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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 Cuproline 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.

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Keywords

Extracellular matrix; cornea; corneal organ culture; confocal fluorescence microscopy; stroma

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). 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; Cannon 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 3mm-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 35mm²-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 hours 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 (0.5mM 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% CO₂. At the designated time, stromal constructs were maintained in either normoxic or hypoxic conditions for 24 or 48 hours.

2.3. Normoxic and Hypoxic culture

Both models were either incubated under hypoxia (1% O₂, 5% CO₂, and 94% N₂) using a hypoxic incubator (New Brunswick Scientific; Enfield, CT) or under normoxic conditions (21% O₂, 5% CO₂, and 74% N₂; Controls) for 24 or 48 hours. 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 2min at 50°C and 10min 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 Ct 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 minutes, 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 hour, blocked in 1% BSA in PBS + 0.1% Triton-X for 1 hour, 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) were used to label all cell nuclei, and coverslips were mounted with Vectashield (Vector Laboratories; Burlingame, CA). Negative controls, where the primary antibody was omitted, were run with all experiments. Images were obtained in Z with optical sections taken at 0.5-micron step intervals using either a Zeiss 700M Axiovert inverted laser scanning confocal microscope (Zeiss; Thornwood, NY) or a TCS-SP2 confocal microscope (Leica Microsystems; Bannockburn, IL). Negative controls were

imaged prior to experimental images to set a baseline detection level of fluorescence. Fluorescence intensity was quantified using ImageJ software (<http://rsb.info.nih.gov/ij>; NIH; Bethesda MA).

2.6. Transmission Electron Microscopy (TEM) and Cuprolinic Blue

Stromal constructs were processed for TEM or Cuprolinic Blue followed by TEM, as previously described (Mankus et al., 2012; Ren et al., 2008). Cuprolinic blue stain (0.2%) containing 0.3M MgCl₂ was used to examine the size and directional orientation of glycosaminoglycan (GAG) chains (Mankus et al., 2012). TEM, as previously described (Ren et al., 2008), was performed using a Philips 300 TEM (Eindhoven, The Netherlands). Cuprolinic blue-stained proteoglycan filaments were measured using ImageJ software (Mankus et al., 2012; Ren et al., 2008).

2.7. Mass Spectrometric Analysis

Stromal constructs were processed for mass spectrometric analyses, as previously described (Zaarur et al., 2015). Briefly, the tissue was lysed, protein concentration was determined, protein was reduced, free cysteines were alkylated, concentration of urea was reduced, proteins were digested, and the resulting peptides were analyzed by Liquid Chromatography Mass Spectrometry (LC-MS/MS), with a sensitivity of <1ng starting material for a given protein. Mass spectrometer results were matched via computer algorithms to protein sequence databases. The Mass Spectrometry and analysis was performed at the Mass Spectrophotometry Core (Boston University Medical Center; Boston, MA) with the following instrumentation: the nanoACQUITY ultra-performance liquid chromatography (UPLC) system (Waters; Milford, MA) and a Q Exactive mass spectrometer (Thermo Scientific; San Jose, CA) with a TriVersa NanoMate electrospray ionization (ESI) source (Advion; Ithaca, NY).

2.8. Statistical analysis

Data represents a minimum of three independent experiments and is presented as the mean $\pm$ SEM. Statistical significance was determined by either Student's t-test with a stringency of $p < 0.05$ or ANOVA test followed by Tukey's post-hoc.

3. Results and Discussion

Recent studies have shown that changes in the microenvironment sends signals to cells to alter the deposition, remodeling, and organization of the ECM. The cornea is unique in that it is exposed to hypoxia transiently during development, and there is a gradual transition to a normoxic environment over time, from anterior cornea to posterior, as eyelid opening occurs. In addition, unlike many other tissues, the adult cornea is exposed to daily switches between the atmospheric environment while awake and reduced oxygen environment during sleep. To examine changes in the major ECM proteins that are present in the cornea after exposure to episodes of acute hypoxia, we took advantage of two 3-D corneal models: 1) Stromal constructs, which consist of HCFs and self-assembled ECM and becomes multi-layered, achieving a thickness of ~ 65 microns by 8 weeks (Guo et al., 2007); and 2) Corneal organ culture, which is a wounded adult rat cornea that is allowed to heal in culture.

