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# Oral mucosa model based on a collagen–elastin matrix

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*Background and Objective:* The collagen-elastin matrix (Matriderm®) is used to treat deep and full-thickness burns and was recently described as a suitable scaffold for tissue engineering. The aim of the present study was to investigate the biocompatibility of Matriderm® for gingival use through creation of an oral mucosa model *ex vivo*.

*Material and Methods:* Gingival fibroblasts and keratinocytes were cultured. A dermal area on the base of the collagen–elastin matrix was repopulated with fibroblasts. After 14 days, keratinocytes were seeded on this dermal area to engineer a multilayered mucosa. Analysis of the architecture was performed using light and electron microscopy. Immunohistochemical detection of collagen IV and cytokeratin was carried out.

*Results:* Based on this scaffold we generated a multilayered oral mucosa-like structure. Histological, immunohistochemical and electron microscopic analysis of the dermal/epidermal junction showed a typical basement membrane and hemidesmosomal structures. Neighboring keratinocytes formed desmosomes in the epidermal sections. Cytokeratin was detectable in all epidermal layers. These experiments revealed that the collagen–elastin matrix was highly biocompatible with gingival cells under *ex vivo* conditions.

*Conclusion:* Employing tissue-engineering techniques with dermal and epidermal cells from the gingiva, a multilayered oral mucosa was generated and characterized with respect to biocompatibility for Matriderm®. The results indicate that Matriderm® is suitable for the *ex vivo* growth of gingival tissue cells and is a useful scaffold with possible applications in periodontal therapy.

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Both, dermal and epidermal tissues, in the skin as well as in the oral cavity, have a unique barrier function in which they separate the body from its environment. Essential functions of oral mucosal barriers include resistance against pathogens, exogenous substances and mechanical stress (1). Treatment of periodontal recession using several different periodontal, oral or craniofacial surgical approaches (i.e. enlargement of the gingival width or as a pre-prosthodontic or pre-implant treatment, etc.) require adequate amounts of suitable grafting materials (2,3).

Regeneration of the structure and function of oral mucosa is one of the main objectives in periodontal health care. Tissue engineering represents an excellent approach to overcome difficulties encountered in periodontal health care such as diseased sections of soft tissues in the oral cavity and a limited amount of gingival tissue. Originating in the culture of keratinocytes by Rheinwald and Green (4) there were found to be many advantages of soft tissue models. Tissue-engineered skin replacements, including biological substances such as fibrin sealant, various types of collagen and hyaluronic acid, were cultured on acellular and cellular matrices. These components were brought together as scaffolds (5) and were found to perform essential structural and physiological functions during the construction of *in vitro* three-dimensional tissue substitutes (6).

In the generation of oral mucosa models, Alloderm® was employed as an underlying (dermal) structure for the development of fully differentiated oral tissue (7,8). In further studies this *ex vivo* oral mucosa equivalent was also used for intra-oral grafting in oral surgery (9). In addition, the function of this scaffold was investigated *ex vivo* as a test system to determine radiation-induced effects on oral keratinocytes (10).

The primary indication of the collagen–elastin matrix (Matriderm®) as a dermal substitute is for the treatment of deep partial and full-thickness burn wounds (11–13). Matriderm® has been proved to be an effective dermal substitute and was recently described as a suitable scaffold for tissue engineering in the generation of three-dimensional skin scaffolds (14). Based on this technique we tested the biocompatibility of Matriderm® with gingival fibroblasts as well as with keratinocytes. In further experiments we generated a three-dimensional oral mucosa model using tissue-engineering techniques and investigated the architecture of this model by histological analysis and electron microscopy. Important characteristics and measures of tissue quality are multilayered epithelia, interactions between keratinocytes by desmosomes as well as the assembly of a basement membrane during co-culture.

