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Bi-layered collagen nano-structured membrane prototype (collagen matrix 10826®) for oral soft tissue regeneration: an "*in vitro*" study

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Abstract

Aim: To evaluate fundamental cell functions, such as adhesion, IL-6 production and proliferation of human gingival keratinocytes cultured on a newly engineered collagen matrix (CM-10826) and to assess the degree of specific biocompatibility of this new device.

Materials and methods: Primary cultures of human keratinocytes were derived "*in vitro*" from biopsies of independent donors. Their true epithelial origin was ensured by the expression of cytokeratin 14. Adhesion, proliferation and production of IL-6 cytokine was then measured in the presence or absence of CM-10826 activity or of its relevant components.

Results: Functional tests revealed that keratinocytes adhered to CM-10826 and up-regulated their basal IL-6 production. The type of keratinocytes used expressed cytokeratin 14. Proliferation experiments demonstrated that the best cellular response was observed in the presence of Collagen I, the main component of CM-10826. No undesired effects were observed as for keratinocyte viability, morphology or differentiation.

Conclusions: Our results demonstrate that CM-10826 has a favourable biological effect on the *"in vitro"* response of gingival keratinocytes in terms of IL-6 production, cell growth and adhesion. These findings may encourage a possible use of this collagen membrane as a tissue which, alone, may substitute for autologous gingival grafts thereby overcoming the limitations of autologous tissue.

Keratinized gingiva (KG) is a part of oral mucosa covered with keratin or parakeratin that includes the free and attached gingiva (Gartner 1994). KG extends from the gingival margin to the muco-gingival junction. Keratinocytes account for 90% of the cell population of KG. The more undifferentiated, highly proliferating elements reside in the basal stratum, linked to each other and attached to the basal lamina by multiple adhesion receptors including members of the integrin family. Of particular interest are α6β4 and α3β1 integrins, almost invariably expressed on the surface of keratinocytes and strategically localized at the lateral cell-cell boundaries (α3β1) or at the cell-lamina interface (α6β4). These integrins are able to recognize multiple components of the extracellular matrix (collagen, fibronectin, laminin) and therefore to activate intracellular signalling pathways, which regulate migration, proliferation, survival and differentiation (Giancotti & Ruoslahti 1989; Sastry & Horwitz 1996; Giancotti 1997; Mainiero et al. 1998; Smith & Fuchs 1998). Keratinocyte differentiation progresses from the basal to the stratified superficial layer, with cells progressively acquiring size and expression of higher molecular weight cytokeratins, meanwhile decreasing proliferation. This process is governed by both intra- and extracellular stimuli. Among them, detachment of α6β4 integrin from its natural ligand, for example laminin 5, is one of the most effective (Symington & Carter 1995; Tennenbaum et al. 1996). In addition, epithelium growth factor (EGF) stimulates basal keratinocyte mitosis (Shirasuna et al. 1991; Whitcomb et al. 1993) and interleukin 6 (IL-6) stimulates keratinocyte proliferation (Brauchle et al. 1994). The end of the differentiation program is the formation of keratinized (KG) or parakeratinized gingiva, characterized by the expression of cytokeratin 14, which can be considered a

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biochemical marker of the stratified squamous epithelium (Moll et al. 1982; Coulombe et al. 1989).

Some studies suggested that a minimum width of KG is necessary to maintain gingival health (Lang & Löe 1972), whereas several other have shown that the absence of KG is compatible with the maintenance of periodontal health (Dorfman et al. 1980; Wennström et al. 1981; Wennström 1983; Wennström & Lindhe 1983; Kennedy et al. 1985). However, clinical studies have shown that lack of gingiva may occur in cases when the absence of KG is combined with orthodontic treatment or when vestibular depth is needed in patients with removable partial dentures or with subgingival crown margins in patients with fixed prosthetic restorations (Karring et al. 1971; Rateitschak et al. 1979; De Trey & Bernimoulin 1980; Hangorsky & Bissada 1980).