Previously, we demonstrated that collagens, along with proteoglycan core proteins and associated GAG chains, were secreted into the ECM (Guo et al., 2007; Ren et al., 2008). The stromal construct model allows us to examine the development and regeneration of the corneal stroma, and its ability to respond to and alter the deposition of matrix proteins under a controlled environment. The corneal organ culture model allows us to examine events of wound repair in its 3-D conformation. This may involve deposition of proteins or information on how cells move across the substrates. The dramatic changes that occur indicate that hypoxia plays a major modulatory role in the organization of stromal matrix proteins during development and again after injury. Using these models, we tested whether short-term hypoxic exposure modulates the assembly and secretion of the ECM.

3.1. Changes in Assembly of Stromal Construct

The alignment of F-actin filaments throughout the stromal construct was examined after they were cultured for 1, 2, and 4 weeks and then exposed to either normoxic or hypoxic conditions for 48 hours. Stromal constructs were stained for F-actin with rhodamine-phalloidin and imaged throughout in optical steps of 0.5 microns. The enface images represent the middle optical section of each stromal construct (Figure 1A₁–F₁). Since the stromal constructs varied in thickness, the middle optical section was determined by dividing the total number of optical steps collected per sample by 2. An orthogonal slice demonstrates the changes in localization of the F-actin filaments throughout the entire thickness of the stromal construct for each time point (Figure 1A₂–F₂). There was an increase in intensity of F-actin from the first to second week in the same y-plane in the normoxic stromal constructs. After 4 weeks, both the hypoxic and normoxic conditions demonstrated F-actin staining throughout the stromal construct, as depicted by the orthogonal images (Figure 1A₂–F₂). The staining revealed a more orderly organization of cells under hypoxic conditions. The orthogonal and *enface* images revealed that under hypoxic conditions, numerous cells displayed less intense F-actin staining.

In addition, an increase in beta-actin (1.56 fold) and alpha-actinin (3.08 fold) was detected under hypoxic conditions using mass spectrometry (Table I). As beta-actin responds to stressed conditions and an increase in nitrous oxide production, it may be a reliable marker for hypoxic conditions. To further examine the stromal constructs, analysis of Ki67-mRNA expression demonstrated that the hypoxic stromal constructs had decreased proliferation with values that were 70% of the normoxic at 2 weeks and 40% of the normoxic at 4 weeks (data not shown).

3.2 Ultrastructural Changes in the 3-D Stromal Construct

The role of hypoxia on the assembly of the ECM was further analyzed using TEM. After 1 week, stromal constructs exposed to either normoxic or hypoxic conditions for 48 hours had a multi-layered matrix (Figure 2A.a–d) and displayed cells with an elongated morphology; however, those exposed to hypoxic conditions had both flatter cells and a thinner stromal construct (Figure 2A.c), with the mean thickness 40% less than the normoxic ($p < 0.05$). Interestingly, both the hypoxic and normoxic cultures showed evidence of collagen secretion (Figure 2A.b,d: arrows). However, collagen fibrils were more prominent in the hypoxic stromal constructs (Figure 2A.d, arrowheads).

To determine potential reasons for the difference in stromal construct thicknesses, we examined the expression of lysyl oxidase1 (LOX1) mRNA, which is an enzyme that cross-links collagen fibrils and facilitates crosslinking by catalyzing the oxidation of peptidyl lysine and hydroxylysine residues, and the diameter of collagen fibrils. Values for LOX1 mRNA expression were significantly higher under hypoxic conditions (12-fold, $p < 0.05$; Figure 2B); however, even though the collagen diameter decreased in the hypoxic stromal constructs at the 1-week time point, it was not significantly different ($p = 0.11$; Figure 2C). LOX1 is often enhanced under hypoxic conditions when Hif-1 α is upregulated in tumor cells. Furthermore, it contributes to the invasive properties of hypoxic cancer cells (Erler and Giaccia, 2006). However, in the stromal constructs, preliminary experiments indicated that the transcription factor, Hif-1 α , was not upregulated (data not shown). While it is not understood, the cornea may be under more stringent control, as oxygen levels regularly fall during the diurnal cycle of sleep. Moreover, Xing and colleagues demonstrated that preconditioning with acute hypoxia prevented the degradation of the transcription factor Hif-1 α and produced protection against UV-induced apoptosis (Xing et al., 2006). It is not known if Hif-1 α is mediated during corneal development prior to eyelid opening, but the hypoxic environment may give it protection.

