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J Liu
Z Bian
AM Kuijpers-Jagtman
JW Von den Hoff

Skin and oral mucosa equivalents: construction and performance

Authors' affiliations:
Jiarong Liu, Anne M. Kuijpers-Jagtman, Johannes W. Von den Hoff, Department of Orthodontics and Oral Biology, Radboud University Nijmegen Medical Centre, HB Nijmegen, The Netherlands
Jiarong Liu, Zhuan Bian, Key Laboratory of Oral Biomedical Engineering of Ministry of Education, School and Hospital of Stomatology, Wuhan University, Wuhan, China

Correspondence to:
Dr J.W. Von den Hoff
Department of Orthodontics and Oral Biology
Radboud University Nijmegen Medical Centre
PO Box 9101, 6500 HB Nijmegen
The Netherlands
E-mail: h.vondenhoff@dent.umcn.nl

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Abstract

Authors – Liu J, Bian Z, Kuijpers-Jagtman AM, Von den Hoff JW

The skin and the oral mucosa act as a barrier against the external environment. Loss of this barrier function causes dehydration and a high risk of infection. For the treatment of extensive skin wounds such as in severe burns, autologous skin for transplantation is often not available in sufficient amounts. Reconstructions in the oral cavity, as required after tumor resections or cleft palate repair, are often complicated by similar problems. In the last two decades, the field of tissue engineering has provided new solutions to these problems. Techniques have been developed for the culture of epithelial grafts, dermal substitutes, and the combination of these two to a 'functional' skin or mucosa equivalent. The present review focuses on developments in the field of tissue engineering of skin and oral mucosa. The performance of different types of engineered grafts in animal models and clinical studies is discussed. Recent developments such as the use of epithelial stem cells, and gene therapy with transduced skin grafts are also discussed.

Key words: fibroblasts; keratinocytes; oral mucosa; skin; tissue engineering

Introduction

The skin and the oral mucosa play a crucial role as a barrier against exogenous substances, pathogens, and mechanical stresses (1). Defects in this barrier cause water and protein loss, and allow bacteria to invade the underlying tissue. Extensive burn wounds in the skin therefore need to be covered as early as possible (2, 3). The conventional treatment uses split-thickness grafts from the patients' own skin. However, the amount of unburned skin is a limiting factor in extensive burn wounds. The morbidity of the donor site may present an additional problem. Allogeneic skin grafts are commonly used for temporary wound coverage. Unfortunately, these allografts have two main problems: immunologic rejection and the risk of viral transmission (4). In the oral cavity, reconstructions after tumor resection, vestibuloplasty, or the treatment of gingival recessions also require suitable grafting materials (5, 6). Furthermore, the surgical closure of a cleft palate is also hampered by a shortage of oral mucosa. Oral mucosa is limited in supply and the use of skin grafts in the oral cavity has some disadvantages. The keratinized surface of the grafted skin tends to macerate and is easily infected by fungi. Hair growth may also occur after the transplantation of skin into the oral cavity (7, 8).

In full-thickness skin wounds, both the epithelial and dermal layers are lost. These wounds heal by secondary intention, which is characterized by extensive granulation tissue formation followed by wound contraction and scar formation (9). In exposed areas of the body scar tissue causes aesthetic problems, and around the joints it may impair function (10). The mechanical effects of scar tissue in children can even restrict growth (11). In cleft palate repair, autologous grafts are generally not used because of the limited availability of oral mucosa. Therefore, large open wounds exposing the palatal bone often remain after surgery. These wounds also heal by second intention and cause wound contraction and scar tissue formation. The scar tissue is mainly responsible for the growth inhibition of the dento-maxillary complex after cleft palate repair (12–14).

To solve the problem of the availability of autologous skin or oral mucosa for reconstructive surgery and to minimize scar formation, several techniques of tissue substitution have been developed. This review focuses on tissue engineering of skin and oral mucosa. The first part briefly recapitulates the general structure of skin and oral mucosa, and their differences. Subsequently, developments in the preparation of epithelial grafts, cellular dermal substitutes, and composite skin and mucosa equivalents are discussed. In addition, the potential use of epithelial stem cells for tissue engineering and gene therapy is highlighted.