When dental implants are considered, it has not been demonstrated that the presence of keratinized mucosa (KM), the implant counterpart of KG, is a prognostic factor for their survival (Adell et al. 1986). However, experimental studies in monkeys and humans have demonstrated that the lack of KM increases plaque accumulation around implants, (Warrer et al. 1995; Chung et al. 2006) but the influence of this condition on the survival of the dental implants has not been demonstrated in humans (Chung et al. 2006). Furthermore, Zigdon & Machtei (2008), have shown that both the peri-implant width and thickness of KM may be inversely correlated with recession of the mucosa.

To augment gingival tissue dimensions, the free gingival graft (Sullivan & Atkins 1969) and free connective tissue grafts have been proposed (Edel 1998) with similar clinical predictability (Roccuzzo et al. 2002). Cellular dermal allografts have also been used to overcome the limitation of source of donor's tissue, to reduce morbidity and to allow a better colour and texture integration, but the increase of KM may be less than with autogenous transplant (Wei et al. 2000; Gapski et al. 2005; Park 2006; Moharamzadeh et al. 2007; Thoma et al. 2009). Besides, *in vitro* analysis suggests that fibroblast seeding on to a cellular dermal allograft can allow for favourable conditions for cell adhesion and proliferation (Rodrigues et al. 2010).

The use of a tissue created by seeding oral keratinocytes on collagen membranes of porcine origin, has also been evaluated (Xiong et al. 2008). The fabricated palatal mucosa equivalent may exhibit the characteristics of the native counterpart, but this procedure may be time consuming and expensive.

More recently the use of a collagen matrix of porcine origin has proven to be as effective and predictable as the connective tissue graft for increasing the width of KG and to be associated with a significantly lower patient morbidity (Sanz et al. 2009; Herford et al. 2010; McGuire & Scheyer 2010; Jung et al. 2011).

The objective of this study is to test "*in vitro*" a new collagen matrix of porcine origin (CM-10826) produced by Geistlich Pharma AG (Wolhusen, Switzerland) on seeded cells from three human gingival biopsies of keratinized mucosa. Initially, we investigated if cells growing on the membrane were of epithelial origin and would retain the features of normal keratinized gingiva by evaluating the expression of human MCH class I antigens (MHC-I), EGF receptors (EGFR) and the presence of cytokeratin 14 (Moll et al. 1982; Coulombe et al. 1989) and integrins α6β4 and α3β1 (Giancotti & Ruoslahti 1989; Sastry & Horwitz 1996; Giancotti 1997; Mainiero et al. 1998; Smith & Fuchs 1998). Thereafter, we investigated the effect of CM-10826 on fundamental cell processes as adhesion, IL-6 production and proliferation of keratinocytes. The ultimate aim of the membrane is to be a material suitable for being grafted alone, without any previous treatment, on a recipient site, instead of autologous gingival tissue.

Materials and methods

The experiments were undertaken with the understanding and written consent of each subject and according to ethical principles, including the world medical association Declaration of Helsinki (2008) and was independently reviewed and approved by the ethical board of the University of Verona (protocol number 6640, project number 1085).

CM-10826 membrane, chemicals and antibodies

Collagen matrix 10826 made up of Collagen I and III obtained from healthy pigs was kindly provided by Geistlich Pharma AG. Sterility was ensured by gamma irradiation. Media were purchased from Seromed (Berlin, Germany), supplements from Sigma (Milan, Italy) and EGF from Austral Biological (San Ramon, CA, USA). Foetal bovine serum (FBS) was from Celbio (Pero, Milan, Italy); (Fab)2 goat anti-mouse immunoglobulins (Ig), anti-integrin α3, α6 and β1 (CD29) were all from Immunotech (Marseille, France). FITC-labelled (Fab)2 goat anti-mouse Ig were from Becton-Dickinson (San Josè, CA, USA) and mAb B9-12 (anti MCH Class I) were gifted by Dr. R.S. Accolla, University of Pavia at Varese, Italy. mAb 3E1 (anti- β4) was from Calbiochem (La Jolla, CA, USA). Collagen I, collagen IV, fibronectin and laminin, all from pig origin, anti-EGFR and anti-cytokeratin 14 (CK14) were from Sigma.

