Construction and characterization of a multilayered gingival keratinocyte culture model: the TURK-U model

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Abstract In construction of epithelial cells as multilayers, the cells are grown submerged to confluence on fibroblast-embedded collagen gels and, then, lifted to air to promote their stratification. We recently demonstrated that gingival epithelial cells form uniform monolayers on semi-permeable nitrocellulose membranes, supported with a semi-solid growth medium, which allows the cells to grow at an air–liquid–solid interface from the beginning of the culturing protocol. In this study, the aim was to further

develop our previous model to form a multilayered gingival epithelial culture model. Two different epithelial cell lines (HaCaT from skin and HMK from gingiva) were used in all experiments. Both cell lines were grown first as monolayers for 3 days. After that, keratinocytes were trypsinized, counted and seeded on a sterile semi-permeable nitrocellulose membrane placed on the top of a semi-solid growth medium, forming an air–liquid–solid interface for the cells to grow. At days 1, 4, and 7, epithelial cells were fixed, embedded in paraffin, and sectioned for routine Hematoxylin-Eosin staining and immunohistochemistry for cytokeratin (Ck). At day 1, HMK cells grew as monolayers, while HaCaT cells stratified forming an epithelium with two to three layers. At day 4, a stratified epithelium in the HMK model had four to five layers and its proliferation continued up to day 7. HaCaT cells formed a dense and weakly proliferating epithelium with three to four layers of stratification at day 4 but the proliferation disappeared at day 7. At all days, both models were strongly positive for Ck5, Ck7, and Ck 19, and weakly positive for Ck10. Gingival epithelial cells stratify successfully on semi-permeable nitrocellulose membranes, supported with a semi-solid growth medium. This technique allows researchers to construct uniform gingival epithelial cell multilayers at an air–liquid–solid interface, without using collagen gels, resulting in a more reproducible method.

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Introduction

The stratified squamous epithelium of gingiva is the first line of defense against constant bacterial accumulation at gingival margins of teeth. It consists of three structurally different sections, the junctional, sulcular, and oral gingival epithelium (Schroeder and Listgarten 1997). The junctional epithelium has a non-keratinized structure with high turnover rate and limited differentiation. It mediates the adhesion of gingiva to tooth surface. The sulcular epithelium (a non-keratinized epithelium) and oral epithelium (an orthokeratinized or parakeratinized epithelium) form a barrier against bacterial challenge by producing antimicrobial peptides, such as beta-defensins (Gursoy and Könönen 2012).

It has been over 40 years since keratinocyte cells were cultured in monolayers for the first time (Rheinwald and Green 1975). Culturing keratinocyte sheets on glass or plastic surfaces allowed researchers to analyze the biology of these cells and their responses against different stimuli, e.g., bacteria or mediators of inflammation. However, performing research using non-differentiating cell monolayers does not reflect the real situation in the oral cavity, because oral epithelial cells proliferate at the basal layer of a stratified epithelium, where daughter cells of the basal cells differentiate toward the surface of the epithelium (Moharamzadeh et al. 2007). Introduction of keratinocyte cultures with stratification was a milestone in cell biology studies. First *in house* multilayer models were constructed on permeable membranes at an air–liquid interface (Breitkreutz et al. 1984; Rosdy and Clauss 1990). These models were successfully used to test invasive characteristics of oral bacteria and *Candida* species (Andrian et al. 2004; Yadev et al. 2011). A gingival epithelium model was later developed with the implementation of gingival fibroblast embedded collagen layer and dental tissue components into keratinocyte multilayer models (Oksanen and Hormia 2002). These three-dimensional tissue engineered oral mucosa equivalents were further developed by implementing oral bacterial biofilms (Gursoy et al. 2012; Belibasakis et al. 2015) or bone tissue (Almela et al. 2016) into the culture models.

