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Research article

Temporal and spatial analysis of stromal cell and extracellular matrix patterning following lamellar keratectomy

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ABSTRACT

Extracellular matrix (ECM) supplies both physical and chemical signals to keratocytes which can impact their differentiation to fibroblasts and/or myofibroblasts. It also provides a substrate through which they migrate during wound repair. We have previously shown that following transcorneal freeze injury (FI), migrating corneal fibroblasts align parallel to the stromal lamellae during wound repopulation. In this study, we compare cell and ECM patterning both within and on top of the stroma at different time points following lamellar keratectomy (LK) in the rabbit. Twelve rabbits received LK in one eye. Rabbits were monitored using in vivo confocal microscopy at 3, 7, 21 and 60 days after injury. A subset of animals was sacrificed at each time point to further investigate cell and matrix patterning. Tissue was fixed and labeled in situ with Alexa Fluor 488 phalloidin (for F-actin), and imaged using multiphoton fluorescence and second harmonic generation (SHG) imaging (for collagen). Immediately following LK, cell death occurred in the corneal stroma directly beneath the injury. At 7 and 21 days after LK, analysis of fluorescence (F-actin) and SHG results (collagen) indicated that fibroblasts were co-aligned with the collagen lamellae within this region. In contrast, stromal cells accumulating on top of the stromal wound bed were randomly arranged, contained more prominent stress fibers, and expressed alpha smooth muscle actin (α-SMA) and fibronectin. At 60 days, cells and matrix in this region had become co-aligned into lamellar-like structures; cells were elongated but did not express stress fibers. Corneal haze measured using in vivo confocal microscopy peaked at 21 days after LK, and was significantly reduced by 60 days. Cell morphology and patterning observed in vivo was similar to that observed in situ. Our results suggest that the topography and alignment of the collagen lamellae direct fibroblast patterning during repopulation of the native stroma after LK injury in the rabbit. In contrast, stromal cells accumulating on top of the stromal wound bed initially align randomly and produce a fibrotic ECM. Remarkably, over time, these cells appear to remodel the ECM to produce a lamellar structure that is similar to the native corneal stroma.

1. Introduction

Stromal keratocytes play a central role in mediating the corneal response to injury or refractive surgery (Netto et al., 2005). During wound healing, quiescent corneal keratocytes surrounding the area of injury generally become activated, proliferate, and transform into a fibroblastic phenotype (Jester et al., 1999c; Stramer et al., 2003). In certain wound types, fibroblasts further differentiate into myofibroblasts, which generate stronger forces and synthesize a disorganized fibrotic extracellular matrix (ECM) (Blalock et al., 2003; Jester et al., 1999a). Following vision correction procedures such as photorefractive keratectomy (PRK) or laser assisted in situ keratomileusis (LASIK), cellular force generation and fibrosis can alter corneal shape and reduce corneal transparency. In addition, a decrease in the concentration of keratocyte-specific "corneal crystallin" proteins has been associated with an increase in cellular light scattering during wound healing, which also contributes to clinical haze (Jester et al., 1999b, 2012). Both PRK and LASIK result in a region of keratocyte death beneath the laser-treated area (Mohan et al., 2000; Møller-Pedersen et al., 1998; Wilson, 2002). Stromal cell death can also be induced by toxic injury (Jester et al., 1998; Maurer et al., 1997) as well as UV cross-linking of the cornea in

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keratoconus patients (Knappe et al., 2011; Mencucci et al., 2010; Wollensak et al., 2004). Ideally, repopulation of damaged stromal tissue following these insults should occur via intra-stromal migration of keratocytes from the surrounding stromal tissue, without generation of contractile forces that could disrupt the collagen architecture or the production of fibrotic ECM which can reduce transparency. Previous work has shown that myofibroblast transformation of corneal keratocytes during wound healing is mediated by transforming growth factor beta (TGF-β) in combination with other growth factors (Chen et al., 2009; Etheredge et al., 2009; Funderburgh et al., 2001; Jester et al., 1995, 1999a, 1999c, 2002); however, less is known about the biochemical and biophysical signals that regulate cell and matrix patterning during wound healing.

We recently used in vivo confocal microscopy to assess keratocyte backscattering, alignment, morphology and connectivity during intra-stromal wound healing, following a full-thickness corneal freeze injury (FI) in the rabbit (Petroll et al., 2015). We also correlated these findings with en bloc 3-D confocal fluorescence imaging of cellular patterning, and second harmonic generation (SHG) imaging of the corneal collagen lamellae. Interestingly, we found that keratocyte alignment during wound repopulation was highly correlated with the structural organization of the lamellae, suggesting contact guidance of intra-stromal cell migration.

