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In vivo behavior of complete human oral mucosa equivalents: characterization in athymic mice

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Background and Objective: The interest in tissue engineering as a way to achieve repair of damaged body tissues has led to the carrying out of many studies whose results point to the potential effectiveness of these methods. In a previous study, we reported the obtaining of complete autologous oral mucosa equivalents (CAOMEs), characterized by oral immature keratinocytes and stem cells on an autologous plasma and fibroblast scaffold. The purpose of this study is to show their behavior *in vivo*, by using them as free grafts in experimental animals, and to demonstrate their potential capacity to regenerate oral mucosa.

Material and Methods: We engineered CAOMEs, as previously described. All CAOMEs thus obtained were used as free grafts in *nu/nu* mice. To assess their evolution *in vivo*, we studied their histological and immunohistochemical features by using AE1/AE3 pancytokeratin, the 5/6 cytokeratin pair, cytokeratin 13, laminin 5, collagen IV, vimentin, p-63 and Ki-67, at 7, 14 and 21 d.

Results: The structure became progressively closer to that of oral mucosa samples. Cytokeratin 5/6 staining became increasingly intense in the basal and suprabasal layers, and cytokeratin 13 was exclusively positive in the superficial layers. The basal membrane was completed in 21 d. Vimentin showed a correct formation of the chorion. The increasingly positive staining of p-63 and Ki-67 indicated that the regeneration process was taking place.

Conclusion: The present study shows the potential regenerative capacity of the CAOMEs by their ability to reach maturity similar to that seen in oral mucosa.

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The growing interest in the field of tissue engineering as a way to achieve the *ad integrum* repair of damaged body tissues has led to the carrying out of many studies whose results point to the potential effectiveness of these methods in modern medicine. Within this field, the development of oral mucosa equivalents has experienced considerable progress in recent years (1–6).

Previously, we reported the obtaining of complete autologous oral mucosa equivalents (CAOMEs), characterized by oral immature keratinocytes and stem cells on an autologous plasma and fibroblast scaffold, and their theoretical potential for *in vivo* reconstitution of damaged oral tissue (7).

The aim of the present study was to show their behavior *in vivo*, by using them as free grafts in experimental animals, and to characterize their evolution by histological and immunohistochemical markers using the AE1/AE3 pancytokeratin, the cytokeratin 5/

6 pair, cytokeratin 13, laminin 5, collagen IV, vimentin, p-63 and Ki-67 in different time periods. Our hypothesis is that CAOMEs could reach a structure close to that seen in oral mucosa and could potentially become useful in clinical practice.

Material and methods

Engineering of CAOMEs

Two biopsies were taken from healthy oral mucosa (a 5 mm × 5 mm biopsy to obtain the equivalents and a 2 mm × 2 mm biopsy as a control) of patients amenable to oral surgery and blood donors. A 27 mL blood sample was also taken. All patients received detailed oral and written information, and informed consent was obtained. The study was approved by the Clinical Research Ethics Committee of the Princedom of Asturias. The samples were divided into small fragments and subjected to four washes with 5 mL of phosphate-buffered saline containing 0.05% trypsin and 0.02% EDTA (Gibco, Invitrogen, Barcelona, Spain). The resulting cell pellet was used to obtain the primary keratinocyte culture. The remaining tissue fragments were subjected to a fifth wash with collagenase (Sigma, Madrid, Spain) and Dulbecco's modified Eagle's medium at a concentration of 2 mg/mL, in order to obtain the cell pellet to use for the primary fibroblast culture.