Whilst the current multilayer models mimic the gingival tissues relatively well, they are still not actively used in all laboratories. Firstly, they require well trained laboratory staff. Secondly, constructing

an organotypic model is time-consuming, as it takes at least 2–3 weeks before the culture is ready for experiments (Dongari-Bagtzoglou and Kashleva 2006; Moharamzadeh et al. 2012). Thirdly, the use of an organotypic model is expensive, especially when the study protocol includes several test and control groups. Therefore, there is an obvious need for a fast, simple, and reliable method to create epithelial multilayer models for *in vitro* studies. Culturing epithelial cells on floating collagen gels to induce their differentiation is a known method that has been used for long time (Enami and Nandi 1978). The use of permeable supports as scaffolds, such as nitrocellulose membranes, instead of collagen gels allows analyzing of morphological characteristics of the cells without disturbing their integrity (Minchinton et al. 1997). Moreover, the proliferation and differentiation of keratinocytes to form multilayers and the synthesis of keratins have been found to be dependent on cell number density (Ryle et al. 1989). By combining this information, our group recently demonstrated that gingival keratinocytes can survive, form monolayers, and produce adhesion proteins at least for 24 h when they are cultured at high densities on semi-permeable nitrocellulose membranes at a floating position on a semi-solid growth medium (Gursoy et al. 2016). In the present study, we hypothesized that keratinocyte stratification can be accelerated by culturing them at high cell densities at an air–liquid–solid interface, omitting the submerged growing and air lifting steps of common multilayer cell culture techniques. Therefore, our aim was to develop a multilayered cell culture model at an air–liquid–solid interface, using semi-permeable nitrocellulose membranes floating on a semi-solid growth medium.

Materials and methods

In all experiments, human gingival keratinocytes (HMK cell line) (Mäkelä et al. 1999) were cultured in a keratinocyte-SFM medium supplied with pre-qualified human recombinant epidermal growth factor 1–53 and bovine pituitary extract (cat 17005075, Fisher Scientific, Paisley, Scotland) and human skin keratinocytes (HaCaT cell line) (Boukamp et al. 1988) in cell culture media composed of Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Paisley, UK) supplemented with 10 % fetal calf

serum, 1 % essential amino acids, and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml, Fisher Scientific, Bleiswijk, The Netherlands). Both cell lines were maintained as frozen stocks and cultured as monolayers for 3 days. Semipermeable nitrocellulose membranes with a 0.22-µm pore size (GSP02500, Millipore, County Cork, Ireland) were pre-cut to disks of 6 mm in diameter and sterilized. A semi-solid growth medium was produced by mixing the above described culture media with 1.17 % methyl cellulose (M0262, Sigma, St. Louis, MO, USA) and 10 µg/ml fibronectin (F2518, Sigma). The sterile nitrocellulose disks were placed on the semi-solid growth medium in a floating position and 100,000 keratinocytes were seeded on the floating membranes in 5 µl cell culture medium. After 1, 4, and 7 days of incubation at 37 °C, epithelial cells with the membranes were removed, fixed in 4 % formalin for 24 h, and embedded into paraffin for immunohistochemical analyses.

The embedded samples were cut to 5-µm-thick sections and mounted onto slides for immunohistochemistry. The samples were deparaffinized and immunostained for Ki-67 (M7240, Dako, Glostrup, Denmark), cytokeratin (Ck) 5 (MON3029, Sanbio/Monosan, Uden, The Netherlands), Ck7 (M7018, Dako), Ck10 (M7002, Dako), Ck19 (M0888, Dako), and collagen IV (M0785, Dako). Immunostainings were performed by using routine procedures and an automated immunostainer (TechMate, Dako, Glostrup, Denmark). Briefly, antigen retrieval in a microwave twice for 5 min in 1 mmol/l citrate buffer (pH 6.0) was followed by endogenous peroxidase blocking with 3 % H₂O₂. Primary antibodies were detected by a biotinylated secondary antibody, coupled with streptavidin–horseradish peroxidase (HRP), and visualized with 3,3′-diaminobenzidine tetrahydrochloride in HRP buffer. A computer-assisted image analysis system was used to get color images from the areas of interest. The image analysis system comprised a true-color red–green–blue video camera (2088 × 1550 pixels, Leica DC 300 V 2.0, Leica, Wetzlar, Germany) connected to a microscope (Leica DMLB, Leica). High-power images of the sections were acquired and evaluated at a magnification of ×50.

An intensity histogram for each cytokeratin secretion profile was calculated by using the Image J program (NIH, Bethesda, MD, USA).

Statistical analyses

Student's *T* test was used to compare the number of cell layers, number of proliferating cells, and intensities of Ck5, Ck10, and Ck19 between HaCaT and HMK cell lines at each test day.