## Material and methods

The collagen–elastin matrix (Matriderm®; the underlying structure on which the gingival tissue was cultured) was purchased from Skin & Health Care (Dr. Suwelack, Billerbeck, Germany). This material supports dermal regeneration and contains dermal collagen types I, II, III and V from bovine dermis and elastin derived from bovine nuchal ligament. For the isolation and growth of primary human gingival keratinocytes, biopsies of the marginal gingiva from the distal region of the mandibula were taken, using a 5-mm-diameter disposable biopsy punch (Stiefel Laboratorium, Offenbach, Germany), from 15 healthy volunteers given local anesthesia. The tissue was collected in sterile transport medium [Dulbecco's modified Eagle's minimal essential medium (DMEM); Life Technologies, Karlsruhe, Germany], containing 10% fetal calf serum (Greiner, Frickenhausen, Germany), 100 U/mL of penicillin and 0.1 mg/mL of streptomycin (Life Technologies). The tissue was washed extensively with $Mg^{2+}$- and $Ca^{2+}$-free phosphate-buffered saline ($PBS^{-/-}$) (Life Technologies), containing 100 U/mL of penicillin, 0.1 mg/mL of streptomycin and 0.25 g/mL of amphotericin B. The tissue was sliced into small pieces (of approximately 3 $mm^2$). The dermis and the epidermis were separated by incubation overnight at 4°C with 10 mg/mL of dispase II (Roche, Penzberg, Germany) in $PBS^{-/-}$.

The epidermal part of the tissue biopsy was then removed with forceps and incubated in a 0.05% trypsin/0.02% EDTA solution for 20 min at 37°C (Invitrogen, Karlsruhe, Germany) until a single-cell suspension was obtained. Keratinocytes were resuspended and incubated in cell culture flasks. Human gingival keratinocytes were cultured as previously described (15). Briefly, the cells were cultured in a serum-free medium containing DMEM/Ham's F12 [4 : 1, volume per volume (v/v)], HEPES and penicillin/streptomycin (Invitrogen), and basal substances (15).

The dermal cells were dissociated by incubation for 2 h at 37°C in a solution of 0.1% collagenase (Sigma-Aldrich, Munich, Germany). The isolated fibroblasts were harvested and seeded in cell-culture flasks containing fibroblast growth medium [DMEM containing 100 U/mL of penicillin, 0.1 mg/mL of streptomycin and 10% fetal calf serum (Greiner)].

## Ethical considerations

The study was approved by the ethical committee of the Justus Liebig University of Giessen (request no. 52/00; renewal no. 22/05). All participating volunteers were instructed and gave their written informed consent before samples of oral mucosal tissue were obtained. All experiments followed the guidelines of good clinical/laboratory practice (GCP/GLP) and the Helsinki Declaration of the World Health Organization (1964), update Seoul 2008 (59th World Medical Association, General Assembly, Seoul, October 2008, http://www.wma.net/en/30publications/10policies/b3/17c.pdf).

### Generation of the oral mucosa model

The collagen–elastin matrix (commercially available in a size of 148 × 105 × 2 mm) was sliced into distinct pieces of 30 × 30 mm and stored under sterile conditions in six-well plates ready for use.

After 24 h of culture in DMEM, fibroblasts in early passages (five or fewer) were seeded on the matrices in fibroblast growth medium ($3 \times 10^5$ fibroblasts per $cm^2$). For the next 14 d, culture was performed submersed in fibroblast growth medium and the culture medium was renewed daily. Keratinocytes in early passages (five or fewer) were seeded on the collagen–elastin matrix ($6 \times 10^5$ keratinocytes per $cm^2$). Culture was performed in keratinocyte growth medium. One week later the gingival tissue model was lifted to the air–liquid interface (ALI). Oral mucosa models at the ALI were cultured in Epilife™ (Cascade Biologics, Portland, OR, USA) with human keratinocyte growth supplement, 1.4 mM $Ca^{2+}$, 73 μg/mL of ascorbyl-2-phosphate (Sigma-Aldrich), 100 IU/mL of penicillin, 0.1 mg/mL of streptomycin and 10 ng/mL of human keratinocyte growth factor (CellSystems, St Katharinen, Germany). After culturing the model at the ALI for 10 d ($n \geq 3$ individual experiments), samples were further cut, using sterile scissors, into individual pieces and then prepared for histological, immunohistochemical and electron microscopic analyses.

### Histological analysis

The morphological properties of the gingival tissue model were assessed using histological techniques. For light microscopy analyses, samples were fixed in 4% formalin for 1 h, then dehydrated and embedded in paraffin. Paraffin sections (of 5–10 μm thickness) were obtained using a rotary

microtome (Leitz 1512; Leitz, Wetzlar, Germany). For analysis of the gingival tissue model architecture, sections were stained with hematoxylin and eosin and with periodic acid-Schiff (PAS) to detect the polysaccharide-rich basement membrane. The number of epidermal cell layers was visually determined from hematoxylin and eosin-stained sections using an Olympus Vanox-T AH-2 microscope (Olympus, Hamburg, Germany).