The keratinocyte growth medium was QN (Dulbecco's modified Eagle's medium [Gibco, Invitrogen] and Ham'S F12 [Gibco, Invitrogen] in a 3:1 ratio, with 10% fetal bovine serum [Gibco, Invitrogen, MERCK, Madrid], insulin [5 g/mL; Sigma], cholera toxin [8 ng/mL; Sigma], adenine [24 mg/mL; Sigma)], triiodothyronine [1.3 ng/mL; Sigma] and hydrocortisone [0.4 μg/mL; Sigma]) and required the use of lethally irradiated 3T3 cells as a feeder layer. For the cultivation of fibroblasts, Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum was used. After 3 d of culture, epidermal growth factor (10 ng/mL; Austral Biologicals, San Ramon, CA, USA) was added to the keratinocyte culture. When cells reached 90% confluence, a first pass (P1) was made into T-25 flasks for keratinocytes and T-12.5 flasks for fibroblasts. It took P1 keratinocytes 7–8 d to reach confluence. During that period, a second pass (P2) was needed for fibroblasts into T-75 flasks. We employed fibrin glue obtained from the patient's blood sample as scaffolds. To construct a 25 cm^2 CAOME we used the following materials: 4 mL of plasma, 22×10^3 fibroblasts, 0.7 mL of calcium chloride at 1% 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 from P1 (one third of the total amount of T-flask cells). The remaining cells were frozen as previously described (8). From a primary culture, we obtained three CAOMEs of oral mucosa, each 25 cm^2. In order to avoid keratinocyte differentiation, we employed the submerged method. The handling of CAOMEs became easier following the method previously described by Meana *et al*. (9).

Grafting of CAOMEs into experimental animals

For the handling of animals, we followed the animal care guidelines established by the Faculty of Medicine, University of Oviedo, which follow the ICLAS procedures and Directive 86/609/EEC. Eight- and 10-wk-old male NIH Swiss *nu*/*nu* mice weighing 20 g each were used as experimental animals.

The total number of 25 cm^2 CAOMEs used in the study was 12. Each of these CAOMEs was further subdivided into four pieces. From all the available CAOME fragments, we randomly selected 12, which were then used as grafts in an equal number of mice. Each of these mice was randomly assigned to a study group, so there were three study groups with four mice each. The first group was made up of mice which kept the grafts for 7 d; the second group kept them for 14 d; and the third one, for 21 d. The grafting procedure was performed following the methodology described by Barrandon *et al*. (10), with some modifications. Mice were anesthetized by intraperitoneal injection of a mixture of sodium pentobarbital (dose, 0.038 mg/g) and xylazine (dose, 0.075 mg/g). With the anesthetized mouse under a flow hood, we raised a skin flap on the dorsum.

A square, cephalic-pedicled flap was designed in order to ensure proper irrigation and avoid necrosis. We randomly selected a CAOME fragment from the 12 available and placed it subcutaneously on the back of the mouse, positioning its base on the muscle and the epithelium facing upwards. No sutures or Silastic® (Dow Corning GmbH, Wiesbaden, Germany) sheets were applied in order to avoid the introduction of any materials other than the CAOME. Instead, we conducted a thorough apposition of the flap over the graft to prevent any movement and then sutured with vicryl® 4/0 (Ethicon, Madrid, Belgium). The process took 10–15 min per mouse (Fig. 1). The aftercare was limited to feeding and cleaning the cage. In order to recover the graft, once the time frame of each group concluded, the animal was killed by CO_2 inhalation. Then, the flap with the graft was removed.

Histological and immunohistochemical study of CAOME as a graft

The morphological study was carried out via hematoxylin and eosin staining (MERCK, Madrid, Spain). The immunohistochemical staining was carried out using a Cytomation Autostainer (Dako, Carpinteria, CA, USA). Previously, an antigen recovery buffer (Dako) was manually applied. The Autostainer was programmed as follows. The first stage comprised a first wash in buffer (Dako), Dual Endogenous Enzyme Block for 5 min (Dako), then another buffer wash and, finally, application of the corresponding primary antibody for 15 min, except for p-63 and Ki-67 (10 min). The second stage comprised a buffer