Structure of skin and oral mucosa

The basic structure of human skin and oral mucosa is similar (Fig. 1). They both are composed of two layers: a covering epithelium and an underlying connective tissue separated by a basal membrane (15, 16). The epithelium acts as a barrier against exogenous substances and pathogens and, mainly in skin, dehydration. The main cells are keratinocytes that are tightly attached to each other by desmosomes, and arranged in a number of distinct layers. The connective tissue supports and nourishes the epithelium, and connects it to the underlying structures.

The mucosa of the oral cavity is somewhat different from skin. The two main tissue components of oral mucosa are termed the oral epithelium and the lamina propria, the underlying connective tissue (17). In contrast to the epidermis of skin, which is orthokeratinized, all three major differentiation patterns of keratinocytes occur in normal oral epithelia. In regions subject to mechanical forces associated with mastication such as the gingiva and the hard palate, a keratinized epithelium resembling that of the epidermis occurs. The pattern of maturation of keratinized epithelium mostly is orthokeratinization. Parts of these keratinized areas show a variation of keratinization, known as parakeratinization, in which the nuclei of the cornified layer are still recognizable (17). The floor of the mouth and the buccal regions, which require flexibility to accommodate chewing, speech, or swallowing, are covered with a lining mucosa with a non-keratinizing epithelium. The specialized mucosa on the dorsum of the tongue contains numerous papillae and is covered by an epithelium, which may be either keratinized or non-keratinized (16).

In many regions of the oral mucosa a layer of loose fatty or glandular connective tissue, the submucosa, underlies the lamina propria. It determines the

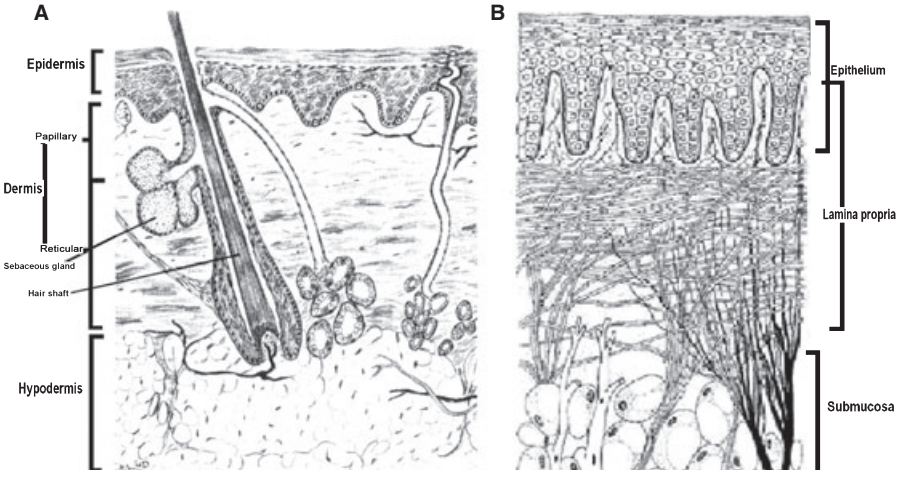

Fig. 1. Normal structure of skin and oral mucosa. This figure illustrates the main tissue components of skin (A) and oral mucosa (B).

flexibility of the attachment of the oral mucosa to the underlying structures. In the gingiva and the hard palate, the oral mucosa is directly attached to the periosteum of the underlying bone, without a submucosa. This is called a mucoperiosteum and it provides a firm, inelastic attachment (16, 17). The appendages of the oral mucosa are mainly small salivary glands and few sebaceous glands (17). The normal appendages of skin such as sweat glands and hairs are completely absent.

Both the epithelium and the underlying connective tissue have an important function in skin and oral mucosa. In the field of tissue engineering, techniques have been developed for the culture of epithelial grafts either or not combined with an underlying dermal component. Several of the constructs have also been evaluated in animal experiments and/or clinically.

