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Histological and histochemical evaluation of human oral mucosa constructs developed by tissue engineering

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Summary. Reconstruction of large oral mucosa defects is often challenging, since the shortage of healthy oral mucosa to replace the excised tissues is very common. In this context, tissue engineering techniques may provide a source of autologous tissues available for transplant in these patients. In this work, we developed a new model of artificial oral mucosa generated by tissue engineering using a fibrin-agarose scaffold. For that purpose, we generated primary cultures of human oral mucosa fibroblasts and keratinocytes from small biopsies of normal oral mucosa using enzymatic treatments. Then we determined the viability of the cultured cells by electron probe quantitative X-ray microanalysis, and we demonstrated that most of the cells in the primary cultures were alive and had high K/Na ratios. Once cell viability was determined, we used the cultured fibroblasts and keratinocytes to develop an artificial oral mucosa construct by using a fibrin-agarose extracellular matrix and a sequential culture technique using porous culture inserts. Histological analysis of the artificial tissues showed high similarities with normal oral mucosa controls. The epithelium of the oral substitutes had several layers, with desmosomes and apical microvilli and microplicae. Both the controls and the oral mucosa substitutes showed high suprabasal expression of cytokeratin 13 and low expression of cytokeratin 10. All these results suggest that our model of oral mucosa using fibrin-agarose scaffolds show several similarities with native human oral mucosa.

Key words: Fibrin-agarose, Constructs, Tissue engineering.

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This work is dedicated to Prof. J. Gómez on his 85th anniversary.

Introduction

Different surgical procedures carried out in the oropharyngeal region frequently result in large tissue defects (Schultze-Mosgau, 2004). Reconstruction of these defects is challenging, and oral and maxillofacial surgeons are often confronted with a shortage of oral mucosa to replace the excised tissues (Song et al., 2004). Although it has been demonstrated that primary reconstruction of large oral defects is always more advantageous than secondary reconstructions, primary surgical closure of large oral defects is extremely difficult. In these cases, various types of skin-bearing flaps have been proposed as autologous substitutes of the oral mucosa (Baumann et al., 1996). In some patients, however, the use of skin-bearing flaps is often associated to complications, such as the presence of adnexal structures causing hair growth on the implanted graft or excessive keratinisation of the reconstructed tissue (Toft et al., 2000). All these disadvantages sometimes cause a significant morbidity and aesthetic and functional limitations for the patients submitted to these techniques.

Construction of biological substitutes of the human oral mucosa by tissue engineering could contribute to solve these problems and complications. By using these techniques, it is possible to develop efficient substitutes of different organs and tissues for therapeutic use, including skin (Meana et al., 1998; Llames et al., 2004, 2006), cornea (Nishida, 2003; Alaminos et al., 2006), urothelium (Wunsch et al., 2005) and blood vessels (Pascual et al., 2004). In this sense, some researchers have recently proposed different tissue engineering techniques for construction of an organotypic substitute of the oral mucosa (Lauer and Schimming, 2001; Schultze-Mosgau et al., 2004; Song et al., 2004). Evaluation of the viability of the cells kept in culture is very important before these cells can be used for clinical purposes and, especially, for construction of artificial organs and tissues by tissue engineering. In addition, the construction of artificial organs and tissues is highly

dependent on the availability of an appropriate biocompatible scaffold that allows the cells to attach and grow both in vitro and in vivo.

Evaluating the viability of cultured cells is not easy, and different approaches have been proposed to date with that purpose. A number of methods are based on the detection of permeability alterations in the cell membrane by trypan blue or propidium iodide staining (Bergert et al., 2005), or quantification of intracellular enzymes in the culture medium (Chen and Wagner, 2001). However, most of these techniques are not accurate enough to detect early cell damage, but only identify cell alterations once they have become irreversible. In most cases a positive result with these techniques reveals that the integrity of the cell membrane has been lost. For these reasons, such methods are not sensitive enough to detect cells that are prone to death but do not display any cell membrane alterations yet.

In contrast, one of the most sensitive techniques for determination of cell viability is quantification of the ionic content of the cultured cells, especially potassium and sodium (Fernandez-Segura et al., 1999; Roomans, 2002a,b). Histochemical x-ray microanalysis associated with electron microscopy is the most powerful approach to measure total elemental composition, making it possible to simultaneously determine the concentrations of different elements and the ultrastructure of the cells (Somlyo et al., 1989; Krep et al., 1996). By using this combined biochemical and morphological technique, our group has previously quantified the ionic content of different cell types, including U937 cells (Fernandez-Segura et al., 1999), epithelial tumor cells (Fernandez-Segura et al., 1997) and K562 cells (Warley, 1994). However, the microanalytical pattern of isolated human oral keratinocytes and fibroblasts kept in culture for tissue engineering purposes has not been determined so far.

