to postoperative pain or adhesion of the graft to the underlying tissue while epithelial coverage occurred more rapidly in Group 1 (Table 2). Patients who had lesions excised from the tongue, in either group, experienced occasional paraesthesia (dull sensation and/or numbness). AlloDerm® control grafts experienced more tethering and indurations of the tongue, secondary to wound contraction, than EVPOME grafts. Complete epithelial coverage was achieved in the EVPOME grafts by $27.4 \pm 1.2$ days while the AlloDerm® grafts required $46.0 \pm 2.8$ days. The difference was statistically significant ($P<0.01$).

At 6 days post-surgery, after removal of pressure dressings, a cytological sample was taken at the centre of the graft, which revealed healthy single and aggregates of epithelial cells on the surface of EVPOME, indicating the persistence of cultured oral keratinocytes on the grafts (Fig. 3A). In contrast, the cytological smear taken at the centre of the AlloDerm®, showed only numerous neutrophils within a mucinous exudate but no evidence of viable epithelial cells (Fig. 3B). The surface of the transplanted EVPOME exhibited a dark red hue that was found to correlate with vascular ingrowth into the dermal equivalent from the underlying host tissue (Fig. 4A). The AlloDerm® grafts, in contrast, were faintly red, indicating a lesser degree of vascular infiltration (Fig. 4B). At the second week post-grafting, after removal of the bolus or stent, host granulation tissue was seen to extend over the wound periphery in all patients (Fig. 4A,B). In several patients a portion of the surface of the AlloDerm®, was seen to have a ‘feather’ appearance during the second week after surgery (postoperative days 8 to 14). Histological examination showed this to be necrotic tissue infiltrated with bacteria (not shown).

A biopsy taken at 2 weeks post-grafting, from an area that clinically appeared epithelialized in both groups, showed histologically the submucosal layer to have an intense inflammatory infiltrate among a bed of immature granulation tissue with diluted, leaky venules (Fig. 5A,B). The overlying epithelial layer in Group 1, EVPOME, showed a well-ordered, thick spinous layer, indicative of hyperplasia (Fig. 5C), whereas that in Group 2, AlloDerm® alone, showed a more random irregular regenerative pattern (Fig. 5D). At the 4 week biopsy, taken from an epithelialized area in both groups, Group 1, EVPOME, showed a more differentiated, overlying epithelium in contrast to, Group 2, AlloDerm®, which lacked the uniformity and epithelia regularity (Fig. 6A,B). An inflammatory response was noted within the dermal layer of both groups as documented.

![Fig. 3. Cytological smear of grafts. A: Squamous cells seen on the smear, suggestive of the presence of cultured keratinocytes (Papanicolaou staining). B: Numerous neutrophils within mucinous exudates with no evidence of keratinocytes being present (Papanicolaou staining).](image-url)
by the plethora of blood vessels perpendicular to the overlying epithelium (Fig. 6A,B). In Group 1, the dermal component showed fewer numbers of inflammatory cells and large venules laden with ‘tall’ endothelial cells (Fig. 6C), and the presence of elongated spindle-shaped fibroblasts among collagen bundles (Fig. 6E). Group 2, in contrast, showed evidence of a persistent intense inflammatory response with numerous giant cells and eosinophils present within the dermis (Fig. 6D,F, compared with Fig. 6C,E).

There were no clinical signs of a foreign body response to either the EVPOME or AlloDerm® grafts with healing eventually occurring uneventfully. Total serum chemistry profile after grafting showed no systemic evidence of an immunologic or systemic response to the transplants.

Discussion

Recently, tissue-engineered oral mucosa substitutes have been applied onto open wounds in the oral cavity. These oral mucosa substitutes can be classified into three types: (1) sheets of cultured autologous keratinocytes, (2) an artificial dermis composed of collagen and/or glycosaminoglycans, or (3) a composite oral mucosa, containing both an epithelial and dermal layer. These studies have shown favorable outcomes using the different types of grafts in reconstructive oral and maxillofacial surgery but have several major deficiencies in their experimental design and protocol; the absence of a control group and a small sample size, respectively. To our knowledge, our investigation is the first study evaluating the clinical application of a composite human ex vivo produced oral mucosa equivalent (EVPOME) with a parallel control group, AlloDerm®, without epithelium with a sufficient sample size to perform statistical analysis.

The EVPOME utilized in our clinical protocol is a composite or full-thickness oral mucosa equivalent fabricated with an epithelial and dermal layer. To date, there has only been one other report on the intraoral use of a bilayered cultured oral mucosa. In that study the dermal equivalent utilized was composed of a Type I collagen gel repopulated with fibroblasts. A major disadvantage of the use of collagen, intraorally, is its fragility and early breakdown secondary to the plethora of lytic enzymes present within the oral cavity. In addition, the resurfacing of the oral cavity is more difficult to accomplish than skin because of a moist environment laden with numerous microbial organisms, and an undulating surface which can compromise graft stability. The use of AlloDerm®, as a dermal equivalent, enhances the durability and compliance of the EVPOME as well as improving the handling characteristics assisting in manufacture, intraoral placement and stabilization of the graft at the surgical site.