3.3. Expression of Collagens

Previously, we demonstrated that the major corneal collagens were expressed and deposited in the stromal constructs under normoxic conditions (Erler and Giaccia, 2006). Proper collagen synthesis and deposition, which are important for the integrity of the stroma, require the antioxidant vitamin C, which is added to the stromal construct medium. Vitamin C also serves as a co-factor for the enzymes prolyl and lysyl hydroxylase, which are responsible for the proper hydroxylation of collagen.

Expression of Type III collagen, a marker of wounded cornea and corneal scarring, is not usually detected in the unwounded corneal stroma (Trinkaus-Randall et al., 1991). Figure 3 shows a middle optical section (Figure 3A₁–F₁) and an orthogonal image (Figure 3A₂–F₂) of hypoxic and normoxic stromal constructs stained for Type III collagen. In the hypoxic cultures, fluorescence was similar to normoxic cultures at 1 week (Figure 3A,D), but decreased at 2 and 4 weeks (Figure 3B,C,E,F). This data agrees with previous findings that cells become more keratocyte-like with increased time in culture (Karamichos et al., 2014). Further analyses of the stromal constructs demonstrated that the expression of Type III collagen mRNA (Figure 3G) decreased significantly ($p < 0.05$, 2.6 fold) with exposure to hypoxic conditions, as compared with normoxic controls at 2 and 4 weeks. We also examined for the expression of Type V collagen, as it plays a major role in the assembly of collagen fibrils. Previously, vitamin C was shown to enhance levels of cell-layer associated pepsin-resistant Type V collagen, with only a minor induction in the levels of Types I and III collagen (Kypreos et al., 2000). In our study, all stromal constructs were cultured in the presence of 0.5 mM 2-O- α -D-glucopyranosyl-L-ascorbic acid, and the normoxic values were set to 1, to normalize the data. As seen in Figure 3H, exposure to hypoxia did not cause a significant change in the expression of Type V collagen mRNA. These data suggest that the stromal constructs under these conditions are not indicative of a wounded environment.

While it is known that collagen fibril formation can occur in the absence of cells, investigators have examined the role of non-collagenous proteins in fibrillogenesis and speculated that the binding partners generated the diversity of fibril patterns. For example, Kadler and colleagues (Kadler et al., 2008) proposed that fibronectin played a role in regulating the site of assembly, while the collagens mediated nucleation. While there is a distinct pattern in blood vessels or tendons, in the cornea the fibrils are present in unique orthogonal patterns (Kadler et al., 2008).

3.4. Hypoxia Inhibits the Secretion of Fibronectin and Delays Migration

Fibronectin is a glycoprotein, which is a component of a provisional matrix for epithelial cells to migrate on. It's presence was detected in early stages of corneal development prior to eyelid opening and then decreased over time (Cintrón et al., 1984). In addition, after injury of an adult cornea under normoxic conditions, fibronectin was elevated transiently in the stroma and along the basal lamina (Zieske et al., 1987). These data led us to examine the role of hypoxia on the localization of fibronectin in the stromal constructs.

In Figure 4, representative middle optical *enface* confocal images (Figure 4A₁–F₁) of fibronectin at all time points and conditions are shown, as well as orthogonal sections (Figure 4A₂–F₂), which demonstrate fibronectin localization throughout the stromal construct. At 1 week, fibronectin was detected in both normoxic and hypoxic conditions (Figure 4A,D). Under normoxic conditions, fibronectin increased at 2 weeks (Figure 4B); however, in the hypoxic samples (Figure 4B), fibronectin was reduced. After 4 weeks, fibronectin deposition was negligible under both environments (Figure 4C,F), indicating a state of minimal perturbation. Interestingly, fibronectin displayed a polarity in the orthogonal section under hypoxic conditions at 1 week (Figure 4D₂, arrows) that was not detected in the normoxic stromal constructs (Figure 4A₂). This reflects a change in fibronectin deposition throughout the stromal construct. Similar changes were detected in development when the stroma was hypoxic (Cintrón et al., 1984). The presence of fibronectin in the early weeks of ECM formation in the stromal constructs supports the transient appearance during development. It is possible that the appearance of fibronectin is necessary for the formation and organization of collagen fibrillogenesis in the stromal constructs (Hubbard et al., 2016).