### Immunofluorescence staining

After deparaffinization, tissue sections were treated with 0.1% Trypsin II-S (Sigma-Aldrich) for 30 min, washed three times with PBS, then incubated overnight (at 4°C) with primary antibody: collagen IV (1 : 50 dilution, monoclonal mouse; DakoCytomation, Hamburg, Germany) and pan-cytokeratin (1 : 40 dilution, polyclonal rabbit; Dianova, Hamburg, Germany). After washing three times in 15 ml enploying glass cuvettes PBS, the sections were incubated for 1 h at room temperature with secondary antibody: fluorescein isothiocyanate (FITC)-conjugated anti-rabbit or anti-mouse depending on the primary antibody (1 : 100 dilution; Dako). For visualization of the nucleus, sections were incubated for 15 min with a 1 : 1000 dilution of TOPRO-3 iodide in PBS (Invitrogen). The sections were analyzed by microscopy using a Zeiss Axiophot (Zeiss, Oberkochen, Germany). All experiments were repeated at least twice.

### Transmission electron microscopy

The gingival tissue model was fixed for 60 min in Karnovsky solution [2 g of paraformaldehyde in 25 mL of distilled water, 10 mL (25%) of glutaraldehyde and 15 mL of 0.2 cacodylate buffer]. Samples were osmicated for 60 min in cacodylate buffer containing 0.5% $OsO_4$. After washing in 0.1 M cacodylate buffer, the samples were dehydrated through an increasing ethanol series and embedded in agar low-viscosity resin (Plano, Wetzlar, Germany). Ultrathin sections were prepared using an LKB II microtome (LKB, Bromma, Sweden) and collected on 200-mesh hexagonal copper grids. Uranyl acetate and lead citrate were used for contrast. The sections were examined using an LEO 906 transmission electron microscope (LEO Elektronenmikroskopie GmbH, Oberkochen, Germany).

### Scanning electron microscopy

For scanning electron microscopy, oral mucosa models were rinsed with 0.1 M sodium cacodylate and fixed for 24 h at 4°C in a 0.1 M sodium cacodylate buffer containing 2.5% glutaraldehyde and 0.6% paraformaldehyde. Samples were then dehydrated in a graded series of ethanol, dried in a critical-point dryer (CPD 030 Critical Point Dryer; Balzers Union, Balzers, Lichtenstein) sputter-coated with gold (CPD 040; Balzers Union) and examined in a Hitachi S2300 scanning electron microscope (Hitachi, Tokyo, Japan) operated at 15 kV.

## Results

After 30 d of culture, an *ex vivo* differentiated oral mucosa model was generated on Matriderm® (Figs 1 and 2). In this model, fibroblasts were seeded onto Matriderm®. Then, after 14 d of incubation, keratinocytes were introduced onto the fibroblast scaffold. Histological analysis, using standard hematoxylin and eosin staining, showed that the fibroblast scaffold had characteristics of human gingiva (Fig. 2). Apically, a stratum basale was visible and characterized as a single layer consisting of a cuboidal shape, and cells in the superficial layer appeared extended and compacted (Fig. 2).

To characterize the epidermal junction zone, PAS staining was performed that clearly showed the basement membrane underneath the stratum basale (Fig. 3A). Immunohistochemical staining revealed a basement membrane component – collagen IV – juxta-positioned between the dermal and epidermal layers (Fig. 6B).

Transmission electron microscopy analysis showed a multilayered formation of gingival keratinocytes (Fig. 4A)

