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PERFUSION CULTURE PROMOTES DIFFERENTIATION OF ORAL KERATINOCYTES *IN VITRO*

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In order to reconstruct the mucosal lining of the oral cavity tissue engineered autologous mucosa grafts could be of great benefit. In conventional stagnant cultures cells often tend to dedifferentiate. Perfusion culture has been demonstrated to reestablish differentiation in various epithelial cell types. Thus, in secondary cultures of human oral keratinocytes from ten patients conventional stagnant culture versus perfusion culture technique was compared. Proliferation and state of differentiation as expressed morphologically and immunohistochemically were assessed. After 14 days oral keratinocytes in a perfusion culture system tend to be further differentiated. They build up a thicker epithelium (3.4 ± 1.0 *vs*. 2.4 ± 0.4 layers), form microridges, express cytokeratins 1, 2, 10, 11, 19 but not 13 in all cells and cytokeratins 5, 6 particularly in cells attached to the carrier membrane. In contrast, oral keratinocytes in stagnant culture do not form microridges and rather express cytokeratins 13, 14, and 19 in mattering amounts.

K e y   w o r d s : *gingiva, keratinocytes, perfusion cell culture, cell differentiation, cytokeratins, cell proliferation, intracellular matrix*

INTRODUCTION

Human oral keratinocytes have been studied *in vitro* (1, 2) as well as *in vivo* (*e.g.* autogenous graft in preprosthetic surgery, peri-implant soft tissue management) (3, 4). In spite of technical improvements in tissue engineering of oral mucosa like collagen sheets as carriers for cultured keratinocytes (5) or optimized growth medium for primary culture of human oral keratinocytes (6) cells tend to dedifferentiate *in vitro*, although conventional culture conditions encourage proliferation sufficiently (7, 8). Dedifferentiation could be due to attachment of oral keratinocytes to the artificial surface of polystyrene culture dishes but may be provoked by discontinuous exchange of medium in stagnant cell cultures, too (9). Concentrations of nutrients, toxic metabolites and paracrine factors/cytokines alter rapidly while feeding cells (10) exposing oral keratinocytes to biochemical stress that causes loss of morphological and functional characteristics or generation of atypical marker proteins (11).

Dedifferentiation is less apparent *e.g.* in keratinocyte culture at liquid-air interface (12) or in coculture with fibroblast or endothelium (13-16). Perfusion culture also has been shown to limit dedifferentiation of different epithelial cells (8, 17). Hence, we tested the effect of perfusion culture on proliferation and differentiation of oral keratinocytes, as there is only little comparable experience published so far (18).

MATERIALS AND METHODS

Culture medium

DMEM/F12 medium was prepared as described by LAUER (3, 4): Dulbecco's Modified Eagle Medium/Ham's Nutrient Factor 12, ratio 3:1, (Invitrogen/GIBCO, Karlsruhe, Germany), 10 µg/L epidermal growth factor, 8.5 µg/L cholera toxin, 5 mg/L insulin (Sigma, Steinheim, Germany), 1% (v/v) penicillin/streptomycin and 10 % (v/v) inactivated fetal calf serum (Biochrom AG, Berlin, Germany).

KCSFM/DF Medium consisted of Keratinocyte-SFM (Invitrogen/GIBCO, Karlsruhe, Germany)/ DMEM/F12 Medium, ratio 1:1, 2.5 % (v/v) inactivated fetal calf serum, 1 % (v/v) Penicillin/Streptomycin and 7 % (v/v) HEPES-Buffer (Invitrogen/GIBCO, Karlsruhe, Germany).

Primary keratinocyte culture

Experiments were performed in agreement with the Ethical Commission of the Medical Faculty, University of Dresden. Gingival biopsies of 5 mm in diameter were taken from 10 consenting patients (4 female/6 male, aged 15 to 65 years) undergoing third molar surgery or implant surgery. Biopsies were cut into small pieces and explant cultures initiated in T25-Flasks (Nunc, Wiesbaden, Germany) using DMEM/F12 Medium.

Perfusion culture and stagnant culture

After 14 days of primary culture oral keratinocytes were trypsinized (0.25%/0.02% Trypsin/EDTA, Biochrom AG, Berlin, Germany) and seeded at a concentration of 150000 cells/cm^2 pipetting concentrated single cell suspension onto porous polycarbonate membranes (diameter 13 mm, pore size 3 µm; Corning-Costar, Bodenheim, Germany) mounted in sterile carrier rings (Minucells and Minutissue, Bad Abbach, Germany). With KCSFM/DF Medium added carefully cells became adherent and were left in stagnant culture for 48 hours. Membranes at this stage of cell culture will be referred to as initial secondary culture.

