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Expression of keratinocyte biomarkers is governed by environmental biomechanics

Philipp Eberwein[a], Thorsten Steinberg[b,*], Simon Schulz[b], Dominik Zimmermann[c], Rosita Accardi[d], David Beck[b], Thomas Reinhard[a], Pascal Tomakidi[b]

[a] Department for Ophthalmology, University Eye Hospital Freiburg, Kilianstrasse 5, 79106 Freiburg, Germany
[b] Department of Oral Biotechnology, Dental School, University Hospital of Freiburg, Hugstetterstrasse 55, 79106 Freiburg, Germany
[c] Laboratory for Process Technology, Department of Microsystems Engineering (IMTEK), University of Freiburg, Georges-Koehler-Allee 103, 79110 Freiburg, Germany
[d] Infections and Cancer Biology Group, International Agency for Research on Cancer (IARC), 150 Cours Albert Thomas, 69372 Lyon Cedex 08, France

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ABSTRACT

In solid body tissues, environmental biomechanics is indispensable for tissue homeostasis. While characteristics of homeostasis include morphogenesis, proliferation and differentiation, the influences through biomechanics in corneal keratinocytes are poorly understood. Here we show for the first time that corneal keratinocytes, established in a defined biomechanical microenvironment of micropatterned soft pillars, exhibit favoritism of late and terminal differentiation at large pillar patterns of 11 μm with matched small 5 μm arrays. At 11 μm, epithelial cells expressed decreased levels of early differentiation marker cytokeratin 19 (KRT19), which was antagonized by an increase in biomarkers of late and terminal differentiation, i.e. cytokeratin 12 (KRT12), involucrin and filaggrin. Keratinocytes showed proper morphogenesis on 5 μm arrays, whereas 11 μm yielded in morphological disorders. While the propensity of keratinocyte proliferation appeared attenuated at large pillar patterns, stem cell marker ABCG2 was weak though homogeneous at 5 μm, but strong at 11 μm. Thus, corneal keratinocytes reveal interference of biomarker expression, morphogenesis and proliferation, which are at least in part characteristics of tissue homeostasis by mechanisms, depending on environmental biomechanics of micropattern-allocated cell adhesion points in vitro.

Introduction

Cells of solid body tissues are capable of feeling and responding to their extracellular environment (Discher et al., 2005). This perception of environmental biomechanics, similar to growth factors induces intracellular signaling cascades, which lead to behavioral cellular responses. These responses, critical in tissue homeostasis and disease, include features such as the development of regular cell morphology, proliferation and differentiation (Ingber, 2003; Stark et al., 2006).

Response to the extracellular environment is determined by the stiffness of the extracellular matrix (ECM), reflected by the elasticity or Young's modulus (D'Sa et al., 2009). This modulus depends on the type of tissue, i.e. whether the matrix constituents harbor cells of soft, e.g. fibroblasts of soft connective tissues, or hard tissues, e.g. mineralized tissues, like cartilage or bone (Discher et al., 2005). Feeling of the biofunctional extracellular environment is based on adhesion of focal contact integrins to ECM molecules, which allocate distinct patterns of nano- and microscaled cell adhesion or anchor points (Mussig et al., 2010; Nicolas and Safran, 2006). By synergism, the ECM's Young's modulus and patterning of cell adhesion points substantiate cornerstones of environmental biomechanics.

