The corneal epithelial basement membrane (EBM) modulates myofibroblast generation and fibrosis (scarring or “late haze”) via regulation of epithelium- and tear film- derived profibrotic growth factors, including TGF-β and platelet-derived growth factor (PDGF).1-3 Defective corneal EBM regeneration has been shown to underlie the development of myofibroblast-mediated stromal fibrosis that occurs after injuries, such as photorefractive keratectomy1,2,4 or pseudomonas corneal ulcers,5 likely by facilitating TGF-β and PDGF penetration into the stroma at sufficient concentrations to drive myofibroblast development from precursor cells. Regeneration of the EBM triggers apoptosis of myofibroblast precursors and mature myofibroblasts by depriving them of a requisite supply of epithelium- and tear film- derived TGF-β and PDGF—allowing repopulation by keratocytes, removal of disordered extracellular matrix produced by the myofibroblasts,5,6 and restoration of corneal transparency.

Keratocytes, and possibly other stromal cells, contribute to EBM regeneration after corneal injury through the production of EBM components, such as perlecan, nidogens, and laminins.2,7-10 Similarly, fibroblasts or mesenchymal cells produce basement membrane components in skin, gut, lung, kidney, and other organs that likely contribute to the maintenance or regeneration of basement membranes in those organs.11-18 Perlecan is a large, five-domain proteoglycan that is a critical component of basement membranes (BMs), which binds to and crosslinks many extracellular matrix components in BMs and also cell-surface molecules, such as integrins.19-22 It is a critical organizer of the BMs in many organs and is known to modulate growth factor functions via sequestration by its domain 1.22 Similarly, nidogens are critical BM components that also interact with other BM components, such as laminins, perlecan, and collagen type IV.23-24 Although laminins are the major early drivers of BM regeneration via their novel capacity to self-assemble, the addition of perlecan and the nidogens to the regenerating BMs in many organs appears to have a critical role in regeneration of the structurally and functionally mature BM. The cytokine and growth factor regulation of expression of these important BM components has been poorly characterized in the context of wound healing and repair processes. IL-1α,
Regulation of Expression of Basement Membrane Components

TGF-β, and PDGF regulate and orchestrate critical aspects of the wound-healing response of the cornea, including epithelial cell proliferation, motility and differentiation, myofibroblast development, keratocyte and myofibroblast viability and apoptosis, as well as keratocyte and corneal fibroblast production of chemokines, metalloproteinases and collagens. The purpose of this study was to determine whether IL-1α, TGF-β, or PDGF modulate the production of EBM components nidogen-1, nidogen-2, or perlecans in keratocytes, corneal fibroblasts, or myofibroblasts in vitro. This study also examined perlecans-protein expression and localization in rabbit corneas after 4.5-D photorefractive keratectomy (PRK), when EBM ultrastructure was normally regenerated and the stroma remained transparent, compared with 9-D PRK when the EBM ultrastructure was not regenerated and corneas developed stromal fibrosis.

**Methods**

Isolation of Rabbit Corneal Keratocytes, Fibroblasts, and Myofibroblasts

Primary keratocytes were isolated and cultured from fresh rabbit corneas (Pel Feeze, Rogers, AR, USA) without serum as previously described with Dulbecco’s modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA). Identical methods were used to produce corneal fibroblasts, except the cells isolated from fresh corneas were cultured with 10% fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO, USA) and fibroblast growth factor (FGF-2, 40 ng/mL; Sigma Aldrich) in DMEM. Corneal myofibroblasts were produced by culturing corneal fibroblasts in DMEM with 1% FBS and 2 ng/mL TGF-β1 (R&D, Minneapolis, MN, USA) for at least 72 hours. The medium was changed every 48 hours in all cultures. In preliminary experiments, advanced DMEM with 2.5 mg/L ascorbic acid (ThermoFisher Scientific, Rockland, IL, USA) was used for the cell culture for quantitative real-time PCR (qRT-PCR).