Cell-seeded membrane carriers for perfusion culture were then transferred into a perfusion chamber (Minucells and Minutissue, Bad Abbach, Germany) mounted on a heated plate and covered with a styrofoam hood. Culture was continued for 14 days with continuous flow of KCSFM/DF medium at 0.7 ml/h (IPC-N8 peristaltic pump, Ismatec, Zürich, Switzerland). Membranes at the end of this culture period will be called terminal perfusion culture.

Remaining membrane carriers were kept in stagnant culture for the same time incubating them in 6 well-plates at 37°C, 95% air humidity and 5% CO_2 atmosphere (Heraeus Instruments, Osterode, Germany) the KCSFM/DF medium being changed every 72 hours. Membranes resulting from this culture are named terminal stagnant culture. Cells from each patient were analyzed microscopically (surface view and cross-section) after initial secondary culture, terminal perfusion culture and terminal stagnant culture.

Fixation and embedding

Polycarbonate membranes were stained with hematoxylin/eosin, mounted directly on glass slides and covered with cover slips for rating of proliferation in surface view.

Cells on remaining membranes were fixed in 70% ethanol for 1 hour and dehydrated transferring them through solutions of increasing alcohol concentration (70%, 80%, 90%, 100%; 30 minutes each). Specimens were embedded in methylmethacrylate (Technovit 8100, Kulzer, Wehr, Germany) at 4°C. Semithin sections of 1.5 µm were prepared on coated slides for immunohistochemistry and for counting cell layers in cross sections (19).

Immunohistology for cytokeratins

Epitopes of interest were exposed as described previously (20). In brief, semithin sections were predigested with 0.1% trypsin (Biochrom AG, Berlin, Germany) solution for 15 minutes and rinsed with phosphate buffered saline. Unspecific bindings were blocked with 10% horse serum (Boehringer, Mannheim, Germany). With primary antibodies added (diluted according to manufacturers instructions, at the most 1:5), specimens were incubated for 1 hour at room temperature and then rinsed with phosphate buffered saline. Antibody reactivity on methylmethacrylate embedded tissue sections had been tested previously (20). Slides without primary antibody or with unspecific immunoglobulins added (ICN, Meckenheim, Germany) served as negative controls. Sections were incubated for 1 hour with a secondary biotin-conjugated antibody (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) and rinsed again. Antibody binding sites were revealed by avidin-biotin-conjugated peroxidase (30´) and diaminobenzidine solution (0.05 mg/l DAB/0.05 M Tris-HCl pH 7.3/0.01% H_2O_2, 5´). Specimens were counterstained with hematoxylin for 10 seconds and mounted

in 40% glycerin (Merck, Darmstadt, Germany) in phosphate buffered saline.

Light microscopy

The slides were examined under a BX-61 (Olympus, Japan) and digital images taken using Analysis-software (Soft Imaging Systems, Münster, Germany).

Proliferation and counting of cell layers

Proliferation was rated in surface view as confluent (+++), partially confluent (++), colony-like growth (+) with respect to the surface of entire membranes covered by oral keratinocytes. Cell layers were counted in histological cross sections of membranes evaluating 20 fields of vision of each specimen with mean and standard deviation being calculated.

Scanning electron microscopy

Cells were fixed in 3% buffered paraformaldehyde for 20 minutes. After critical point drying (CPD 030, BAL-TEC GmbH, Witten, Germany) according to the standard protocol using liquid carbon dioxide, and sputtering with gold-palladium in a sputter coater (SCD400, BAL-TEC GmbH, Witten, Germany) probes were examined with a scanning electron microscope (LEO 435 VP, LEO Elektronenmikroskopie GmbH, Oberkochen, Germany).

RESULTS

Proliferation

Oral keratinocytes from initial secondary culture covered the membranes with cell colonies (*Fig. 1a*) that grew either as monolayers or as heaps of cells (*Fig. 1d*). Locally, protrusions of basal cells into the membrane´s pores were visible under the light microscope and more clearly in scanning electron microscopy (*Fig. 2a,b*).

Oral keratinocytes on membranes from terminal stagnant culture showed confluent (n=5), partially confluent (n=2) or colony-like growth (n=3) (*Fig. 1b*). In histological cross sections an average of 2.4±1.0 for the number of cell layers was found (*Fig. 1e*). Cells and nuclei had a flat shape. Their cytoplasm contained numerous vacuoles (*Fig. 1g*). The luminal surface of the cellular coat was smooth. Scanning electron microscopy of confluent areas of the membrane revealed polygonal cobblestone-like cells that adhered tightly to each other (*Fig. 2c*). Numerous microvilli protruded from luminal cell membranes without any specific pattern (*Fig. 2d*).