On the other hand, different biomaterials have been used as stromal substitutes. Ideally, a good biomaterial should be biocompatible and consistent, and epithelial cells should be able to adhere and grow on and within it. Currently, the biomaterials that have been used most often to construct epithelial/stromal tissue substitutes are type I collagen and fibrin. Type I collagen has been extensively used as a scaffold in tissue engineering for the construction of artificial skin, oral mucosa, corneas and other tissues (Orwin and Hubel, 2000; Reichl and Muller-Goymann, 2003). However, collagen is an expensive material and tends to shrink and lose volume when the cells are seeded within the scaffold (Tegtmeyer et al., 2001). In addition, stromal substitutes made of collagen are not stable and are quickly degraded (Chen et al., 2005). However, human fibrin has been proposed as a stromal substitute to construct different tissue substitutes, especially human skin (Meana et al., 1998; Llames et al., 2004, 2006), and has the advantages of low cost, availability and good tolerance to cells. In contrast with collagen gels, fibrin gels are not contracted by stromal cells (Meana et al., 1998; Llames et al., 2004, 2006), although its consistency may be sometimes poor. Other materials such as agarose are commonly used in tissue engineering of the cartilage (Aufderheide and Athanasiou, 2005), but barely used in combination with other biomaterials. By using a mixture of human fibrin and agarose, our research group was able to construct an efficient substitute of the rabbit cornea with good results in terms of consistency, transparency and cell growth (Alaminos et al., 2006).

In this work, we have developed a new model of artificial oral mucosa using viable human fibroblasts and keratinocytes and a hydrogel scaffold made of a mixture of fibrin and agarose.

Materials and methods

Human tissue samples

Twenty small biopsies corresponding to normal human oral mucosa were obtained from healthy donors undergoing minor oral surgery under local anesthesia in the School of Dental Sciences of the University of Granada. Average size of the tissue samples was 3x3x2 mm. Immediately after extraction, all tissues were kept in a transport medium at 4°C (Dulbecco's modified Eagle's medium DMEM; 100 U/ml of penicillin G, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B) and processed in the following 24 h.

All patients provided consent to participate in the study. The work was approved by the local research committee (number 01/04).

Primary cultures of oral fibroblasts and keratinocytes

On arrival at the laboratory, all samples were washed twice in phosphate buffered saline (PBS) and incubated overnight at 37°C in a mixture of DMEM and 2 mg/ml of *Clostridium histolyticum* collagenase I (Gibco BRL Life Technologies, Karlsruhe, Germany). This enzymatic treatment is able to digest all the extracellular matrix of the oral chorion and release the fibroblasts entrapped there. Once the samples were digested, and to obtain primary cultures of human oral fibroblasts, detached connective tissue components, including fibroblasts, were collected by centrifugation and expanded in culture flasks containing DMEM medium supplemented with antibiotics (100 U/ml of penicillin G, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B) and 10% of fetal bovine serum (FBS) using standard culture conditions. Then, undigested oral epithelium was washed in PBS, cut into small pieces and co-cultured with a layer of mitomycin C-treated (10 mg/ml) 3T3 feeder cells (8-10x10^3 cell/cm^2) (Rheinwald and Green, 1975). The medium used in this case was a 3:1 mixture of DMEM and Ham's F12 supplemented with 10 % fetal calf serum, 1% antiobiotics, 24 μg/ml adenine, 0.4 mg/ml hydrocortisone, 5 mg/ml insulin, 10 ng/ml epidermal growth factor, 1.3 ng/ml triiodothyronine and

8 ng/ml of cholera toxin.

In all cases, the cells were incubated at 37°C in 5% carbon dioxide. The medium was changed every three days, and subcultivation of the cultured cells was carried out using a trypsin 0.5 g/l-EDTA 0.2 g/l-solution at 37°C for 10 min. Keratinocytes and fibroblasts used for tissue engineering of the tissue models always corresponded to the three first cell subcultures.

Histochemical X-ray microanalytical evaluation of keratinocytes and fibroblasts cells

For histochemical X-ray microanalysis, cultured fibroblasts and keratinocytes cells were subcultured on gold grills covered with a thin layer of pioloform (Ted Pella Inc., CA, USA), sterilized overnight under UV light, as a physical support to the cells. Cultured cells were seeded at a density of 5000 cells per grid and incubated for 24 h using specific culture media for fibroblasts or keratinocytes. After 24 h of culture, gold grids containing the cultured cells were washed with ice-cold distilled water for 5 s to remove the extracellular medium and immediately plunge-frozen in liquid nitrogen following previously published protocols (Abraham et al., 1985; Warley, 1994; Fernandez-Segura et al., 1997). After cryofixation the grills were placed in a precooled aluminum specimen holder at liquid nitrogen temperature and freeze-dried at increasing temperatures for 24 h in a E5300 Polaron freezer-drier apparatus equipped with a vacuum rotatory pump system. Freeze-dried gold grills were carbon-coated in a high-vacuum coating system and microanalyzed within the following hours.