The differences in localization of Type III collagen and fibronectin observed in the normoxia and hypoxia stromal constructs suggested that SMA might be elevated. Therefore, SMA was examined in the center of the stromal construct using *enface* optical sections (Figure 5A₁–F₁) and throughout the stromal construct using orthogonal sections (Figure 5A₂–F₂). At the 1-week time point, SMA was elevated in the normoxic stromal constructs (Figure 5A), as compared with the hypoxic stromal constructs (Figure 5D). At 2 weeks, SMA was detected throughout the normoxic stromal constructs (Figure 5B₂), and present more apically in the hypoxic cultures (Figure 5E₂). When the expression of SMA mRNA was evaluated at 2 weeks, no significant difference was detected (Figure 5G). Interestingly, by 4 weeks, the middle of the stromal constructs displayed equivalent localization (Figure 5C₁,F₁); however, the hypoxic orthogonal sections showed elevated localization within the anterior and posterior regions of the stromal construct (Figure 5F₂, arrows). Since TGF-beta1 is

associated with elevated SMA and alters the expression of matrix proteins causing fibrosis (Karamichos et al., 2011b), we examined the stromal constructs for changes in TGF-beta1 receptor, and found that TGF-beta1 receptor mRNA was elevated 4 fold after 48-hour exposure to hypoxic conditions (data not shown). However, when we examined the protein, there was only a 1.5-fold increase in TGF-beta1 receptor using mass spectrophotometric analysis (Table I). The minor change in TGF-beta1 receptor protein may be indicative of the stability of the stromal construct. These data support the minor changes in SMA.

3.5. Regulation of Proteoglycans in Stromal Constructs

Proteoglycans are the other major ECM protein family that is present and modified during stromal wound repair. As they are known to modify expression during development and injury, we examined expression and GAG association with collagen. Proteoglycans, along with sulfated-GAG chains, dynamically interact with collagen fibrils in the cornea, which is critical to the characteristic corneal structures and functions. The charged-GAG chains of the proteoglycans interact with cell-surface binding molecules. Previously, we demonstrated that the GAG chains were present along the collagen fibrils in the stromal constructs (Ren et al., 2008). In addition, other investigators showed that proteoglycan localization in the corneal stroma changed during development, and they hypothesized that it changed as the environment became more normoxic (Cintrón and Covington, 1990).

In the stromal constructs, there was a significant decrease in the expression of the small leucine rich proteoglycan (SLRP) mRNA: lumican, decorin, and keratocan (Figure 6A,B,C). We speculate that the negligible expression of keratocan in the hypoxic samples (Figure 6B) was due to the use of fibroblasts in the stromal constructs. Previously, we demonstrated that an increase in keratocan was detected over time when cells were treated with TGF-beta III, suggesting that the cells had become keratocyte-like when cultured under normoxic conditions (Karamichos et al., 2014). Our results suggest that this speculation is true. While the SLRPs uniformly showed a decrease in response to hypoxia, there was a significant increase in the expression of perlecan mRNA (Figure 6D) at both time points (1.5–2 and 4 fold, respectively; $p < 0.05$). In the unwounded cornea, investigators showed that perlecan was present along the epithelial basal lamina but not in the stroma (Mankus et al., 2012). This was consistent with the lack of perlecan expressed throughout the stromal construct cultured under normoxic conditions (Figure 6D). In contrast, perlecan was detected throughout the stroma in the wounded cornea (Torricelli et al., 2015), and was a minor component in the stromal construct. The role of perlecan was demonstrated in studies where a decrease in perlecan along the basal lamina was associated with a fragility in the epithelial cell-basal lamina interface, thinner wing cells, and reduced Ki67. (Inomata et al., 2012; Mayo et al., 2008).

