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Tissue and cellular biomechanics during corneal wound injury and repair

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ABSTRACT

Corneal wound healing is an enormously complex process that requires the simultaneous cellular integration of multiple soluble biochemical cues, as well as cellular responses to the intrinsic chemistry and biophysical attributes associated with the matrix of the wound space. Here, we document how the biomechanics of the corneal stroma are altered through the course of wound repair following keratoablative procedures in rabbits. Further we documented the influence that substrate stiffness has on stromal cell mechanics.

Following corneal epithelial debridement, New Zealand white rabbits underwent phototherapeutic keratectomy (PTK) on the right eye (OD). Wound healing was monitored using advanced imaging modalities. Rabbits were euthanized and corneas were harvested at various time points following PTK. Tissues were characterized for biomechanics with atomic force microscopy and with histology to assess inflammation and fibrosis. Factor analysis was performed to determine any discernable patterns in wound healing parameters.

The matrix associated with the wound space was stiffest at 7 days post PTK. The greatest number of inflammatory cells were observed 3 days after wounding. The highest number of myofibroblasts and the greatest degree of fibrosis occurred 21 days after wounding. While all clinical parameters returned to normal values 400 days after wounding, the elastic modulus remained greater than pre-surgical values. Factor analysis demonstrated dynamic remodeling of stroma occurs between days 10 and 42 during corneal stromal wound repair.

Elastic modulus of the anterior corneal stroma is dramatically altered following PTK and its changes coincide initially with the development of edema and inflammation, and later with formation of stromal haze and population of the wound space with myofibroblasts. Factor analysis demonstrates strongest correlation between elastic modulus, myofibroblasts, fibrosis and stromal haze thickness, and between edema and central corneal thickness.

Statement of significance

Tissue biomechanics during the course of corneal wound healing is documented for the first time through atomic force microscopy, and is correlated with advanced clinical imaging and immunohistochemistry. Parameters obtained from the study are applied in a multivariate statistical model to cluster the data for better classification and monitor the wound repair process. Elastic modulus of the anterior corneal stroma is dramatically altered following wounding and correlates initially with the development of edema and inflammation, and later with formation of stromal haze and population of the wound space with myofibroblasts. Importantly, the occurrence of myofibroblasts is preceded by changes in tissue mechanics, which is important to consider in light of crosslinking procedures applied to treat corneal diseases.

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1. Introduction

Corneal opacities are one of the leading causes of blindness worldwide [1]. There is an increasing emphasis on the incorporation of biophysical and biochemical stimuli, intrinsic to tissues, for better implant design [2]. Currently available artificial corneas focus on integration of the device into the existing stromal tissue. For this, a part of the native tissue is removed resulting in a wound. However, little is known about how the intrinsic biophysical microenvironment of the cornea is altered during wound healing, and/or how these changes may influence cell differentiation to in turn predict the success of prosthetic integration [3].

Keratoablative surgical procedures such as laser-assisted *in situ* keratomileusis (LASIK) and photorefractive and phototherapeutic keratectomies (PRK and PTK) that necessitate wounding of the central cornea are widely performed to correct refractive errors and treat anterior stromal disorders [4]. While LASIK largely spares the individual constituents of the anterior cornea, PTK and PRK remove substantial portions of the anterior stroma as well as the epithelium, basement membrane and Bowman's layer. Renewal of an intact epithelium and basement membrane, replenishment of stromal cells, and precise remodeling of stromal collagen fibers and lamellae are some of the main events that are critical for corneal restoration. Upon corneal stromal wounding, significant remodeling of the stroma occurs, thus altering the microenvironment of the wound space to promote transformation of the quiescent keratocyte to the activated fibroblast and subsequently the differentiated myofibroblast (KFM transformation) [5]. Myofibroblasts also arise from differentiation of bone marrow-derived cells that migrate into the corneal stroma following wounding [6]. These events are orchestrated precisely by cross-talk between biophysical and biochemical stimuli, provided by the remodeling matrix as well as the inflammatory, stromal and epithelial cells in the wound environment [7]. Dysregulation of the wound healing process, such as excessive numbers and/or prolonged persistence of activated fibroblasts and myofibroblasts within the remodeling wound space, can result in the formation of stromal haze or scar formation associated with decreased corneal crystalline expression, increased light scatter and production of disorganized extracellular matrix [8,9]. In such situations there is reduced corneal transparency that can lead to clinically significant visual compromise.

