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Hypoxia modulates the development of a corneal stromal matrix model

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ABSTRACT

Deposition of matrix proteins during development and repair is critical to the transparency of the cornea. While many cells respond to a hypoxic state that can occur in a tumor, the cornea is exposed to hypoxia during development prior to eyelid opening and during the diurnal sleep cycle where oxygen levels can drop from 21% to 8%. In this study, we used 2 three-dimensional (3-D) models to examine how stromal cells respond to periods of acute hypoxic states. The first model, a stromal construct model, is a 3-D stroma-like construct that consists of human corneal fibroblasts (HCFs) stimulated by a stable form of ascorbate for 1, 2, and 4 weeks to self-assemble their own extracellular matrix. The second model, a corneal organ culture model, is a corneal wound-healing model, which consists of wounded adult rat corneas that were removed and placed in culture to heal. Both models were exposed to either normoxic or hypoxic conditions for varying time periods, and the expression and/or localization of matrix proteins was assessed. No significant changes were detected in Type V collagen, which is associated with Type I collagen fibrils; however, significant changes were detected in the expression of both the small leucine-rich repeating proteoglycans and the larger heparan sulfate proteoglycan, perlecan. Also, hypoxia decreased both the number of Cuprolinic blue-positive glycosaminoglycan chains along collagen fibrils and Sulfatase 1, which modulates the effect of heparan sulfate by removing the 6-O-sulfate groups. In the stromal construct model, alterations were seen in fibronectin, similar to those that occur in development and after injury. These changes in fibronectin after injury were accompanied by changes in proteoglycans. Together these findings indicate that acute hypoxic changes alter the physiology of the cornea, and these models will allow us to manipulate the conditions in the extracellular environment in order to study corneal development and trauma.

1. Introduction

The cornea is exposed to a deficit in oxygen levels, known as hypoxia, during development, the sleep portion of the sleep/awake diurnal cycle after eyelid opening, and wound healing at the site of tissue damage. The cornea is a unique tissue in that it is avascular and transparent. To maintain this transparency, some of the conditions that are required are as follows: the proper balance in nutrient supply and diffusion of atmospheric oxygen to maintain an oxygen tension of 21%. When the cornea is damaged, corneal cells attempt to increase oxygen delivery in order to facilitate wound repair. Upon sleeping, the oxygen level is altered, often dropping to ~8%, and the carbon dioxide levels rise, thus causing the microenvironment to become acidic (Liesegang, 2002). It is speculated that rapid eye movements and the occasional eye openings during sleep cycles attenuate the effects of hypoxia, enabling corneal swelling to dissipate within an hour of awakening (Morgan et al., 2010). In addition to development and injury, disease or pathology can also induce a hypoxic state (Boost et al., 2017; Kanda et al., 2017; Kim et al., 2017; Sanyal et al., 2017).

During development, proteoglycan expression first appears in the anterior corneal stroma; however, as development progresses and lid opening approaches, the proteoglycan expression localizes in the posterior stroma (Cintron and Covington, 1990; Gregory et al., 1988). The glycoprotein, fibronectin, is detected as fine lines within the corneal stroma in early development and remains through birth; however, in the unwounded adult cornea, it is absent (Cintron et al., 1984).

Abbreviations: F-actin, filamentous actin; Sulf1, sulfatase 1; LOX1, lysyl oxidase; SMA, smooth muscle actin; TGF-beta1, transforming growth factor beta 1; GAG, glycosaminoglycan; DMEM, Dulbecco's modified Eagles medium; EMEM, Eagle's minimum essential medium; TEM, transmission electron microscopy; PCR, polymerase chain reaction

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Interestingly after corneal injury, fibronectin protein is transiently expressed, thus supporting the premise that the response to injury bears similarities to development (Zieske et al., 1987). Likewise, as corneal organization and clarity return after injury, so do the proteoglycan levels, which resemble those that occur in unwounded adult corneas (Hassell et al., 1983). In addition, injury that alters proteoglycan profiles and properties are associated with changes in the organization of collagen fibrils (Brown et al., 1999; Connon and Meek, 2003; Funderburgh et al., 1988).

Since it is a relatively simple tissue, the cornea is an excellent model to study collagen-proteoglycan interactions. The development of the three-dimensional (3-D) stroma-like construct model (stromal construct model) demonstrates that assembly of the stromal matrix can be followed and manipulated (Karamichos et al., 2011a). In addition, the corneal organ culture model allows us to examine extracellular matrix (ECM) deposition and wound repair in a similar 3-D configuration as in the stroma of the cornea (Minns and Trinkaus-Randall, 2016). As the stromal matrix is constantly in flux, the changes in oxygen tension throughout the day due to the diurnal cycle may contribute to these differences by altering the protein expression in the ECM. Therefore, in this study, we tested the effect of hypoxia on the secretion and assembly of ECM proteins in the cornea using both models.

In this study, we demonstrated that short-term exposure to hypoxia altered the deposition and expression of matrix proteins, such as fibronectin, collagens, and proteoglycans. In addition, there was a decrease in the expression of sulfatase 1 (Sulf1), which modulates changes in sulfation of glycosaminoglycan chains. In the corneal organ culture model, there was a change in the deposition of fibronectin that was similar to development along with a delay in migration. These responses allow us to predict changes that occur in trauma and development when the intracellular environment is altered.