Sulfation of GAGs was detected using Cuprolic Blue, an anionic dye (Figure 6E). While there was no detectable difference at 1 week (Figure 6E.a,b), at 2 weeks (Figure 6E.c,d), the number of chains along collagen fibrils decreased under hypoxic conditions (Figure 6E.b,d and F). These were denoted as proteoglycan units (PG units) and determined along 10nm of a collagen fiber (Figure 6F; $p < 0.05$). From this data, we speculated that the addition of sulfated-GAG chains was inhibited by the hypoxic conditions at longer time points (Figure

6F). Results from other investigators indicate that chondroitin, dermatan, and keratan sulfates were present at early time points, and their presence should be considered in Figure 6F (Ho et al., 2014).

The biosynthesis of GAGs is a complex process. Investigators have shown that sulfotransferases modify the sulfated heparan sulfate sequences and that Sulf1 removes some of the 6-O-sulfate groups from trisulfated IdoA2S-GlcNS6S (D2S6) and disulfated GlcA-GlcNS6S (D0S6) disaccharides (Ai et al., 2003). In the stromal constructs, there was almost a 50% reduction in the expression of Sulf1 after 2 weeks under hypoxic conditions (Figure 6G; $p < 0.05$). This reduction may be a factor that explains the lower amount of decoration of the collagen fibrils at later times.

3.6. Corneal Organ Culture Wound Model

Changes in exposure of corneal organ cultures to hypoxia provide investigators with a model of corneal development that can be easily manipulated. To examine for alterations in the substrate, corneal abrasions were performed on adult rat corneas. Then, the corneas with scleral rims were removed and cultured for up to 24 hours in either normoxic or hypoxic conditions. To examine changes in proteoglycans, corneal organ cultures were cultured for 18 hours, fixed, incubated without (Figure 7A,B,E,F) or with (Figure 7C,D,G,H) heparinase III, and stained with either 3G10 (Figure 7A–D) or 10E4 (Figure 7E–H). As predicted, there was no staining observed with 3G10 in corneal organ cultures without heparinase treatment (Figure 7A,B); however, in the heparinase-treated hypoxic corneal organ cultures (Figure 7C), fluorescence was detected along the basal lamina (arrows) and throughout the stroma (arrowheads), indicating the presence of heparan sulfate cores. Staining was markedly less in the normoxic corneal organ cultures (Figure 7D); however, 3G10 was still detected along the basal lamina (arrows) and in the anterior stroma. When the samples were probed with 10E4, there was detectable fluorescence along the basal lamina in the hypoxic and normoxic corneal organ cultures in the absence of heparinase (Figure 7E,F; arrows), indicating the presence of heparan sulfate chains. As predicted, there was no detectable fluorescence when heparinase-treated corneal organ cultures were probed with 10E4 (Figure 7G,H).

In addition, we examined the morphology of epithelial cells in the wound area after incubations were conducted under normoxic or hypoxic conditions (Figure 8A). By 18 hours, the normoxic tissue was healed (Figure 8A.e); however, the hypoxic tissue remained open (Figure 8A.f). Asterisks indicate the leading edge (Figure 8A.a–d,f). As seen in Table II, near complete repair took 6 hours longer under hypoxic conditions than normoxic. The greatest delay in wound healing was observed during the first 12 hours of repair, and at 12 hours under hypoxia, there was a high degree of migration variability (Table II). Therefore, we hypothesize that the delay was associated with a change in the morphology of the leading edge and/or a change in the substrate.

Since heparan sulfate proteoglycans are involved in the fibrillogenesis of fibronectin (Mitsi et al., 2006) and bind to collagen, we examined for the presence of fibronectin in the rat corneal organ culture 18 hours after wounding. Fibronectin was detected along the wound edge/area after injury in epithelial cells in both normoxic and hypoxic conditions (Figure 8B.a,b). In the stroma, transient fibronectin was detected in normoxic corneal organ cultures

(Figure 8B.a), which was similar to what was previously reported (Zieske et al., 1987); however, fibronectin was not detected in the matrix of hypoxic corneal organ cultures, and secretion and/or deposition was diminished (Figure 8B.b). This data is supported by Kadler and colleagues' hypothesis that fibronectin is involved in the assembly of collagen fibrils (Kadler et al., 2008). We speculate that the diminished fibronectin impaired the ability of the epithelial cells to pull along the fibronectin of the provisional matrix, thus causing migration to be delayed (Figure 8B). In fact, a study by Hubbard and colleagues supports this idea by elegantly demonstrating a change in a cell's morphology as it moved along a single fibronectin molecule (Hubbard et al., 2016).

