The influence of substrate topography on the migration of corneal epithelial wound borders

Bernardo Yanez-Soto [a], Sara J. Liliensiek [a], Joshua Z. Gasiorowski [b],
Christopher J. Murphy [c,d], Paul F. Nealey [e,*]

[a] Department of Chemical and Biological Engineering, School of Engineering, University of Wisconsin-Madison, Madison, WI 53706, USA
[b] Department of Biomedical Sciences, Midwestern University, Downers Grove, IL 60515, USA
[c] Department of Veterinary Surgical and Radiological Sciences, School of Veterinary Medicine, University of California Davis, Davis, CA 95616, USA
[d] Department of Ophthalmology and Vision Sciences, School of Medicine, University of California Davis, Davis, CA 95817, USA
[e] Institute for Molecular Engineering, University of Chicago, 5747 South Ellis Avenue, Jones 217, Chicago, IL 60637, USA

ABSTRACT

Currently available artificial corneas can develop post-implant complications including epithelial downgrowth, infection, and stromal melting. The likelihood of developing these disastrous complications could be minimized through improved formation and maintenance of a healthy epithelium covering the implant. We hypothesize that this epithelial formation may be enhanced through the incorporation of native corneal basement membrane biomimetic chemical and physical cues onto the surface of the keratoprosthesis. We fabricated hydrogel substrates molded with topographic features containing specific bio-ligands and developed an *in vitro* wound healing assay. In our experiments, the rate of corneal epithelial wound healing was significantly increased by 50% in hydrogel surfaces containing topographic features, compared to flat surfaces with the same chemical attributes. We determined that this increased healing is not due to enhanced proliferation or increased spreading of the epithelial cells, but to an increased active migration of the epithelial cells. These results show the potential benefit of restructuring and improving the surface of artificial corneas to enhance epithelial coverage and more rapidly restore the formation of a functional epithelium.

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1. Introduction

Worldwide, the supply of corneal donor tissue remains insufficient [1], motivating the design, fabrication and use of artificial corneas as a therapy for corneal disorders that cause visual impairment [2]. The artificial corneas currently available focus mainly on the biointegration of the device into the stromal component, with less emphasis on restoring the epithelial layer of the tissue. Inadequate regeneration of a fully functional epithelial covering can lead to problems such as epithelial downgrowth [3], infection [4] and extrusion due to stromal melting [5]. It has been suggested that in order to reduce the aforementioned problems, the ideal artificial cornea should support the normal processes that allow the formation and maintenance of a stratified epithelium over the implant [6]. We hypothesize that the incorporation of biochemical and biophysical cues that have been previously characterized and quantified from the anterior corneal basement membrane [7] will promote epithelial coverage of the implant.

Corneal epithelial wound healing is a highly organized series of events, where the basement membrane has many functions to help maintain a normal stratified epithelium [8–13]. There are several phases during the corneal epithelial wound healing process, including a lag phase (between wounding and initiation of cell migration) where cells alter their metabolic status; a migration phase to cover the bare surface; a proliferation phase and a differentiation phase, where cells stratify and re-establish multiple layers of distinct cells [9,13,14]. In addition to morphological and behavioral alterations, specific signaling components involved in the reformation of the basement membrane (BM) have been reported. Several molecules have been shown to be upregulated in corneal epithelial cells after injury, including fibronectin [15], collagen [16], and laminin-332 (LN332) [17]. These molecules all serve as potential markers to determine whether biophysical and biochemical cues influence the wound healing process.

* Corresponding author. Tel.: +1 773 702 9143.
E-mail addresses: yanezsoto@ucdavis.edu (B. Yanez-Soto), nealey@uchicago.edu (P.F. Nealey).

Previous research from our group, as well as others, has demonstrated that mimicking chemical and physical aspects of the BM of the cornea [7] influenced behaviors essential to the wound healing process in the corneal epithelium, such as adhesion [18], proliferation [19] and migration [20]. These behaviors suggest that topographic cues will have an effect on important phases of the corneal epithelial wound process, translating into an improved rate of wound healing with subsequent stratification and maintenance of a healthy epithelium.

