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Human corneal epithelial cell response to substrate stiffness

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ABSTRACT

It has been reported that mechanical stimulus can affect cellular behavior. While induced differentiation in stem cells and proliferation and directional migration in fibroblasts are reported as responses to mechanical stimuli, little is known about the response of cells from the cornea. In the present study, we investigated whether changes in substrate stiffness (elastic modulus) affected the behavior of human corneal epithelial cells (HCECs). Polyacrylamide substrates with different elastic moduli (compliant, medium and stiff) were prepared and HCECs were cultured on them. HCEC responses, including cell viability, apoptosis, intercellular adhesion molecule-1 (ICAM-1) expression, integrin-$\alpha_3\beta_1$ expression and changes in cytoskeleton structure (actin fibers) and migratory behavior, were studied. No statistically significant cell activation, as measured by ICAM-1 expression, was observed. However, on compliant substrates, a higher number of cells were found to be apoptotic and disrupted actin fibers were observed. Furthermore, cells displayed a statistically significant lower migration speed on compliant substrates when compared with the stiffer substrates. Thus, corneal epithelial cells respond to changes in substrate stiffness, which may have implications in the understanding and perhaps treatment of corneal diseases, such as keratoconus.

1. Introduction

Chemical signals, such as signaling molecules, growth factors and chemoattractants, can induce a response in cellular behavior, such as differentiation, proliferation and directional migration. It is now well recognized that cells can respond not only to these chemical signals, but also to mechanical signals [1]. Mechanotransduction is the process during which cells detect mechanical stimuli from the environment and convert them into intracellular chemical signals [2]. These mechanical signals can either be a stimulus from the environment that cells are exposed to, such as shear flow on endothelial cells [3,4], or can originate from the mechanical properties of the environment cells live on/in. Substrate stiffness (elastic modulus) has been known to be the primary mechanical property affecting cellular behavior [5,6]. Cameron et al. [7] have recently reported that loss modulus also has an influence on differentiation and proliferation of stem cells.

Mechanotransduction, whether the stimulus is substrate stiffness or mechanical loading of cells both on 2-D substrates and within 3-D matrices, has been reported for different cell lines, such as fibroblasts [5,6,8–11], embryonic [12–14] and mesenchymal [15–17] stem cells, endothelial cells [3,4,18] and cardiac myocytes [19–22]. Various molecular mechanisms have also been suggested to play a role in mechanotransduction in these cell lines, such as signaling through G-membrane proteins [23], changes in focal adhesion proteins binding [24] and signal transduction through gene expression [25].

In the response of cells to substrate stiffness, it is believed that mechanotransduction is an "outside-in and inside-out" mechanism [26]. This signaling loop is completed through cell adhesion spots to the substrate which are called focal adhesions (FAs) [27]. FAs are complex structures that consist of many different molecules, with one of the main components being integrin. Integrin is a heterodimer molecule containing α and β subunits. There are 18 different α subunits and eight different β subunits, allowing for 24 different combinations [28] with different affinities to various extracellular matrix (ECM) molecules; for example, integrin-$\alpha_3\beta_1$ can interact with collagens, laminin and fibronectin [29]. Integrins are shown to connect to ECM ligands on one side and attach to cell cytoskeleton filaments, specifically actin filaments, on the other side through various molecules, such as talin [30]. Focal adhesion full formation or "maturation" is known to depend on mechanical forces, which can be either internal contractile forces or external loads applied at adhesion points [31,32].

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Mechanotransduction studies have generally focused on stem cells to understand the role of environmental mechanical signals in differentiation and on fibroblasts in wound healing and scar formation. Little is currently known about how mechanical stimulus affects corneal epithelial cells, which can be relevant to ocular diseases such as keratoconus (KC), where high enzyme activities result in a mechanically weaker cornea [33,34]. While some studies suggest that corneal epithelial cells have a more elongated morphology in keratoconic corneas [35], it is not clear how the decrease in mechanical stability of the cornea during the disease can affect these cells.

In the present paper, following a preliminary study [36], we further investigated the responses of human corneal epithelial cells (HCECs) to changes in substrate stiffness, which mimics potential changes that HCECs may be exposed to during weakening of the cornea in KC. We hypothesized that decreased stiffness of the substrate may interfere with actin polymerization and cytoskeleton structure. This, in turn, may affect HCEC migration. To thoroughly study the response of HCECs to substrates with varying elastic modulus, a biocompatible synthetic polymer was selected for this proof-of-concept study and cellular behavior was studied from various perspectives. Pelham and Wang [5] first used polyacrylamide (PAAm) gels for mechanotransduction in the late 1990s. Since then, PAAm gels have been extensively studied and used in mechanobiology research [15,37,38] because of their optical clarity, chemical inertness and wide range of mechanical properties. Accordingly, PAAm samples with elastic moduli comparable to human cornea were fabricated and HCECs were cultured on them. Cell viability and apoptosis, cytoskeleton structure, adhesion molecule expression and migratory behavior were then assessed.

