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Research Article

Substrate elasticity as biomechanical modulator of tissue homeostatic parameters in corneal keratinocytes

Katharina Moers[a], *Thorsten Steinberg*[a,*], *Günther Schlunck*[b], *Thomas Reinhard*[b], *Pascal Tomakidi*[a], *Philipp Eberwein*[b]

[a]*Department of Oral Biotechnology, Dental School, University Medical Center Freiburg, Hugstetterstrasse 55, 79106 Freiburg, Germany*
[b]*University Eye Hospital, University Hospital Freiburg, Kilianstrasse 5, 79106 Freiburg, Germany*

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ABSTRACT

This study aimed at identifying putative modulations of tissue homeostatic parameters in corneal keratinocytes in response to biomechanical cues as basis for innovative cornea biomechanical-tailored biomaterials. Since cornea epithelial biomechanics is already described for contacts on nanostructures, we herein analyzed cell response to mechanical substrate elasticity. Therefore, corneal keratinocytes were established on physiologically-relevant elastic substrates of 40 kPa, 130 kPa but also on non-physiological stiff substrates of 1.74 MPa for 3 days. qPCR revealed that changes in gene expression were only marginal between 40 kPa and 130 kPa, while significant modulations were seen on 1.74 MPa substrates for most tissue-innate biomarkers under study. Gene expression fairly coincided with the protein, with differentiation progression biomarkers involucrin and fillagrin being already significantly increased between elasticities of 40 kPa and 130 kPa. Regarding focal adhesions, reinforcement was seen for ß1 integrin and phospho- p^{125FAK} between 40 kPa and 130 kPa, while from 130 kPa to 1.74 MPa actin redistributed and phospho-p^{125FAK} was strikingly up-regulated. These findings suggest elasticity dependence for differentiation progression and focal adhesion dynamics of corneal keratinocytes, supporting the concept of biomechanics governed regulation of tissue homeostasis. Moreover, this concept in turn can be translated into prospective cornea-tailored biomaterials for therapeutic approaches in ophthalmology.

Introduction

In addition to biochemical molecules such as growth factors and hormones, biomechanical cues render pivotal for tissue homeostasis, since cells of solid body tissues are capable to feel and respond to the biomechanics of their environmental substrate [1]. Herein, patterning of extracellular anchor points for cell adhesion [2,3] and the stiffness of the cells' environmental adhesion substrate also being referred to as substrate rigidity or -elasticity [4] are key players. Hence, biomechanics is able to govern behavior of various cell entities, as exemplified by reports on fibroblasts [5] and also for pluripotent human mesenchymal stem cells [6]. With emphasis on human cornea, the following physiological elasticity moduli (E-Modulus/Young's Modulus) values have been described for tissue matrix interfaces: 109.8 +/− 13.2 kPa for Bowman's layer, i.e. interface between cornea

*Corresponding author. Fax: +49 761 270 47440.
E-mail address: thorsten.steinberg@uniklinik-freiburg.de (T. Steinberg).

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epithelium and cornea stroma, and 50.0+/− 17.8 for Descemet's membrane, i.e. interface between stroma and endothelium, the latter also being in the range of skin and oral mucosa in conjunction with their underlying connective tissues [7,8].

Among the plethora of features substantiating cell behavior, proliferation and differentiation are hallmark-parameters which essentially orchestrate lifelong homeostasis in body tissues, including the cornea epithelium. While in tissues or cultures harboring corneal epithelial keratinocytes, proliferation can be visualized by detection of reliable proliferation markers such as Ki-67 [9], differentiation is indicated by a panel of biomarkers, discriminating progressive stages of keratinocyte maturation. In concordance with other stratified epithelia, corneal keratinocyte maturation can be discriminated morphologically and by the expression of certain biomarkers, among which the intermediate filament cytokeratin (CK) proteins indicate early CK19 and progressive CK12 cell differentiation [10–12]. As in skin and/or keratinized oral mucosa, the cornified envelope constituent involucrin [13] and the keratin filament aggregating protein filaggrin [14] indicate terminal differentiation. Though expressed in a variety of tissues, including several types of stem cells, the plasma membrane decorating protein ABCG2, identified as multidrug transporter in the cancer drug-resistance phenotype, is described as indicator for stem cells in the cornea epithelium as well. Interestingly, currently published data on ABCG2 point to a widened functional range of this molecule, since ABCG2 has been shown to be up-regulated in human embryonic stem cells exposed to different external stress stimuli like physical stress, drugs, and UV exposure [15].