The use of an irradiated xenogeneic 3T3 mouse feeder layer, utilized in other studies, has been shown to optimize the in vitro culturing of keratinocytes, while effecting stem cell maintenance resulting in a longer life-span, and a more proliferative cell population. The ability to increase and maintain the number of stem cells to sustain proliferation and
renewal of the keratinocyte population would be beneficial in enhancing the manufacture of the EVPOME. Unfortunately the optimization of keratinocyte growth should not be at the cost of an increase risk of cross contamination of the cultured human keratinocytes with xenogeneic material from the irradiated 3T3 feeder layer, especially in light that the majority of oral and maxillofacial surgical procedures are elective in nature and the surgery can be timed to assure the availability of an adequate population of autologous keratinocytes.

In our investigation, several observations indicated that the grafted EVPOME had advantages over the use of AlloDerm® without epithelium. First, was the early revascularization noted by the increase in ‘redness’ of the EVPOME graft; second, cytological evidence of the presence of intact squamous cells within the deeper portion of the submucosal layer (original magnification × 400). C: Epithelial layer demonstrates a thick spinous layer and elongated rete ridges. Immature, highly vascular granulation tissue is present within the subjacent epithelial layer (original magnification × 50). D: The basal cell layer shows an increase of nucleus/cytoplasm ratio and disordered cellular arrangement. Note prominent inflammatory cell infiltration is evident beneath epithelium (original magnification × 75).

On postoperative day 6 after removal of the pressure dressing, it was observed that the grafted AlloDerm®, without epithelium, appeared to have an ‘ulcerative’ appearance secondary to the lack of reduction in dermal inflammatory response, histologically, over time when compared to the AlloDerm® alone, and fourth, the more rapid and mature epithelial coverage, observed clinically and noted histologically, of the EVPOME graft.

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Fig. 6. Photomicrograph of histologic findings of the postoperative biopsy at 4 weeks in EVPOME (A,C,E) and AlloDerm® (B,D,F) grafts (haematoxylin and eosin staining). A: In the EVPOME a thick differentiated epithelial layer is present. A decreased inflammatory cell infiltration is present within the dermal layer in which immature is being replaced by fibrous connective tissue (original magnification × 50). B: There is disarray of the epithelial layer of the AlloDerm®. As well an intense inflammatory response is still persistent. Numerous diluted blood vessels are noted by their arrangement perpendicular to the overlying epithelium (original magnification × 60). C: A decrease in number of inflammatory cells is seen within the EVPOME. The thickness and diameter of the blood vessels beneath the epithelium are reduced (arrows) (original magnification × 300). D: The AlloDerm® alone shows the continued presence of neutrophils, lymphocytes, macrophages and plumped fibroblasts beneath epithelium (original magnification × 300). E: The EVPOME shows few plumped fibroblasts and eosinophils within in the deeper portion of the dermis (original magnification × 300). F: In the deeper portion of the AlloDerm®, an intense cellular infiltration containing numerous eosinophils (arrows) is observed (original magnification × 300).
an epithelial layer. In contrast, the grafted EVPOME had a ‘slough off’ appearance of its superficial epithelial layer as is usually seen in free gingival grafts. It is known that this ‘sloughed off’ tissue usually represents loss of the more differentiated superficial stratified cells of the transplant. Unfortunately, a biopsy was not taken of the centre of the EVPOME graft to confirm the presence of an intact basal layer. It is, therefore, assumed that basal cells of the epithelial layer continued to remain attached to the underlying dermal substrate. It is most likely the presence of the persistent epithelial basal cells of the EVPOME grafts that were influential in allowing a more rapid healing to occur. This, in turn, resulted in a better colour rendition of the EVPOME graft with the adjacent mucosa as well as a marked reduction in the underlying inflammatory response within the dermal component.

The advantages noted with the use of the EVPOME grafts may be the result of the presence of an intact and mature epithelial layer secreting keratinocyte-derived cytokines, such as vascular endothelial growth factor (VEGF) that effect dermal vascularity. We have observed that human oral keratinocytes can naturally secrete high endogenous levels of VEGF in culture (data not shown). The release of VEGF and associated cytokines may have moderated the inflammatory response, enhanced cell growth, neoangiogenesis, and wound healing while minimizing graft contracture. According to Young et al., the initial 2–3 days after transplantation are critical to graft survival and is predicated on vascular ingrowth into the transplants via the process of mosoulization in association with neoangiogenesis. The presence of numerous intact acellular blood vessel channels, within the dermal layer, that are maintained within the architecture of the AlloDerm® after preparation may also have assisted in the early revascularization of the dermal equivalent in both groups.