Topographically-molded PEGDA hydrogels functionalized with the adhesive peptide RGD, a sequence found in wounded corneal epithelium BM [21], can provide human corneal epithelial cells (HCECs) with specific biomimetic cues inspired by the BM [22]. Here, we report the use of topographically and biochemically controlled poly(ethylene glycol) diacrylate (PEGDA) hydrogel substrates as an artificial BM simulant to improve the wound healing and functionality of the corneal epithelium.

2. Materials and methods

2.1. Fabrication of biochemically functionalized PEGDA hydrogel substrates with topographic features

Precursor solutions of 20% (w/w) PEGDA were prepared by dissolving PEGDA 3400 in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH 8.0, with 0.067% (w/v) lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) as the photoinitiator. RGD peptide (Cys–Gly–Gly–Arg–Gly–Asp–Ser–Pro) (UW-Madison Biotechnology Center, WI) was added to the precursor solutions to reach a final concentration of 10 mM. The hydrogel substrates were molded with groove-and-ridge topographic features of different ranges (400 nm, 1400 nm and 4000 nm pitch) or with flat surfaces using a replica-molding technique, as previously reported [22]. Briefly, in a nitrogen atmosphere, a 30 μL drop of the precursor solution was placed on top of a degassed polydimethylsiloxane (PDMS) stamp containing the desired topography with 0.5 mm PDMS spacers. The precursor solution was then covered by a glass coverslip previously treated with 3-(trichlorosilyl) propyl methacrylate (TPM, Sigma-Aldrich, UK) to ensure adhesion of the gels to the surface. The construct was polymerized under UV-light (364 nm for 900 s at 7.0 mW/cm^2) and the PDMS stamps were peeled off, transferring the pattern to the surface of the hydrogel. Hydrogel substrates were sterilized for 24 h by soaking in 5% isopropyl alcohol (IPA) in 1× phosphate buffered saline (PBS, pH 7.2), rinsed for 24 h in 1× PBS and pre-incubated for 2 h in cell culture media for full equilibration.

2.2. AFM imaging of molded hydrogels

To ensure the incorporation of topographic cues, molded hydrogels containing topographic features were imaged by atomic force microscopy (AFM), using a Nanoscope IIIa Multimode scanning probe microscope (Veeco Instruments Inc., CA). Samples were hydrated in 1× PBS for at least 48 h at room temperature, and scanned in a fluid cell in contact mode using a SNL-10 silicon nitride cantilever with a silicon tip (Veeco Probes, CA).

2.3. HCEC culture

HCECs were harvested from human cadaver corneas graciously provided by the Lions Eye Bank of Wisconsin, Madison or the Missouri Lions Eye Bank (Columbia, MO) as previously reported [23]. Cells from two to four corneas were centrifuged and re-suspended in epithelial medium (EP medium). EP medium is composed of a 3:2 ratio of Ham's F12:Dulbeco's Modified Eagle medium (DMEM) (Invitrogen, CA),

supplemented with 2.5% (v/v) fetal bovine serum (FBS), 0.4 μg/mL hydrocortisone, 8.4 ng/mL cholera toxin, 5 μg/mL insulin, 24 μg/mL adenine, 10 ng/mL epidermal growth factor, 100 units penicillin and 100 μg/mL streptomycin [24]. All HCECs were incubated on plates seeded with a feeder layer of Swiss mouse 3T3 fibroblasts previously treated with 4 μg/mL of mitomycin-C for 2 h. HCECs were incubated at 37 °C and 5% CO$_2$ until they reached approximately 70% confluence. HCECs were used between passages 1 and 4.