"*In vitro*" derivation of human gingival keratinocytes long-term cultures

Three independent gingival biopsies were obtained from three independent healthy patients under informed consent. Biopsies were 5–10 mm wide specimens taken from areas of KG and were executed in the Department of Oral and Maxillofacial Surgery of the University of Verona under local anaesthesia.

Primary cultures of gingival keratinocytes were obtained as previously described (Green et al. 1979) with minor modifications. Briefly, tissue specimens were minced and trypsinized (0.05% trypsin/0.01% EDTA) at 37°C for 3 h. Cells were collected every 30 min, pooled, plated onto lethally irradiated 3T3-J2 cells (gift of Prof. H. Green, Harvard Medical School, Boston, MA) at $2.5 \times 10^4/cm^2$ and cultured in humidified atmosphere of 5% CO2 in DMEN (Dulbecco's Modified Eagle's Medium) e Ham's F12 medium (2:1) with 10% FCS (Foetal Calf Serum), insulin (5 μg/ml), transferrin (5 μg/ml), adenine (0.18 nM), hydrocortisone (0.4 μg/ml), choleric toxin (0.1 nM), triiodotironin (2 nM), EGF (epithelial growth factor) (10 ng/ml), glutamine (4 mM) and penicillin-streptomycin (50 IU/ml). Primary cultures were expanded into secondary cultures by plating on feeder-layer cells before performing experiments.

Immunofluorescence and immunohistochemistry

Immunofluorescence analysis was performed according to standard techniques by using FACScantocytofluorimeter (BD Bioscience, San Jose, CA, USA). Samples stained with mouse monoclonal antibodies anti-integrins were mounted onto a glass cover coverlid and examined using an Axioscope microscope equipped for epifluorescence (Zeiss, Oberkochen, Germany). Images were recorded using the Noah imaging software (Euroimmun, Munich, Germany) and files assembled with Adobe Photoshop 5.5 (Adobe System Inc., Mountain View, CA, USA).

Cell treatments

To perform adhesion tests keratinocytes were detached by trypsin-EDTA treatment and maintained in suspension (0.5×10^6 in

1.5 ml of complete medium) for 6 h in the incubator with or without fragments of CM-10826 (3–5 mm^2). Conical tubes were used to avoid keratinocyte adhesion to the plastic. Samples were stopped at 1, 3 and 6 h to number adherent cells and to evaluate cell viability by Trypan Blue exclusion. For IL-6 production assays keratinocytes were plated at 0.5×10^6 cell/well on 24 wells microtiter plates previously coated with collagen I or collagen IV, or fibronectin or laminin or CM-10826. CM-10826 was carefully cut to fit the well. Casein represented the experimental control. Coating was carried by incubating the plates for 18 h at 4°C with 10 μg/ml of either protein in PBS. Unbound proteins were extensively washed out with PBS. After 24 h of culture the various supernatants were collected, cell-cleared by centrifugation at 258 *g* for 20 min and stored at −20°C. Adherent keratinocytes were lysed for 30 min on ice with a buffer composed of Tris 50 mM pH 7.5, NaCl 150 mM, Na pyrophosphate 2.5 mM, EDTA at 1% NP40 and protease inhibitors at 1 mM concentration. Lysates were cleared by micro centrifugation at 4°C for 10 min, supernatants were aliquoted and stored at −80°C. Total proteins recovered from each well were quantified by Coomassie protein assay reagent (Pierce, Rockford, IL, USA). IL-6 concentration in cell-free supernatants was measured with an ELISA kit used according to the manufacturer's instructions (Endogen, Woburn, MA, USA). Optical density (OD) values were plotted onto a standard curve and expressed as pg/ml. Having measured the volume of cell-free supernatants of each sample, IL-6 could be re-express as total IL-6 for each and then calculated as pg IL-6 or CCL5/mg of protein lysate. Proliferation assays were performed by plating keratinocytes (2×10^4 cell/well) in flat bottom 96-well microtiter plates in complete medium in the presence or absence of the various ECM proteins coated as for IL-6 experiments. Cultures were pulsed for 6H with 1μCi [methyl-^{3}H] thymidine (^{3}H-TdR) (6.7 Ci/mmol) from NEN Dupont (Boston, MA, USA). Cells were harvested onto glass-fibre filters and radioactivity measured in a beta-spectrometer. Results were expressed as the percentage of incorporation of the untreated control.