An intact epithelium has been noted to create a barrier effect of the overlying dermis protecting it from bacterial invasion. In addition, the intact keratinocyte layer on the EVPOME could have been a source of other released growth factors, such as transforming growth factor alpha (TGFα) and epidermal growth factor (EGF) that could influence host epithelial cells proliferation and migration from the margin of the wounds thus enhancing epithelial coverage resulting in less of an inflammatory response within the dermal component. The presence of a persistent epithelium on the EVPOME transplants would also potentially make them useful as a device for grafting of genetically modified keratinocytes that may be beneficial for various therapeutic applications.

There were no systemic complications seen in either Group 1 or 2 after grafting. Postoperative pain was minimal in both groups of patients with evidence of slight neurosensory deficits observed in some of the cases involving reconstruction of the tongue. Kangesi noted that nerve regeneration, into a grafted skin equivalent, composed of de-epidermized dermis and cultured keratinocytes, followed re-vascularization. Thus, since the tongue contains a rich nerve plexus within its musculature longer follow up may see a resolution to this adverse outcome.

In our study, it was noted that the AlloDerm® grafts, without epithelium, resulted in a more indurated and contracted wound than seen with the EVPOME grafts, post transplantation. Severe wound contraction, which is seen in healing by second intention, can result in post-grafting morbidity, leading to functional disturbances in speech by restriction of the mobility of the tongue. Garner demonstrated that early epidermal coverage prevented excessive collagen synthesis within the underlying dermal fibroblasts and can minimize wound contracture. The smaller degree of wound contracture seen with the EVPOME grafts may be the result of the establishment of an intact and mature epithelial layer influencing underlying collagen synthesis within the dermal component, AlloDerm®. It also appears that the persistence of the cultured autologous keratinocytes on the EVPOME played a key role in assisting in its integration into the underlying host tissue. In addition the inflammatory cellular infiltrate within the dermis decreased more dramatically from week 2 through 4, postoperatively, in the EVPOME, in contrast to the persistent inflammatory infiltrate seen in Group 2, AlloDerm® without an epithelial layer. The evidence of less induration at the wound site in the EVPOME graft may be related to a more physiologic repair of the underlying dermal layer secondary to the presence of an intact epithelial layer that has the capability to secrete pro-inflammatory cytokines and growth factors resulting in a more moderate and shortened inflammatory phase of wound repair.

Retrospective evaluation of our clinical study with grafted EVPOMEs, revealed several design and logistical deficiencies:

1. Only one biopsy was allowed and it was not taken at the same time period.
2. Biopsies were taken from the periphery of the graft (from areas that ‘appeared’ to have an epithelial layer) and not from the graft centre. This did not allow us to assess the absence or presence of a basal epithelial layer on either type of graft at a site that would be less influenced, the centre, by migration of cells from the periphery of the reconstructed wound.
3. The use of specific stains may have added additional information.
4. A blinded evaluator did not clinically assess the grafts and would have added more objectivity to the clinical evaluation.
5. A thinner dermal equivalent may have resulted in a faster vascular ingrowth but may also have impaired the handling characteristics (curling up of the margins during manufacture). This issue will have to be evaluated in future studies.
6. The majority of the surgical sites were the tongue, a highly mobile organ. A non-mobile bony recipient site, such as a maxillary or mandibular ridge, may have been more predictable in stabilization of the grafts thus enhancing re-vascularization and maintaining the epithelial layer.
7. Elimination of the use of pituitary extract in the manufacturing process of the EVPOME to create a more defined and ‘clean’ system. This point has, in fact, been accomplished in our laboratory and will be incorporated into future investigations.
8. We are planning, in the future, a randomized, controlled multi-centre study using patients with non-malignant lesions that would allow more similarity between the groups being evaluated.

In summary, it appears 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 than the AlloDerm® graft without an epithelial layer. The advantage of the EVPOME over the
AlloDermin® may have been due to the persistence of a transplanted epithelial basal cell layer resulting in the development of a more rapid and mature epithelium. The persisted basal layer of grafted keratinocytes and/or the initial presence of an intact epithelial layer, developed in vitro, at the time of grafting of the EVPOME, may have given it a decided advantage in eventual repair and healing of the surgical wound by the release of cytokines and growth factors that are known to be secreted by keratinocytes. Additional studies will give us more insight into the potential benefits of the use of a tissue engineered human oral mucosal equivalent as an aid in surgical reconstruction and as a vehicle for use in gene therapy.

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