In our body tissues, homeostasis is characterized by the controlled balance of cell proliferation and differentiation, whereat in solid tissues, like the corneal epithelium, ECM-driven anchorage-dependent regular cell morphogenesis is mandatory (Li et al., 1999). In epithelia, division or proliferation of stem cells and transient amplifying cells substitutes such cells, which were lost due to terminal differentiation, thereby guaranteeing lifelong maintenance of tissue renewal (Slack, 2000). Similar to skin or the oral cavity epithelia, the corneal epithelium comprises of several cell layers, thereby belonging to the complex stratified epithelia. Unlike skin or the oral cavity, in corneal epithelial differentiation the spatial trajectory of progressively differentiating cells is not reflected by an exclusive bottom-up migration, but rather an out-inside modus, i.e. centripetal from the limbal margins to the cornea center. Consequently, the proliferative compartment of the corneal epithelium localizes preferentially at the limbal margins and in the basal cell layer, but not in parabasal cell layers, like in oral epithelia (Papini

* Corresponding author. Tel.: +49 761 270 4746; fax: +49 761 270 4744.
E-mail address: thorsten.steinberg@uniklinik-freiburg.de (T. Steinberg).

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et al., 2005). Despite these differences, progressive stages of corneal epithelial cell differentiation are characterized by the expression of tissue-specific biomarkers, among which members of the intermediate filament polypeptide family, the cytokeratins (KRT), indicate early and late stages of corneal keratinocyte differentiation. While limbus-associated KRT19 identifies early corneal keratinocyte differentiation (Espana et al., 2004; Schlotzer-Schrehardt and Kruse, 2005), progressive, i.e. later stages are indicated by KRT12 expression (Espana et al., 2004; Li et al., 2008; Liu et al., 1993). Biomarkers of terminal corneal differentiation are involucrin and filaggrin (Adhikary et al., 2004; Tong et al., 2006). As known from skin, involucrin, covalently cross-linked at inner plasma membrane sites, renders a protein scaffold, which in interplay with additional precursor proteins yields in the formation of the cornified envelope (Kalinin et al., 2002). In this envelope, the covalently cross-linked proteins provide reinforcement against external insults (Steinert and Marekov, 1995). Enzymatically, cornified envelope formation is managed by transglutaminase type I activity, with involucrin being a highly efficient enzyme substrate (Lambert et al., 2000).

During cornified envelope formation, transglutaminases are also involved in the cross-linkage of the condensed keratin cytoskeleton upon keratinocyte terminal differentiation. Cytoskeletal condensation is mediated by filaggrin (filament aggregating protein), which rapidly aggregates to the keratin cytoskeleton (Smith et al., 2006). As in other self-renewing tissues, also ocular surface epithelia, including cornea, are suggested to contain "side population" (SP) cells, which display features seen in multiple adult stem cell types. Among these features, efflux of the Hoechst fluorescence dye indicates SP cells, while the efflux driver, the G2 subtype member of the ATP Binding Cassette (ABC) transporter ABCG2, serves as a putative stem cell marker (Budak et al., 2005; Zhou et al., 2001).

In line with keratinocytes of other complex human epithelia, also cells derived from human corneal epithelium can hardly be propagated in long-term in vitro cell cultures, due to the cells' innate limited life span. This problem can be abandoned by immortalization of primary human keratinocytes with viral DNA or distinct viral oncogenes, respectively. As shown by our own group for, one-time infection of human keratinocytes with the E6/E7 oncogenes of the human papilloma virus type 16 (HPV 16) has been proven an established principle for successful and smooth immortalization (Pear et al., 1993), i.e. gain of non-limited in vitro propagation in conjunction with preservation of the cell- and tissue-specific differentiation program (Roesch-Ely et al., 2006).

In this study, we show for the first time that defined environmental biomechanics governs features of cell behavior in immortalized human corneal keratinocytes (IHCK). By comprising large micropatterns of 11 μm, biomechanics revealed favoritism in expression of late and terminal keratinocyte differentiation biomarkers, concomitant with reduced proliferation propensity, increased levels of stem cell marker ABCG2 and less proper morphogenesis. The results suggest that these biomechanical conditions reflect a more inconvenient stressful extracellular microenvironment for IHCK.