While the impact of soluble signaling molecules such as transforming growth factor-β (TGF-β) on corneal wound healing processes are well-studied [10,11], there is a knowledge gap in regards to the participation of biophysical cues in determining wound healing outcomes. This knowledge gap is particularly relevant due to the expanding use of strategies to stabilize the corneal matrix using cross-linking (CXL) which have been reported to stiffen the corneal matrix [12,13]. The use of cross-linking was initially motivated by efforts to slow progression of progressive corneal degenerative diseases such as keratoconus [14–16] but its use has expanded to include treatment of numerous corneal diseases including infectious keratitis [17,18]. Crosslinking is reported to induce anterior keratocyte apoptosis [19–21] and stimulate stromal fibroblast to myofibroblast transformation [19,21].

We have previously demonstrated that biophysical cues profoundly modulate a host of fundamental corneal cell behaviors that are integral to corneal wound healing including adhesion, migration, proliferation, differentiation and response to growth factors [22–29]. Specifically, we have demonstrated that substratum topography [30] and compliance [31] have a marked effect on fibroblast to myofibroblast transformation and are as potent as TGF-β1, the most well-studied soluble signaling factor affecting corneal stromal cells, in modulating KFM transformation. A better understanding of the biophysical signaling environment that participates in the genesis, persistence and subsequent removal of the myofibroblast within the corneal wound space is critical to identifying new strategies for the management of stromal haze and fibrosis. Here, we report the changes in the corneal biophysical environment over the course of wound healing and the role they play in KFM transformation *in situ*.

2. Materials and methods

2.1. Animals

The study design was approved by the Institutional Animal Care and Use Committee of the University of California-Davis and performed according to the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. Thirty New Zealand White female rabbits (3 per group Charles River Laboratories, Wilmington, MA) with a mean ± SD body weight and age of 3.6 ± 0.1 kg and 1.2 ± 0.0 years, respectively, were utilized in this study. A complete ophthalmic examination (slit lamp examination & indirect ophthalmoscopy), applanation tonometry (Tonopen XL, Medtronic, Minneapolis, MN, USA), Fourier-domain optical coherence tomography (FD-OCT; RTVue 100, software version 6.1; Optovue Inc., Fremont, CA, USA), ultrasonic pachymetry (Accupach VI; Accutome Ultrasound Inc., Malvern, PA, USA) and fluorescein staining were performed prior to inclusion into the study; only animals free of ocular disease were used. Applanation tonometry and USP were performed following application of 0.5% proparacaine (Alcon Inc., Fort Worth, TX, USA) to the cornea.

2.2. Excimer laser PTK and post-operative treatment

Rabbits were pre-medicated with midazolam (0.7 mg/kg) and hydromorphone (0.1 mg/kg) administered intramuscularly (IM) followed by ketamine (10–30 mg/kg) IM for induction and maintenance of anesthesia. Following administration of proparacaine hydrochloride 0.5% ophthalmic solution (Bausch and Lomb, Rochester, NY, USA), an 8 mm diameter corneal trephine (MSI Instruments, Pheonixville, PA, USA) was used to mark the central cornea and the epithelium of the right eye (OD) was removed with an excimer spatula (BD Visitec, Franklin Lakes, NJ, USA) within the marked region. An excimer laser (Nidek Excimer Laser Corneal Surgery System EC-5000, Fremont, CA) was used to perform a phototherapeutic keratectomy (PTK) by photoablating the superficial stromal elements of the right cornea (6 mm diameter, 40 Hz, 167 pulses, 100 μm depth) in the center of the epithelial wound. The left eye remained unwounded and served as a control. Rabbits were treated OD with ofloxacin 0.3% ophthalmic solution (Alcon, Hunenberg, Switzerland) twice daily (BID) until re-epithelialization was complete; buprenorphine (0.03–0.06 mg/kg) was administered IM BID for 3–7 days post-wounding to provide analgesia. A slit lamp examination with Modified McDonald-Shadduck scoring, and fluorescein stain was performed daily for the first week post-wounding. A slit lamp examination with Modified McDonald-Shadduck scoring including a semi-quantitative score for stromal haze using a modification of a previously defined system [32], applanation tonometry, FD-OCT, USP, and fluorescein stain were also performed on days 1, 3, 7, 10, 14, 21, 28, 35, 42, and 70 following PTK. Also, a single rabbit was followed for 400 days after wounding.

Fourier-Domain (FD-OCT) imaging (RTVue® 100, software version 6.1; Optovue Inc., Fremont, California, USA; 26,000 A scan/sec, 5 μm axial resolution, 840 nm superluminescent diode) of the central cornea was performed using a corneal adaptor module.