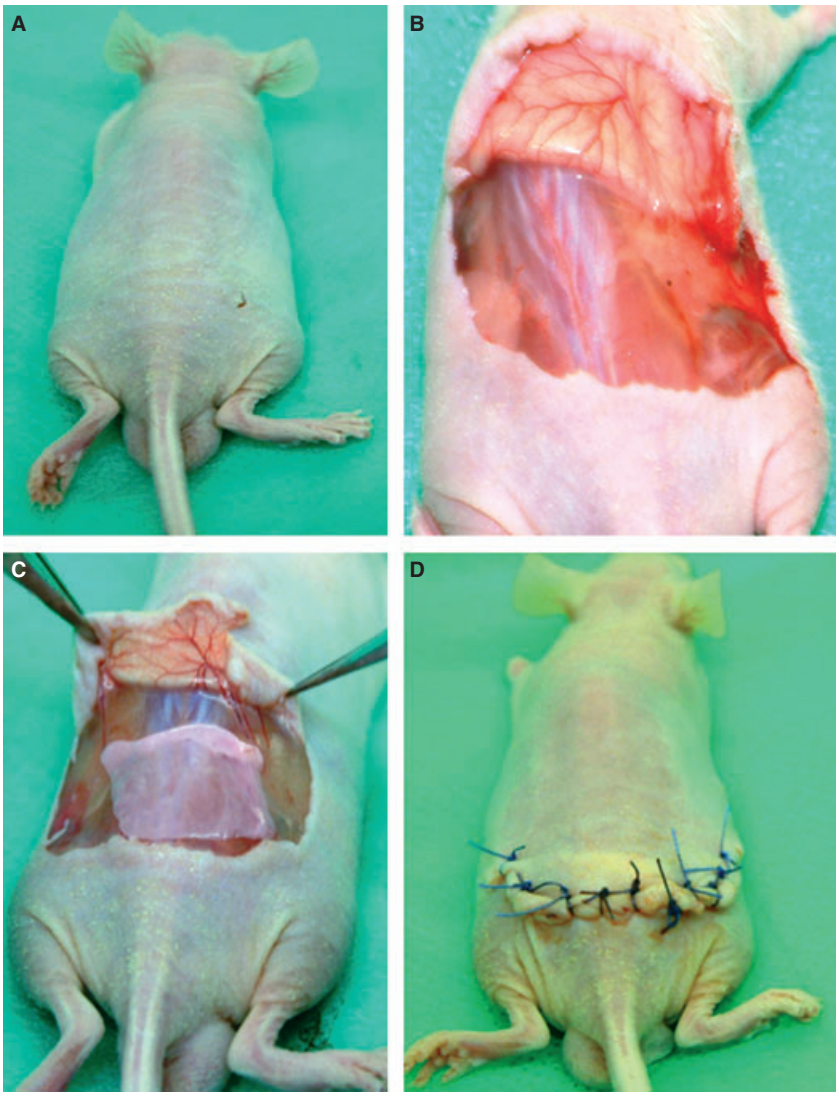

Fig. 1. Procedure for grafting a CAOME fragment subcutaneously on the dorsum of a mouse following Barrandon's technique. (A)Anesthetized mouse, positioned prone. (B) A Barrandon's flap was elevated. (C) A CAOME fragment was grafted. Notice the aspect of the CAOME close to a split-thickness mucosa graft. (D) After the grafting procedure, the wound was closed with absorbable suture material.

wash and an application of universal secondary anti-mouse antibody (1:10 dilution). The next stage included EDL Labeled Polymer for 30 min (Dako Envision + Dual Link), a buffer wash, then 3-3′-diaminobenzidine for 10 min (Dako DAB+), a buffer wash and, finally, hematoxylin for 7 min (Dako Automation Hematoxylin). After another buffer wash, a dehydration process with ethanol 96% was carried out (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.

Results

Histological study

In the first week of development, we observed the presence of three or four layers of cells forming the epithelium, which was in intimate contact with the chorion and the fibroblasts. The basal epithelial cells exhibited a cuboidal appearance, while the cells of the upper layers were fusiform in shape, their most superficial layer having a more elongated shape. Structurally, no organized stratification was observed, contrary to what happened in control oral mucosa. There was an increase of fibroblasts surrounded by fibrillar networks. We were able to dertermine the onset of vascular infiltration into the chorion for all the CAOMEs.