Western Immunoblotting and Immunohistochemistry (IHC) for Stromal Cell Markers

To verify the specificity of each cultured cell type of the stroma, Western immunoblotting and immunofluorescence were performed to confirm expression of markers specific for these cell types. Primary keratocytes were verified based on their expression of keratocan by Western immunoblotting. We observed parallel studies of effects in the corresponding corneal stromal cells. Passage one keratocytes or 2nd to 3rd passage corneal fibroblasts or myofibroblasts were used for all experiments.

Cell Growth Factor Treatment

Cells were treated with or without IL-1α (10 ng/mL), IL-1β (10 ng/mL), TGF-β1 (2 ng/mL), TGF-β3 (10 ng/mL), PDGF-AA (10 ng/mL), or PDGF-AB (10 ng/mL) for 8 or 12 hours, as indicated in the figures. The concentrations of cytokines and growth factors used in these experiments was based on previous studies of effects in the corresponding corneal stromal cells.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), reverse transcription and qPCR amplification was performed as described previously. The relative amount of target mRNA was measured using the comparative Ct (ΔΔCt) method by normalizing target mRNA Ct values to those of 18S. The sequences of primers used in this study were described previously. Quantitative real-time experiments were repeated three times and results were consistent in the different experiments. In each case, means for a cytokine or growth factor at each time point were determined from three independent experiments.

**Western Blot Analysis**

Primary cultures of keratocytes were treated with or without IL-1α (10 ng/mL) or TGF-β1 (2 ng/mL) for 16 hours. Cells were then moved to ice, rinsed three times with 2 mL of ice-cold PBS buffer and extracted by adding 4 M guanidine chloride containing 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM PMSC (Sigma), 10 μg/mL aprotonin, and protease inhibitor cocktail (Roche, Mannheim, Germany). Extracts were then dialyzed into 50 mM Tris/HCl, pH 7.5, containing 10 mM CaCl2, 19 for 4 hours, and the precipitates (extracellular matrix proteins) were collected and dissolved in 0.1 M Tris-acetate solution (pH 6.0) containing 6 M urea and protease inhibitor. Protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific). For keratocan, a 100 μg protein aliquot was incubated with endo-β-galactosidase (0.1 U/mL, Sigma-Aldrich) in 50 mM sodium phosphate (pH 5.8) at 37°C overnight. After digestion, the protein was lyophilized, dissolved, and underwent Western blot analysis for keratocan. For perlecans Western blots, the dialyzed extract was digested...
with 1 mU/mL of heparitinase III (Cat No. H8891; Sigma-Aldrich) at 37°C for 3 hours. For Western blot analysis, 10 μg of cellular protein was separated on 4% to 15% SDS-PAGE gels and then transferred to PVDF membranes for immunoblotting. The membranes were blocked with 5% nonfat milk and probed with primary antibodies at 4°C overnight. The primary antibodies used were anti-perlecan (1:1,000, sc377219; Santa Cruz Biotechnology), anti-nidogen-1 (1:500, AF2570, R&D System), anti-nidogen-2 (1:1,000, sc-373859; Santa Cruz Biotechnology), and keratocan (cat. no. sc353242, 1:2000; Santa Cruz Biotechnology). Western blotting for β-actin (1:5,000, A5441; Sigma-Aldrich) was used as a loading control. Western blot analysis was performed using enhanced chemiluminescence for signal detection. Western blot signal intensities were quantified by densitometry using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Animals and Surgery

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic Foundation. All animals were used in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Anesthesia was performed with intramuscular injection of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg). Topical proparacaine hydrochloride 1% (Alcon, Fort Worth, TX, USA) was applied to each eye just prior to surgery. Euthanasia was performed with intravenous injection of 100 mg/kg heptanal sodium (Shering-Plough, Kenilworth, NJ, USA) with the animal under general anesthesia.

Female 12- to 15-weeks-old New Zealand white rabbits weighing 2.5 to 3.0 kg were included in this study. One eye of each rabbit was selected at random to have PRK. Contralateral eyes were used for control groups. Rabbits underwent epithelial scrape and then incision PRK with a 6-mm diameter ablation zone using a VISX Star 54 IR excimer laser (Abbott Medical Optics, Irvine, CA, USA), according to a previously published method. One drop of Vigamox (Alcon, Ft. Worth, TX, USA) was applied to the PRK and control cornea four times a day until the epithelium healed in eyes that had surgery and control eyes. Time points for –9- or –4.5-D PRK were 1, 2, 4, 7, 14, and 30 days after surgery with three eyes in each group at each time point.