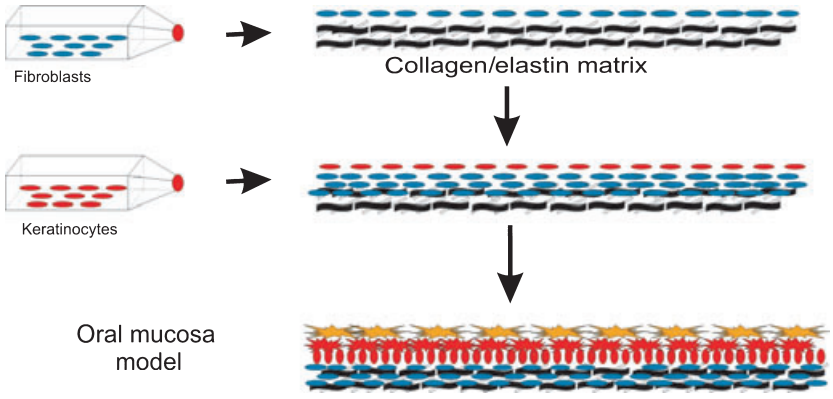


*Fig. 1.* Fibroblasts were cultured on Matriderm® and proliferated into the collagen–elastin matrix in fibroblast growth medium. During the submersed culture, medium was changed daily. After 14 d, gingival keratinocytes were seeded on the matrix and the culture was continued in keratinocyte growth medium. After a total of 3 wk of culture under submersed conditions, the gingival tissue model was lifted to the air–liquid interface (ALI). Following 10 d of culture at the ALI, a viable multilayered oral mucosa scaffold was detectable.

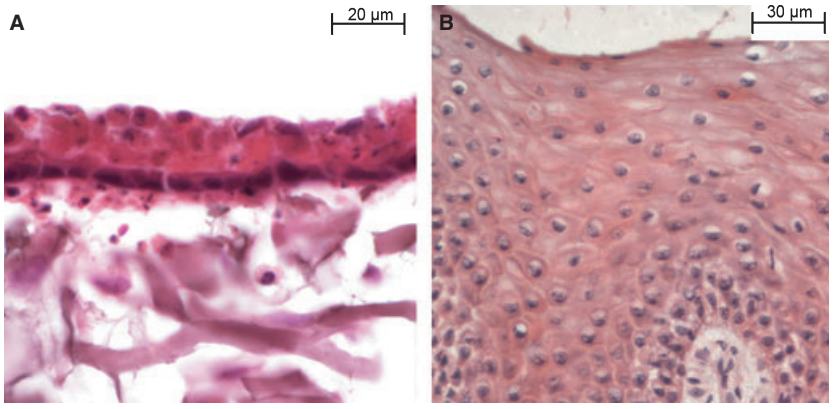


*Fig. 2.* Hematoxylin and eosin staining of an oral mucosa model. (A) Oral mucosa after 10 d of culture at the air–liquid interface (ALI) in comparison with naïve gingival tissue. (B) The stratum basale was identified as the bottom layer of keratinocytes on the matrix. Cells that moved towards the apical region (into upper layers) changed from a columnar shape to a polygonal shape.

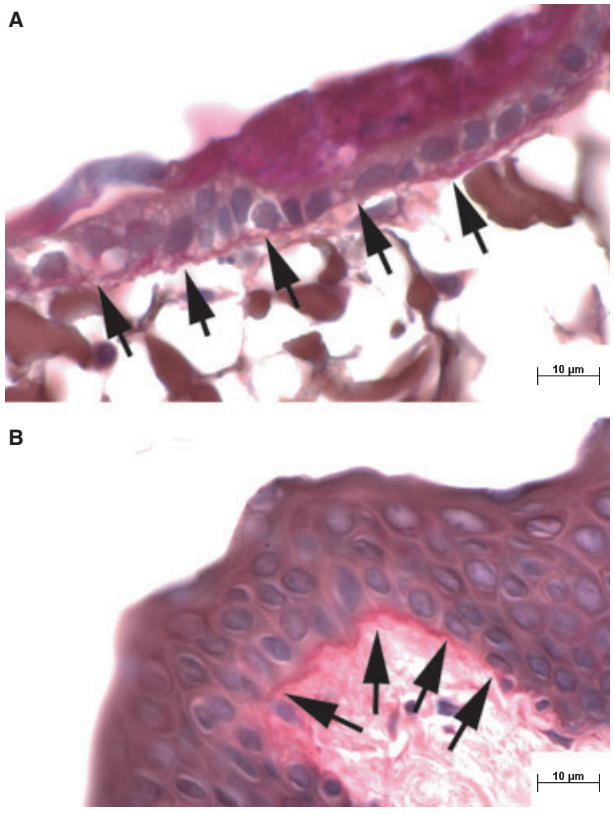


*Fig. 3.* Periodic acid-Schiff (PAS) staining of an oral mucosa model showing the basement membrane (indicated by arrows) between the dermis and the oral mucosa. (A) Matriderm® and (B) naïve gingival tissue biopsy.

that was comparable with the arrangement demonstrated by hematoxylin and eosin staining (Fig. 2A). Dermal fibroblasts were detected inside this matrix (Fig. 4B). Electron microscopy analysis revealed a basement membrane in the epidermal junction zone and hemidesmosomal structures (Fig. 4D) as well as desmosomal cell–cell connections (Fig. 4C). It was not possible to engineer a three-dimensional oral mucosa model without the presence of an underlying fibroblast layer (data not shown).

