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Epithelial basement membrane proteins perlecan and nidogen-2 are up-regulated in stromal cells after epithelial injury in human corneas

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ABSTRACT

The epithelial basement membrane (BM) is a specialized extracellular matrix that has been shown to have a critical role in corneal development, wound healing, and disease. Although the epithelial BM contributes to corneal homeostasis, relatively little is know about non-epithelial production of its components that may be important in defective regeneration of the epithelial basement membrane associated with opacity after photorefractive keratectomy. The purpose of the current study was to investigate stromal production of corneal epithelial BM proteins in wounded human corneas using immunohistochemistry. A total of five unwounded control eyes and five 30-min epithelial-wounded corneas were obtained from fresh corneoscleral buttons removed from human eyes enucleated due to choroidal melanoma with normal anterior segments. In the wounded corneas, an eight mm patch of central corneal epithelium and epithelial BM was removed with a Beaver blade when the patient was under general anesthesia. Immunohistochemical analyses were performed to detect perlecan and nidogen-2 proteins—important components of the epithelial BM lamina lucida and lamina densa zones. Perlecan and nidogen-2 proteins were detected in the BM itself and at low levels in keratocytes in all unwounded corneas. After epithelial injury, both perlecan and nidogen-2 were expressed at high levels in stromal keratocytes, including superficial keratocytes in the early phases of apoptosis. Thus, after epithelial and epithelial BM injury, stromal keratocytes contribute important perlecan and nidogen-2 components to the regenerating epithelial BM.

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Basement membranes (BM) are specialized forms of extracellular matrix that regulate biological events, including local concentrations of growth factors and cytokines (Kruegel and Miosge, 2010). The formation of BM is a prerequisite for normal tissue development and function (Smyth et al., 1999; Yurchenco et al., 2004a; 2004b).

In corneal wound healing, the epithelial BM has been shown to have a critical role in regulating the generation of severe late corneal opacity after corneal surgeries such as photorefractive keratectomy (PRK) (Fini and Stramer, 2005; Fujikawa et al., 1984; Netto et al., 2006; Sta Iglesia and Stepp, 2000; Torricelli et al., 2013a; 2013b; Torricelli and Wilson, 2014; Wilson et al., 1999). Prior studies have shown that structural integrity of the regenerated BM after stromal injury is an important determinant of whether a particular cornea develops haze—likely by limiting access of epithelium-derived growth factors, such as transforming growth factor beta (TGFβ), that modulate myofibroblast development from precursors cells, and persistence of these cells, in the subepithelial stroma (Fini and Stramer, 2005; Netto et al., 2006; Stramer et al., 2003; Torricelli et al., 2013a; 2013b).

Epithelial BMs are composed of diverse extracellular matrix molecules and the specific composition varies somewhat in different tissues (Kruegel and Miosge, 2010). In general terms, the corneal epithelial BM is assembled from four primary components: laminins, collagens, heparan sulfate proteoglycans, such as perlecan, and nidogens (Yurchenco et al., 2004a; 2004b; Torricelli et al., 2013b). The laminia lucida and lamina densa layers that are not regenerated in rabbit corneas with haze (Torricelli et al., 2013a) are thought to be composed of specific components that include perlecan, nidogen-1, nidogen-2 and laminin 332 (Yurchenco et al.,

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2004a; 2004b; Kruegel and Miosge, 2010). Perlecan has an important function in maintaining cell adhesion and integrity of the corneal matrix (Vittitow and Borras, 2004) and also functions to maintain the selective basement membrane barrier (Rossi et al., 2003; Smith and Hassell, 2006). There are two different nidogen genes, nidogen-1, also called entactin (Timpl et al., 1983), and nidogen-2, also called osteonidogen or entactin-2 (Kohfeldt et al., 1998). Nidogen-1 and nidogen-2 have similar structure and affinity for other extracellular matrix proteins (Fox et al., 1991; Salmivirta et al., 2002 Hohenester and Engel, 2002), and have been shown to compensate for each other in single-gene knockouts (Schymeinsky et al., 2002). Double nidogen knockouts have numerous defects and perinatal lethality (Bader et al., 2005). Nidogens act as bridging molecules linking different components of the BM, including perlecan (Schittny et al., 1988; Kvansakul et al., 2001; Hopf et al., 2001) and also have strong affinity for laminins and collagen type IV Ho et al., 2008). Both nidogen-2 and perlecan contribute to the lamina lucida and lamina densa that show defective regeneration in corneas with severe haze (Torricelli et al., 2013a; 2013b).

Despite the importance of the epithelial BM in corneal wound healing, relatively little is known about non-epithelial biosynthesis of these components; with many studies concluding exclusively the epithelial cells themselves produce them. However, there has been data published suggesting underlying stromal cells produce some of the components (Kohfeldt et al., 1998; Maguen et al., 2008). The present study aimed to investigate stromal production of corneal epithelial BM proteins that are components of the defective zones noted with TEM after PRK—perlecan and nidogens—in wounded and unwounded human corneas. Several commercially available antibodies for nidogen-1 were tested but did not yield adequate staining in human corneas. Therefore, this study was limited to immunohistochemistry for nidogen-2 and perlecan proteins.

Informed consent for tissue donation and use in research was obtained and the study adhered to the tenets of the Declaration of Helsinki for experiments involving human tissues. Approval to perform this study was also obtained from the Institutional Review Board at the Cleveland Clinic.