Tissue Fixation, Sectioning, and Immunohistochemistry

The corneoscleral rims were collected, cryopreserved in optimal cutting temperature (OCT) compound (Sakura FineTek, Torrance, CA, USA), within a 24 × 24 × 5-mm mold (Fisher Scientific, Pittsburgh, PA, USA), bisected, and 7-μm thick central corneal cut with a cryostat, as previously described in detail. Sections were placed on 25 × 75 × 1-mm microscope slides (Superfrost Plus; Fisher) and kept at −80°C until IHC was performed. IHC for perlecan was performed using a mouse monoclonal anti-human perlecan antibody (Clone E6; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) that was previously shown to work in IHC against rabbit antigen at 1:50 dilution in 1% BSA in PBS for 1 hour at room temperature. The secondary antibody, goat anti-mouse Alexa flour 650, was used at 1:200 dilution in 1% BSA in PBS for 1 hour at room temperature.

Duplex IHC was performed to simultaneously detect the keratocan marker for keratocytes and the α-SMA marker for myofibroblasts. Briefly, for keratocan, a goat anti-human primary antibody raised against keratocan peptide H2N-LRLDGNEKPIPIDILAVC-OH (a gift from Winston W. Kao, Cincinnati, OH, USA). For duplex α-SMA with keratocan, a mouse monoclonal anti-human α-SMA clone IA4 (Cat. No M6651; Dako, Carpinteria, CA, USA) was used. The slides were washed with PBS containing 0.01% Tween-20 and blocked with 5% donkey serum in PBS. The slides were incubated overnight with anti-keratocan antibody at 1:200 dilution and anti-α-SMA antibody at 1:50 dilution in 5% donkey serum containing 0.05% NP-40. The slides were three time washed with PBS containing 0.01% Tween-20 and then incubated at room temperature with secondary antibodies—Alexa Flour 488 donkey anti-goat IgG (Cat# A-11055; ThermoFisher Scientific) and Alexa Fluor 568 donkey anti-mouse IgG (Cat# A10037; ThermoFisher Scientific), both at a dilution of 1:200 in 5% donkey serum containing 0.05%NP-40 for 1 hour. The slides were washed three time with PBS with 0.01% Tween-20 and coverslips were mounted with Vectashield containing DAPI (Vector Laboratories Inc.) to allow visualization of all nuclei. The sections were viewed and photographed with a Leica DM5000 microscope equipped with Q-imaging Retiga 4000RV camera and ImagePro software.

Duplex IHC for vimentin and α-SMA was performed using the method described above, except the primary antibody for vimentin was mouse monoclonal anti-vimentin (Cat. # M7020; Dako) used at 1:50 dilution for 60 minutes and the primary antibody for α-SMA was goat polyclonal anti-α-SMA (Cat. # NB300-978; Novus Biologicals USA, Littleton, CO, USA) used at 1:100 dilution for 60 minutes at room temperature. The secondary antibodies were donkey anti-mouse IgG (H+L) highly cross-adsorbed, Alexa Fluor 488 (Cat. # A21202; Thermo Fisher Scientific) and donkey anti-goat IgG (H+L) cross-adsorbed, Alexa Fluor 568 (Cat. # A11057; Thermo Fisher Scientific), respectively, both used at 1:250 dilution and incubated for 60 minutes at room temperature.

Similarly, duplex IHC for CD45 and α-SMA was performed using the method described above, except slides were blocked with 5% donkey serum in PBS for 90 minutes at room temperature and the primary antibody for CD45 was mouse anti-rabbit CD45 monoclonal (Cat. # MCA808GA; Bio-Rad, Hercules, CA, USA) used at 1:50 dilution and the primary antibody for α-SMA was the goat anti-α-SMA polyclonal (Cat. # NB300-978; Novus Biologicals, Centennial, CO, USA) used at 1:100 dilution for 60 minutes at room temperature. The secondary antibodies were the donkey anti-mouse IgG (H+L) highly cross-adsorbed, Alexa Fluor 488 (Cat. # A21202; Invitrogen) used at 1:200 dilution and the donkey anti-goat IgG (H+L) cross-adsorbed, Alexa Fluor 568 (Cat. # A11057; Invitrogen) used at 1:300 dilution, respectively, for 60 minutes at room temperature.