Scanning electron microscopy showed the changes that occurred during the creation of an oral mucosa model over a 30 d time period. The naïve matrix presented irregularly shaped pores of approximately 50 µm in diameter (Fig. 5A) and was not suitable as an underlying structure for freshly seeded keratinocytes alone. Incubation of Matriderm® in fibroblast growth medium led to macroscopic shrinkage (≈30%) and reduced the pore diameter (Fig. 5B). Fibroblasts were then seeded onto the scaffold, almost to confluence, and the remaining gaps closed after 14 d of incubation (Fig. 5C,D). Thereafter, the gingival keratinocytes were seeded on top of the fibroblast layer and cultured submersed for 6 d. After an additional 10 d incubation period in close proximity to the ALI, the gingival keratinocytes formed a continuous epidermal layer (Fig. 5E,F). Immunohistological analysis with an antibody against pan-cytokeratin revealed that cytokeratin was expressed in all layers (Fig. 6D).

## Discussion

To date, the uses of Matriderm®, derived from the fields of dermatology and wound surgery, have been found to lie in tissue regeneration as well as in the esthetic and predictable treatment of patients with deep and partial burn wounds (11–13). When compared with conventional skin grafts, the take-rates of Matriderm® and the color of post-therapeutic sites are probably comparable. In addition, no blisters or unstable or hypertrophic scars occurred (11). The characteristics of Matriderm® included the potential for use in the generation of an engraftable skin equivalent for the purpose of transplantation (14).

The results presented here indicate that Matriderm® was a suitable scaffold for the *in vitro* generation of an oral mucosa model. Gingival fibroblasts and keratinocytes proliferated and differentiated *ex vivo* in and onto the matrix for up to 30 d of incubation.