After 2 wk, the number of epithelial layers increased and their organization improved, so that both a basal and a suprabasal stratum could be distinguished. We observed an increase of the intercellular network surrounding fibroblasts, which were becoming increasingly numerous and better organized in relation to the epithelium, and positioning parallel to it. The presence of larger vessels was objectively more abundant and uniform throughout the chorion than that found in 1 wk grafts.

After 3 wk, qualitative and quantitative changes were evident. The number of layers varied between 10 and 13, with a clear epithelial division into basal, spinous and superficial layers. Like the control oral mucosa, all grafts showed a basal layer consisting of large core cuboidal cells. Above it, the existence of a spinous cell layer that gradually lengthened until it flattened was apparent. The chorion itself featured an organization similar to that observed in the control oral mucosa, in which fibroblasts were surrounded by connective tissue and numerous vessels.

There was no presence of epithelial ingrowth into the underlying chorion in any of the developmental stages (Figs 2 and 3).

Immunohistochemical study

Pancytokeratin AE1/AE3 positivity was observed in every layer of the epithelium, in all the preparations investigated throughout the grafting period.

At 1 wk of CAOME grafting, we observed the existence of a marked immunopositive staining against the cytokeratin 5/6 pair in cells of the basal and upper layers. During the second week, the immunolabeling in the basal

Table 1. Immunohistochemical markers

Primary antibody	Dilution	Function	Company
Pancytokeratin AE1/AE3	1:200	Human epithelial marker	Dako
Cytokeratin 5/6 pair	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
Vimentin	1:200	Fibroblast marker	Biogenex

Biogenex (Barcelona, Spain)

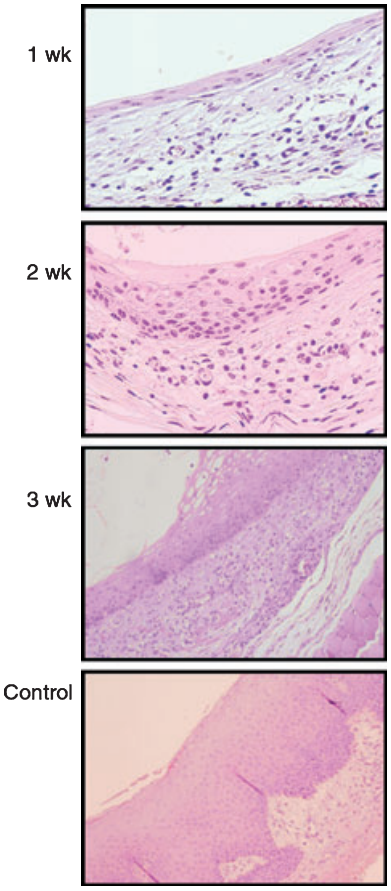

Fig. 2. Histological evolution of CAOMEs over 21 d compared with a control sample (hematoxylin and eosin staining, 20×).

and suprabasal layers continued, with weakly stained cells in the outermost layers. Throughout the third week, both the basal and middle layers preserved their immunopositivity, which was more pronounced in the former layer, whereas the most superficial layers showed very weak or no staining.

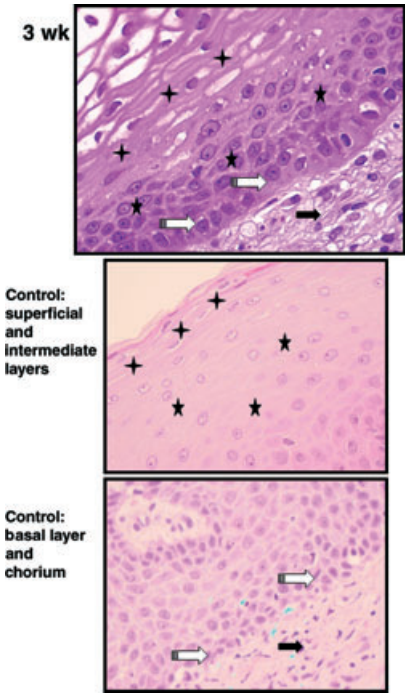

Fig. 3. Epithelial and chorion morphology of the CAOME at the third week compared with a control sample. Layers: superficial (black four-pointed stars), intermediate (black five-pointed stars) and basal (white arrows); and chorion (black arrows) (40×).