Histochemical X-ray microanalysis of the specimens was performed using a Philips XL30 scanning electron microscope (SEM) equipped with an EDAX DX-4 microanalytical system and a solid-state backscattered electron detector. The samples were examined with SEM using a combination of secondary electron (SE) and backscattering imaging modes.

For X-ray microanalysis, the analytical conditions were: tilt angle 0°, take-off angle 35° and working distance 10mm. All spectra were collected in the spot mode at 10,000x for 200 s live time, and the number of counts per second by the detector was about 500. To determine total ion content, we used the peak-to-local-background (P/B) ratio method (Statham and Pawley, 1978; Boekestein et al., 1984; Fernandez-Segura et al., 1997) with reference to standards composed of 20% dextran containing known amounts of inorganic salts (Warley, 1990). In total, we quantified the ionic content of 25 cultured keratinocytes and 25 cultured fibroblasts.

Fabrication of a fibrin-agarose stromal substitute

Fibrin was obtained from frozen plasma of human blood donors (kindly provived by Dr. Fernandez-Montoya, Human Tissue Bank of Granada). To produce a fibrin gel (Meana et al., 1998; Llames et al., 2004, 2006), 21 ml of human plasma were added to 250,000 cultured fibroblasts resuspended in 2 ml of DMEM with 10% FCS. To prevent degradation of the scaffold by fibrinolysis, the mixture was supplemented with 200 μl of tranexamic acid. Finally, 2 ml of 1% $CaCl_2$ were added to the solution to precipitate fibrin polymerization. At the same time, type VII agarose was melted and solved in PBS and added to the fibrin mixture at a final concentration of 0.1%, immediately after the $CaCl_2$ was added. Finally, the mixture was seeded in culture inserts and allowed to solidify at 37°C for 2 hours.

Construction of oral mucosa substitutes by tissue engineering

Organotypic oral mucosa constructs were developed by using a sequential culture technique as described below. To ensure both a submerged cultivation and a proper differentiation of the multilayered epithelium, we used the air-liquid culture technique (Reichl and Muller-Goymann, 2003). For that purpose, bioengineered oral mucosa was assembled in Transwell culture inserts with 0.4 μm porous membrane (Costar, Corning Inc., Corning, NY, USA). This pore size allows the nutrients to cross through the membrane of the insert but prevents migration of the cells to the other compartment.

To construct an artificial oral mucosa, the stromal substitute was developed directly on the porous membrane of the culture inserts. Twenty-four hours after the stromal matrix substitute had solidified, human oral keratinocyte cells were seeded on top of the constructed stroma (approximately 1,000,000 keratinocytes per 25 ml construct), and cultured for 10 days submerged in keratinocytes culture medium. When keratinocyte cells reached confluence, the air-liquid culture technique was used for 21 more days. The whole process is schematically shown in Figure 1.

Microscopic evaluation of artificial oral mucosa

Samples for scanning electron microscopy were fixed in cacodylate-buffered 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide for 90 minutes. After fixation, the samples were dehydrated in increasing concentrations of acetone (30%, 50%, 70%, 95% and 100%), critical point dried, mounted on aluminum stubs, sputter coated with gold according to routine procedures (Sanchez-Quevedo et al., 1994), and examined in a scanning electron microscope (Quanta 200; FEI, Eindhoven, The Netherlands) using a high vacuum mode. For transmission electron microscopy, samples were fixed, postfixed and dehydrated as described above for scanning electron microscopy, and then embedded in Spurr's resin and cut into ultrathin sections with an ultramicrotrome. For analysis, the sections were stained with aqueous uranyl acetate and lead citrate and examined with a transmission electron microscope (EM902; Carl Zeiss Meditec, Inc., Oberkochen, Germany).

Immunohistochemistry

The expression of cytokeratins 10 and 13 was determined by immunohistochemistry in paraffin-embedded tissue sections corresponding to controls and oral mucosa constructs using prediluted, ready-to-use, mouse monoclonal antibodies against cytokeratin 10 and cytokeratin 13 (Master Diagnostica, Spain). First, paraffin was removed from the tissue sections using xylene, and the samples were then rehydrated in water. To quench endogenous peroxidases that could affect the results of the hybridization, all samples were incubated in 3% H_2O_2 for 15 min. Then, we used 0.01 M citrate buffer (PH 6.0) at 98°C for 5 min for antigen retrieval. After blocking any unspecific binding sites with bovine serum albumin, we incubated the samples with the primary antibodies for 1 h at room temperature. After 3 rinses in 0.5% Triton-X100 in PBS, the biotin-conjugated antimouse IgG secondary antibody was applied for 30 min, whereas a horseradish peroxidase-conjugated streptavidin solution was applied for 40 min. Finally, the colour was developed with a commercial DAB kit (Vector Laboratories, Burlingame, CA) and samples were then counterstained in Mayer's haematoxylin and mounted on coverslips for optical evaluation. As positive controls, sections of normal human oral mucosa were used. For the negative controls normal horse serum was used instead of the primary antibody.

