Journal of
PERIODONTAL RESEARCH

J Periodont Res 2010; 45: 375–380

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2009.01248.x

In vitro engineering of complete autologous oral mucosa equivalents: characterization of a novel scaffold

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Peña I, Junquera LM, Meana Á, García E, García V, De Vicente JC. In vitro *engineering of complete autologous oral mucosa equivalents: characterization of a novel scaffold. J Periodont Res 2010; 45: 375–380. © 2010 John Wiley & Sons A/S*

Background and Objective: Restoration of oral mucosa defects by means of *in vitro*-cultured equivalents has become a valid alternative in the field of oral and periodontics surgery. Although different techniques have been described, none has been able to provide an equivalent with an autologous scaffold for the epithelium. The purpose of this study was to obtain complete autologous oral mucosa equivalents (CAOME) using the patient's own fibroblasts and plasma and to characterize these equivalents both morphologically and immunohistochemically.

Material and Methods: We acquired cell types (keratinocytes and fibroblasts) from the same mucosal samples, which were taken from healthy patients who underwent oral surgery. To construct the CAOME, a small sample of blood was obtained from the patient and subsequently processed to obtain a fibrin glue scaffold. All CAOME thus obtained were stained using the standard hematoxylin and eosin method to study their morphological characteristics. To establish the type of cells in the epithelial layer, CAOME were stained with pancytokeratin AE1/AE3, cytokeratins 5/6 and 13, p-63 and Ki-67. Finally, laminin 5 and collagen IV were used to reveal the presence of a basal membrane.

Results: The CAOME featured a monolayer of cube-shaped epithelial cells similar to that found on the basal layer of the oral mucosa. Close to the epithelial layer lay the fibrin and fibroblasts-embedded scaffold. The CAOME was positive to pancytokeratin AE1/AE3, cytokeratin 5/6 and p-63. No reaction was found to cytokeratin 13 and Ki-67. There was staining to laminin 5 but not to collagen IV.

Conclusions: It is possible to engineer a CAOME with an epithelium of basal-like and immature keratinocytes, which could potentially reconstruct *in vivo* loss of tissue.

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Key words: oral; tissue engineering; autologous; fibrin

Accepted for publication July 17, 2009

During clinical practice, oral surgeons must face up to the challenge of restoring tissue losses using well-known techniques such as skin grafts, regional and local flaps or microvascular free flaps (1). However, these methods produce morbidity at the donor site and the eventual outcomes are functionally and aesthetically incomplete. Restoration of oral mucosa defects by means of *in vitro*-cultured equivalents has become a valid alternative in the field of oral and periodontics surgery. Although different techniques have been described, none has been able to produce a scaffold without xenogenic or allogenic materials. Their usage entails severe risks, and they are exceedingly expensive to obtain (2–5). Previously, we reported successful results using fibrin as scaffold in the

treatment of burns with skin equivalents (6–8).

In the present paper, we describe our tissue-engineering technique that was used to obtain a complete autologous oral mucosa equivalent (CAOME). In addition, complete histological and immunohistochemical characterization was carried out, using haematoxylin and eosin staining, pancytokeratin AE1/AE3, cytokeratins 5/6 and 13, laminin 5, collagen IV, p-63 and Ki-67 as markers.

Material and methods

Obtaining the samples

Healthy patients previously subjected to third molar surgery, and blood donors from the Blood and Tissues Community Centre of the Princedom of Asturias, were selected. All patients were presented with detailed oral and written information, and signed the pertinent informed consents. This work was approved by the Ethics Committee of the Regional Clinical Investigation of the Princedom of Asturias, Spain. Two nonkeratinized oral mucosa samples were obtained from each patient: one of 5 mm^2 (to obtain a CAOME) and one of 2 mm^2 (as a control). Both samples were taken from the nearby area surrounding the surgically intervened area without provoking any additional morbidity. Samples were then transported in sealed bottles containing 100 mL of RPMI (Gibco, Invitrogen, Paisley, UK) and 2 mL of an antibiotic–antimycotic solution (penicillin, streptomycin and amphotericin B; Gibco, Invitrogen) in standard doses for cell culture. To develop the scaffold, a 27 mL blood sample was extracted from the patient 1 wk before surgery.