Statistical analysis

Results were obtained in three independent experiments performed in duplicate, or, in the case of thymidine incorporation, in triplicate, and analysed by paired *t*-test after checking for the normality assumption. A total of 18 stimulated cultures (i.e. with CM-10826) and 18 untreated control (i.e. without CM-10826) were paired for all the biological assays. Significance was considered when $P < 0.05$.

Results

Physical and chemical features of CM-10826

CM-10826 is classified as a implantable, resorbable medical device class II, allowed to permanent contact with the bone and the periosteum. Cellular components and other molecules are removed with a standardized purification process, whereas collagen is kept in a native form. Collagen fibres are made up of 50–80 nm fibrils. As shown in Fig. 1 the membrane is build up as 3D-matrix with two juxtaposed layers: soft stratum is structured with open pore sponge system in which pores are organized in a 3-D network with a diameter ranging from 40 to 60 μm; hard stratum is composed of compact collagen fibres which allow suturing procedures. The micropores width is intended to facilitate fibroblast migration, angiogenesis and to stimulate resident keratinocytes' growth (Rutkowski 2006).

Biological features of human gingival keratinocytes cultures

As shown in Fig. 2a cultured keratinocytes maintained "*in vitro*" the ability to form cohesive monolayers. As shown in Fig. 2b, results of FACS analysis showed that virtually all these cells co-expressed high amounts of human MHC-I, EGFR and cytokeratine CK-14 (CK14) together with α3 integrin, partner of β1 chain in the formation of the fibronectin adhesion receptor α3β1, and α6 integrin, partner of β4 chain in the formation of the laminin receptor α6β4. Moreover, as shown in Fig. 2c cell distribution of the fibronectin and the laminin receptors (α3β1 and α6β4 integrin heterodimers) was similar to that displayed by normal epithelia "*in vivo*" (De Luca et al. 1990).

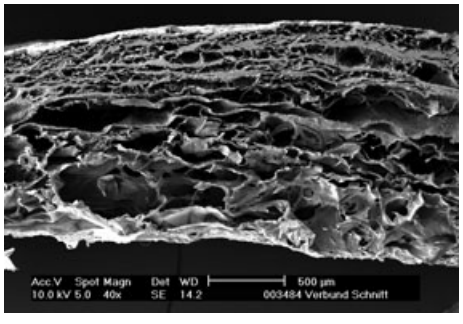

Fig. 1. Electron-micro photography of new collagen matrix 10826 showing the hard layer and soft layer (Kindly supplied by Geistlich Pharma AG Wolhusen, Switzerland).

Gingival keratinocytes adhere to CM-10826

Biological outcomes of the CM-10826/keratinocyte interaction were examined. As shown in Fig. 3a keratinocytes adhered to CM-10826 in a time-dependent manner, from the 25% observed at 1 h to the 60% at 3 h and almost the 70% ($67 \pm 5\%$, $P < 0.05$) of the starting population attached to Collagen Matrix 10826 6 h later following the first contact. The CM-10826/keratinocytes adhesion was further examined by thin layer microscopy (Fig. 3b,c). Keratinocytes adhered firmly to CM-10826, grow on the surface, spread their large cytoplasm and interconnect with neighbouring elements.

