Tissue Engineering of a Human Oral Mucosa for Tissue Repair and Regeneration

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Summary: Within the last decade a new field of 'tissue engineering' or 'regenerative medicine' has emerged that offers a new and exciting alternative for soft tissue reconstruction. This review discusses the advantages and disadvantages of different approaches to development of an oral mucosa equivalent. It also includes future research directions in this exciting area of repair and regeneration of oral mucosa.

Key words: tissue engineering, oral mucosa, stem cells

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INTRODUCTION

Skin and mucosa substitutes have been developed in the area of tissue engineering to assist tissue regeneration and enhance repair procedures for trauma, ablative oncologic resections and reconstructive surgery. Biologic substitutes of human skin and mucosa have several other prospective applications including: models of skin and mucosal biology and pathology, treatment and closure of skin and mucosal wounds, alternatives to animals for safety testing of consumer products, and for delivery and expression of transfected genes (Boyce, 1996). To date, the development of oral mucosa substitutes has not advanced as rapidly as the technology for the production of skin equivalents.

Both skin and mucosa substitutes have a common set of requirements for the duplication of anatomic structures and physiologic functions that they are to emulate. For use in wound closure, the first definitive requirement is re-establishment of the epidermal barrier to fluid loss and microorganisms as well as alleviating pain and enhancing wound healing. In full-thickness skin loss, replacement of both the epidermis and dermis is the preferred approach. Replacement of these tissue compartments must also minimize scar formation and restore acceptable function and esthetics. A major advantage of the use of substitutes in wound closure is to reduce or eliminate the donor site for skin and mucosa grafts. If one is successful in the elimination or minimization of donor site morbidity this could shorten recovery time and reduce the length of operative procedures.

Various approaches that have been used in the fabrication, manufacturing and 'tissue engineering' of skin and mucosa substitutes can be classified as: 1) *in vitro* culturing of autologous or allogeneic keratinocytes, 2) *in vitro* tissue engineering of dermis composed of either artificial (collagen, glycosaminoglycans, polymers of polyglycolic and polylactic acid) or allogeneic and acellular dermis, and 3) a bilayer of skin or mucosa from a combination of 1) and 2). Three essential components are known to be necessary to tissue engineer human skin or mucosa: cells, an extracellular matrix and cytokines (Bell, 1995).

ORAL MUCOSA SUBSTITUTES

Oral mucosa is an excellent intraoral graft material but is available in a limited supply. Split-thickness skin grafts, which are used, are available in ample supply but may contain adnexal structures and express a different pattern of surface keratinization that can lead to the development of abnormal tissue texture in the oral cavity that could interfere with function (Dellon et al, 1976; Moller and Jolst, 1972). The elective nature of the majority of oral and maxillofacial surgical procedures, as opposed to

burn injuries, would allow the flexibility and timing to develop an *ex vivo* tissue- engineered oral mucosa that could be used for intraoral grafting procedures. The recent developments of oral keratinocyte culture techniques have paralleled those of skin keratinocytes, which has enabled the development of tissue-engineered autogenous oral mucosa suitable for intraoral reconstructive procedures (Izumi et al, 1999, 2000, 2003).

In Vitro Culturing and Fabrication Techniques

A majority of studies have utilized an irradiated layer of a transformed 3T3 fibroblastic cell line as a feeder layer to propagate and expand their oral keratinocyte population to generate oral keratinocyte (epithelial) sheets for intraoral grafting (De Luca et al, 1990; Raghoebar et al, 1995; Tsai et al, 1997). All of the studies demonstrated successful clinical outcomes and regeneration of normal oral mucosa in the post-grafting biopsies, the longest being four months postoperative. Hata et al (1995) and Ueda et al (1995) reported that oral mucosa keratinocytes grew more rapidly and differentiate less than skin keratinocytes. Dermal substitutes for burn injuries have also been applied into the oral cavity, such as the bilayered membranes with a collagen-GAG/silastic sheet, similar to the Burke and Yannas' Integra® (Bertolami et al, 1991; Omura et al, 1997). Although they showed successful postoperative appearances and an advantage of easy sterilization and cost-effectiveness, this material is very difficult to handle. The silastic sheet does not present a problem with handling but the collagen sponge becomes 'sticky' when it absorbs blood, resulting in difficulty during suturing. It appears that the environment of the oral cavity, a moist area laden with bacteria and lytic enzymes, may not be conducive to the collagen-rich dermal components used in skin equivalents. In addition, an oral mucosa equivalent must not only be anatomically similar to mucosa but must also possess the mechanical and handling characteristics of the mucosa to be useful within the intricate confines of the oral cavity.