Primary cultures

Samples were divided into small fragments and washed in 5 mL of phosphate-buffered saline containing 0.05% trypsin and 0.02% EDTA, at 37°C in an atmosphere of 5% CO2, and agitated for 30 min. Trypsin was inactivated by QN medium (37.5 mL) [Dulbecco's modified Eagle's minimal essential medium (DMEM)] (Gibco, Invitrogen) and Ham's F12 (12.5 mL) (Gibco, Invitrogen), in a 3:1 ratio, containing 10% fetal bovine serum (Gibco, Invitrogen; Australia), insulin (5 μg/mL; Sigma, Madrid, Spain), cholera toxin (8 ng/mL; Sigma), adenine (24 μg/mL; Sigma), triiodotironine (1.3 ng/mL; Sigma) and hydrocortisone (0.4 μg/mL; Sigma). Then, the product was centrifuged and a cellular pellet was obtained, which was resuspended in QN medium (50 mL). Each sample was washed four times (with trypsin and EDTA). All cellular pellets thus produced were used for keratinocyte primary cultures. The remaining fragments of the sample were washed with DMEM containing collagenase (Sigma), at a concentration of 2 mg/mL, as described for keratinocytes but for 2 h. After centrifugation, a cellular pellet was obtained and resuspended in DMEM (50 mL) supplemented with 10% fetal bovine serum. This pellet was used for primary culture of fibroblasts. Cells were cultured first in 24-well plates (Falcon, Esparza De Galar, Navarra, Spain), at a density of $27–30 \times 10^3$ keratinocytes/cm^2, in wells of 9 cm^2 containing QN medium and a gamma-irradiated 3T3 feeder layer, following the method described by Rheinwald & Green in 1975, and at a density of 85×10^3 fibroblasts/cm^2, in wells of 2 cm^2 containing DMEM and 10% fetal bovine serum. After 3 d of culture, epidermal growth factor (10 ng/mL; Austral Biologicals, San Ramon, CA, USA) was added to keratinocytes. When cells reached 90% confluence, a first-pass (P1) was made into T-25 flasks for keratinocytes and into T-12.5 flasks for fibroblasts. Time until confluence for P1 keratinocytes was 7–8 d. During that period a second pass (P2) was needed for fibroblasts into T-75 flasks. The control of the confluence of the cultures was made using an inverted optical microscope.

CAOME construction

We employed fibrin glue obtained from the patient's blood as scaffolds. To construct a CAOME of 25 cm^2 we used the following: 4 mL of plasma, 22×10^3 fibroblasts, 0.7 mL of 1% calcium chloride in saline serum, 70 μL of tranexamic acid (FIDES-Ecofarma, Almacera, Spain) and saline serum. The mixture was then allowed to solidify at 37°C for 30–60 min with 4–7 mL of QN medium. After that period, keratinocytes were seeded. Fibroblasts were from P2 and keratinocytes were from P1 (one-third of the total amount of cells in T-25 flasks). The rest of the cells were frozen as previously described (6). From a primary culture we obtained three CAOME of oral mucous, 25 cm^2 each. To avoid differentiation of keratinocytes we employed the submerged method. Handling of CAOME was made easier by following the method previously described by Meana *et al.* (9).

Histological and immunohistochemical study of CAOME

The morphological study was carried out using the haematoxylin and eosin staining method (Merck, Barcelona, Spain). Immunohistochemical staining was carried out using a Cytomation Autostainer (Dako, Carpinteria, CA, USA). First, an antigen-recovery buffer (Dako), was manually applied. The Autostainer was programmed as follows: first wash in buffer (Dako), Dual Endogenous Enzyme Block for 5 min (Dako), another buffer wash and, finally, application of the corresponding primary antibody for 15 min, except for p-63 and Ki-67 (10 min). Second, a buffer wash was performed and universal secondary anti-mouse antibody (dilution 1:10) was applied. The next step included incubation in EDL Labeled Polymer for 30 min (Dako Envision + Dual Link), buffer wash, incubation in 3-3′-diaminobenzidine for 10 min (Dako DAB+), buffer wash and, lastly, incubation in haematoxylin for 7 min (Dako Automation Hematoxilin). After buffer wash, a process of dehydration was made with 96% ethanol (3 min), absolute alcohol (two 5-min passes) and xylene (another two 5-min passes). Primary antibodies, dilutions, function and origin are detailed in Table 1.