4. Conclusion

These results collectively demonstrated that exposure of 3-D stromal constructs and corneal organ cultures to transient hypoxia altered the expression and deposition of matrix molecules, such as collagens, proteoglycans and glycoproteins. These allowed for the development of a controlled model to study both development and the onset of disease, such as diabetes. We propose that the use of hypoxia as a pretreatment or for different intervals or extents of time would provide investigators with a model of corneal development that may be manipulated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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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Highlights

- The stromal construct response to hypoxia was similar to that observed in development.
- Changes in proteoglycan and fibronectin with hypoxia mirrored changes in development.
- Type III collagen synthesis was decreased with hypoxic exposure.
- Decoration of collagen fibrils with sulfated glycosaminoglycans decreased in response to hypoxia.

Figure 1. Organization of cells in stromal constructs as indicated by F-actin filaments
A–F) Immunofluorescent images of rhodamine-phalloidin (red), a marker of F-actin filaments, in 1, 2, and 4-week stromal constructs after 48 hours of normoxic or hypoxic exposure. Stromal constructs were counterstained with To-Pro-3 (blue), a marker of cell nuclei. Single optical sections representing the middle of each stromal construct (A₁–F₁) and orthogonal images (A₂–F₂) are presented. The orthogonal images indicate localization of rhodamine-phalloidin within the stromal construct. Scale bar = 50 microns. N = a minimum of three experiments.

Figure 2. Stromal constructs produce an abundant amount of matrix

A) Organized collagen fibrils were assembled by corneal fibroblasts. Multiple layers of the matrix are present under normoxic (A.a,b) and hypoxic (A.c,d) conditions. Collagen fibrils adjacent to cells suggest active secretion (A.b,d, arrows). Scale bar = 5 microns (A.a,c) and 20 microns (A.b,d) **B)** Lysyl Oxidase1 mRNA (2 weeks) is presented as fold change in expression normalized to normoxic values. Graph indicates that Lysyl oxidase1 expression was elevated under hypoxic conditions (* $p < 0.05$). **C)** Graph of collagen fibril diameters at 2 weeks indicates no significant difference between normoxic and hypoxic conditions. Independent experiments were repeated a minimum of 3 times.

Figure 3. Hypoxia induces changes in the regulation of collagens Type III and V in the stromal construct model over 4 weeks

A–F) Type III Collagen (green) in 1, 2, and 4-week stromal constructs after 48 hours of normoxia or hypoxia exposure. Stromal constructs were counterstained with To-Pro-3 (blue), a marker of all cell nuclei. Single optical sections representing the middle of each stromal construct (A₁–F₁) and orthogonal images (A₂–F₂) are presented. The orthogonal images indicate localization of Type III collagen within the stromal construct. Type III collagen localization decreased with time under hypoxic conditions (D–F). Representative secondary antibody control for 4-week stromal construct is presented as a single middle optical section (C₁, inset) and orthogonal image (C₂, inset). Scale bar = 50 microns. **G)** Graph of Type III collagen mRNA expression at 2 and 4 weeks presented as fold change in expression normalized to normoxic values (*p<0.05). **H)** Graph of Type V collagen mRNA expression at 2 and 4 weeks presented as fold change in expression with hypoxic values

normalized to normoxic. No significant difference was detected. Data represents a minimum of 3 experiments.

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Figure 4. Hypoxia induces changes in fibronectin in the stromal construct model over 4 weeks A–F) IF of fibronectin (green) in 1, 2, and 4-week stromal constructs after 48 hours of normoxia or hypoxia exposure. Stromal constructs were counterstained with To-Pro-3 (blue), a marker of all cell nuclei. Single optical sections representing the middle of each stromal construct (A₁–F₁) and orthogonal images (A₂–F₂) are presented. The orthogonal images indicate localization of fibronectin within the stromal construct. Fibronectin localization decreased with time under hypoxic conditions (D–F). Arrows indicate a high-density localization of fibronectin in 1-week hypoxia stromal constructs (D₂). Scale bar = 50 microns. Data represents a minimum of 3 experiments.