2.4. Wound healing assay

HCECs at 60–70% confluence were exposed to 0.01% EDTA in 1× PBS for 1 min to allow detachment of feeder cells. For live fluorescence staining, cells were incubated with a 6 μM live dye solution of CellTracker green CMFDA (5-Chloromethyl fluorescein diacetate, Life Technologies, NY) in DMEM for 45 min at 37 °C and 5% CO$_2$ as recommended by the manufacturer. The dye solution was then replaced with fresh EP medium, and cells were incubated for another 45 min at 37 °C and 5% CO$_2$. After live staining, cells were harvested using 0.025% Trypsin/0.01% EDTA, centrifuged and re-suspended in EP medium, and plated at a density of 150,000 cells/cm^2 on hydrogel surfaces around a 5 mm diameter cloning cylinder to act as an 'exclusion zone' (Fig. 1). After incubation for 24 h, the cloning cylinders were removed and the fluorescently labeled cells on the hydrogel samples were imaged at 0 h, 24 h, 48 h and 72 h using a Stemi SV11 dissecting microscope (Zeiss, NY) with a 1.2× objective lens. The area of the wound was measured using AxioVision software version 4.8 (Zeiss, NY). The size of the wound was defined as the radius (R) of the circle of the measured area (A), using the equation $R = (A/\pi)^{1/2}$, and the wound closure was defined as the difference of the radius from the starting point ($\Delta R = R_0 - R$). From the wound closure data, a linear regression was performed, and the slope was reported as the wound healing rate in mm/hour. An experimental set consisted of at least four replicates of each hydrogel substrate. Each experimental set was repeated in triplicate.

2.5. Proliferation assay

The assessment of cell proliferation was measured through the incorporation of 5-ethynyl-2′-deoxyuridine (EdU) with the Click-iT EdU Cell Proliferation Assay Kit (Invitrogen, CA). HCECs were plated using the wound healing method described above. For the starting time point, cells were incubated in 10 μM EdU for 12 h before removal of the cloning cylinders. For subsequent time points (12 h, 24 h and 36 h), the cloning cylinders were removed and HCECs were incubated for 12 h in 10 μM EdU prior to the corresponding time point. At each corresponding time point, cells were fixed and permeabilized and the EdU was stained according to the manufacturer's protocol. The nuclei of cells that replicated in the 12 h period of incubation with EdU were labeled in red. Cells were also stained with 0.1 μg/mL 4′,6-Diamidino-2-phenylindole (DAPI) (Invitrogen, CA) in 1× PBS for 30 min, which permitted the detection of all nuclei (blue).

Fluorescence microscopy was used to quantify the percentage of cells that incorporated EdU in the 12-hour incubation period with respect to the total number of cells. The area of the confluent layer was measured, and the cell density was reported as the total number of cells/area. At least three images were analyzed for each topography in each experiment, and the experiments were repeated in triplicate.

2.6. Immunocytochemistry and analysis of LN332 expression and location

For the purpose of evaluating the migratory status of cells upon epithelial wounding, we analyzed the expression and location of LN332 (also designated as laminin-5) in HCECs. Following each time point, cells were fixed with a 1% para-formaldehyde solution in 1× PBS at room temperature for 20 min. Cells were then permeabilized with 0.1% (w/v) Triton X-100 (Sigma–Aldrich, St Louis, MO) in 1× PBS for 7 min, followed by exposure to a solution of 1% (w/v) bovine serum albumin (Sigma–Aldrich, St Louis, MO) and 1% goat serum (Sigma–Aldrich, St Louis, MO) in
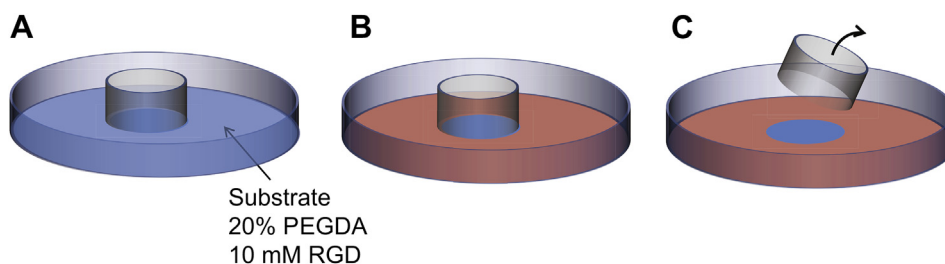

Fig. 1. Wound healing assay for the testing of topographic substrates. A) The bottom of a culture dish is covered with the PEGDA substrates patterned with topographic ridges and grooves. A cloning cylinder is placed on top to act as an exclusion zone. B) HCECs are cultured on the substrates, until confluent. C) The cloning cylinder is removed, creating a simulated wound.