Table 1. Immunohistochemical markers

Primary antibodies	Dilution	Function	Company
Pancytokeratin AE1/AE3	1:200	Human epithelial marker	Dako
Cytokeratin 5/6	1:200	Basal keratinocyte (5) and early differentiation (6) markers	Dako
Cytokeratin 13	1:200	Suprabasal keratinocyte marker	Dako
p-63	1:200	Stem cell marker	Dako
Ki-67	1:800	Proliferative activity marker	Dako
Laminin 5	1:2000	Basal membrane marker	Dako
Collagen IV	Prediluted	Basal membrane marker	Dako

Results

Primary cultures

Three to four days after culture, it was possible to identify the presence of stable keratinocyte colonies in all cultures from all samples. On the seventh day, 3T3 cells were driven towards the periphery of the cultures. Cultures reached confluence in 12–14 d. At that point, 3T3 cells had all but disappeared (Fig. 1). None of the cultures became contaminated with fungi or bacteria.

CAOME construction

Secondary cultures reached 90% confluence in 7–8 d. The total time taken to obtain a CAOME was between 26 and 30 d. Quantitative evolution of both primary and secondary cultures is shown in Table 2. CAOME were similar to a split-thickness skin graft in volume (Fig. 2) and were easy to handle thanks to Histoacryl® (B/Braun, Barcelona, Spain) and vaseline gauze. Hence, none of the CAOME suffered a division between the epithelial layer and the scaffold.

Histological evaluation

CAOME had a monolayer of cube-shaped epithelial cells that was interspersed with some elongated cells. In some areas of the epithelial sheet, there were two layers of cells. Cube-shaped cells had a nuclei/cytoplasm ratio greater than that of elongated cells. Close to the epithelial layer was the fibrin and fibroblasts embedded scaffold. There was no evidence of rête ridges or other cell types typically found in mature oral mucous (Fig. 3).

Immunohistochemical results

To identify human epithelium in our CAOME, AE1/AE2 human pancytokeratine was used. A positive result led us to determine the type of keratinocytes. We applied both cytokeratins 5/6 and 13. Epithelium was positive to cytokeratin 5/6 and no staining was observed to cytokeratin 13 (Fig. 4). To demonstrate the presence of basal membrane, we used laminin 5 and collagen IV. Staining only occurred to laminin 5, and this was discontinuous, in any case. Ki-67 was negative for all CAOME but p-63 was positive in some areas of the epithelium (Fig. 5).

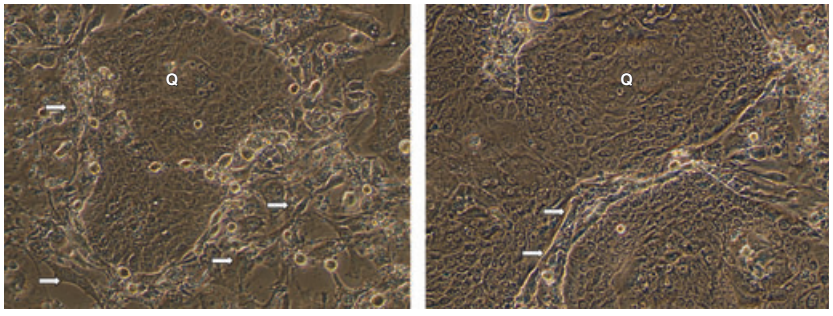

Fig. 1. As keratinocytes reach confluence (Q), 3T3 feeder cells (white arrows) disappear.