Following FI, the epithelial basement membrane remains intact, and stromal healing involves fibroblast migration into the injured tissue, without myofibroblast transformation, fibrosis or matrix remodeling. In contrast, healing following keratectomy wounds in the rabbit has three phases: stromal repopulation (migration), fibrosis, and regeneration and/or remodeling. Specifically, using in vivo confocal microscopy, Jester and coworkers demonstrated that following PRK, corneal fibroblasts migrated into the wounded stromal tissue by 7 days after injury, without transforming into myofibroblasts (Moller-Pedersen et al., 1998). By 21 days, significant sub-epithelial haze, myofibroblast transformation and associated fibrosis were detected. Interestingly, by 6 months, both stromal thickness and haze values returned to near pre-operative levels, suggesting regeneration and/or remodeling of corneal tissue. In addition to changes in cell phenotype and backscatter during stromal wound healing, others have studied ECM organization following keratectomy wounds (Farid et al., 2008; Fitzsimmons et al., 1992; Latvala et al., 1995; Moller-Pedersen et al., 1998; Rawe et al., 1992); however, the temporal and spatial correlation between cell alignment and ECM patterning during the fibrosis, remodeling and/or regeneration phases of wound healing has not been established.

In this study, we investigate the relationships between cell and ECM organization during healing following lamellar keratectomy (LK). Using a custom modified Heidelberg Retinal Tomograph with Rostock Corneal Module (HRT-RCM; Heidelberg Engineering, GmbH, Dossenheim, Germany) in vivo confocal microscope (Petroll et al., 2013), we assess keratocyte patterning, tissue growth, and corneal haze development throughout the full thickness of the cornea at various time points post-injury. Additionally, we use in situ multiphoton fluorescence and SHG imaging to determine the correlation between cell and ECM alignment in the migratory, fibrotic and regenerative/remodeling phases of wound healing (Han et al., 2005; Morishige et al., 2006).

2. Materials and methods

2.1. Animal model

All animal procedures were in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research as well as approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee. 12 New Zealand white rabbits were anesthetized using 50 mg/kg intramuscular ketamine and 5.0 mg/kg xylazine and locally anesthetized in the left eye with 1 drop of proparacaine. LK was performed following anesthesia. A speculum was placed into the left eye and a 150 μm deep incision was made in the peripheral cornea using a diamond knife. A spatula was used at the base of the incision to separate the layers of collagen within the stroma. Once a resection plane was created, a 5 mm trephine punch was used to remove the anterior corneal tissue in the central cornea. Immediately following surgery, 0.3 mg/kg of buprenorphine SR (slow release) was injected. Gentamicin eye drops were administered as an antibiotic in the left eye twice a day for 7 days following injury.

2.2. In vivo confocal microscopy

Rabbits were monitored using an HRT-RCM in vivo confocal microscopy with Confocal Microscopy Through Focusing (CMTF) software for analysis as previously described (Petroll et al., 2013, 2015). Rabbits were scanned 1 week before LK (Pre-Op), and at 3, 7, 21, and 60 days after LK. Rabbits were anesthetized prior to scanning with 50 mg/kg intramuscular ketamine and 5.0 mg/kg xylazine and locally anesthetized in each eye with 1 drop of proparacaine. Since the reflection from the Tomocap can obscure images of the superficial epithelial cells, a thin PMMA (poly(methyl methacrylate)) washer was placed on the Tomocap to eliminate these reflections, as previously described (Zhivov et al., 2009). The objective was positioned on the cornea to create a flat field-of-view image in the central cornea. CMTF scans were collected by starting the scan in the anterior chamber and finishing above the epithelium with a constant speed of 60 μm per second. Images were acquired with the rate set to 30 frames per second. To allow quantitative assessment of haze, scans were collected using a constant gain setting, by unchecking the “auto brightness” box in the HRT software interface. Each scan was conducted using a gain of 6, which was set by moving the horizontal slider under the “auto brightness” box six mouse clicks to the right. At least 3 scans were collected within the central area of the cornea where LK was performed. Each scan contained a 3-D stack of 384 × 384-pixel images (400 × 400 μm), with a step size of approximately 2 μm between images. Additional scans were collected closer to the wound edge in some animals. In some cases, a portion of the scan was saturated when using manual gain settings due to strong cell/matrix reflectivity. In these cases, additional scans were taken using the “auto brightness” enabled so that changes in cell patterning and morphology could be documented. Only the scans taken with a gain of 6 were used for quantitative analysis.

After image acquisition, scans were saved as “.vol” files, which could be opened into our in-house CMTF software to analyze the 3-D changes in cell morphology and cell/ECM reflectivity (Petroll et al., 2013). The program generates an intensity vs. depth curve, corresponding to the average pixel intensity of each image and the z-depth of that image within the scan, respectively. The relative amount of backscatter, or haze, associated with the stromal keratocytes and ECM was measured by taking the area under the curve between the location of the basal lamina peak (top of the stroma) and the endothelial peak. A baseline of 13 was chosen for haze calculations, since this value was below the baseline intensity for the normal stroma and above the intensity of the anterior chamber. The thicknesses for the epithelial and stroma layer were also calculated by the CMTF program using the interfaces (peaks on CMTF curve) between each layer in the cornea.