Since the majority of reconstructive procedures in oral and maxillofacial surgery are of an elective nature this gives one the ability to time the biopsy of autogenous mucosa for the planned surgical reconstruction. This will allow sufficient time to grow the necessary number of cells to fabricate the desired size graft needed for surgery. In developing a methodology to tissue-engineer any tissue it is necessary to abide by the requirements and restrictions imposed by the FDA. The cultivating technique of Rheinwald and Green (1975) utilizes a xenogeneic irradiated fibroblast cell line, 3T3, as a feeder-layer to enhance keratinocytes growth. During the culturing period to expand human cells they will be exposed to a transformed murine cell line. This contact could potentially contribute to cross-contamination or transfection of the mutational or xenogeneic DNA into the co-cultured human keratinocytes. Serum and pituitary extracts, as well as a xenogeneic feeder-layer, contain undefined factors such as slow viruses (Creutzfeldt Jakob disease or bovine spongiform encephalopathy, etc) and foreign contaminants (Smola et al, 1999). The importance of not using a feeder layer, pituitary extract or serum to culture oral mucosa autografts is obvious especially in an elective surgery because of the potential of the introduction of undetermined risks to the patient (Lauer, 1995).

In development of a mucosal equivalent two basic components exist: the superficial portion or epidermis that contains the keratinocytes, and the deeper portion or dermis. Investigators, in the past, have attempted to graft skin and oral mucosal defects with epithelial sheets. These epithelial sheets have been friable and difficult to handle with a low engraftment rate. They also needed to be conveyed to the surgical site or wound with a carrier vehicle such as petrolatum gauze that is secured in place with either a stent or metallic surgical clips. There have been reports of "oral mucosa" equivalent-like Apligraf® but these equivalents are still experimental and have not been used in clinical studies (Oda et al, 1998; Odioso et al, 1995). Another type of "oral mucosa" equivalent composed of de-epidermized dermis and cultured oral mucosa keratinocytes from buccal mucosa and hard palate was studied in Korea (Cho et al, 2000; Chung et al, 1997). These oral mucosa substitutes were developed for toxicological and pharmacological studies and not for use in a clinical setting.

Studies have shown that the concurrent grafting of a dermal component aids in enhancing the quality and time of wound healing (Kangesu et al, 1993; Marks et al, 1991; Martin et al, 1995). Sabolinski et al (1996) showed that the rate of closure of the wound and the increase in percentage of wound repair is enhanced with the presence of a dermis. In addition, the maturation process and biologic events of skin regeneration are accelerated with the presence of a dermal substrate (Compton, 1993). Inokuchi et al (1995) have found that autogenous fibroblasts, within the grafted dermal matrix, facilitated the long-term maintenance of the reorganized cultured epidermis by supporting self-renewal of the epithelium *in vivo*. Clugston et al (1991) noted that the absence of a grafted dermis resulted in a contracture of cultured keratinocyte autografts on the order of 50%. Thus, the development and grafting of a full-thickness oral mucosa graft with a dermis can assist in epithelial graft adherence, minimize wound contraction and assist in epithelial maturation while encouraging the formation of a basement membrane (Gallico and O'Conner, 1995). Auger et al (1995) showed that a dermal equivalent would be best made out of human,

rather than animal, collagen. The human collagen (dermis) helps to promote deposition of additional basement membrane constituents, resulting in a more optimal pattern of keratinocyte differentiation and less immunogenicity than animal collagen. Our laboratory at the University of Michigan has been successful in the *ex vivo* production of an oral mucosa equivalent (EVPOME) using oral keratinocytes seeded onto a human cadaver dermal matrix, AlloDerm® (Izumi et al, 1999, 2000, 2003). AlloDerm® is an acellular, biocompatible, human connective tissue matrix, with an unaltered extracellular matrix and intact basement membrane, which consistently integrates into the host tissue. The AlloDerm®, most importantly, trims, adapts, and sutures like autologous tissue. Human de-epidermized dermis that has retained its basal lamina consisting of keratinocytes combined with a mesenchymal or dermal component, has successfully shown enhanced epithelial morphogenesis and an increase in expression of differentiation markers when it is grown at an air-liquid interface (Regnier et al, 1986).