Cultured epithelial grafts

Keratinocytes can be grown in culture to produce thin epithelial sheet grafts. Briefly, the technique of Rheinwald and Green is based on the use of serum-containing media and a feeder-layer of irradiated murine fibroblasts (18). Another approach relies on serum-free media. Serum-free media often contain bovine pituitary extract and may therefore still expose the recipients to animal pathogens. Recent studies describe serum-free media without any animal proteins (19). The results show that growth of keratinocytes occurs, but the expansion rate is much lower. Up to now, serum-containing media still provide the most efficient way to expand the keratinocytes. Epithelial grafts can be cultured from autologous or allogeneic keratinocytes. Autologous grafts are not rejected (20), but require 2-to-3 weeks culture time. Allogeneic grafts can provide immediate wound coverage, but they may be rejected and may transmit diseases. The freezing of allogeneic grafts is suggested to reduce the risk of disease transmission (21).

The disadvantages of cultured epithelial grafts are that they are difficult to handle and fail to prevent wound contraction and scarring in full-thickness wounds (21, 22). It is also possible to grow the keratinocytes on a suitable carrier that is detached from the culture vessel together with the epithelium, and transferred to the wound bed. Several biocompatible and biodegradable materials are used as carriers including fibrin (19, 23), collagen (24), hyaluronic acid and synthetic polymers (25). The carrier film may also provide a barrier against bacterial infection (25). The epithelia grown on the carrier are easy to handle and retain their original size when detached, whereas epithelial grafts alone may shrink to one-third of the original size. Alternatively, keratinocytes can be sprayed onto the wound bed suspended in fibrin glue (26, 27). In this case, the culture time is shorter because the keratinocytes can be transplanted before they reach confluence, but a differentiated epithelium is not present immediately after application.

Substances present in the fibrin used for application have been shown to either inhibit or stimulate growth of the keratinocytes. These conflicting results seem to depend on the method of fibrin preparation, and the method used for determining keratinocyte growth (19, 28). Fibrin-based epithelial grafts have also been evaluated in animal models (25, 29) and human full-thickness burns (23, 30). These specific studies show that fibrin enhances keratinocyte attachment to the wound bed, which may increase the take rate (29). No differences in wound closure and wound contraction were observed.

Similar to skin keratinocytes, oral keratinocytes can be grown in serum-containing or serum-free medium (31). Oral keratinocytes in culture generally reach confluence more rapidly than skin keratinocytes (32). Immunohistochemical data indeed show that cultured oral epithelium contains more proliferating cells than cultured skin epithelium (33). The cell morphology and keratin expression of cultured oral epithelial grafts seem to depend on the site of origin in the oral cavity (32). Intra-oral wounds that remain after palatal surgery or carcinoma resection have been treated with cultured mucosal epithelium (34–36). The cultured keratinocyte grafts enhanced the healing process and promoted re-epithelialization of the defects.

Epithelial stem cells

A new development is the use of epithelial stem cells for tissue engineering. In addition, stem cells in an epithelial graft can possibly be used as a vehicle for gene therapy in inborn metabolic skin diseases [(37); see paragraph 6]. Multi-potent stem cells are found in the bulge region of the hair follicle, while uni-potent

stem cells reside between the normal basal keratinocytes (Fig. 2). The uni-potent stem cells can only give rise to a lineage of differentiating keratinocytes but the multi-potent stem cells can also form new hair follicles and sebaceous glands. However, the identification of stem cells in the epithelium by molecular markers remains a controversial issue. Until now, the only accepted methods for identifying stem cells are the analysis of their replicative behavior *in vitro,* and the detection of long living, slowly cycling cells *in vivo* (38). The keratinocytes that retain nucleotide labels *in vivo* are believed to be epithelial stem cells. Epithelial stem cells seem to express high levels of the β1 and α6 integrin subunit, and low levels of the transferrin receptor (39). Their capacity to self-renew makes stem cells highly interesting for tissue engineering purposes. An enriched population of epithelial stem cells has been obtained from human oral mucosa (40). However, the isolation of a pure population of epithelial stem cells has not succeeded up to now. This is the main reason that epithelial stem cells are not yet routinely used for tissue engineering.

Several authors attempted to isolate epithelial stem cell populations from mouse skin (41–43). The isolated cells reached confluence only after a long culture time suggesting that they indeed were stem cells. The cells also formed larger and more expandable colonies in culture than normal keratinocytes. In a composite culture, the epithelium derived from these cells also maintained the epithelial morphology longer than normal epithelial cells.