Materials and methods

Cell culture and immortalization

After informed consent and approval by the institutional ethics committee of the Medical Faculty, University of Freiburg (Vote number 307/09), primary human corneal keratinocytes were grown as explant culture from the limbus, which remained after keratoplasty procedures and was obtained from the Cornea Eye Bank of Freiburg. Small tissue fragments were grown as explant cultures with the epithelial side downward directed using DME medium (PAA, Cölbe, Germany) supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany), 2 mM L-glutamine (Invitrogen, Karlsruhe, Germany), and antibiotics (kanamycin, 50 μg/ml; Roche, Mannheim, Germany). As soon as the first keratinocytes grew out of the explant, medium was switched to Keratinocyte Growth Medium 2, containing supplements (KGM2, Promo Cell, Germany, Heidelberg) and antibiotics (kanamycin, 50 μg/ml; Sigma–Aldrich, Munich, Germany). After 7–9 days cultivation time under standard cell culture conditions, sub-confluent cells were immortalized using the open reading frames of the E6/E7 oncogenes of the human papillomavirus type 16, according to the protocol established by Pear et al. (1993). After successful immortalization human corneal keratinocytes (IHCK) were propagated in KGM2 under antibiotics (neomycin 100 μg/ml, Sigma–Aldrich, Munich, Germany) selection pressure for a further 40 passages. Following immortalization, IHCK were used for different experimental setups in this study between passage 25 and 30. In the case of all experiments, requiring IHCK establishment on micropillar surfaces, cells were routinely cultured for 72 h (h), while inoculation density was constantly 5×10^4 per surface (growth area 64 mm^2).

Manufacturing of PDMS micropillar surfaces and biofunctionalization

For micropillar interface production, the protocols published by Mussig et al. (2010) were slightly modified, whereat the wafer technology was kindly provided by the group of Prof. Dr. Holger Reincke head of the laboratory of Process Technology of the Department of Microsystem Engineering University Freiburg. In brief, moulds with holes of defined height and diameter were created to achieve a homogeneous thick layer of photoreactive polymers, which defines the structural height onto a solid silicon wafer. The epoxy based SU-8 10 resin (Microresist Technologies, Germany) was spin-coated onto a clean silicon wafer with a defined speed. Then, the solvent was evaporated by a two step baking process. The wafer was exposed to UV light through a customized designed negative chromium mask (Masken Lithographie & Consulting GmbH, Jena, Germany). The photo-initiated ring opening reaction was accomplished at 95 °C on a hotplate. Developing is performed in a propylenglycol–monoethylether–acetate (PGMEA) containing developer (mr-Dev.600, Microresist Technologies, Germany) for 1 min, followed by blow drying with a stream of nitrogen. For generating the micropillar structures, the silicon-based polymer PDMS (polydimethylsiloxane, Dow Corning, Midland, Michigan, USA) was mixed vigorously with a hydrosilane cross-linker in the ratio 10:1. To remove trapped air, the mix was degassed for 30 min under vacuum at 7×10^{-2} mbar. The pillar arrays with different pillar interspaces were fabricated by pressing the moulds upside down onto a drop of PDMS on a 24 mm × 24 mm coverslip, followed by curing for 4 h at 65 °C. After cooling to RT, the substrates were peeled with a razor blade and overlapping PDMS residues were removed under sterile conditions. Each pillar array presented a dimension of 8 mm × 8 mm with a cell growth area of 64 mm^2. The pillars had a height of 15 μm, a diameter of 5 μm and were arranged in squares with pillar interspaces of 5, 7, 9 and 11 μm. For depositing fibronectin (FN) only to the pillar tops, drops (100 μl) of a FN solution (1 mg/ml, Sigma–Aldrich GmbH, München, Germany) in PBS were pipetted carefully to the surfaces and incubated for 30 s at RT. Immediately after withdrawing the drop, the surface was rinsed with PBS, kept moistened with PBS containing 0.05% TWEEN-20 (Sigma–Aldrich GmbH, Munich, Germany) and subsequent overnight incubation with PBS containing 4% bovine serum (BSA, Biochrom, Berlin, Germany) for 12 h. After this biofunctionalization procedure, which limits cell adhesion to the