Oral keratinocytes on membranes from terminal perfusion culture had proliferated better (confluence n=6, partial confluence n=3, colony-like growth n=1) (*Fig. 1c*). The number of cell layers in histological cross section was 3.4±0.4 and hence the keratinocyte coat thicker (*Fig. 1f*). Cell protrusions had invaded many pores of the membrane. Basal cells directly attached to the polycarbonate membrane were of rather cuboid shape with round nuclei, whereas cells of upper layers looked flat with ellipsoidal nuclei. The cells' cytoplasm contained fewer vacuoles (*Fig. 1h*). The luminal surface of the epithelial coat was not as smooth as after stagnant culture as single cells seemed to be attached only loosely to the continuous cell layer underneath. Scanning electron microscopy of such cells revealed long cellular protrusions, microspikes, and a dense microvilli pattern (*Fig. 2e,f*). Microvilli of the continuous cell layer formed plenty of microridges.
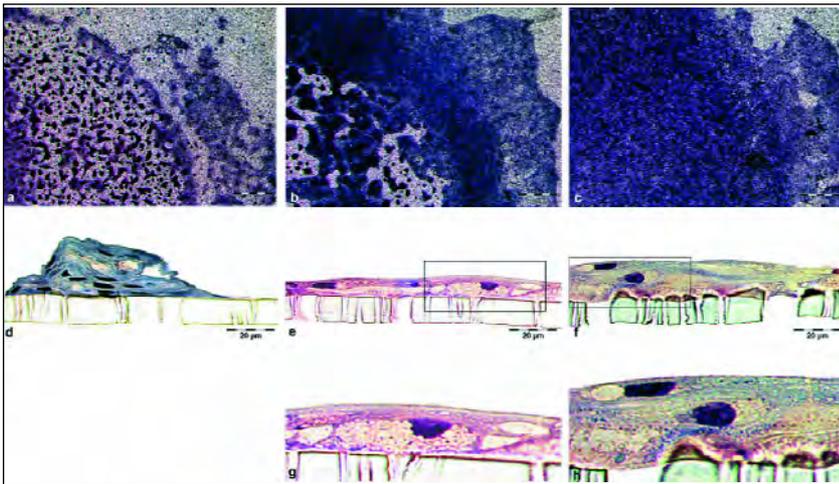
Fig. 1 a - h. Surface view (*a-c*) and semithin cross sections (*d-f; g, h* details of *e, f*) of oral keratinocytes on polycarbonate membranes, HE stain. (*a, d*), initial secondary culture membrane with colony-like distribution of cells and keratinocyte heaps. (*b, e, g*) terminal stagnant culture membrane with a mainly confluent and partially confluent cell layer. The cytoplasm contains numerous vacuoles. (*c, f, h*) Terminal perfusion culture membrane with a confluent cell layer and beginning stratification of the epithelium. The cytoplasm contained only few vacuoles.
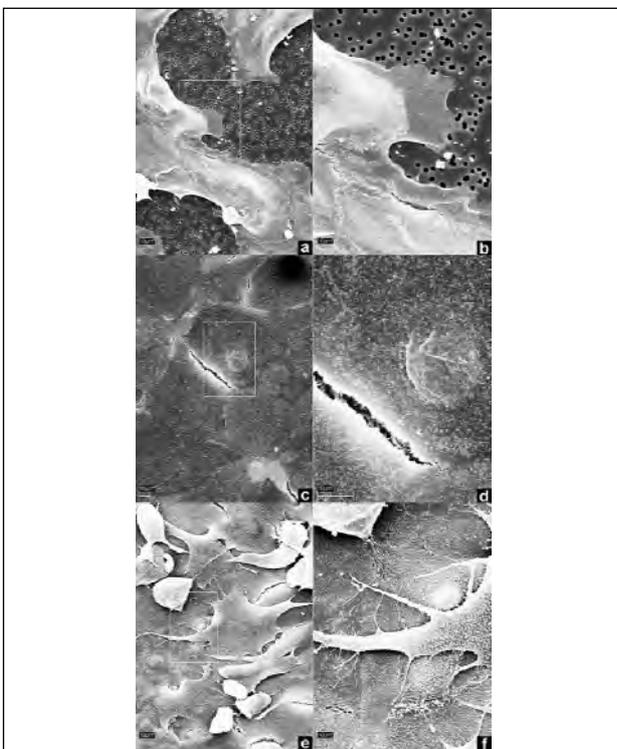

Fig. 2 a - f. Scanning electron microscopy of (*a, b*) oral keratinocytes on initial secondary culture membrane. Cells are spread out on the polycarbonate membrane covering its pores. (*c, d*) Oral keratinocytes on terminal stagnant culture membrane. Polygonal cells in a cobblestone pattern with smooth surface, microvilli protruding from the luminal membranes. (*e, f*) Oral keratinocytes on terminal perfusion culture membrane. Irregular surface pattern with desquamating cells possessing long cell protrusions and microspikes. Microridges as well as microvilli are visible.