In conclusion, cultured epithelial grafts can provide adequate coverage of the wound bed. It is possible to cover a large area with grafts derived from only a small biopsy. However, epithelial grafts without a carrier material or a dermal substrate are difficult to handle. In addition, epithelial grafts do not reduce wound contraction and scarring in full-thickness wounds. A dermal component seems to be required to achieve this goal.

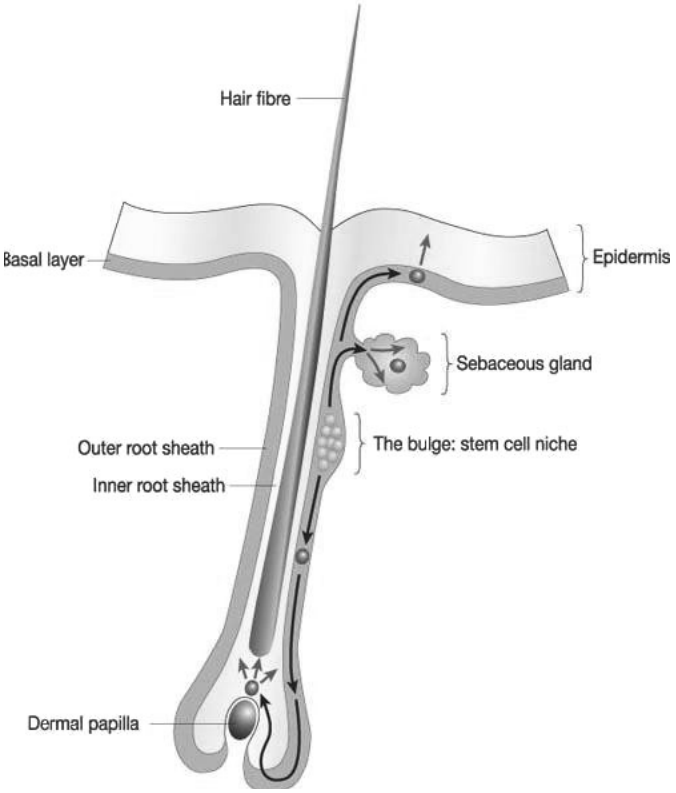

Fig. 2. Stem cells in the hair follicle. Hair follicles are formed by an invagination of the epithelium that penetrates into the dermal tissue. Multipotent epithelial stem cells are located in the bulge area and can migrate upwards to populate the sebaceous gland and the interfollicular epithelium. They can also migrate downwards and enter the matrix surrounding the dermal papilla to form the hair. Reprinted by permission from Macmillan Publishers Ltd.: Reya T, Clevers H. Wnt signalling in stem cells and cancer. *Nature* 2005;434(7035):843–50.

Dermal substitutes

The dermal component plays an important structural and physiological role in both the skin and the oral mucosa. Various acellular materials have been used to construct a substitute for the dermal tissue. The dermal substrate can be synthetic or from biological origin. Fibroblasts, the main cell type in dermal tissue, may be seeded into the substrate.

Acellular dermal substrates

Synthetic materials that have been used as a dermal substrate include polymers such as poly (L)-lactic acid and poly glycolic acid in the form of films, foams, and sprays (44). Some synthetic substrates are composed of two or more layers of polymers. The advantages of synthetic materials are their reproducible mechanical and physical properties, and their unlimited availability (45). However, these materials tend to elicit a foreign body response and may facilitate infection, leading to the formation of dense scars and fibrosis.

Biological substrates are derived from human or animal tissues, and are mainly produced from purified collagen or derived from skin. Collagen is a natural substrate for cellular attachment, growth, and

differentiation. In addition to its structural properties, it is biocompatible and non-toxic (44). Purified collagen has been used in the form of gels, sponges, meshes and membranes. To enhance its structural properties, other synthetic or biological molecules are often included. A collagen type-I substrate with chondroitin-6-sulphate, Integra Artificial Skin, was developed in the eighties (46, 47). The advantages of Integra are, as stated by Pomahac et al. (1998), good cosmetic results, easy to use, and fast healing. The main disadvantages are the low take rate and the risk of infection (48–50).