When cytokeratin 13 was used, we observed immunostaining only in the uppermost layer at 1 wk. In the grafts obtained 2 wk after implantation, immunolabeling was present in the superficial layer and the upper part of the middle layer. Immunohistochemical characterization against this antibody in the samples obtained at 3 wk after grafting was similar to that observed in the control oral mucosa (Fig. 4).

During the study of the formation of the basement membrane through the use of laminin 5 and collagen IV, we observed the progressive continuity of its structure in intimate contact with the epithelial basal areas over the weeks.

Immunostaining against vimentin was positive every week and in all samples analysed. We observed a progressive increase in the thickness of the chorion underlying the epithelium and a better organization of the fibroblasts, which, in the third week, had formed a lamina propia very similar to that present in the control oral mucosa. In samples from the first 2 wk, we were also able to properly identify immunolabeling in all basal keratinocytes. However, those grafted for 3 wk only showed immunopositivity for certain basal keratinocytes (Fig. 5).

Ki-67 was negative in all samples obtained a week after grafting. In those obtained after 2 wk, we identified sporadic immunostaining in some basal layer cells, which clearly increased in number in the samples obtained after 3 wk of grafting. However, the number of cells positive for this marker observed in our study was low compared with findings in the control oral mucosa.

CAOMEs at 1 wk featured sporadic and widely dispersed areas of immunolabeling for p-63. In the case of 2 wk grafts, cell immunolabeling in the basal layer was influenced by cell immunostaining of the layers directly above it. The 3 wk grafts showed some striking results. We found that almost all basal cells were positive for p-63, as well as having a significant increase in the immunolabeling of cells located directly above them; even some cells from surface layers were stained. These findings were similar to those seen in the native oral mucosa (Fig. 6). Table 2 summarizes the immunohistochemical results observed in our study.

Discussion

The presence of positive markers for stem cells in the equivalents is not sufficient to support the presence of such cells. Confirming their presence would require the CAOMEs to be able to regenerate mucosa *in vivo* (11). We

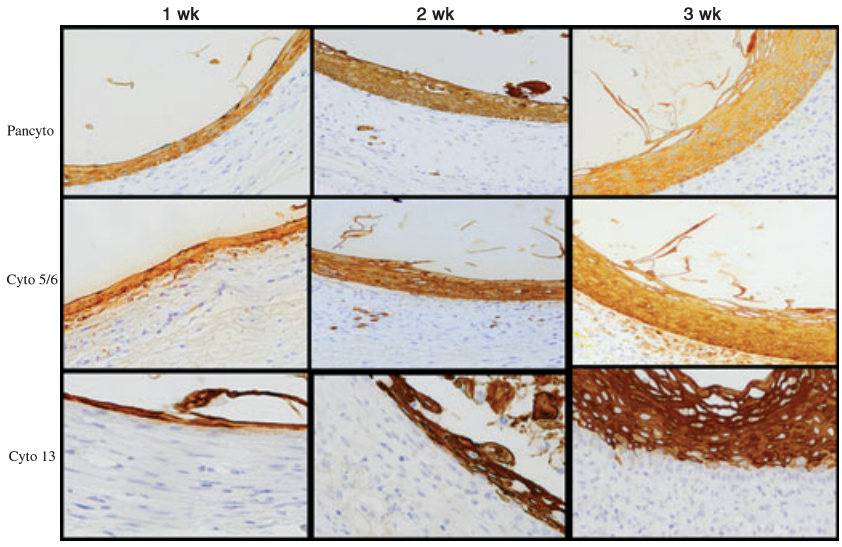

Fig. 4. The cytokeratin 5/6 pair pattern was present in the first and second week in both basal and suprabasal layers; in the third week, cells from the suprabasal layers were more weakly stained. Only the basal layer showed intense positive staining. In contrast, the cytokeratin 13 pattern was more stable, showing negative staining in the basal layer in all weeks.