For expressing the above-described proliferation and differentiation biomarkers, adhesion, perceptive feeling and internal processing of corneal keratinocytes in response to an extracellular environmental substrate of adequate or optimally cell convenient biomechanical elasticity is mandatory, as recently described for T cells [16]. Hence, molecules involved in substrate biomechanics-related cell response are also parameters of tissue homeostasis, since they render prerequisites for their establishment and maintenance. In this context, important molecular constituents are cell adhesion-mediating integrin components like the ß1 subunit, found in cell-environmental substrate contacts like those, facilitating keratinocyte-basement membrane interactions [17]. The cell's feeling of the substrate occurs *via* molecules like p^{125FAK}, located in focal cell–matrix-adhesion complexes [18], and involved in integrin-mediated mechano-transduction. Depending on environmental stiffness, the biomechanical stimulus, e.g. reflected by shear stress, promotes actin cytoskeletal deformation, thereby involving p^{125FAK} as an integrin receptor-related molecular switch to modulate cell shape, adhesion and motility [19]. Similar to motility, cell spreading and changes in cell shape are directly linked to the actin cytoskeleton and require focal adhesion dynamics including turn over. Regarding this turn over, recent studies have demonstrated that phosphorylation of p^{125FAK} is also important for the disassembly of such focal contacts, presumably by p^{125FAK}-exclusion from focal adhesions concomitant with its delocalization to membrane ruffles [19–21].

In the context of the aforementioned biomechanical issues with emphasis on patterning of cell adhesion anchor points, cornea-related studies have reported dependence of cell behavior from sub-micron environmental topography on wave-like nanostructures [22], but also on micro-patterned surfaces [10]. With emphasis on the present study, the advantage of 2D-cultures over 3D lies in simplification of the complexity of the 3D-situation. Using this approach, detection of the direct impact of the applied biomechanical cues to corneal keratinocytes is considerably facilitated. For consecutive extrapolation of data elaborated in 2D-monolayer cultures to the 3D-in vivo situation, analysis of cell-behavioral biomarkers, which contribute to tissue homeostasis, appears mandatory. By employing already cell culture proved [23,24], polyacrylamide- and polydimethylsiloxane-derived substrates of various types, i.e. cornea-physiologic but also non-physiologic elasticity, we have therefore aimed at identifying putative modulation of cornea epithelium-innate biomarkers, indispensable for 3D-cornea tissue homeostasis in vivo. Interestingly, clear favoritism on differentiation progression could be observed on substrates at stronger rigidity, while phospho-p^{125FAK}-reinforcement as further intriguing finding provides evidence for accelerated focal adhesion dynamics including turn over, concomitant with actin cytoskeletal redistribution.

Materials and methods

Cell culture and immortalization

Primary human corneal epithelial cells were derived from the corneoscleral rim following keratoplasty procedures and obtained from the Cornea Eye Bank of the University Eye Hospital Freiburg. This proceeding was conducted after informed consent and approval by the institutional ethic committee of the Medical Faculty, University of Freiburg (Vote number 307/09). In detail, small tissue fragments of the rim were grown as explant cultures with the epithelial side directed downward and some tissue pieces were cultivated the other way around 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). Once the first keratinocytes grew out of the explant, corneal epithelial cell culture 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). Following corneal epithelial cell isolation procedure, after a cultivation period of 7–9 days under standard cell culture conditions, subconfluent primary corneal 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. [25]. Use of this modus operandi for keratinocyte immortalization has been shown in our previous studies, to yield cells with prevailing normal diploid chromosome status, and lack of any tumorigenicity, i.e. benign or malignant tumor growth, following xenografting onto athymic nude mice [26]. After successful immortalization human corneal keratinocytes (IHCK) were cultivated in KGM2 under antibiotics (neomycin 100 µg/ml, Sigma Aldrich, Munich, Germany) selection pressure for further 40 passages. For experiments, IHCK were used between passages 100 and 125 and pre-evaluated with respect to their in vivo-like morphology and tissue-specific biomarker expression compared to their primary counterparts (primary human corneal keratinocytes). For IHCK cultivation on stiffness-defined gel substrates, cells were routinely cultivated for 72 hours (h), taking into