Discussion

Since Rheinwald & Green established, in 1975, the criteria to develop *in vitro* keratinocyte sheets (10), several advances have been made in tissue engineering. As seen in skin regeneration, an equivalent must regenerate tissues *in vivo* and, to do so, it must bring in stem cells (11). The majority of clinical failures, according to some authors, occur because such stem cells are not present (12). When planning the regeneration of the oral mucosa, most authors create stratified mature equivalents (2–5,13,14). Such an approach may be useful when planning *in vitro* studies, but for *in vivo* clinical trials, the same problems have been described as those occurring in skin procedures, especially when dealing with the loss of superficial layers (15,16). Animal trials with mature equivalents have revealed degeneration of the epithelium construct, but this did not occur with monolayer equivalents cultured using the submerged method (17). Authors tend to explain such findings as resulting from the fact that immature monolayered submerged equivalents have basal progenitor cells and stem cells that still divide, grow and stratify *in vivo*. Thus, in order to regenerate oral mucosa properly, it is fundamental progenitor cells that are implanted, and this can be accomplished by the submerged culture method used in our study. Other advantages of this method are a shorter culture period (18) and clonogenic capacity preservation (12). The time needed to construct our equivalent was shorter than that publicized by those authors in favour of stratification (14,19). Even in the submerged scenario, stratification and maturation of stem cells is possible, so we developed immature monolayer equivalents because stratification and maturation after transplantation should be possible notwithstanding how thin the layers are (18).

Together with the monolayer and the submerged concepts, we used cells from P1. It has been shown that cells start to differentiate in P3 and usually

Table 2. Quantitative evolution of primary and secondary cultures

Keratinocyte number (and origin)	Primary culture period (d)	No. of keratinocytes after primary culture	Equivalents (cells/cm^2; expansion ratio 1:3)	Total culture period (d)
270,000 (buccal)	12	3,500,000	129,630	27
250,000 (buccal)	14	2,100,000	77,778	30
260,000 (buccal)	13	3,800,000	140,741	30
275,000 (buccal)	12	4,100,000	151,852	26

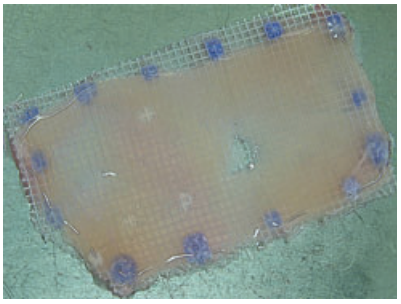

Fig. 2. Macroscopic view of a complete autologous oral mucosa equivalent (CAOME) after keratinocytes reached confluence. Scale: 6.5 × 3.5 cm.

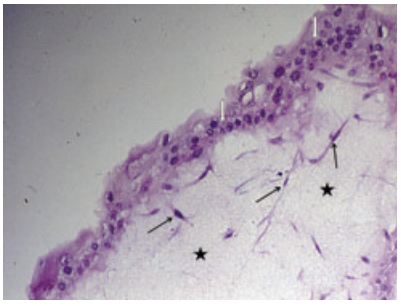

Fig. 3. Complete autologous oral mucosa equivalents (CAOME) have an epithelial monolayer (white arrows) under an autologous fibrin glue scaffold (black stars) in which fibroblasts are embedded (black arrows) close to keratinocytes. Haematoxylin and eosin (100× magnification).

degenerate in P5 (20). The more passes, the fewer holoclonic cell lines (21). Most authors use P2 and P3 cells, in contrast to the P1 cells used in our study.

To construct our scaffold, we used autologous fibroblasts taken from the same sample as the keratinocytes. The presence of a subepithelial substratum has been proven to promote *in vivo* epithelial maturation and organization (5). In addition, fibroblasts are essential to inhibit the apoptosis of epithelial basal cells (22) and to promote the formation of a full basal membrane. In our opinion, it is mandatory to use autologous fibroblasts to improve epithelial growth and to avoid extracting a second sample from the patient (6). Until now, all clinical trials with oral mucosa equivalents have shown results without the use of fibroblasts. Lauer *et al*. (13,14) used collagen scaffolds without fibroblasts, which resulted in a poor epithelial organization, which some authors related (23) to the absence of fibroblasts. Other authors used acellular dermis from tissue banks; however, epithelial loss also occurred because of the absence of fibroblasts (16). Fibroblasts are also fundamental in preserving the coherence of the three-dimensional structure of our fibrin scaffold because they produce collagen fibers that replace the fibrin matrix similarly to collagen glues (5,24,25).