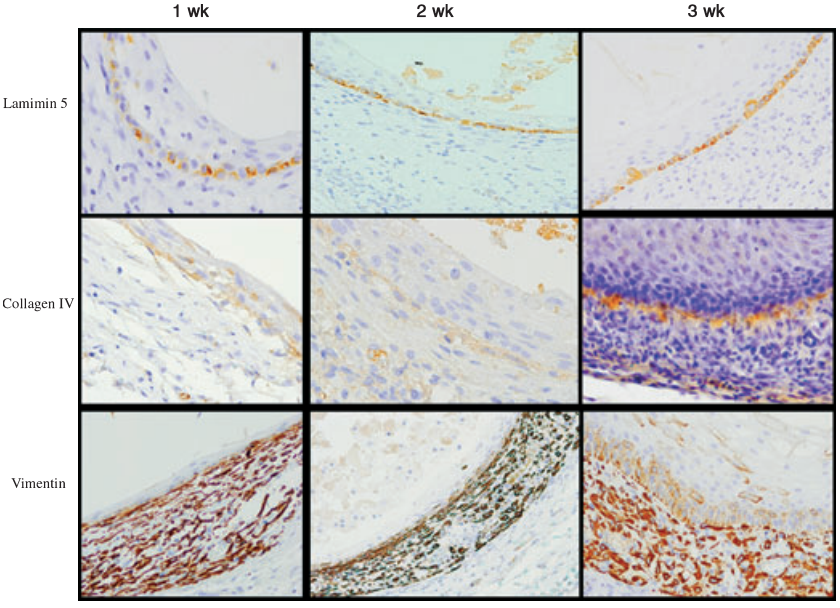

Fig. 5. Basal membrane formation was not clearly continuous until the third week. In the second week, laminin 5 immunostaining was virtually continuous, and in the third week, it was completely continuous. Until the third week, we did not witness continuity throughout the epithelium for collagen IV. Together with the progressive organization of the epithelial layer and basal membrane, the chorion became more structured, with fibroblasts aligning parallel to the epithelial layer.

worked with immature CAOMEs. In our view, maturation *in vitro* prolongs culture time at early stages. In this regard, in the first week of development, our findings are consistent with those reported by other authors (12–14), whose CAOMEs were ripened using an air–liquid interface for 7 d. Fourteen days after grafting, we observed a progressive increase in the number of layers; the most notable feature was the remarkable organization the epithelium achieved, with a basal layer and some suprabasal layers not yet arranged. The epithelium began to mature in the second week as long as the basal layer proliferation was preserved. In the third week after grafting, our equivalents featured an epithelium organized into three distinct layers, each showing a proper cell morphology similar to what would be expected from a nonkeratinized oral mucosa (15). Our work highlights the possibility of achieving *in vivo* the same results other authors only obtain *in vitro* (16,17). Furthermore, by employing submerged CAOMEs instead of air–liquid interface differentiated CAOMEs (12,13), we did not observe any hydropic degeneration or epithelial loss at 21 d after grafting, which are issues alluded to in the cited studies. We believe this is due to the lack of progenitor cells as a consequence of CAOME differentiation before grafting.

At 1 wk after grafting, fibroblasts started to organize and vessels to appear, a fact that other authors have not detected until the second week (18). It was during the second week that the fibroblasts of our CAOMEs positioned themselves parallel to the epithelium, probably related to the collagen fibers (19). In addition, we found a significant increase in the vascularization of the graft, which is crucial to ensure its viability *in vivo*.