Microarray gene-expression analysis

Total RNA corresponding to three primary cell cultures of oral mucosa fibroblasts (two samples) and keratinocytes (one sample) and three oral mucosa constructs was extracted by using the Qiagen RNeasy System (Qiagen, Mississauga, Ontario, Canada), according to the manufacturer's recommendations. Quality and integrity of the RNAs was verified by checking 28S and 18S rRNA after ethidium bromide staining of total RNA samples on 1.2% agarose gel electrophoresis. Total cDNA was synthesized with a T7-polyT primer and a reverse transcriptase (Superscript II, Life Technologies, Inc., Carlsbad, CA), and labeled cRNA were synthesized by *in vitro* transcription with biotinylated UTP and CTP (Enzo Diagnostics, Farmingdale, NY). Labeled nucleic acid target quality was assessed by test-2 arrays and then hybridized (45°C for 16 h) to Affymetrix Human Genome U133 plus 2.0 oligonucleotide arrays. After automated washing and staining, absolute values of expression were calculated and normalized from the scanned array by using Affymetrix Microarray Suite. To identify statistically significant differences of expression of different genes, we used the non-parametric test U of Mann-Whitney.

Results

Isolation and culture of human oral mucosa cells

Human oral mucosa fibroblasts were efficiently cultured by using the enzymatic treatment technique described in the methods section. Oral fibroblasts attached to the culture flask very early, showing a good growth rate in culture and reaching confluence around the 8th day of cultivation (8.32±3.23 days). In culture, oral fibroblasts displayed a spindle-like shape, with cells tending to become very elongated when subconfluency was reached.

On the other hand, the outgrowth technique from tissue explants allowed us to obtain primary cultures of human oral keratinocytes. Initially, keratinocytes attached to the bottom of the culture flasks and formed small aggregates of 4 to 5 cells (cells islets) within the layer of feeder cells around the 8th day of culture (mean 8.2±3.1). Then, the cell islets tended to grow and expand

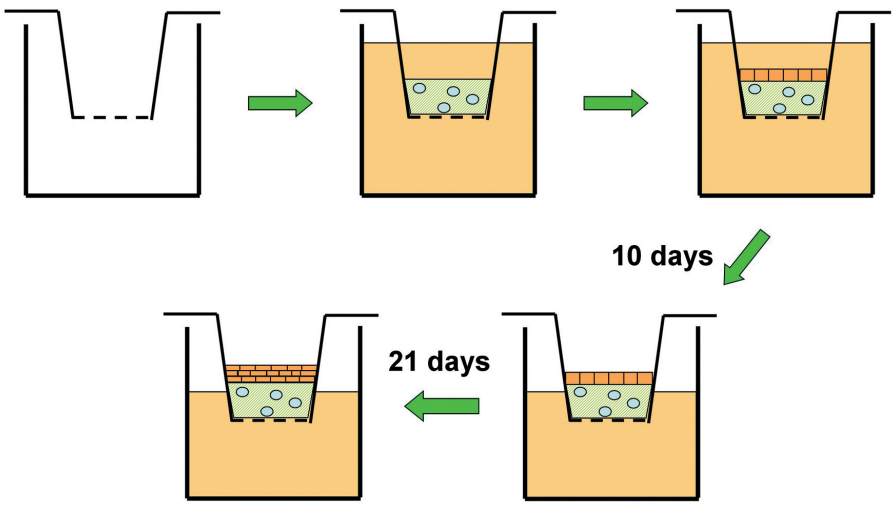

Fig. 1. Schematic representation of the method used to construct substitutes of the human oral mucosa by tissue engineering using porous culture inserts. First, a fibrin-agarose stromal substitute with fibroblasts embedded was constructed on the porous membrane. Then, the keratinocytes were seeded on top, and the air-liquid culture technique was used to promote epithelial stratification.

centrifugally and substitute the 3T3 feeder cells. After 28 days (28±13.1), all feeder cells had been substituted by the keratinocytes. On confluency, oral keratinocytes showed a polygonal shape with a pavement-like appearance.

Development of three-dimensional artificial oral mucosa

Construction of oral mucosa substitutes by tissue engineering was efficiently carried out by using porous culture inserts. First, we developed a stromal substitute composed of human fibrin and 0.1% agarose. Embedded in these scaffolds, human oral mucosa fibroblasts displayed a rapid proliferation rate, becoming elongated and spreading out in the stromal lattice after 1 to 3 days of culture. No contraction of the fibrin-agarose gels was observed in any case. Once the stromal substitute had been constructed, keratinocytes cells were seeded on top of the chorial matrix, and a cell monolayer of keratinocytes was observed after 7 to 10 days of submersed culture. Stratification of the oral mucosa epithelial layer was observed after 2 weeks of exposure of keratinocytes cells to air in the culture inserts (air-liquid technique).