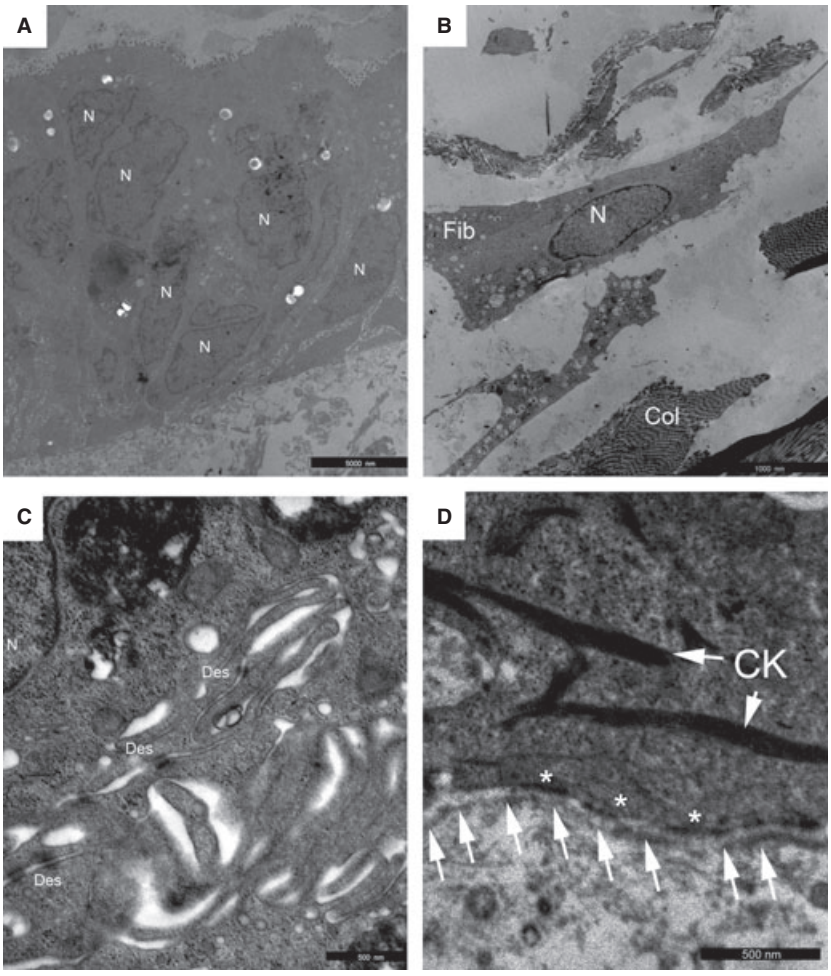


*Fig. 4*. Analysis of the oral mucosa model by transmission electron microscopy. (A) Transmission electron microscopy of a multilayered oral mucosa model: gingival keratinocytes with nuclei (N) are shown. (B) Gingival fibroblasts (Fib) were grown inside the matrix, and nuclei (N) and collagen fibers (Col) are visible. (C) Gingival keratinocytes were connected via desmosomes (Des). (D) A basement membrane (arrows), hemidesmosomal structures (*) and cytokeratin filaments (CK) were detectable between the dermal and the epidermal parts of the mucosa.

The biocompatibility with gingival cells documented in this study indicates that Matriderm®, as a supportive structure for the formation of tissue scaffolds, might become valuable for the treatment of oral cavity defects.

Based on comparable techniques, an oral mucosa model was developed (Figs 1–6). Two weeks after seeding, Matriderm® was successfully populated by fibroblasts that formed the dermal area (Fig. 5C). In addition, these cells rendered the surface of the collagen–elastin matrix receptive for the settling and growth of gingival keratinocytes (Fig. 5E). Analysis of the naïve matrix by scanning electron microscopy showed large pores, which rendered its potential as a solely underlying scaffold as inadequate (Fig. 5A). Using our method to introduce a dermal area, large-scale surface inhomogeneity of the matrix (cf. Fig. 5A and 5B) was filled by gingival fibroblasts (Fig. 5C), which made the surface suitable for growth of the epidermal layer of gingival keratinocytes (Fig. 5E). In experiments with other collagen scaffolds in which the aim was to create an oral mucosa model, the absence of fibroblasts led to poor epithelial organization (16–18). *In vitro* studies indicated that fibroblasts secrete soluble factors to initiate keratinocyte proliferation and differentiation (19,20). Dermal fibroblasts were found to promote the development of identifiable keratinocyte layers into basal, prickle, granular and cornified strati, in addition to mediating keratinocyte proliferation (20). The essential role of fibroblasts is the production of basement membrane components (among them collagen type IV and laminin 5) (14,21,22). Considering the necessity for dermal cells, the oral mucosa model presented here clearly shows a basement membrane between the dermal–epidermal junction zone, as depicted by PAS staining (Fig. 3A), transmission electron microscopy analysis (Fig. 4D) and immunohistochemistry (Fig. 6B). Fibroblasts incorporated into a dermal substrate may play a role in the formation of a basal membrane and provide a suitable environment to support the formation of epidermis (20,23). Hitherto, oral keratinocytes in monoculture produce only a thin epidermal layer, and research to date has been unable to create a multilayered oral epithelium in the absence of fibroblasts or basement membrane components (e.g. collagen IV) (7,24). The presence of a unique basement membrane in our model (Figs 3A, 4D and 6A) might corroborate the findings of these investigations (23). Correspondingly, micro-environmental *ex vivo* studies performed with skin substitutes revealed the need for dermal components (fibroblasts) for the generation of a stratified epithelium (20,25). In our study, keratinocytes were triggered, by the addition of fibroblasts, to form a multilayer epithelium (Figs 2A, 3A, 4A and 6D). Without fibroblasts we could not create multilayered epithelia. This emphasizes the complex role of the cellular interaction in the differentiation of keratinocytes and the bilateral influence of neighboring dermal/epidermal layers and/or their metabolites in *ex vivo* tissue engineering.