Manufacture of a Tissue-engineered Oral Mucosa

The University of Michigan approach of tissue-engineering an oral mucosa equivalent is to use a serum and pituitary extract-free culture system without a xenogeneic feeder-layer thus having a completely defined culture medium for the manufacture of our ex vivo produced oral mucosa equivalent (EVPOME) (Izumi et al, 2004). Our EVPOME is composed of autogenous oral keratinocytes and a cadaver acellular, AlloDerm® (Fig 1). Electron microscopic evaluation of the EVPOME shows that the AlloDerm® retains an intact basement membrane and anchoring fibrils on the papillary surface (Izumi and Feinberg, 2002). After culturing four days submerged our EVPOME shows several layers of keratinocytes adherent to one another via desmosomal attachments while specific junctional structures between basal cells and the basement membrane of the AlloDerm (were not seen at this time. At day 11 EVPOMEs, cultured four days submerged and seven days at an air-liquid interface, numerous rudimentary hemidesmosome-like structures were seen incorporated into the anchoring fibrils of the basement membrane of the AlloDerm. This seems to indicate that the basal cells layer was firmly attached to the underlying dermal equivalent of the day 11 EVPOME suggesting an ability of the epithelial layer to withstanding shear stress.

Human Clinical Trials

In a study preformed in Japan, autogenous oral keratinocytes were harvested from a punch biopsy four weeks prior to surgery, placed in a serum-free culture system and seeded onto a human cadaveric dermal equivalent, AlloDerm® (Izumi et al, 2003). Thirty