Gingival keratinocytes increase their IL-6 production in the presence of CM-10826 and proliferate in the presence of collagen I, a major component of this device

It is known that cell adhesion activates intracellular signalling pathways governing a multiplicity of cellular processes including the gene expression of cytokines, such as IL-6 (Ramarli et al. 1998). As evident from Fig. 4a the basal IL-6 production was doubled in the presence of CM-10826, strongly increased in the presence of a major component of CM-10826, whereas collagen IV appeared almost unaffected. Collagen I was also found to act as the best stimulator of proliferation of keratinocytes, measured by thymidine uptake (Fig. 4b). CM-10826 could not be directly tested in proliferation assays because it was impossible to melt it in the reagent required to measure the H3-thymidine incorporation without quenching the results. However, the pro-proliferative activity of Collagen I strongly suggests that the CM-10826 may act in the same way.

Discussion

The aim of our study was to evaluate the activity of a newly engineered collagen membrane Collagen Matrix 10826 (CM-10826) on fundamental cell functions of human gingival keratinocytes, such as adhesion, IL-6 production and proliferation, and therefore to establish the degree of specific biocompatibility of this new device.

Initially cytofluorimetric and immunohistochemical analysis on keratinocyte cultures were done. They showed the epithelial keratinized phenotype of the cells by the presence of CK14 and EGFR, as confirmed in literature