Results

All experiments were done in triplicates and repeated four times on separate days. The morphological and proliferative differences in multilayer structures of HMK (human gingival keratinocyte cell line) and HaCaT (human skin keratinocyte cell line) cells grown on an air–solid–liquid interface were evident at all three time points of the present study. At day 1, HaCaT keratinocytes stratified and formed a dense and highly proliferative epithelium with two to three layers. At day 4, the thickness of the epithelium did not increase, while the cell proliferation was markedly decreased. At day 7, cell proliferation in the HaCaT multilayer model was lost and cells started to lose their integrity. HMK cells formed a very thin layer (one to two cell layers or only one layer with disconnections) at day 1. At day 4, HMK cells formed homogenous stratification with four to five layers of proliferative epithelial cells. The integrity of the model was still visible at day 7, HMK cells continuing their proliferation (Figs. 1, 2).

The HMK and HaCaT models showed strong Ck5 (Fig. 3) and Ck7 immunostaining at all test days. Ck19 was barely visible in the HMK model at day 1, becoming more evident at days 4 and 7. A weak Ck10 and moderate Ck19 immunopositivity was seen in the HaCaT model as well (Figs. 4, 5). In both models, collagen IV was barely visible at day 1. At day 4, collagen IV became visible at the cell-membrane interface in the HMK model and it remained up to day 7.

The number of cell layers and proliferating cells at region of interest (ROI = 717 µm²) were given for both cell lines at each test day, (Fig. 6a, b). In addition, cytokeratin secretion profiles were quantified by using histograms and given as mean intensity values (Fig. 6c–e). Statistical differences (*p* < 0.05) between HaCaT and HMK cell lines at each day are marked with an asterisk on HMK bar. All antibodies are commonly used in our routine service laboratory for histopathology (University of Turku, Finland), thus their specificity with positive controls were always

Fig. 1 Hematoxylin and eosin staining of the two (HaCaT and HMK) multilayer epithelium models (*scale bar indicates 50 μ m*)

monitored. As a negative control, the primary antibodies were omitted resulting in a negative immunostaining (Fig. 6f).

Discussion

Keratinocyte cell cultures in conventional monolayer models lack of stratification, vertical cell contacts, and differentiation. Thus, monolayers do not offer the opportunity to simulate the gingival epithelium *in vitro*. In current organotypic raft models, fibroblasts are used to regulate the behavior and stratification at

an air–liquid interface (Merne and Syrjnen 2003). Here we demonstrate for the first time that even in the absence of fibroblasts, gingival keratinocytes can develop a reproducible stratified epithelium with several layers within a few days and keep their proliferative character up to day 7.

In the present study two keratinocyte cell lines, HaCaT from skin and HMK from oral mucosa, were used. Both HMK and HaCaT keratinocytes are non-tumorigenic, spontaneously immortalized cell lines and do not contain any human papilloma virus DNA (Boukamp et al. 1988; Turunen et al. 2014). Although the origin of HaCaT cells is skin, this cell line has been

Fig. 2 Ki-67 immunostainings of the multilayer epithelium models. While HaCaT cells lost their proliferative capacity at day 4, cell proliferation in HMK cells was evident at all test points (days 1, 4, and 7) of the study (scale bar indicates 50 μ m)

widely used in multilayered cell culture models of the oral cavity and infection-related responses of this cell line against periodontal pathogens were demonstrated (Gursoy et al. 2010, 2012, 2016; Zeidán-Chuliá et al. 2014). For these reasons, HaCaT cell line was included in the present study and characterized in the model together with HMK cells. As result, behaviors of the two cell lines in our test conditions, especially in cellular proliferation, differed considerably. These differences may be attributed to their genetic origins; gingival keratinocytes have significantly higher proliferation and migration rates in

comparison with skin keratinocytes (Rowat and Squier 1986). Moreover, HaCaT cells have hypotetraploid karyotypes, while HMK cells have total tetraploid karyotypes (Turunen et al. 2014).