Cell–cell interactions via desmosomes were detected by electron microscopy (Fig. 4C), known to be cadherin-based and highly specialized anchoring junctions that link intermediate filaments (26). These junctions are particularly important for main-

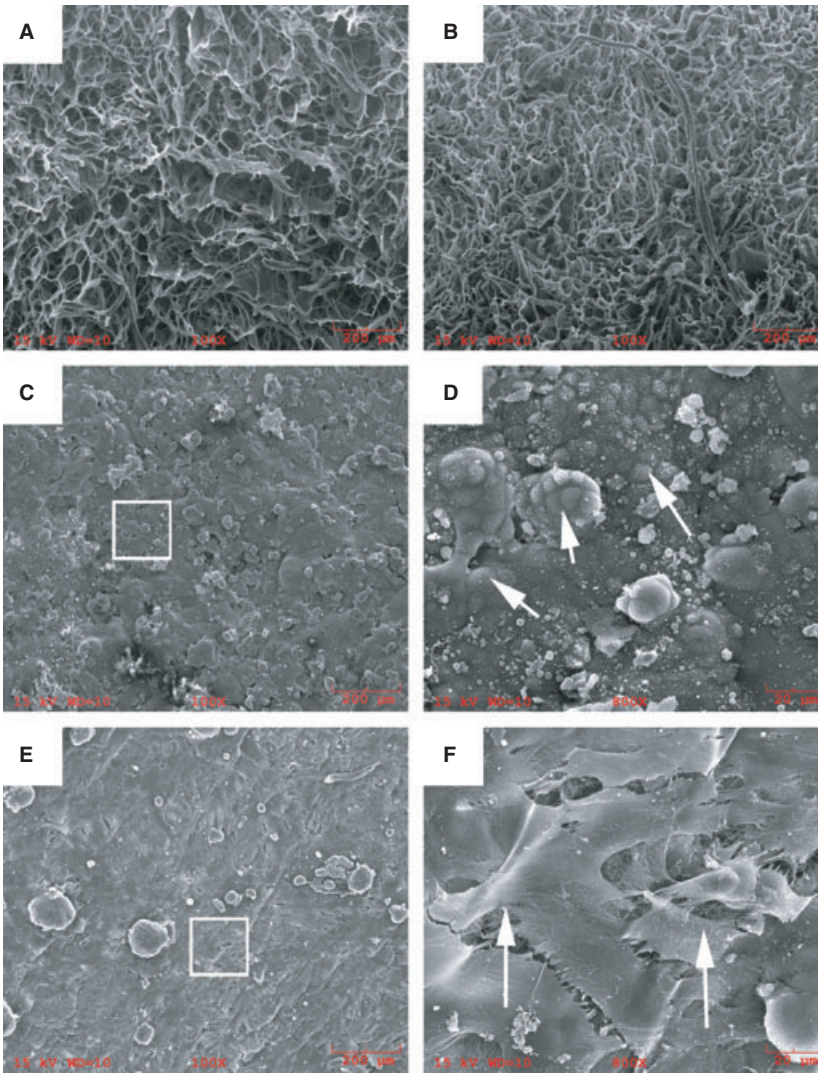


*Fig. 5.* Analysis of an oral mucosa model by scanning electron microscopy. (A) Naïve collagen–elastin matrix (Matriderm®) without media or cells. (B) Matrix without cells, after 1 d of incubation in fibroblast growth medium. (C) Gingival fibroblasts cultured for 14 d; the white square marks the area of higher magnification shown in panel D. (D) Magnification (800×) of the white square shown in panel C; fibroblasts are indicated with arrows. (E) Oral mucosa model after 30 d of culture with differentiated gingival keratinocytes; the white square marks the area of higher magnification shown in panel F. (F) Magnification (800×) of the white square shown in panel E; keratinocytes are marked with arrows.

taining the integrity of tissues that endure physical stress, a rationale for the stability of our scaffold (27).

Keratin filaments form a complex network that extends from the surface of the nucleus to the cell membrane and interact with desmosomes and hemidesmosomes, thus collaborating via cell–cell adhesion and basal cell-underlying connective tissue connections (Figs 4C and 6D). In further investigations we intend to employ our model in pharmaceutical testing or grafting (e.g. periodontal surgery, or surgical reconstruction in the oral cavity).

In a recent publication, Pena *et al.* (28) propagated a fibrin-based oral mucosa scaffold. Their reports lacked evidence of tissue formation: in particular, their histological illustrations were deficient of basement membrane and stratification. It was controversially discussed whether stratification and cornification are mandatory and feasible *ex vivo* for the creation of an oral mucosa model (28). However, an intended use for pharmaceutical testing mandates a comparable and thus fully stratified model containing dermal and epidermal structures. Therefore, elucidation of structural characteristics in models with an intended use for pharmaceutical applications becomes mandatory (10).