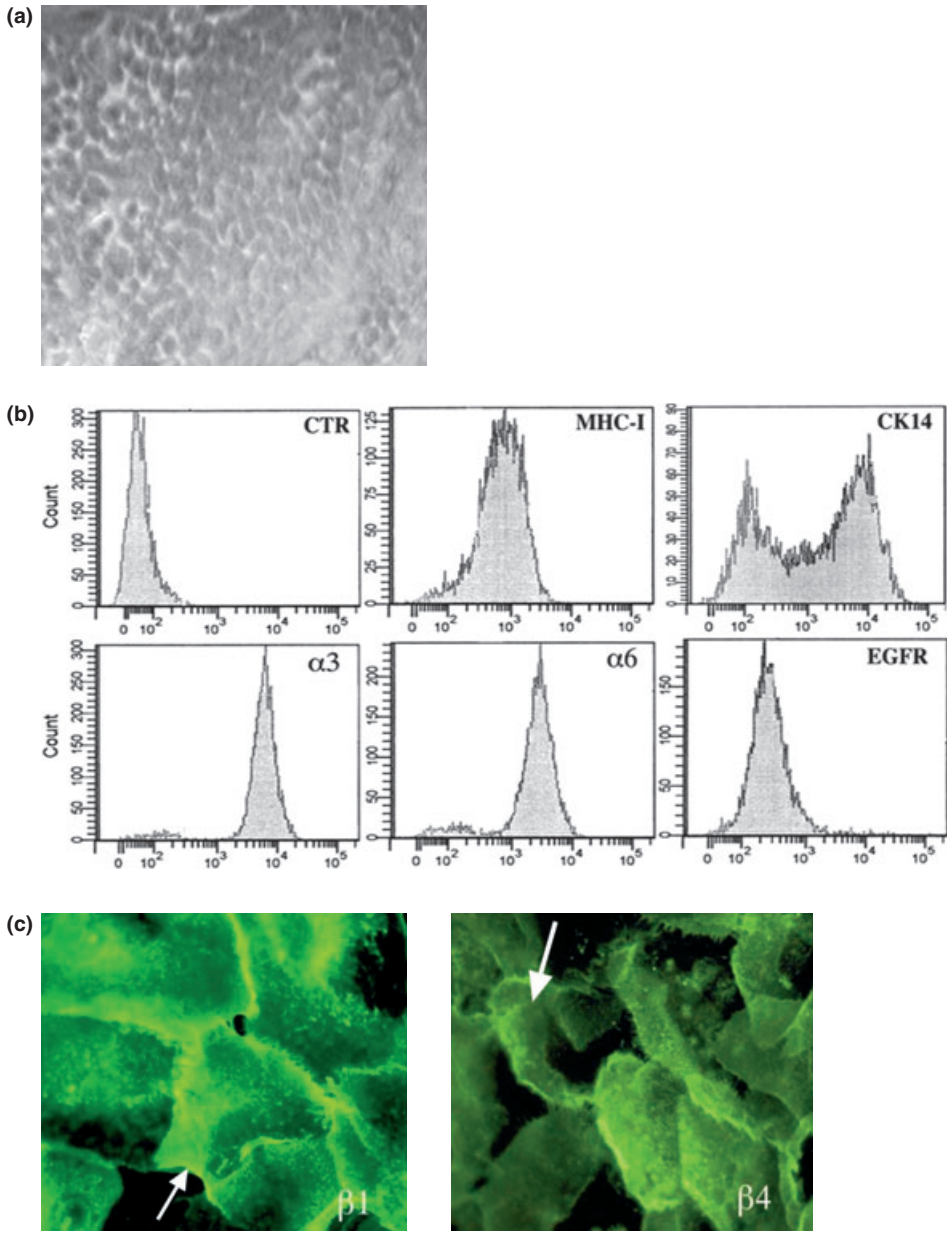

Fig. 2. Representative morphology and phenotype of keratinocytes at the second passage of culture. All results are representative of three independent experiments. (a) Cohesive monolayer of gingival keratinocytes examined at the inverted microscope (20× magnification). (b) Phenotype assessed by FACS analysis considering MHC-class I molecules (MHC-I), α3 (3) or α6 (6) integrands, EGF receptors (EGFR) and cytokeratin (CK14). (c) s1 (1) or s4 (4) expression at the surface of keratinocytes examined at 40× magnification. Arrows indicate peculiar site of localization.

(Moll et al. 1982; Coulombe et al. 1989; De Luca et al. 1990). Secondarily the keratinocytes' capacity of making cohesive monolayers by the expression of fibronectin and laminin receptors, cytokines, chemokines and cell growth factors (De Luca et al. 1990) was also demonstrated. Cell distribution of the fibronectin and the laminin receptors (α3β1 and α6β4 integrin heterodimers) was similar to that displayed by normal epithelia "*in vivo*" (De Luca et al. 1990). Results obtained by thin layer microscopy demonstrated that keratinocytes adhered firmly to CM-10826, grew on the surface, spread their large cytoplasm and interconnects with neighbouring elements.

These results suggested that gingival keratinocytes derived in culture displayed the typical features of normal keratinized epithelium.

In addition, keratinocytes were used as cohesive and homogeneous monolayers to obtain a representation of "*in vivo*" physiological situation which let us perform functional tests as the adhesion, IL-6 cytokine production and keratinocyte proliferation.

Adhesion was efficient as it occurred rapidly and the mortality was low. Collagen matrix 10826 and collagen I adherent cells were stimulated to produce a significant increase of IL-6 their production. Thus, significantly high production of IL-6, an anti-apoptosis molecule, could increase the cell-protection from their spontaneous apoptosis ($212 \pm 32\%$, $P < 0.01$ compared with untreated controls). Moreover, a similar effect was observed in the presence of Collagen I, a major component of Collagen Matrix 10826.

The cell-proliferation test, by tritiated thymidine cell-incorporation measurement (^{3}H-Thymidine), showed a significant increase in proliferation of plated keratino-