Histological evaluation of the artificial oral mucosa revealed that the epithelial cells tended to form a normal, stratified epithelium with desmosomes (Fig. 2A), and that the superficial cells showed different patterns of differentiation characterized by microvilli and parallel rows of microplicae (Fig. 2B). In addition, immunohistochemical analysis of the constructed oral mucosa demonstrated phenotypic similarities with normal oral mucosa, with expression of cytokeratin 13 in suprabasal layers and absence of expression of cytokeratin 10 (Fig. 3). The macroscopic appearance of the reconstructed oral mucosa showed that it was a relatively consistent tissue, allowing the surgeon to apply simple stitches to it.

Evaluation of the cell viability of cultured oral fibroblasts and keratinocytes and oral mucosa constructs

Microanalyses of 25 keratinocytes and 25 fibroblast cells showed that cultured oral epithelial and stromal cells were characterized in general by high K/Na ratios (10.72 and 9.11 respectively) (Fig. 4). In addition, the profiles of ionic distribution for each cell type were similar to those found for live, vital cells, with a high amount of Cl, K and P and low contents of Ca, Mg, Na and S (Table 1). All these findings suggest that all the cells analyzed displayed a good viability and can be used for tissue engineering purposes.

In addition, the gene expression analyses of different caspases (*CASP1*, *CASP2*, *CASP3*, *CASP4*, *CASP6, CASP7*, *CASP9*, *CASP10*, *CASP12*, *CASP14*) revealed that the expression of these genes with an important role in apoptosis was in general very low in both the primary cultures and the oral mucosa constructs (average expression 157 fluorescent units), being slightly higher in primary cultures (163 fluorescent units) than in the

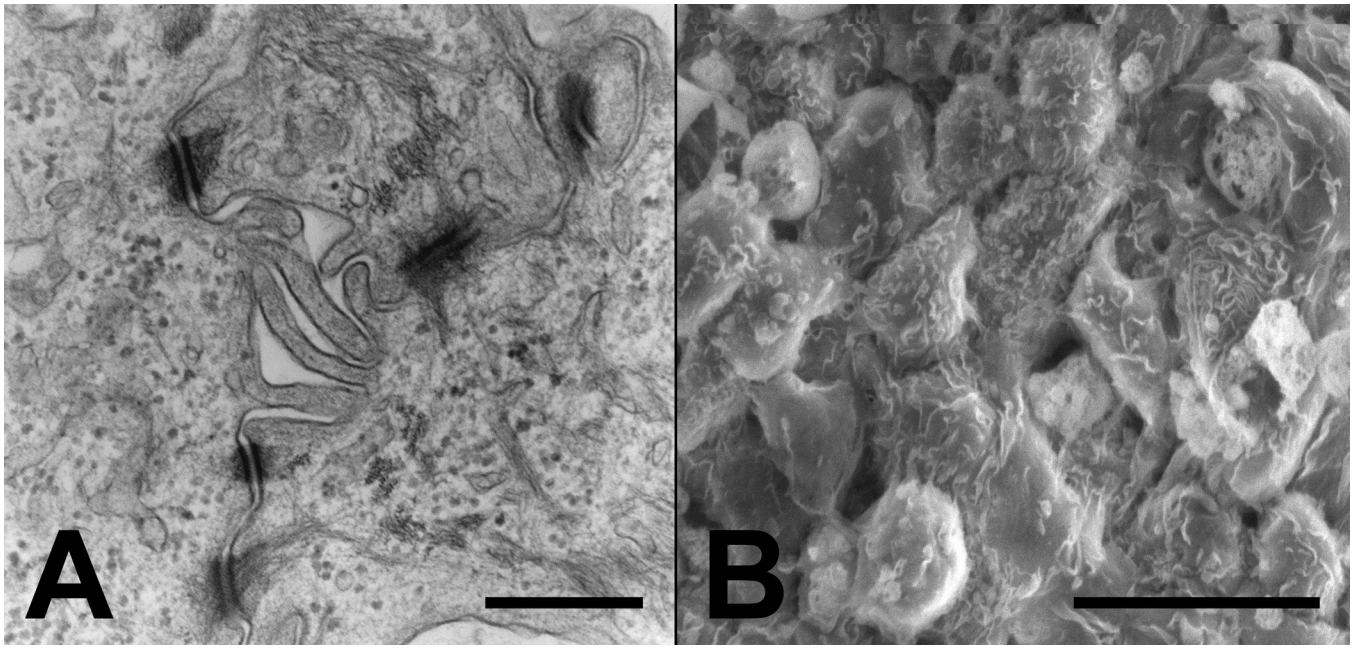

Fig. 2. Microscopic evaluation of the constructed oral mucosa. **A.** The ultrastructural analysis of the constructed oral mucosa reveals the presence of several desmosomes joining adjacent cells. Scale bar: 100 nm. **B.** Scanning electron microscopy image of the surface of the constructs shows different patterns of cell differentiation, including microvilli and microplicae. Scale bar: 20 μm.

oral mucosa substitutes (151 fluorescent units). On the other hand, expression of several proliferation markers (*PCNA*, *KI67* and *MIB*) was very high in primary cell cultures, where cells proliferate actively (average 638 fluorescent units) and lower in the oral mucosa substitutes (average 299 fluorescent units), where cells tend to differentiate rather than proliferate. The differences were not significant ($p>0.05$ for all genes compared). All these findings suggest that cells in the oral mucosa constructs were not experiencing apoptosis during the period of study.