A unique feature of our protocol was the use of the first autologous scaffold. We employed fibrin from the patient's plasma, and only a small blood sample was needed. The method is fast, cheap and simple, compared with synthetic fibrin (26). Fibrin is the ideal matrix because it can stimulate fibroblast proliferation (27) while preserving stratification and differentiation of keratinocytes *in vivo* (12). The CAOME may be removed manually, but doing so demands the use of vaseline gauze and Histoacryl® (9). Some of the most commonly used scaffolds are collagen glues (5,13,24,25), but these matrixes suffer significant retraction and they are not autologous. Other alternatives include amniotic membranes (28) and acellular dermis (15,18,19). The problem is still their nonautologous origin and a complex pretreatment is required to avoid any eventual immunorejection of the equivalent. In our case, autologous fibrin glue led to no potential rejection by the host.

CAOME are 25 cm^2 in size. From a single 5-mm^2 punch, we obtained three CAOME in < 30 d. If fewer CAOME are needed, the period could be shorter. The total surface was 75 cm^2, so we were able to restore almost all the oral mucosa tissue. During secondary culture, none of the scaffolds suffered surface retraction, as seen with collagen glues (25). Despite the small thickness of the engineered CAOME, handling was easy and similar to the manipulation of a split-thickness skin graft. It was possible to cut it without detaching the epithelium from the scaffold. This flexibility is an advantage over other equivalents (15,19).

The epithelial cells morphology of CAOME is similar to that observed in progenitor keratinocytes from the basal layer (2,3). Also, few elongated cells with a low nuclei/cytoplasm ratio were observed. In concordance with other authors (29), we considered these cells to be more mature cell types, similar to those found at the suprabasal layers. None of the CAOME obtained had rête ridges, something common in oral cultures (2,4,13,14, 24,25). After histological evaluation, we applied pancytokeratin AE1/AE3 and were able to demonstrate the presence of human epithelium in CAOME (30). In oral mucosa, basal cells are positive to cytokeratin 5 (3,5,14), being cytokeratin 6 an early differentiation marker (5). Cells at the suprabasal layers are positive to cytokeratin 13, a marker of maturity (31). Epithelium in our CAOME stained positive to cytokeratin 5/6 but not to cytokeratin 13. These findings suggest the presence of oral basal cells in our epithelium. Together with these cytokeratins, we used p-63, previously suggested as a marker of oral stem cells (32,33). We identified some positive cells among the other epithelial cells showing the regeneration potential of our equivalents. To corroborate the macroscopic findings of a strong linkage between the epithelium and the