Five unwounded control corneas (donors age: 60 ± 15, range 34–71) and five wounded corneas (donors age: 61 ± 19, range 47–85) were obtained from fresh corneoscleral buttons of human eyes with normal anterior segments enucleated for choroidal melanoma. In wounded corneas, the central epithelium was scraped to remove an eight mm patch of central corneal epithelium and BM when the patients were under general anesthesia. The corneas were collected from the eyes 30 min after injury using a 9.0 mm trephine. Specimens were immediately embedded in liquid optimal cutting temperature (OCT) compound (Sakura FineTek, Torrance, CA, USA) within a 24 mm × 24 mm × 5 mm mold (Fisher Scientific, Pittsburgh, PA, USA). Corneas were centered within the mold so that the block could be bisected and transverse sections cut from the center of the cornea. Frozen tissue blocks were stored at −80 °C until sectioning was performed.

Central corneal sections (seven μm thick) were cut with a cryostat (HM 505M, Micron GmbH, Walldorf, Germany). Sections were placed on 25 mm × 75 mm x 1 mm microscope slides (Superfrost Plus, Fisher) and maintained at −80 °C until staining was performed.

Cryostat-cut sections were dried for 20 min at room temperature, washed in phosphate-buffered saline (PBS) and blocked in PBS with 5% donkey serum for 60 min. The sections were incubated in primary antibodies against perlecan at 1:50 (Santa Cruz Biotechnologies; Santa Cruz, CA, cat# sc-27449) or nidogen-2 at 1:50 (20 micrograms/ml) (Abcam, Cambridge, MA, cat#, ab14513) in 5% donkey serum overnight at 4 °C. Sections were washed with PBS and then incubated at room temperature for 60 min in donkey anti-goat IgG-FITC (Santa Cruz Bio, cat# sc-2024) secondary antibody diluted at 1:200 in PBS for perlecan or goat anti-rabbit IgG (H + L) (Jackson ImmunoResearch, West Grove, PA, cat# 111-545-144) secondary antibody diluted 1:75 in PBS for nidogen-2. In negative control slides, a nonspecific isotypic control antibody was used for nidogen-2 staining (no preabsorption antigen available). Pre-absorption with blocking peptide (Santa Cruz Bio. cat# sc-27449 P) at 10X the antibody concentration was performed in controls for perlecan staining. Coverslips were mounted with Vectashield containing DAPI (Vector Laboratories Inc., Burlingame, CA) to allow visualization of all nuclei in the tissue sections. The sections were viewed and photographed with a Leica DM5000 microscope equipped with Q-Imaging Retiga 4000RV (Surrey, BC, Canada) camera and ImagePro software. Immunoshistochemistry was performed at least three times on sections from each cornea to confirm consistent results.

In human control corneas, immunohistochemical staining for both perlecan and nidogen-2 revealed a sharp and continuous reactivity in the BM and diffuse staining in all layers of the epithelium (Figs. 1 and 2) in each cornea. Faint immunoreactivity was detected in the stroma for both BM components in unwounded corneas (Figs. 1 and 2).

Immunostaining for perlecan was up-regulated in anterior to mid-stromal keratocytes, compared to control unwounded corneas, at 30 min after epithelial and epithelial BM scrape in human corneas (Fig. 1), including in cells in the early stages of apoptosis (not shown). Perlecan was also detected at higher levels in remnants of the epithelial BM or BM that had already begun to regenerate, compared to the unwounded control corneas. Nidogen-2 was up-regulated in keratocytes in the anterior to mid-stroma and stromal connective tissue in all wounded corneas, including keratocytes in the early stages of apoptosis (not shown), as well as in remnants of the epithelial BM or BM that had already begun to regenerate (Fig. 2).

This study reveals that stromal keratocytes up-regulate production of perlecan and nidogen-2 proteins after epithelial and BM injury in human corneas. Thus, keratocytes likely contribute to epithelial BM regeneration after epithelial-basement membrane scrape injury in humans, and both the epithelium and underlying keratocytes contribute components to regenerate the corneal epithelial BM (Marinkovich et al., 1993).

Interestingly, it was noted that both perlecan and nidogen-2 were up-regulated in keratocytes that were undergoing apoptosis detected by the TUNEL assay at 30 min after epithelial scrape. Prior studies on keratocyte apoptosis showed that the dying cells and apoptotic body remnants persist for more than 4 h after epithelial injury (Wilson et al., 1996; Ambrósio et al., 2009), even though anterior stromal keratocytes are found to have ultrastructural changes associated with apoptosis detectible by transmission electron microscopy immediately after epithelial scrape injury. The findings here suggest those cell remnants can continue to generate at least some proteins until apoptosis is completed and they disappear.

The importance of normal regeneration of epithelial BM blocking myofibroblast development and maintenance of corneal stromal opacity has been demonstrated by several studies (Fini and Stramer, 2005; Gipson et al., 1989; Netto et al., 2006; Stramer et al., 2003; Torricelli et al., 2013a). Normal BM barrier function likely modulates myofibroblast development from precursors and persistence in the anterior stroma by limiting penetration of epithelial-derived growth factor such as TGFβ and platelet-derived growth factor (PDGF) (Torricelli et al., 2013b). Mature myofibroblasts are themselves opaque and excrete a large amount of disorganized extracellular matrix that causes a loss of corneal transparency (Jester et al., 1999; Wilson, 2012). Transmission electron microscopic analyses in rabbits showed there was abnormal regeneration of

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