TEM of EBM Ultrastructure

Transmission electron microscopy (TEM) of rabbit corneas at 1 month after –4.5- or –9-D PRK was performed by previously published methods using a Philips CM12 transmission electron microscope operated at 60 kV (FEI Company, Hillsboro, OR, USA).

Statistical Analysis

A sample size of three was used in all experiments. All data are represented as the mean ± SD, and statistical significance was determined using Dunnett’s test from three or more independent experiments and the growth factor–treated groups were compared with the control groups. A P < 0.05 was considered to be a statistically significant difference.
RESULTS

Analysis of Growth Factors/Cytokines Effects on Nidogen-1, Nidogen-2, or Perlecan mRNA with Real-Time PCR

Initially, a series of cytokines and growth factors known to be critical modulators of the early corneal wound healing response (IL-1α, IL-1β, TGF-β1, TGF-β3, PDGF-AA, or PDGF-AB) were screened for modulation of the mRNAs of key BM components by marker-verified keratocytes, keratocyte-derived corneal fibroblasts, or keratocyte-derived myofibroblasts in vitro. Two different time points, 8 and 12 hours of cytokine exposure were included and the data for each cytokine or growth factor were obtained by calculating the means from three independent experiments (Fig. 1). At each time point of exposure, 8 or 12 hours, the cytokine- or growth factor–treated keratocytes were compared statistically to vehicle-treated keratocytes (Fig. 1A). In keratocytes, perlecan mRNA was significantly increased in response to 10 ng/mL IL-1α or 10 ng/mL IL-1β at 12 hours compared with the control cultures without IL-1α or -1β (Fig. 1A). There was a trend for each cytokine to increase perlecan mRNA at 8 hours in keratocytes that did not reach statistical significance compared with control cultures (Fig. 1A). There were trends for 2 ng/mL TGF-β1 or 10 ng/mL TGF-β3 exposure for 12 hours to decrease perlecan mRNA expression in keratocytes (Fig. 1A) but these changes did not reach statistical significance compared with control keratocyte cultures.

Neither 10 ng/mL PDGF-AA or 10 ng/mL PDGF-BB had an effect on perlecan mRNA expression with 8 or 12 hours of exposure (Fig. 1A). In contrast, 2 ng/mL TGF-β1 or 10 ng/mL TGF-β3 significantly inhibited expression of nidogen-1 (Fig. 1B) or nidogen-2 (Fig. 1C) mRNA in the keratocytes after 12 hours of exposure compared with control keratocyte cultures. IL-1α, IL-1β, PDGF-AA, or PDGF-AB did not have significant effects on nidogen-1 (Fig. 1B) or nidogen-2 (Fig. 1C) mRNA expression compared with controls with either 8 or 12 hours of exposure.

None of the tested cytokines had significant effects on perlecan mRNA expression in corneal fibroblasts or myofibroblasts (not shown). Similarly, none of the tested cytokines had significant effects on nidogen-1 or -2 mRNA expression in corneal fibroblasts or myofibroblasts (not shown). Also, use of DMEM culture medium with 2.5 mg/L ascorbic acid in preliminary experiments showed no difference from standard DMEM on qRT-PCR results and, therefore, only the nonascorbic acid results were reported.

These qRT-PCR experiments were repeated three times with each stromal cell types and the results were consistent in the different experiments.