The mesenchymal component of epithelia-fibroblast co-culture models regulates morphological and proliferative characteristics of epithelial cells (Merne and Syrjänen 2003). It has been shown that in the absence of fibroblasts, HaCaT skin keratinocytes form a thin layer of epithelium but then lose their proliferation (Stark et al. 2004). This was the case in our study as well, since HaCaT cells formed a thin layer of

Fig. 3 Ck5 immunostaining of HMK and HaCaT cells grown in the novel multilayer epithelium model. A strong Ck5 immunopositivity was evident at all test points (scale bar indicates 50 μ m)

epithelium at day 1 and lost their proliferation already at day 4. On the contrary, gingival keratinocytes formed a relatively thick layer of epithelium at day 4 and continued to proliferate up to day 7. The dependency of keratinocyte multilayers on fibroblasts is a known feature (Atula et al. 1997; Merne and Syrjänen 2003; Stark et al. 2004). Our present results support the hypothesis of Stark et al. (2004) that the variabilities of the origin of keratinocytes and culture methods used affect their growth pattern.

Multilayer cell cultures require matrices or scaffolds, and collagen, agarose, laminin, gelatin, and fibronectin are the most commonly used scaffolds

(Ravi et al. 2015). In the present model, we used a semi-permeable nitrocellulose membrane as a scaffold. Nitrocellulose membranes are cellulose derivatives and their high cytocompatibility towards human cells has been shown (Li et al. 2013). In our model, the semipermeable character of nitrocellulose allows the growth medium to diffuse through the membrane and to feed the epithelial cells while floating on the semi-solid medium and keeping the cells in contact to air. Moreover, the three-dimensional structure of the porous nitrocellulose membrane provides a greater binding capacity to epithelial cells (Li et al. 2013).

Fig. 4 Ck10 immunostaining of HMK and HaCat cells grown in the multilayer epithelium model. In both models, Ck10 immunostaining was weakly visible especially at day 4

Several tests were performed during the development process of the present model. For example, keratinocytes were grown with different cell densities on membranes, semi-solid culture media were prepared in the absence of fibronectin, or, in the replacement of fibronectin with laminin-332. The results of these preliminary tests revealed that the presence of fibronectin in the semi-solid growth medium and the number of cells seeded on the membrane are of major importance. In the absence of fibronectin, both HaCaT and HMK cells did not spread on the membrane. When fibronectin was replaced with laminin-332, the cells produced

multilayers but did not attach to the membrane. It has been reported that methylcellulose (1.13 %) induced irreversible loss of keratinocyte proliferation but the effect could be reversed by fibronectin (Bose et al. 2012). This finding may explain the dependence of our model on fibronectin. Moreover, when less than 100,000 cells were seeded, the cells formed only localized spherical structures on the membrane.

Cytokeratins are known as differentiation markers of keratinocytes, and the origin of the epithelium regulates their expression (Ouhayoun et al. 1985; Haines and Lane 2012). In the present study, both HMK and HaCaT models stained positively for Ck5

Fig. 5 Ck19 immunostaining of HMK and HaCat cells grown in the multilayer epithelium model. Ck19 immunopositivity in the HMK model increased at every test point, while in the HaCaT model it declined after day 4 (scale bar indicates 50 μ m)

and Ck7. While Ck5 is a marker of the stratified epithelium, Ck7 is often found in single epithelia (Haines and Lane 2012). Interestingly, both Ck5 and Ck7 are present in the junctional epithelium (Jiang et al. 2014). Moreover, Ck19, which is considered a major differentiation marker of the junctional epithelium, stains strongly in the HMK model (Jiang et al. 2014). In the present study, Ck10, an early terminal differentiation marker, was visible only in HaCaT model at days 1 and 4. However, a positive Ck10 staining was observed on the nitrocellulose membrane of both models at day 7. The reason for this phenomenon remained unanswered in this study. It

seems that our multilayer HMK model has a typical cytokeratin expression pattern of the junctional epithelium as was demonstrated by Mackenzie et al. (1991).

In conclusion, an immediate culture of gingival keratinocytes in an air–liquid–solid interface gives rise to a uniform and reproducible stratification of gingival epithelial cells. Our model, named as the TURK-U model, has potential to be used in determination of host–bacteria interactions and cell-to-cell communications in in vitro simulations of periodontal health and disease. It remains to be shown whether it is applicable to other epithelial studies as well.

Fig. 6 Quantitative presentations of the number of **a** cell layers and **b** proliferating cells, and histogram analyses of **c** Ck5 expression, **d** Ck10 expression, and **e** Ck19 expression at regions of interest (ROI = 717 μm^2). **f** Negative staining of the HMK model is also given (scale bar indicates 50 μm). Statistical differences ($p < 0.05$) between HaCaT and HMK cell lines at each test day are marked with asterisk

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