Animal experiments and clinical evaluations of collagen-based dermal substrates show that they are gradually replaced by a neo-dermis (48, 51, 52). The mean 'take' in these studies is about 50–100% depending on the time of evaluation. These materials seem to reduce contraction and hypertrophic scar formation (53, 54). In a long-term follow up study, depigmentation of the epithelium and hypertrophy of the dermis were observed in some wounds (54). In combination with split-skin grafts or cultured keratinocytes, collagen-based substrates are being used for burn wounds and reconstructive surgery (48, 50).

Skin–derived substrates are produced from donor skin by removing the epidermis and the cells from the dermis. This is called de-epidermized dermis (DED). DED is supposed to be free of immunogenic cellular components and to retain the architecture of the original dermis. An important feature of these materials is a structurally intact basement membrane complex, which is beneficial for keratinocyte growth (55). There also is a commercially available human DED, called AlloDerm. After implantation, DED seems to support fibroblast infiltration and neovascularization (56), and the engraftment of cultured keratinocytes (57). It also seems to shorten the healing time and to reduce wound contraction (57, 58). The mean clinical 'take' was over 75% after varying evaluation times (58, 59).

De-epidermized dermis and AlloDerm are also used for intra-oral resurfacing in gingival augmentation procedures, cleft palate repair (60), or reconstructions after tumor resection (6). Gingival augmentation with AlloDerm yields acceptable esthetic results and decreases patient morbidity (5, 61). In one study, DED reduced contraction and scarring of the remaining wounds after cleft palate repair, and also improved soft palatal function (60). A disadvantage of skin-derived substrates is that a secondary procedure for epithelial grafting is often required after implantation. The incorporation of fibroblasts into a dermal substrate may improve dermal regeneration and re-epithelialization.

Cellular dermal substrates

In the engineering of skin and mucosa fibroblasts can be included in the dermal substrate. The presence of fibroblasts stimulates epithelial differentiation (62, 63) and dermal regeneration (64). As discussed above, the dermal substrate is generally prepared from a collagen gel or scaffold, DED, or synthetic materials (45). The fibroblasts proliferate within the substrate, deposit extracellular matrix, and produce growth factors. However, fibroblasts can differentiate into myofibroblasts and cause contraction. Fibroblasts isolated from papillary dermis show a normal morphology and behavior, but adipose-derived fibroblasts are more likely to differentiate into myofibroblasts (64). The origin of fibroblasts for seeding might therefore be crucial.

Autologous or allogenic fibroblasts can be seeded into a dermal substrate. Cultured dermal substitutes can be rendered nonviable by repeated freezing and thawing leaving behind the proteins and growth factors (65). These substitutes essentially function as a reservoir of growth factors and cytokines which may improve healing. The use of viable autologous fibroblasts in substitutes may reduce immunologic reactions (64, 66, 67). In contrast, some authors state that fibroblasts in the dermis are relatively non-immunogenic and do not express HLA-DR markers (68–70). The advantage of a dermal substitute with allogeneic cells clearly is their immediate availability.

Several studies show that a collagen matrix or synthetic scaffold with autologous fibroblasts improves dermal regeneration as compared with an acellular substrate (64, 71). The fibroblasts increase degradation of the substrate, which reduces the inflammatory response (66). Autologous fibroblasts in a dermal substitute also showed a higher rate of cell proliferation than allogeneic fibroblasts after implantation in guinea pigs, and induced less wound contraction (67).

In summary, a dermal substitute provides physical support and a functional tissue at the graft site. In addition, it might reduce wound contraction and scarring. The inclusion of fibroblasts in the dermal

substitute generally improves regeneration and epithelialization. A general disadvantage of the use of a dermal substitute alone is that a split-thickness skin graft or a cultured epithelial may have to be applied in a secondary procedure.

Composite equivalents

The limitations of the above-mentioned techniques led to the development of substitutes composed of keratinocytes cultured on top of a dermal substitute. The epithelial layer may contain autologous or allogeneic keratinocytes, which form a multi-layered epithelium similar to the normal epithelium *in vivo*. The dermal layer consists of a substrate with or without autologous or allogeneic fibroblasts. For the construction of skin and mucosa equivalents comparable techniques have been used. This section focuses on composite equivalents and their animal experimental or clinical evaluation.