Finally, we would like to emphasize how, in the third week of development, the underlying chorion showed little morphological differences with respect to the control oral mucosal chorion, except for the fact that the typical epithelial ingrowth of the oral mucosa was absent. We understand that this may be due to the short period of grafting in the mouse. In this regard, Hotta *et al*. (6) reported that the formation of epithelial ingrowth requires at least 4 wk of grafting. As our study was restricted to 21 d grafts, we cannot be sure whether they would form after that period.

By using the AE1/AE3 pancytokeratin, we established the presence of human epithelium in the grafted CAOMEs. After the 1 wk of grafting, we observed immunopositivity against the cytokeratin 5/6 pair in all samples.

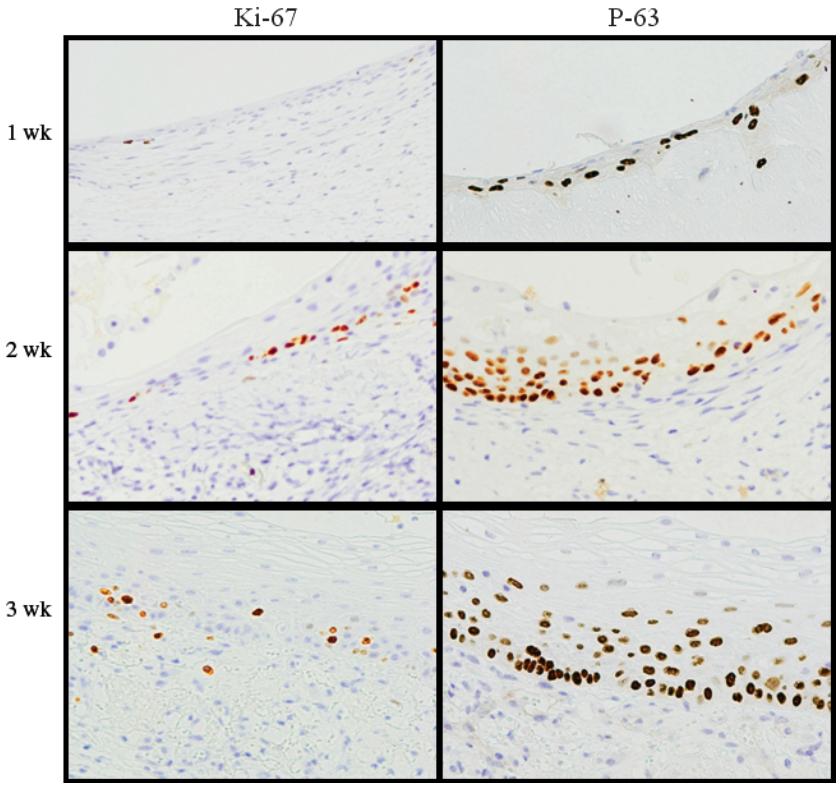

Fig. 6. The Ki-67 pattern was not as expected in a process of epithelial regeneration, in that only some cells were stained with this marker. In contrast, p-63 showed an increase in the number of cells positive to it, which corresponds to a growing epithelium. Near all basal cells and some above them were stained positive for p-63.

Table 2. Immunohistochemical results in *nu*/*nu* mice

Marker	Week 1	Week 2	Week 3
Pancytokeratin	+	+	+
Cytokeratin 5/6 pair	+	+	$+_{b,\ sb}$ $+/-_{s}$
Cytokeratin 13	$+_{s}$	$+_{s}$	$+_{s}$
Vimentin	$+_{f}$ $+/-_{b}$	$+_{f}$ $+/-_{b}$	$+_{f}$
Laminin 5	+	+	+
Collagen IV	+/–	+/–	+
p-63	+	+ +	+ + +
Ki-67	–	+/–	+

Key: +, positive staining; –, negative staining; +/–, weak staining; f, fibroblasts; b, basal keratinocytes; sb, suprabasal keratinocytes; and s, superficial keratinocytes.