Analysis of Perlecan, Nidogen-1, Nidogen-2 Proteins With Western Immunoblotting in Keratocytes

Western immunoblotting was used to confirm that cells exposed to IL-1α or TGF-1 were keratocan+ keratocytes at the beginning of the exposure (Fig. 2A). Western immunoblot-
Regulation of Expression of Basement Membrane Components

FIGURE 2. Regulation of EBM component protein expression by IL-1α and TGF-β1 in primary rabbit keratocytes. Primary keratocan+ keratocytes were cultured and treated with 10 ng/mL IL-1α, 2 ng/mL TGF-β1, or left untreated for 16 hours. Keratocytes to be used in the experiments were lysed and keratocan of the expected size (50 kDa) was detected (A) to confirm these cells were keratocan+ keratocytes at the beginning of the exposure. (B) Perlecan, (C) nidogen-1, and (D) nidogen-2 expression detected by Western blot. Cell extracts used for perlecan Western blots were treated with heparitinase III, as was described in the methods. β-actin was used as a loading control for each experiment. A representative Western blot of the three performed for each BM component is shown. The graphs beneath each Western blot was obtained by densitometry analysis of the bands from each of the three Western blots from different experiments. *The change in BM protein was statistically significant (P < 0.05) compared with the control keratocytes.

All three BM proteins of the expected size (perlecan, Fig. 2B; nidogen-1, Fig. 2C; and nidogen-2, Fig. 2D) were expressed in keratocytes. IL-1α increased the expression of perlecan protein in keratocytes (Fig. 2B). There was a trend for IL-1α to increase nidogen-1 (Fig. 2C) or -2 (Fig. 2D) protein expression in keratocytes after 16 hours of exposure, but the difference compared with control keratocytes did not reach statistical significance.

TGF-β1, in contrast, inhibited the expression of perlecan protein in keratocytes at 16 hours of exposure (Fig. 2B). There was a trend for TGF-β1 to decrease nidogen-1 (Fig. 2C) or -2 (Fig. 2D) protein expression in keratocytes after 16 hours of exposure, but the decreases did not reach statistical significance compared with the control keratocytes.

Analysis of Perlecan Protein Expression in the Injured Corneas Using IHC

To complement the in vitro experiments, −4.5- or −9-D PRK was performed in anterior corneas to study perlecan expression in situ by the stromal cells with IHC during the subsequent corneal wound healing response. In rabbit corneas that had −4.5- or −9-D PRK, the epithelium closed around 4 days after surgery. In corneas that have −4.5-D PRK, the EBM has been shown to fully regenerate to normal morphology with lamina lucida and lamina densa at 8 to 10 days after surgery.6

IHC was performed to detect perlecan in unwounded corneas at 1, 2, 4, 7, 14, and 30 days after −9- or −4.5-D PRK (Fig. 3). By 1 day, and especially at 2 days, after either −9- or −4.5-D PRK, perlecan protein was upregulated in anterior stromal cells (Figs. 3C–F) compared with the control unwounded corneas. As the epithelium was healing, a subepithelial concentration of perlecan appeared at the nascent EBM in either −9- or −4.5-D corneas (Figs. 3E, 3F). At 4 days after PRK, perlecan production in the anterior stromal cells was detectible, but less than at 2 days after PRK in either the −9- or −4.5-D PRK groups (Figs. 3G, 3H). A layer of subepithelial perlecan at the site of the nascent EBM persisted in both groups at 4 days. By 7 days after PRK, a difference was noted in the subepithelial layer of perlecan where the nascent EBM was regenerating in the −9-D PRK corneas destined to develop subepithelial fibrosis (Fig. 3I). This layer became less prominent and had skipped areas with no detectible perlecan in all corneas analyzed in the 7 day −9-D PRK group compared with the −4.5-D PRK group (Fig. 3J). This difference in the nascent EBM perlecan became even more prominent by 14 days after PRK, with no EBM perlecan detectible in the −9-D PRK group (Fig. 3K) in any of the corneas analyzed and persistent EBM perlecan in all of the −4.5-D PRK corneas analyzed (Fig. 3L). Prominent cells with perlecan were present in the anterior stroma at 14 days after −9-D PRK (Fig. 3K), but not in −4.5-D PRK corneas at 14 days after surgery (Fig. 3L). It is known from prior work1,4,5 that z-SMA+ myofibroblast precursor cells develop to become z-SMA+ myofibroblasts in the subepi-
thelial stroma at 14 days after –9-D PRK (not shown) and some of those perlecan+ cells in the anterior stroma in Figure 3K are likely those developing cells. Similarly, at 30 days after –9-D PRK, a change was noted in subepithelial EBM perlecan (arrowheads) in the (J) –9-D PRK corneas compared with the (I) –4.5-D PRK corneas. At 14 days, the difference in the subepithelial perlecan (arrowheads) between (K) –9-D and (L) –4.5-D PRK was even more pronounced. Perlecan was detected in anterior stromal cells in both groups (arrows). In contrast, in corneas (N) at 30 days after –4.5-D PRK, the linear EBM-associated perlecan (arrowheads) was present and there was relatively little perlecan present in stromal cells. Magnification ×200 in all panels.