When a fully differentiated mucosa equivalent was used in a clinical trial, degradation occurred rapidly (29). The researchers hypothesized that the low survival rate of the particular oral mucosa scaffold used for the intra-oral grafting procedure was attributable to the lack of supporting fibroblasts (29). Considering these recent findings, our oral mucosa scaffold incorporates the advantages of the dermal (fibroblasts) and epidermal (keratinocytes) structures in combination with the collagen–elastin matrix (Matriderm®), leading to notable histological as well as electron microscopic, tissue-corresponding depictions of our scaffold. This strategy is adequate for laboratory use and holds perspective for clinical indications (e.g. for oral grafting procedures). Matriderm® with incorporated fibroblasts and overlying keratinocytes might support healing processes (30) and the epidermal structure could act as an immediate and readily employable barrier against environmental impact or oral pathogens. To our knowledge, Matriderm® has not been used in restoration processes in the oral cavity. However, artificial collagen matrices for the treatment of skin defects are established alternatives to acellular human dermis products, if limited or inadequate autologous grafting materials are available (31).

Reconstruction of soft tissue defects in the oral cavity is of importance to improve periodontal health. The majority of raw materials for the production of artificial dermis products have been animal-derived collagens. The prototype of a collagen matrix, Mucograft®, was recently used in restorations of periodontal recessions with good clinical and esthetic results and was also associated with significantly lower patient morbidity when compared with free gingival grafts (32). In the treatment of defects (such as periodontal recession) in the oral

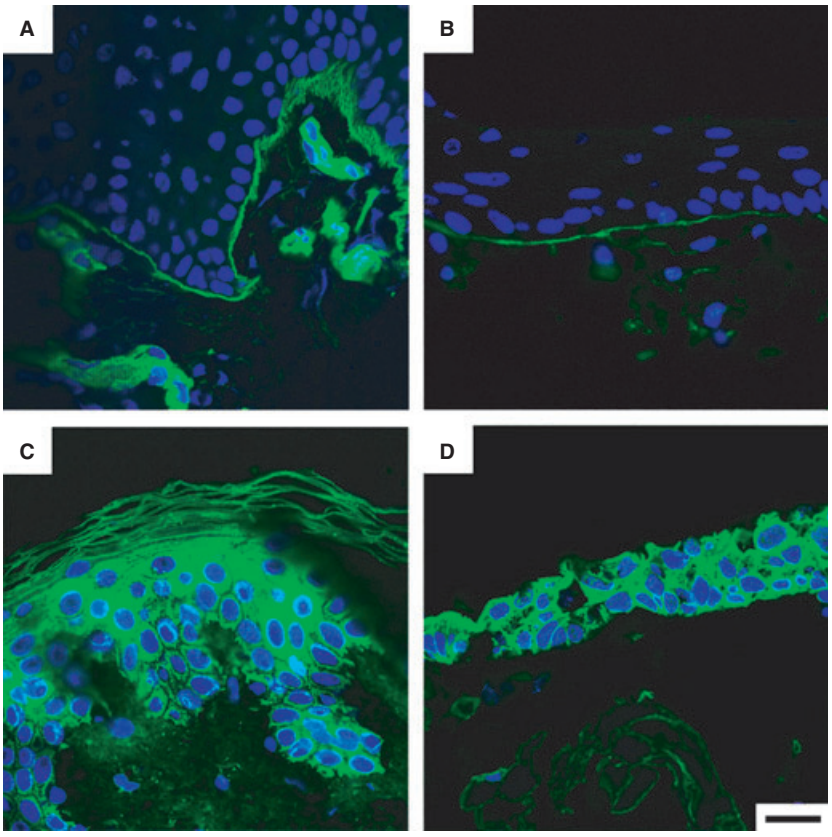


*Fig. 6.* Immunohistochemical analysis of an oral mucosa model after 10 d of culture at the air–liquid interface (ALI) compared with naïve gingival tissue. Collagen IV staining (green) of a basement component in (A) naïve oral mucosa and (B) the oral mucosa model. Pan-cytokeratin-staining (green) of (C) naïve oral mucosa and (D) the oral mucosa model. Nuclei were stained blue using TOPRO-3 iodide. Scale bar = 30 μm.

cavity, acellular dermis products have been frequently used (3).

Our results show that Matriderm® is biocompatible with gingival cells. It has passed the medical product approval of the European Union (ZLG-ZE982.94.12). In future studies we plan to evaluate the potential of this material in periodontal surgery and oral health care.

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