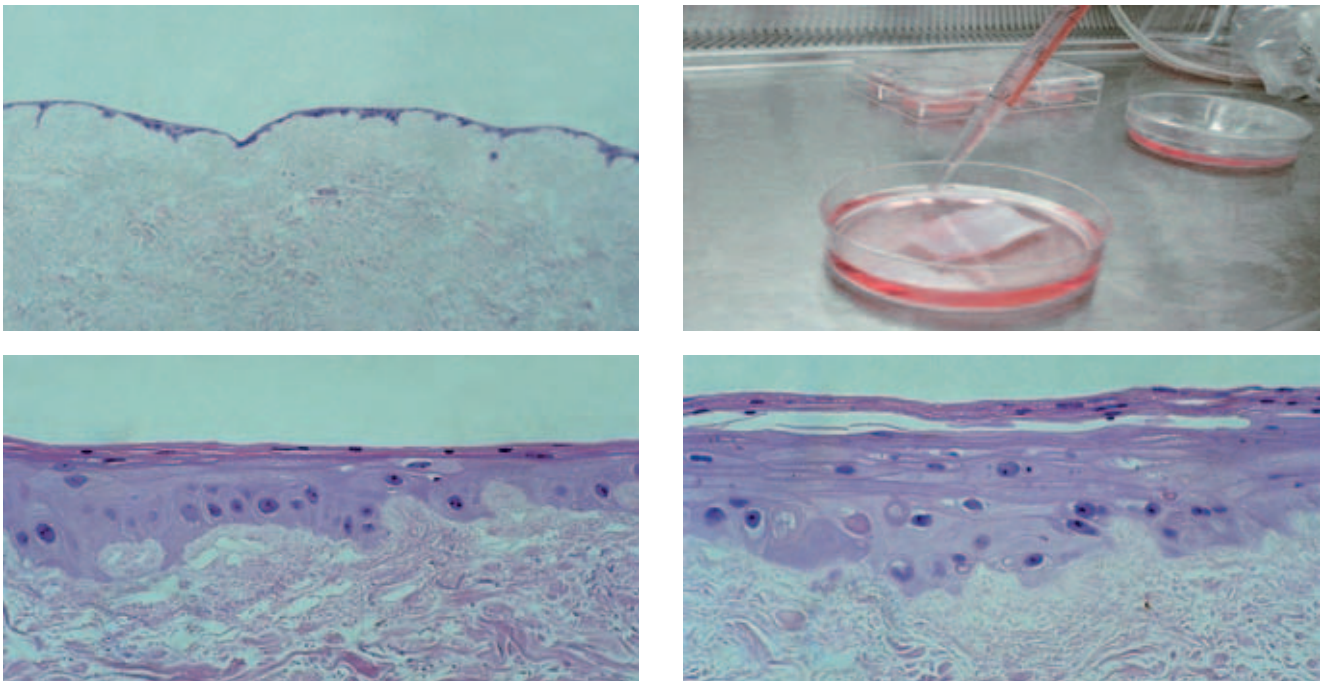

Fig 1 Fabrication stages of development of an *ex vivo* produced oral mucosa equivalent. **(a)** four days submerged creating a contiguous monolayer of cells (10x mag, H&E). **(b)** Raised to an air-liquid interface by floating in a Petri dish. **(c)** 7 days cultured at an air-liquid interface note the beginning of epithelial stratification (40x magnification, H&E). **(d)** Fourteen days cultured at an air-liquid interface, notice the increase in stratification of the epithelial layer (40x mag, H&E).

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 six 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. It thus appears that EVPOME may be an acceptable oral mucosal substitute for human intraoral grafting procedures. In addition, at the University of Michigan we have initiated human clinical trials as well.

FUTURE DIRECTIONS

Future research efforts are being directed to the isolation of progenitor/stem cells from oral mucosa. The ability to isolate an epidermal progenitor/stem cell for use in the fabrication of autologous grafts can result in a more robust engineered tissue as well as an opportunity of using it as a vehicle for gene therapy. The key to successful gene therapy using epidermal cells is the isolation of the putative progenitor/stem cell that can confer sustained expression of the transduced gene. Most stem cell biologists talk about the bulge of the hair follicles, containing follicular stem cells which are bipotent, as they give rise to both keratinocytes of the hair follicle and the interfollicular epidermis (Potten and booth, 2002; Niemann and Watt, 2002; Janes et al, 2002; Fuchs and Raghavan, 2002; Alonso and Fuchs, 2002). Thus, the oral epithelium, in comparison to skin, is quite unique in that it has a higher cell production rate (buccal epithelium: approximately two to three times that of epidermis), with reduced turnover time in both keratinized and nonkeratinized epithelium (buccal epithelium: one to three weeks) compared with the epidermis (range of four to 10 weeks depending on site) (Cutright and Bauer, 1967; Hill, 1984; Weinstein et al, 1984; Ueda et al, 1995; Hata et al, 1995; Thomson et al, 2001). In addition, others have noticed that epithelial cells isolated from the oral mucosa are at a lower stage of differentiation than skin keratinocytes resulting in a higher rate of proliferation *in vitro* in the oral epithelia than in the epidermis (Ueda et al, 1995; Hata et al, 1995; Gibbs and Ponec, 2000). We have, in turn, also noticed that our EVPOMEs showed a keratinocyte population that was highly proliferative, more so than native keratinized mucosa (Izumi et al, 2000). What makes these observation even more interesting is the fact that oral mucosa is devoid of adnexal structures, such as hair and sebaceous glands, thought to be the repositories of stem cells for the epidermis, thus making it a 'large interfollicular area'. This would seem to indicate that the progenitor/stem cell population of oral keratinocytes lies within the basal layers of the mucosa, making them readily accessible for isolation and utilization for fabrication of 'smart' EVPOMEs. This can result in a construct capable of a higher level of gene expression in a significant percentage of cells thus creating a more predictable graft success and functionality for long term use as a vehicle for gene delivery (Bianco and Robey, 2001; Chen et al, 2002; Ortiz-Urda et al, 2002). Studies are in progress to use tissue-engineered oral mucosa as a vehicle for the use of gene therapy to enhance wound healing and/or transmucosally administer systemically needed growth factors.

Lastly, the use of oral mucosa progenitor/stem cells can also potentially be used in the repair/regeneration of other mucosal surfaces such as eyelids/conjunctiva, nasal or extra-cranial tissues such as esophagus, trachea, bladder, urethra or vagina.

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