**Figure 3.** Immunohistochemistry for perlecan protein expression in control unwounded corneas and at time points after –4.5- and –9-D PRK in rabbits. e is epithelium and s is stroma in each panel. Blue is DAPI staining of all nuclei in all panels. In each case, the panel shown is representative of the results noted in three corneas at each time point in each group. (A) Example control staining (this example at 2 days after –9-D PRK) with no primary antibody. (B) In unwounded control corneas perlecan was detected in the EBM (arrowheads) but little was detected in stromal cells. At (C) 1 day after –9-D PRK (D) 1 day after –4.5-D PRK, perlecan protein production was present in some stromal cells (arrows). At (E) 2 days after –9-D PRK, (F) 2 days after –4.5-D PRK, (G) 4 days after –9-D PRK, or (H) 4 days after –4.5-D PRK there was similar perlecan in the nascent EBM (arrowheads) and stromal cells (arrows). At 7 days after –9-D PRK, a change was noted in subepithelial EBM perlecan (arrowheads) in the (J) –9-D PRK corneas compared with the (I) –4.5-D PRK corneas. At 14 days, after the difference in the subepithelial perlecan (arrowheads) between (K) –9-D and (L) –4.5-D PRK was even more pronounced. Perlecan was detected in anterior stromal cells in both groups (arrows). At (M) 30 days after –9-D PRK there continued to be no subepithelial linear EBM perlecan, although perlecan was detected in cells in the anterior stroma (arrows). In contrast, in corneas (N) at 30 days after –4.5-D PRK, the linear EBM-associated perlecan (arrowheads) was present and there was relatively little perlecan present in stromal cells. Magnification ×200 in all panels.

**Multiplex IHC to Detect Keratocan, α-SMA, Vimentin, and CD45 in Corneas at 1 Month After PRK**

To study the localization of α-SMA+ myofibroblasts and keratocan+ keratocytes in the stroma at 1 month after –4.5-D PRK, compared with –9-D PRK, duplex IHC was performed (Fig. 4). In the –4.5-D PRK corneas the stroma was filled with keratocan and keratocan+ keratocytes without α-SMA+ cells (Fig. 4A). Conversely, in –9-D PRK corneas, there was a layer of α-SMA+ myofibroblasts immediately posterior to the epithelium in all three corneas analyzed (Fig. 4B). Further posterior in the stroma, keratocan+ keratocytes were prominent in the –9-D PRK corneas but keratocan stromal staining appeared less homogeneous than in the –4.5-D PRK corneas. Interestingly, between the α-SMA+ myofibroblasts and the keratocan+...
keratocytes was a thin band of α-SMA– keratocan– cells. Duplex IHC for vimentin and α-SMA in a –9-D PRK cornea at 1 month (Figs. 4C, 4D) showed α-SMA+ myofibroblasts (arrows) were also vimentin+, as expected. Keratocytes deeper in the cornea were also weakly vimentin+. Most, if not all, of the cells in the band between myofibroblasts (arrows in Figs. 4C, 4D) and keratocytes (arrowheads) were also vimentin+. Duplex staining for α-SMA and CD45 (Figs. 4E, 4F) showed some, but not all, of the cells in the band beneath the myofibroblasts were CD45+. Because corneal fibroblasts and keratocytes are CD45+, these cells likely included fibrocytes and their progeny that were undergoing differentiation into myofibroblasts. Some myofibroblasts were also CD45+, and these were myofibroblasts that differentiated from fibrocytes and retained the CD45 marker at this point 1 month after surgery, as was shown in the mouse model of fibrosis. Figures 4G and 4F show control IHC without primary antibodies for vimentin/α-SMA and CD45/α-SMA, respectively, showing there was no nonspecific staining. Similar no primary antibody controls for α-SMA/keratocan did not show nonspecific staining (not shown).