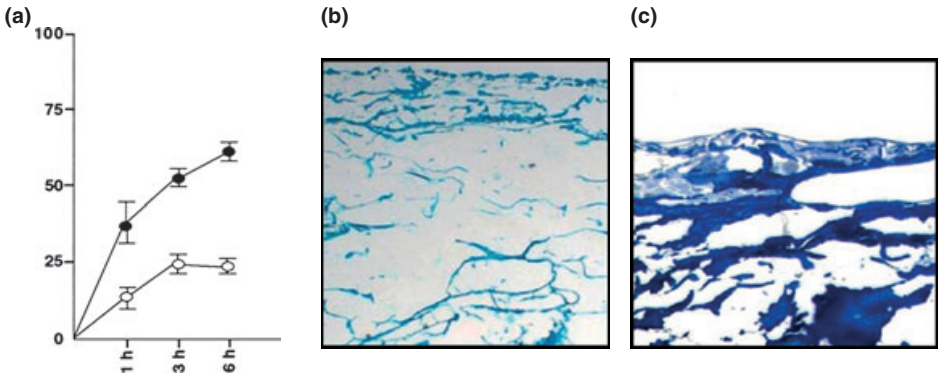

Fig. 3. Adhesion of keratinocytes to CM-10826. (a) Percentage of adhering keratinocytes to CM-10826 at the indicated time points (filled circles). Dying cells at the same time points are also indicated (open circles). Mean ± SD. (b) Optical microscopy of new collagen membrane CM-10826, Gomori's trichrome stain (3,5× magnification). (c) Optical microscopy of keratinocytes spreading into new collagen membrane CM-10826 outer layer, Haematoxylin/Eosin stain (5× magnification).

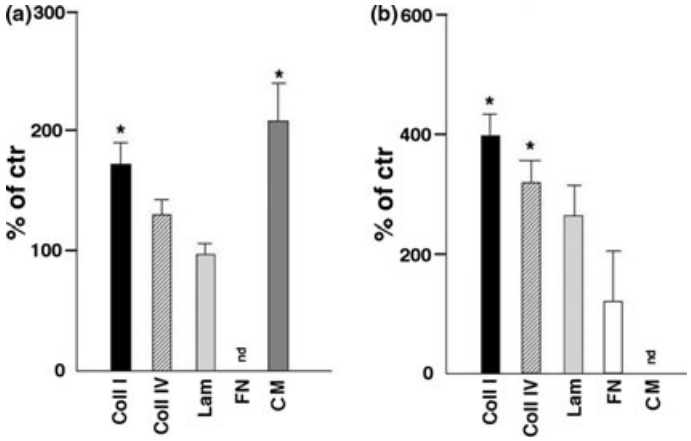

Fig. 4. IL-6 production and proliferation of gingival keratinocytes interacting with CM-10826 or with extra cellular matrix components. Mean values ± SD are shown. *$P < 0.05$. (a) IL-6 released in the supernatants of keratinocytes cultured in the absence or the presence of the indicated ECM proteins or CM-10826. Results were calculated as detailed in the materials and methods and expressed as percentage of the untreated control (46.6 ± 9.4 pg/mg of total cell proteins). (b) Proliferation of keratinocytes cultured in the absence or the presence of Collagen I (Coll I), Collagen IV (Coll IV), Laminin (Lam) or Fibronectin (FN). Results expressed are the percentage of the untreated control (463 ± 89 cpms). Mean values ± SD are shown. All results were obtained by performing at least three independent experiments.

cytes on collagen I and IV. The significant tritiate-thymidine incorporation in keratinocytes during adhesion to collagen I and collagen IV (basal components of collagen matrix 10826) suggested that keratinocyte proliferation on this new membrane is similar (380 ± 52%, $P < 0.001$ compared with untreated controls). Proliferation experiments demonstrated that the best response was observed in the presence of Collagen I, the main component of CM-10826. No undesired effects were observed as for keratinocytes viability, morphology or differentiation.

In summary, CM-10826 demonstrated a positive biological activity on gingival keratinocytes supporting IL-6 production and cell growth and adhesion, without any toxic effect. It appears that CM-10826 alone might function in oral surgery as a substitute for autologous gingival grafts thereby overcoming the limitations of autologous tissue. Besides, the maintenance of the keratinized phenotype of cell-culture may encourage a possible use of this collagen membrane to obtain augmentation of keratinized gingiva. We have tested the effect of CM-10826 on gingival cells "*in vitro*" but the outcomes of this analysis will be part of a wider preclinical study, which is going to test the clinical efficacy of the membrane alone on animals and on patients.

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Conflict of interest and source of funding

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