Discussion

Construction of an efficient artificial oral mucosa by tissue engineering remains a major scientific challenge.

Table 1. Intracellular concentrations of different elements determined by X-ray microanalysis in human oral keratinocytes and fibroblasts.

	KERATINOCYTES	FIBROBLASTS
[Ca]	15.98±9.44	12.92±7.37
[Cl]	174.56±42.31	182.47±21.81
[K]	385.89±85.26	406.70±32.87
[Mg]	20.43±4.41	19.38±5.55
[Na]	35.98±21.12	44.62±13.05
[P]	259.59±48.49	304.40±36.71
[S]	42.43±14.37	69.78±12.72
K/Na ratio	10.72	9.11

All concentrations are shown as average values ± standard deviation of 25 different cells, and are expressed as mmol per kilogram of dry weight.

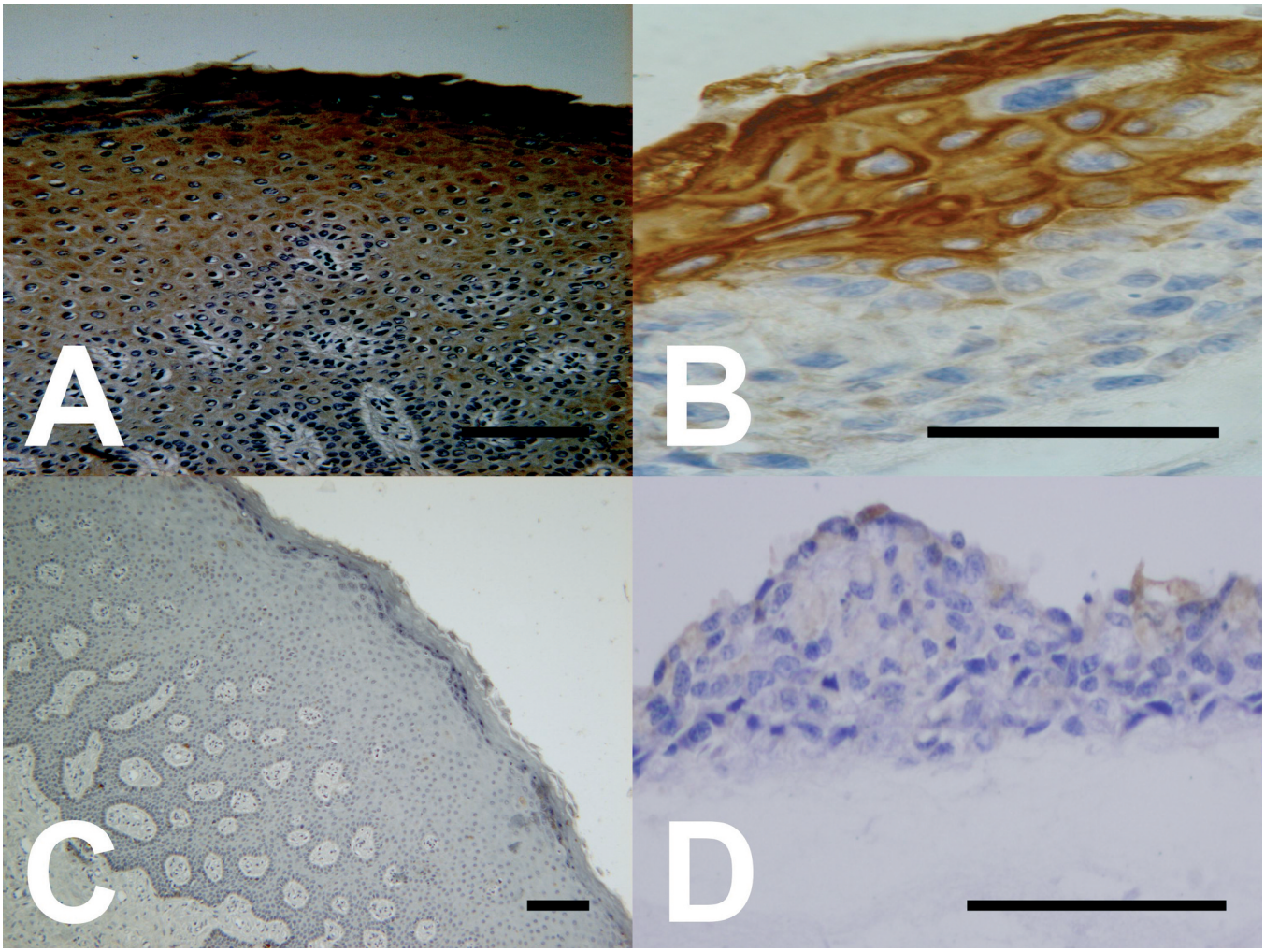

Fig. 3. Immunohistochemical evaluation of the native and constructed oral mucosa. **A.** Expression of cytokeratin 13 in control normal oral mucosa is restricted to suprabasal layers of the epithelium. **B.** Constructed oral mucosa shows strong expression of cytokeratin 13 in suprabasal cell layers. **C.** Negative expression of cytokeratin 10 in normal oral mucosa controls. **D.** Negative expression of cytokeratin 10 in the oral mucosa substitutes. Scale bar: 100 μm in A, C and D and 50 μm in B.

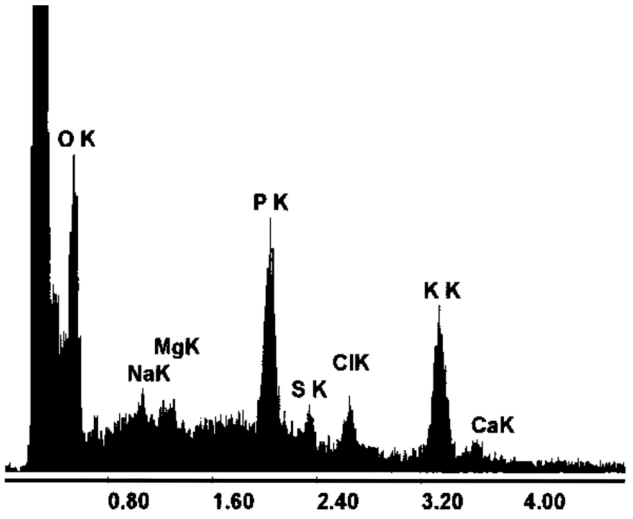

Fig. 4. Illustrative example of a microanalytical spectrum corresponding to a living oral keratinocyte. The peaks correspond to the energy dispersed by electrons located in the k orbitals of sodium (NaK), magnesium (MgK), phosphorus (PK), sulfur (SK), chlorine (ClK), potassium (KK) and calcium (CaK).

The use of these kinds of tissue substitutes could help oral and maxillofacial surgeons to improve the outcome of patients in need of large reconstructions involving the oral cavity (Ueda et al., 1991; Lauer and Schimming, 2001; Schlenz et al., 2001; Izumi et al., 2003). In addition, artificial oral mucosa substitutes generated in the laboratory could be used as in vitro models for investigations of experimental pharmacology or toxicology tests, thus avoiding the need for animal research.

In this work, we first isolated, maintained and evaluated the viability of the two main types of cells in the oral mucosa. Then, we elaborated a stromal substitute made of oral fibroblasts entrapped in a hydrogel of human fibrin and 0.1% agarose, according to a model previously used by our group (Alaminos et al., 2006). Determination of the cell viability of human oral keratinocytes and fibroblasts is a key step in the construction of artificial oral mucosa substitutes by tissue