2. Materials and methods

2.1. Sample preparation

Several steps are involved in preparing PAAm membranes for cell culture: coverslip activation, membrane fabrication, surface functionalization and ECM protein conjugation to the surface [39].

2.1.1. Coverslip activation

In order to fabricate PAAm-coated coverslips, glass coverslips (No. 1, 22 × 22 mm, VWR, Radnor, PA, USA) were chemically activated to allow the polymer to covalently bond to them. Coverslips were first rinsed with ethanol. After drying, they were immersed in 2% (3-aminopropyl) trimethoxysilane solution (Sigma–Aldrich Canada Co., Oakville, ON, Canada) in isopropanol for 10 min. After four washes in distilled water, coverslips were placed in 1% glutaraldehyde solution (Sigma–Aldrich Canada Co.) in distilled water for 30 min. The coverslips were then washed three times with distilled water and air-dried before membrane fabrication.

2.1.2. PAAm membrane fabrication

Membrane fabrication was started with mixing different concentrations of acrylamide (40% w/v, Bio-rad, Hercules, CA, USA) and bis-acrylamide (2% w/v, Bio-rad) monomers and ultrapure water. Variation in elastic modulus was achieved by changing the concentration of acrylamide in the final specimen. The following sample concentrations were used in this study (all concentrations are vol.%): (1) compliant: 7% acrylamide–0.01% bis-acrylamide; (2) medium: 10% acrylamide–0.01% bis-acrylamide; and (3) stiff: 15% acrylamide–0.01% bis-acrylamide. To initiate the polymerization reaction, 10% ammonium persulfate (APS; Bio-rad) solution in water and tetramethylethylenediamine (TEMED; Bio-rad) were added to the monomer mixture. A small volume of each solution (15 μl) was placed on a microscope slide (VWR, Radnor, PA, USA) immediately following the addition of 10% APS solution and TEMED. An activated coverslip was placed on top of the drop so that the solution spread over the coverslip. These assemblies were left for 15–20 min (depending on concentration; lower concentration samples need more time for polymerization). Following polymerization, assemblies were left in ultrapure water for 30 min before peeling the PAAm-coated coverslip from the microscope slide. To remove any unreacted monomer, all membranes were soaked in ultrapure water overnight before surface functionalization.

2.1.3. Surface functionalization of membranes and ECM coupling to the surface

In order to conjugate the ECM protein (in this study, collagen type I) to PAAm membranes, the surface of the samples was functionalized with a heterobifunctional crosslinker, sulfosuccinimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate (sulfo-SANPAH). Sample surfaces were covered with 2 mg ml^{-1} solutions of sulfo-SANPAH (Thermo Scientific, Rockford, IL, USA) in water and membranes were exposed to a UV light source for 10 min. Samples were then thoroughly washed with distilled water to clean the surface from excess sulfo-SANPAH. Following functionalization, membranes were incubated with 0.05 mg ml^{-1} rat tail collagen type I (Sciencell, Carlsbad, CA, USA) solution at 37 °C for 45 min.

2.2. Atomic force microscopy

Atomic force microscope (AFM) using an XE-100 atomic force microscope (Park Systems, Korea) was employed to measure the elastic modulus of the membranes as an indication of their stiffness. Measurements were performed in water, in contact mode, to prevent drying of the membranes. A spherical-tipped indenter (with a radius of less than 10 nm) was used, and a force–displacement curve was obtained for loading and unloading paths. A Hertzian model was then applied to the curve to determine the elastic modulus, based on the assumption that the material is purely elastic [40,41]. Since PAAm is known to exhibit essentially elastic behaviour [41] in the deformation range applied in the present study, this assumption was considered reasonable.

2.3. Cell culture

HPV-immortalized HCECs, kindly provided by Dr Griffith, were maintained in an incubator with keratinocyte medium (KM; Sciencell) supplemented with keratinocyte growth supplement (KGS; Sciencell) and penstrep (Sciencell) at 37 °C, 5% CO_2 and 95% humidity. the cell culture medium was replaced every 2–3 days. After the PAAm-coated coverslips had been prepared, functionalized and collagen-coated, 4×10^4 HCECs were seeded on these surfaces. Samples were kept in 6-well culture plates at 37 °C, 5% CO_2 and 95% humidity.

2.4. MTT assay

Using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium br omide (MTT; Biotium, Hayward, CA, USA), the viability and proliferation of HCECs were determined when cells were cultured on the substrates with different elastic moduli. Following a 48 h incubation of HCECs on substrates of various stiffnesses, PAAm-coated coverslips were transferred to a new 6-well culture plate (BD Falcon, San Jose, CA, USA) to ensure that only cells on the samples were tested. Cells on both substrates and the initial culture plate wells were incubated overnight at 37 °C with 0.5 mg ml^{-1} MTT solution in warm KM/KGS cell culture medium. To dissolve formazan crystals, isopropanol was added and the absorbance was read at 595 and 650 nm using a microplate photometer (Thermo Scientific, Hudosn, NH, USA). Cell viability on the substrates is reported

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