2. Materials and methods

2.1. Corneal organ culture model

A corneal organ culture model was employed, and all studies were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research (Gordon et al., 2010; Lee et al., 2014; Minns and Trinkaus-Randall, 2016). Briefly, Sprague-Dawley rats (Charles River Labs; Wilmington, MA) were euthanized, a 3 mm-diameter epithelial debridement was made, the corneas with scleral rims were removed, and the endothelial side of the corneas were filled with DMEM containing 0.75% low melting point agar. The corneas were placed in 35 mm^2-culture dishes epithelial side up and cultured in DMEM supplemented with 100u/ml penicillin/streptomycin and 100X MEM-Non-essential amino acids (Mediatech; Manassas, VA) at 35 °C for up to 24 h under either normoxic or hypoxic conditions.

2.2. Formation of stromal construct model

The stromal construct models were formed using primary human corneal fibroblasts (HCFs) isolated from human corneas obtained from the National Disease Research Interchange (NDRI; Philadelphia, PA), as described (Guo et al., 2007; Ren et al., 2008). All procedures/methods used in these studies adhered to the tenets of the Declaration of Helsinki, and the study's experimental protocols were judged to be exempt from review by the Institutional Human Studies Committees at the Schepens Eye Research Institute/Mass. Eye and Ear, Harvard Medical School and the Boston University School of Medicine. Briefly, corneal epithelium and endothelium were removed from the donor corneas, and the remaining stromal tissue was cultured as explants. Cells were passaged and plated onto 6-well transwell plates with polycarbonate membrane inserts (0.4-micron pores: Corning Costar; Charlotte, NC) at a seeding density of 1×10^6 cells/ml. Cells were cultured in EMEM + 10% FBS and stimulated with a stable Vitamin C derivative (0.5 mM 2-O-alpha-D-glucopyranosyl-L-ascorbic acid: Wako Chemicals USA, Inc; Richmond, VA) for 1, 2, or 4 weeks at 35 °C and 5% CO_2. At the designated time, stromal constructs were maintained in either normoxic or hypoxic conditions for 24 or 48 h.

2.3. Normoxic and hypoxic culture

Both models were either incubated under hypoxia (1% O_2, 5% CO_2, and 94% N_2) using a hypoxic incubator (New Brunswick Scientific; Enfield, CT) or under normoxic conditions (21% O_2, 5% CO_2, and 74% N_2: Controls) for 24 or 48 h. At the designated time, samples were either processed for indirect immunofluorescence (IF), transmitted electron microscopy (TEM), or quantitative real-time PCR (qRT-PCR).

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted and processed, and qRT-PCR was performed, as previously described (Karamichos et al., 2011b). Briefly, RNA was annealed with oligo dt and random hexamer primers, and first strand synthesis was carried out with MMLV reverse transcriptase (Life technologies; Grand Island, NY). Negative controls were performed without reverse transcriptase. The TaqMan® Gene Expression Master Mix and cDNA template produced were optimized for each assay. The cDNA template was incubated for an initial 2 min at 50 °C and 10 min at 95 °C, followed by 40–50 amplification cycles of 95 °C for 15s and 60 °C for 1min. qRT-PCR was performed using an ABI 7300 (Applied Biosystems; Foster City, CA). Target genes included Col3A1, Col5A1, Keratocan, Perlecan, Decorin, Lumican, Lysyl oxidase, alpha-smooth muscle actin (SMA), and Eukaryotic 18S rRNA Endogenous Control (VIC/MGB Probe: Primer Limited Applied Biosystems; Grand Island, NY). Results were calculated using the ΔΔCt method (Livak and Schmittgen, 2001), using 18S rRNA as the endogenous control. Values were plotted as the mean ± standard error (SEM).

2.5. Indirect immunofluorescence (IF) and confocal microscopy

For IF and confocal microscopy, samples were processed as either tissue sections (corneal organ cultures) or whole mounts (stromal constructs), as previously described (Karamichos et al., 2011a; Lee et al., 2014). Tissue sections were fixed in freshly made 4% paraformaldehyde for 20 min, blocked with 4% bovine serum albumin (BSA) in phosphate buffered saline (PBS), and incubated with a primary antibody of choice in 1% BSA overnight at 4 °C. The antibody, heparan sulfate clone F69-3G10 (3G10: #370260, Seikagaku/Associates of Cape Cod; East Falmouth, MA), was used to stain for heparan sulfate proteoglycans. This antibody recognizes the desaturated hexuronate (glucuronate) that is present at the non-reducing end of heparan sulfate fragments created by Heparinase III digestion. In addition, the clone F58-10E4 (10E4: #370255, Seikagaku/Associates of Cape Cod), which recognizes an epitope present in multiple types of heparan sulfate that contains an N-sulfated glucosamine residue(s), was also used. As controls, parallel slides were pre-incubated with bacterial heparitinase (Flavobacterium heparinum, EC 4.2.2.8). After primary antibody incubation, tissue sections were rinsed with PBS and then incubated overnight with appropriate AlexaFluor secondary antibodies (Invitrogen; Carlsbad, CA). For whole mounts, stromal constructs were fixed in freshly made 4% paraformaldehyde for 1 h, blocked in 1% BSA in PBS + 0.1% Triton-X for 1 h, and incubated overnight at 4 °C with primary antibodies against Type III collagen (Southern Biotech; Birmingham, AL), cellular fibronectin (cFN: Sigma-Aldrich; St. Louis, MO), SMA (Dako North America; Carpinteria, CA), or rhodamine-phalloidin (Invitrogen; Carlsbad, CA). After primary antibody incubation, whole mounts were washed with PBS and incubated overnight with appropriate FITC-conjugated secondary antibody (Jackson Immunolaboratories; West Grove, PA). Counterstains, TO-PRO-3 (whole mount: Life Technologies; Grand Island, NY) and DAPI (tissue sections)