engineering. For that purpose, we used quantitative electron probe X-ray microanalysis associated to electron microscopy. This technique is a well established procedure for evaluation of the viability of cultured cells, since only viable cells are suitable for clinical use (Fernandez-Segura et al., 1999). The main advantage of this method is its ability to detect cells that are prone to death but still keep the integrity of the cell membrane. In contrast with other methods such as trypan blue or propidium iodide staining (Chen and Wagner, 2001), X-ray microanalysis allows the researcher to analyze the ionic profile of the cells by determining the intracellular concentrations of crucial elements like sodium or potassium. Different studies have shown that the intracellular concentrations of sodium and potassium are excellent markers of cell viability, the K/Na ratio being one of the most powerful parameters of cell damage from the microanalytical standpoint (Warley, 1994; Roomans, 2002a).

In this context, the use of human oral epithelial and stromal cells for construction of artificial organs by tissue engineering always requires the previous determination of cell viability to ensure that they are viable and keep their functions. In this study, we investigated the total element content of sodium, magnesium, phosphorus, chlorine, potassium and calcium in human oral keratinocytes and fibroblasts by using quantitative histochemical X-ray microanalysis. According to our results, the primary cultures of keratinocytes and fibroblasts used in this work showed ionic profiles corresponding to cells with good levels of viability. The high intracellular concentrations of phosphorous and potassium were good markers of cell viability, especially in association with low contents of sodium (Roomans, 2002b; Arrebola et al., 2005). In contrast, different studies demonstrated that cells undergoing apoptosis show low intracellular concentrations of potassium and chlorine, even from the first stages of the apoptotic process (Fernandez-Segura et al., 1999; Arrebola et al., 2005). This decrease of the potassium and chlorine levels showed a direct correlation with the loss of cell volume associated to the apoptotic process (Barbiero et al., 1995; Hughes et al., 1997). For those reasons, intracellular ionic quantification by X-ray microanalysis is considered to be an excellent method to determine not only the current viability, but also to predict the future vital status of the cells used in tissue engineering.