In subsequent weeks, we witnessed how this marker strongly stained the basal cells, whereas suprabasal cells were more weakly stained. It is known that cytokeratin 5 acts as marker for basal cells and for those cells directly above them (20). For its part, cytokeratin 6 is positive in those cells which start to differentiate (21). Although, *in vitro*, its pattern of immunolabeling appears to stabilize at 14 d of interphase (17,21), when analysing this pattern *in vivo* (1,2), we see that labeling with the cytokeratin 5/6 pair may be present in the basal and suprabasal layers up to 24 wk after transplantation, featuring a stable pattern in the basal layer and those directly above it at 30 mo. The explanation we give to our findings is that, at 21 d, a stage takes place in which the epithelium regenerates, stratifies and organizes; thus, the finding of numerous cells in the suprabasal layers during early differentiation stages would be expected. Indeed, when we analysed the presentation pattern of cytokeratin 13, we observed how it spread into all the suprabasal layers; some negative cells were found in those layers closest to the base cell line. This distribution pattern of cytokeratin 13 is consistent with findings from other studies *in vitro* (17,20,21) and *in vivo* (1). All these facts confirm the immaturity of the grafted CAOMEs and the developmental stages of the regeneration of epithelium, and demonstrate the regeneration the CAOMEs themselves produce.

To support these findings, we examined the distribution pattern of the p-63 protein, in order to prove how, initially, the number of positive cells was comparable to that obtained *in vitro* at 1 wk (7). However, their number increased steadily over time, and a lot of stem cells were present in the basal layer and directly above it, like the repair and regeneration after a wound (11). By applying vimentin, we verified the following two facts: firstly, we detected the presence of fibroblasts, their quantitative increase and how the chorion became more organized as the weeks passed; and secondly, the test also showed that basal keratinocytes were positive for this intermediate filament. The pattern was homogeneous during the first week, but thereafter only some cells showed immunostaining. Our findings are consistent with those reported by other authors (5,20), which suggests that this filament is present in both mesenchymal cells and undifferentiated keratinocytes.

Of the major components that make up the basement membrane, laminin 5 is the one which appears earliest (17), and it seems to stabilize *in vitro* after 14 d of air–liquid interface (20). However, Ahn *et al.* (14) could not find this positivity at 14 d of grafting in their rabbit model. In our work, we observed discontinuity throughout the first week of grafting,

but in the second week the immunostaining was virtually continuous, and at the third week, completely continuous. For its part, although collagen IV may present a continuous immunolabeling at 14 (20) or 21 d (17) *in vitro*, this continuity is not achieved *in vivo* until the third week (18). In our study model, we started to detect collagen IV in the first week, but it was not until the third that we could witness its continuity throughout the epithelium. Since the presence of a complete and continuous basement membrane promotes epithelial differentiation (17), we believe it is beneficial for regeneration using oral mucosa equivalents that the basement membrane takes as long as possible to complete. Additionally, the fact that laminin 5 appears early provides an adequate anchor to the epithelium for the early repair stages (6).

Finally, in order to assess the existence of cell proliferation and quantify whether it was higher in the third week, in keeping with the increase in the amount of basal stem cells, we used the antibody Ki-67. There are conflicting data on the evolution of Ki-67 in epithelial cultures. The available data from *in vitro* studies (22) show that Ki-67 positivity begins to increase after 14 d of culture, the immunolabeling being located in the basal layer and the one directly above it. By contrast, Tomakidi *et al*. (17) reported that the immunopositivity was higher during the initial stages of culture, while at 14 d, there was a decrease in immunolabeling. To our knowledge, the present study is the first to follow the evolution of this marker *in vivo* and its relation to oral mucosa equivalents. Our findings are consistent with observations of Yoshizawa *et al*. (22) and, at the same time, maintain correlation with other immunohistochemical findings, mainly with the increased p-63 positivity in the basal layer and the one directly above it.

Conclusions

The present study shows the potential regenerative capacity of CAOMEs *in vivo* by their ability to reach maturity with similar characteristics to those of oral mucosa. These findings, together with the fact that CAOMEs developed with our technique are totally autologous, turn them into a potential therapeutic tool for use in clinical practice within the field of tissue regeneration.

Acknowledgements

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