Table 1. Antibodies used to detect intracellular matrix components.

Antigen	Antibody Clone	Isotype	Firm	Reference
CK 14	CK B1	IgM	Sigma	(35)
CK 5/6	D5/16B4	IgG1	Boehringer	(36)
CK 13	Ks 13.1	IgG1	Boehringer	(31)
CK 19	CK 170.2.14	IgG1	Boehringer	(37)
CK 1/2 CK 10/11	AE2	IgG1	ICN	(38)

Cytokeratin expression pattern

1. Cytokeratins 1, 2, 10, 11

Cytokeratins 1, 2, 10, 11 are markers of terminal differentiation of cornified epithelium. On initial secondary culture membranes only some irregularly scattered cells reacted positively to anti-cytokeratins 1, 2, 10, 11 (*Fig. 3j*). Expression patterns in oral keratinocytes from terminal perfusion culture and terminal stagnant culture were different. On terminal stagnant culture membranes there were even less cells (particularly those directly attached to the carrier membrane) staining positive for cytokeratins 1, 2, 10, 11 than after initial secondary culture (*Fig. 3k*). However, on terminal perfusion culture membranes the cytoplasm of all cells showed a homogenous positive staining reaction with that of basal cells being a little more intense (*Fig. 3l*).

2. Cytokeratins 5 and 6

Initial secondary culture cells stained positive for cytokeratins 5 and 6 (*Fig. 3a*). Terminal perfusion culture and terminal stagnant culture cells (*Fig. 3b,c*) stained unevenly with formation of dark cytoplasmic granules in some parts of the specimens, particularly those neighboring pores.

3. Cytokeratin 13

Cytokeratin 13 is a marker for suprabasal cells in non-keratinizing epithelia and was strongly expressed and evenly distributed in oral keratinocytes from initial secondary culture as well as from terminal stagnant culture (*Fig. 3d,e*). In terminal perfusion culture membranes, astonishingly however, cytokeratin 13 expression could be detected only in a few basal cells (*Fig. 3f*).

4. Cytokeratin 14

Being present in all initial secondary culture cells (*Fig. 3g*) cytokeratin 14 could be found mainly close to the porous membrane in terminal stagnant culture cells (*Fig. 3h*). Terminal perfusion culture cells hardly expressed any cytokeratin 14, except some single cells next to pores or protruding into pores (*Fig. 3i*).

5. Cytokeratin 19

On initial secondary culture membranes and terminal stagnant culture membranes cytokeratin 19 could be found in
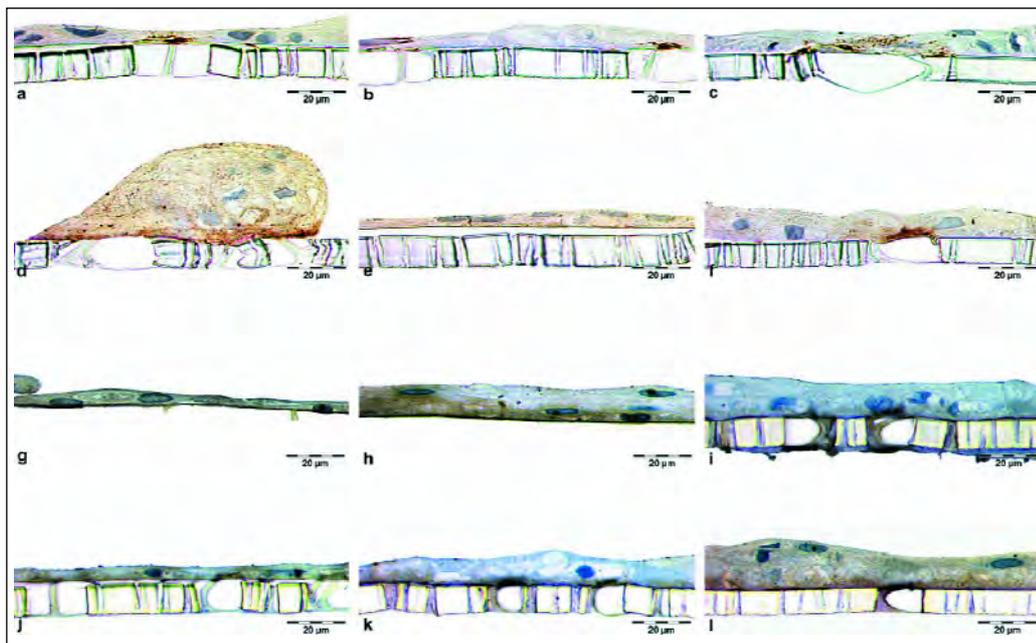
Fig. 3 a - l. Cytokeratin immunohistochemistry of oral keratinocytes on initial secondary culture (*a, d, g, j*), terminal stagnant culture (*b, e, h, k*), and terminal perfusion culture membranes (*c, f, i, l*) for cytokeratins 5 and 6 (*a-c*), cytokeratin 13 (*d-f*), cytokeratin 14 (*g-i*), cytokeratins 1, 2, 10, 11 (*j-l*). See text for details.