Once the cells were cultured and their viability was confirmed by X-ray microanalysis, we developed a model of artificial oral mucosa using stromal substitutes. Several authors have developed different models of artificial oral mucosa using collagen stromal substitutes. However, the use of collagen gels is handicapped in most of the cases by fibroblast-mediated contraction of the hydrogels (Schoop et al., 1999; Bach et al., 2001; Chinnathambi et al., 2003). Other groups, however, have used fibrin matrices as stromal substitutes for different tissues (Meana et al., 1998; Isenberg et al., 2006; Llames et al., 2004, 2006; Peretti et al., 2006; Talbot et al., 2006). Nevertheless, the mechanical properties of pure fibrin polymer gels are not always comparable to those of oral mucosa stroma in terms of consistency and elasticity. Trying to improve the mechanical properties of the artificial oral mucosa, in this work we used a mixture of fibrin and 0.1% agarose (Alaminos et al., 2006). Fibrin-agarose artificial stromas demonstrated better consistency than fibrin alone, allowing us to perform simple sutures on the oral mucosa constructs. Furthermore, fibrin-agarose hydrogels did not contract, as previously reported for collagen gels, even when stromal cells are seeded within (Reichl et al., 2004). In addition, the source of fibrin used in this study (human

plasma) has several advantages, especially the presence of whole-blood sets of cytokines, attachment factors, and platelet-derived growth factors. All these proteins are present and functional in the plasma-based scaffold, offering a highly proliferative environment for keratinocytes (Marx et al., 1998).

To develop an efficient substitute for the human oral mucosa, we used a sequential culture technique using commercially available culture inserts (Reichl and Muller-Goymann, 2003). These culture inserts were used for a two reasons. First, they have a porous membrane that allows the culture media to cross over and reach all the cells in the system. Second, the design of these devices allowed us to use an air-liquid culture technique to promote stratification of the engineered epithelium. Culture inserts of different types have been used to date to construct various types of tissues by tissue engineering (Richard et al., 1991; Geroski and Hadley, 1992; Hutak et al., 2002; Limat et al., 2003; Reichl and Muller-Goymann, 2003; Dai et al., 2005; Iida et al., 2005) as an efficient way to promote epithelial stratification (Casasco et al., 2001; Izumi et al., 2003).

On the other hand, microscopic evaluation of the artificial oral mucosa revealed the presence of a stratified oral epithelium on the surface of the stromal substitutes. The use of fibrin-agarose scaffolds allowed stromal fibroblasts to grow and proliferate within the extracellular matrix and, at the same time, induced the adherence and maturation of a stratified epithelium on top. Fibrin, the major component of the engineered stroma, is a natural protein, very abundant in circulating blood, which is physically recognised by stromal fibroblasts, and it is likely that oral fibroblasts can synthesize collagen fibers and progressively reorganize the stromal mesh. Moreover, our ultrastructural analysis of the artificial tissues showed that oral keratinocytes are able to form a multilayered epithelium with numerous desmosomes and join complexes. Strikingly, different patterns of epithelial cell differentiation were detected on the surface of the engineered epithelium, including microvilli and parallel rows of microplicae. The presence of these kinds of cell surface specializations has previously been associated with the normal differentiation process of oral keratinocytes (Moreu et al., 1993; Sanchez-Quevedo et al., 1994), and suggests that the oral epithelium generated by tissue engineering is very similar to the native oral mucosa epithelium. Furthermore, Immunohistochemistry showed similarities between the native oral mucosa used as control and the constructed epithelium, with a high expression of cytokeratin 13 in suprabasal layers of the epithelium, whereas the microarray analysis demonstrated that the cells in the oral mucosa constructs were not experiencing a process of cell death by apoptosis and still had the capability to proliferate. These results are in agreement with those previously reported by other authors using different types of extracellular matrices (Cho et al., 2000; Igarashi et al., 2003). Altogether, our results indicate that cultured oral keratinocytes are able to physically integrate with the fibrin-agarose lattice used in this work, adhering to it and differentiating on top of the artificial stroma. These results suggest that the oral mucosa constructs based on fibrin-agarose scaffolds progressively acquire histolological and key cytokeratin expression patterns similar to human oral mucosa controls. Therefore, the fibrin-agarose complexes described in this study appear to satisfy the criteria for biomaterials used in tissue engineering of the human oral mucosa.

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