Skin equivalents

Several investigators have tried to construct composite skin equivalents. The substrates used for these studies include collagen-based substrates and DED, either with or without fibroblasts. Collagenous substrates are expected to present suitable binding sites for both keratinocytes and fibroblasts (72–74). When DED is used as a dermal substrate, fibroblasts are seeded on the reticular side and, after culture, the DED is turned over and keratinocytes are seeded on the basal membrane side (75, 76). Skin equivalents with DED seem to provide a stronger dermal-epithelial junction than skin equivalents with collagen-based substrates. This might be related to the presence of an original basement membrane on the DED (77). The keratinocytes and fibroblasts in skin equivalents may be autologous or allogeneic. In theory, autologous keratinocytes provide a more 'permanent' coverage because of their lack of immunogenicity. However, it was shown that, despite the use of autologous keratinocytes, only some of the skin equivalents were permanently engrafted (78, 79). The fact that both the DED and the collagen used for the preparation of the equivalents are allogeneic to some extent might contribute to the final graft loss. The use of allogeneic cells may further enhance the inflammatory response leading to graft rejection (80).

Skin equivalents have been studied both in experimental settings and in the clinic. Many *in vitro* studies using either a collagen substrate or DED show that skin-like equivalents can be produced (75, 77). Keratinocytes were shown to proliferate and differentiate more with fibroblasts included in the dermal substrates than without fibroblasts (22). The epithelium of the skin equivalents usually consists of 5 to 8 stratified cell layers and also expresses structural proteins similar to normal epidermis (29, 81). The formation of a basal membrane is also enhanced by the presence of fibroblasts (77).

Animal experiments and clinical studies show that wound healing is stimulated and contraction is reduced by skin equivalents (73). The take rate of skin equivalents applied to burn patients is between 40% and 70% after two to 3 weeks (78, 79). An autologous skin equivalent based on DED was shown to be suitable for the healing of chronic wounds (82). Skin equivalents based on collagen gels are torn easily during handling and suturing (74). On the other hand, skin equivalents with DED present a risk of virus transmission.

In conclusion, skin equivalents can be constructed that provide an epidermis and a dermis which can be grafted in a one-step procedure. If allogeneic cells are used, the equivalent is directly available but it only functions as a temporary coverage, and has a higher risk of disease transmission. The 'ideal' skin equivalent contains autologous cells and provides a permanent tissue replacement. However, the culture period of several weeks to obtain autologous cells may not be clinically acceptable. In general, long-term results of grafted skin equivalents are not yet available.

Mucosa equivalents

In general, less research has been performed on the construction of mucosa equivalents. As for skin equivalents, mucosa equivalents are generally based on the use of a collagen gel or DED. In reconstructed buccal mucosa the morphology and the expression pattern of proteins such as keratins were quite similar to that in native buccal mucosa, but the epithelium was thinner. In general, the formation of rete ridges was limited and the basement membrane was not fully developed (83, 84). Cultured buccal mucosa

equivalents used for intraoral reconstructions in patients after the resection of mucosal tumors induced no inflammatory reactions (85). Furthermore, the epithelial layer promoted more vascular ingrowth than controls without epithelium.

Other researchers constructed equivalents of gingival and palatal mucoperiosteum using DED (Fig. 3) or collagen gels. Histological analyses showed that the differentiation pattern of the epithelium was similar to that of the mucoperiosteum *in vivo*. However, reconstructed mucoperiosteum appeared to be thinner and lacked the prominent epithelial ridges (86–89). It remains to be established how these equivalents perform *in vivo*. In contrast to skin equivalents for e.g. burns treatment, the time required to culture an autologous mucosa equivalent is mostly less critical because oral reconstructions are generally elective.

Gene therapy with skin grafts

An exciting new development is the introduction of genes into patient keratinocytes to improve genetic skin disorders or to systemically deliver therapeutic proteins for the treatment of diseases affecting other organs (90). These techniques can be combined with tissue engineering methods to produce genetically manipulated skin grafts *in vitro*. Compared to direct *in vivo* gene delivery, this has several advantages. Keratinocytes can be genetically manipulated much easier *in vitro* than *in vivo,* and can be expanded from a small skin biopsy to obtain large grafts. In addition, the *in vitro* approach reduces the risk of systemic spread of the vector. For stable transgene expression it should be integrated in the genome of epithelial stem cells, which prevents its loss during the normal cycling of the epithelium. In other cases such as tissue repair or vaccination, transient expression by normal keratinocytes might be sufficient (90).