most cells. Contrastingly, on terminal perfusion culture membranes not more than very few basal cells close to pores could be stained cytokeratin 19-positive (no images shown).

DISCUSSION

In oral and maxillofacial surgery oral mucosa and gingival grafts regularly needed to cover epithelial defects but sources are limited (21, 29). Tissue engineering of oral soft tissue emerges to be an alternative. However, current culture methods for keratinocytes often lead to loss of cellular differentiation (8). In contrast, perfusion culture technique may help imitate *in vivo* conditions. Along these ideas proliferation of a human oral keratinocytes cell line (OKG4) in three dimensional collagen sponges after 7 days is significantly better under perfusion culture compared to stagnant culture (9.4±1.0 *vs.* 5.0±0.9 cell layers, p<0.005) (18). However, cell differentiation was not examined and moreover, cells from cell lines may behave differently than primary cells from patients (8). Therefore, we compared proliferation and differentiation of oral keratinocytes from ten patients applying either perfusion or conventional stagnant cell culture conditons. Assessment parameters were morphology and cytokeratin pattern.

Both culture methods were not able to generate an oral epithelium with full expression of the organotypical features like stratification with appropriate number of cell layers, cuboidal shape of basal cells or flat shape of cells in upper layers (22). However, oral keratinocytes in perfusion culture formed microridges suggesting a higher degree of gingival differentiation as seen *in vivo* (23) and *in vitro* stagnant culture with low percentage FCS medium (24).

The process of cytoplasmic vacuolization of cultured keratinocytes is not yet fully understood. Apart from the hypothesis that these vacuoles may result from impaired fatty acid metabolism of the cells (25) vacuolization has been considered keratinocytes´ answer to limited cellular damage (26), in particular during in vitro culture (27). According to this idea perfusion culture might impose less cellular stress on oral keratinocytes since less cytoplasmic vacuoles were seen in perfusion culture cells.

In vivo, cytokeratins 1, 2, 10, 11 are expressed in suprabasal layers of gingival epithelium, whereas cytokeratins 5 and 6 are found in the basal cell layer (28). In secondary culture oral keratinocytes hardly bound any anti-cytokeratin 1, 2, 10, 11. However, in perfusion but not in stagnant culture oral keratinocytes expressed cytokeratins 1, 2, 10, 11 indicating differentiation. In contrast cytokeratin 5 and 6 staining patterns did not change after applying different culture conditions keratinocytes next to pores staining the strongest.

Cytokeratin 19 present in simple epithelia and in cultured cells (25) is limited to basal cell layers only in mature oral mucosa and gingiva (29). Thus, loss of cytokeratin 19 expression in suprabasal keratinocytes after perfusion culture - in contrast to permanent presence after stagnant culture - suggests an increase in differentiation.

Cytokeratin 13 found in suprabasal cell layers of gingiva and oral mucosa (29), was demonstrated either in all cells (initial secondary culture and terminal stagnant culture) or in few basal cells (terminal perfusion culture). This cytokeratin 13 shift may indicate a more likely commitment to gingival rather than to mucosal keratinocytes due to perfusion culture.

In conclusion, perfusion culture seems to stimulate proliferation and differentiation of human oral keratinocytes. However, as publications (13, 29, 30) stress the crucial role of fibroblasts for keratinocyte differentiation, such co-cultures should therefore be tested in a perfusion culture system using a more detailed analysis of cellular structure and metabolism (*e.g.* by RT-PCR).

Conflict of interests: None declared.

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