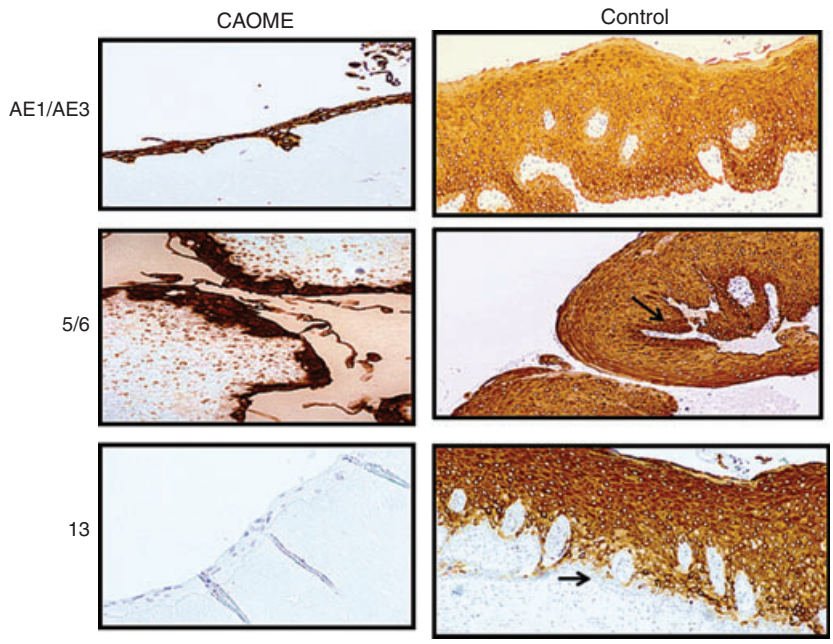

Fig. 4. Comparative cytokeratin pattern results between oral mucosa (control) and complete autologous oral mucosa equivalents (CAOME). Equivalents have human epithelial cells (pancytokeratin AE1/AE3+). Keratinocytes of CAOME stained positive for cytokeratin 5/6, but there was no staining to cytokeratin 13, as seen in the suprabasal layers of the oral mucosa. Thus, the epithelium of the equivalents is composed of oral mucosa-like basal cells (the black arrows point to the basal layer).

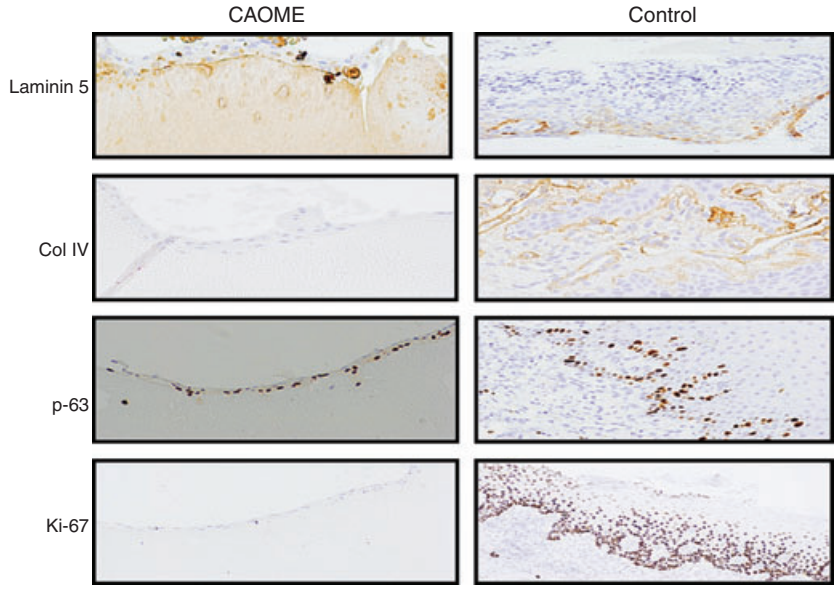

Fig. 5. The basal membrane was undergoing the creation process (laminin 5+; collagen IV−), following the short period of interaction between keratinocytes and fibroblasts. Even if there were some p-63+ cells among the other epithelial cells, there was no evidence of Ki-67+ cells.

fibrin–fibroblasts scaffold, we looked for the formation of a basal membrane using laminin 5 and collagen IV markers. The positive staining to laminin 5 confirmed the existence of an interaction between keratinocytes and fibroblasts that provided sufficient anchoring between the epithelium and the scaffold (19). However, this staining was not continuous, probably because of their brief interaction during the short secondary culture period of 7–8 d. Collagen IV was negative in all CAOME for the same reason as for laminin 5. Usually, collagen IV is absent at the earliest stage (29). Finally, we did not find Ki-67-positive cells among the epithelial cells of CAOME. These data are in agreement with the findings of other authors (18). The reasons for this could be, on the one hand, that the confluence of epithelium could induce a reduced proliferation activity. On the other hand, Bruno & Darzynkiewicz (34) have demonstrated that at the end of the G1 cell cycle phase and at the beginning of the S phase, the amount of Ki-67 antigen is minimal. So, a cell at G1 or S phases could be negative to Ki-67 but be in proliferation.

In conclusion, the present study described an *in vitro* culture technique that allows production of a completely autologous equivalent with progenitor oral mucosa basal cells in the epithelium and a fibrin–fibroblasts scaffold. With just a small sample of biopsy tissue and in a relatively short period of time, it is possible to obtain three CAOME that would permit us to reconstruct almost all the oral mucosa. This technique opens new perspectives in the field of oral mucosa tissue regeneration and can be incorporated into the therapeutic options available to oral surgeons.

Acknowledgement

The present work was funded by CARLOS III HEALTH INSTITUTE (Ministry of Science and Innovation, Spanish Government).

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