This type of approach is being studied for the treatment of different types of epidermolysis bullosa, a genetic skin disorder (91, 92). However, up to now the efficiency of gene delivery is generally insufficient for the treatment of genetic skin disorders. Examples of studies on gene transfer for systemic diseases are the production of growth hormone (for GH deficiency) or proinsulin (for diabetes) by transduced skin grafts (93, 94).

Conclusion

Skin and oral mucosa provide a barrier between the underlying tissue and the external environment. Extensive burn wounds in the skin may require the implantation of a skin equivalent. Also, reconstructions in the oral cavity after tumor resection or cleft palate repair require a suitable grafting material. Ideally, an engineered skin or mucosa equivalent should have the same properties as the original tissue. Consequently, it should possess a protective epithelial layer and a supporting dermal layer. Cultured epithelial grafts provide epithelial coverage of a large wound, but they are difficult to handle and do not contain a dermal component. Grafts from autologous keratinocytes have a high take rate, but the extra

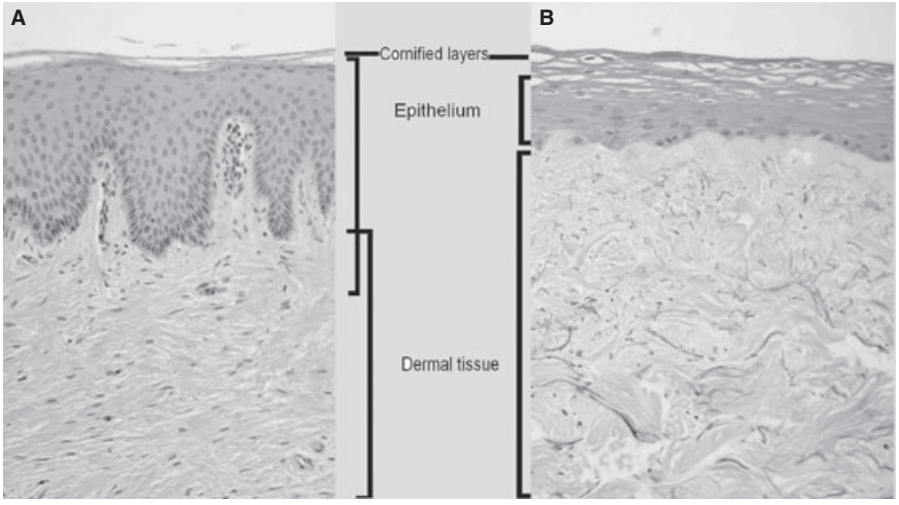

Fig. 3. Morphology of oral mucosa and a mucosa equivalent. (A) Native mucosa of the hard palate (B) Mucosa equivalent constructed with DED and palatal cells. Keratinocytes and fibroblasts were cultured from a palatal biopsy. The fibroblasts were seeded into the DED and cultured for 1 week. Then, keratinocytes were cultured on top for 14 days. The mucosa equivalent contains less epithelial cell layers and the rete pegs are lacking. Haematoxylin-eosin staining (original magnification 200×) (unpublished results J. Liu).

culture time is a large drawback compared with allogeneic grafts. The latter are generally used as a temporary dressing which produces growth factors to stimulate healing.

In full-thickness wounds, epithelial grafts still permit wound contraction and subsequent scar formation. A dermal substitute provides physical support for the epithelial graft and reduces contraction and scarring. Cell-populated dermal substitutes are superior to acellular dermal substrates because the fibroblasts deposit new extracellular matrix, and produce growth factors that promote tissue regeneration. Cultured skin and mucosa equivalents provide an epithelial and dermal substitute in a one-step process. These composite constructs appear to be the optimal replacement for skin and mucosa, although long term evaluations of their clinical efficacy is still lacking.

A promising new development in tissue engineering is the use of epithelial and mesenchymal stem cells. However, up to now the characterization and isolation of true stem cells has not been routinely achieved. Therefore, the use of stem cells in tissue engineering is still in its infancy. The application of tissue engineering techniques for gene therapy of genetic skin disorders and systemic diseases also requires further development to achieve clinical effectiveness.

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