Figure 5. Hypoxia does not alter the expression of SMA in the stromal construct model over 4 weeks

A–F) IF of SMA (green) in 1, 2, and 4-week stromal constructs after 48 hours of normoxia or hypoxia exposure. Stromal constructs were counterstained with To-Pro-3 (blue), a marker of cell nuclei. Single optical sections representing the middle of each stromal construct (A_1 – F_1) and orthogonal images (A_2 – F_2) are presented. The orthogonal images indicate localization of SMA within the stromal construct. No difference in SMA localization between normoxic and hypoxic conditions were noted in the middle optical sections of the stromal constructs (Figure 5C₁,F₁); however, the hypoxic orthogonal sections showed elevated localization within the anterior and posterior regions of the stromal construct (Figure 5F₂, arrows). Scale bar = 50 microns. **G)** Graph of SMA mRNA expression at 2 weeks was presented as fold change in SMA expression normalized to normoxic values. No significant difference was detected. Data represents a minimum of 3 experiments.

Figure 6. Hypoxia decreases the expression of proteoglycan mRNA and sulfation of glycosaminoglycan in the stromal construct

A–D) Graphs of fold changes in the expression of proteoglycan core mRNA induced by 48-hour hypoxia exposure in 2 and 4 week stromal constructs. All values are normalized to the normoxic value at each time point and condition. **E)** TEM of electron dense filaments stained with Cuprolinic blue on 1- (E.a,b) and 2- (E.c,d) week stromal constructs (100,000X). Scale bar is 100nm. Representative images of normoxia (E.a,c) and hypoxia (E.b,d) are shown. **F)** Collagen fibril lengths were measured, and the number of proteoglycan (PG) units along each fibril was counted. Data was normalized to the number of PG units per 100nm collagen. Results were averaged and are presented as $\pm$ SEM. **G)** Graph of sulfatase1 mRNA expression under hypoxic and normoxic conditions. Three independent experiments were performed on all but decorin at 4 weeks, and * $p < 0.05$.

Figure 7. Hypoxia alters regulation of heparan sulfate proteoglycans after epithelial abrasion
 Corneal organ cultures exposed to hypoxia or constant normoxia for 18 hours were probed with either the clone 3G10 (A–D) or 10E4 (E–H) $\pm$ pre-incubation with heparinase. 3G10: heparinase-treated sections (C,D) exposed the core protein that was reduced with normoxia. 10E4: heparinase-treated sections (G,H) eliminated detection of GAGs. Samples were counterstained with rhodamine-phalloidin (red) and DAPI (blue). Arrows and arrowheads indicate positive staining in the basal lamina and stroma, respectively. Images are representative of a minimum of 3 independent experiments. Scale bar = 50 microns.

Figure 8. Hypoxia impairs migration rate and secretion of fibronectin by epithelial cells
A) Epithelial cells stained with rhodamine-phalloidin (red) demonstrate that wound healing is delayed under hypoxic conditions at 4, 6, and 18 hours. Samples were counterstained with DAPI (blue) **B)** Fibronectin (green) was detected in the stroma and epithelium 18 hours after epithelial abrasion. * = leading edge of unhealed wounds. Images are representative of a minimum of 3 independent experiments. Scale bar = 50 microns.

Table I
Mass Spectrometric data of lysates after short-term exposure to hypoxia

Label-free profile of average normalized abundances determined from stromal constructs cultured under normoxic conditions for 2 weeks and exposed to hypoxia or normoxia for an additional 48 hours. N = 3 independent experiments.

Fold	Description (human)	Average Normalized Abundances	
		Hypoxia	Normoxia
1.08	Cytoplasmic actin	2.49E+06	2.69E+06
1.56	Beta-actin	5.06E+05	3.24E+05
3.08	Alpha-actinin	5.76E+04	1.87E+04
1.51	TGF-beta1 receptor	1.15E+05	7.62E+04

Table II
Wound healing is delayed under hypoxic conditions

Percent wound closure after 3mm-debridement wounds were performed on rat corneas. SEM calculated for each time point. N = a minimum of 3 experiments. Time points 2, 12, and 18 hours were significantly different between normoxia and hypoxia (*p<0.05)

Time (hour)	% Closure	
	Normoxia	Hypoxia
0	0 ± 0	0 ± 0
2*	7.75 ± 1	4.5 ± 1
12*	76 ± 5	44.5 ± 10
18*	100 ± 2	76 ± 4
24	100 ± 0	100 ± 4