**DISCUSSION**

The results of this study conclusively demonstrate that IL-1α upregulates perlecan mRNA (Fig. 1A) and protein (Fig. 2B) and IL-1β upregulates perlecan mRNA (Fig. 1A) in keratocytes. Conversely, TGF-β1 and -β3 significantly downregulated nidogen-1 and -2 mRNA (Figs. 1B and 1C) in keratocytes and there was a trend for TGF-β1 and -β3 to downregulate perlecan mRNA (Fig. 1A) in keratocytes, although the latter did not reach statistical significance in this study. However, TGF-β1 did downregulate perlecan protein in keratocytes (Fig. 2B) and there was a trend for TGF-β1 to downregulate nidogen-1 and -2 protein in keratocytes (Figs. 2C, 2D). Thus, this study has again shown IL-1 and TGF-β1 having opposing effects on an important aspect of the corneal wound healing response—in this case epithelial BM component production—as was previously shown for myofibroblast viability. This study is an important demonstration of cytokine and growth factor regulation of perlecan and nidogens production during wound healing. Importantly, none of the cytokines or growth factors that were studied significantly regulated perlecan, nidogen-1, or -2 mRNA production in vitro in corneal fibroblasts or myofibroblasts. A prior study found perlecan mRNA expression was increased by IL-1α in hippocampal glial cultures. Ichimaru and coworkers demonstrated that TGF-β1 induced

IHC for CD45 and α-SMA without either primary antibody shows no nonspecific staining. All magnifications are ×200.
Regulation of Expression of Basement Membrane Components

perlecan deposition by chronic obstructive pulmonary disease airway smooth muscle cells. Warren and coworkers,44 found that TNF-α, but not TGF-β, upregulated production of perlecan in prostrate stromal cell lines. Thus, there appears to be variability in the response to TGF-β in stromal cells from different organs.

This study also found that perlecan protein was upregulated (Figs. 3E, 3F) in anterior stromal cells in situ during the first few days after −9-D PRK (that triggers anterior stromal fibrosis) and −4.5-D PRK (that does not trigger anterior stromal fibrosis) and a thin layer of perlecan appeared beneath the healed epithelium at the site of the nascent regenerating EBM. It is hypothesized that IL-1α and -1β released from the injured and healing epithelium into the stroma triggers this upregulation in stromal cells. Importantly, little perlecan protein was detected in the epithelium in situ after injury—supporting the hypothesis that stromal cells are the major contributor of this critical EBM component during EBM regeneration after corneal injury.10

It is known from prior studies that development of an ultrastructurally mature epithelial BM with lamina lucida and lamina densa does not appear until 8 to 10 days after −4.5-D PRK.6 Up to 4 days after PRK, perlecan localization during EBM regeneration appeared similar in the −9- and −4.5-D PRK groups. However, beginning at 7 days after surgery (Fig. 3I) the linear layer of subepithelial perlecan appeared to break down in −9-D PRK corneas. By 14 and 30 days after −9-D PRK (Figs. 3K, 3M) this subepithelial perlecan layer was undetectable, in contrast to the well-delineated layer in the −4.5-D PRK corneas (Figs. 3L, 3N). This occurred despite the presence of perlecan+ anterior stromal cells in the −9-D PRK corneas at these time points. These perlecan+ stromal cells likely include myofibroblast precursor cells that are predominately α-SMA− at 14 days after −9-D PRK but which continue development into mature α-SMA+ myofibroblasts by 30 days after −9-D PRK (Fig. 4B).6,12,43 These in situ results are consistent with prior in vitro results in cultured cells that showed that myofibroblasts produce perlecan protein.9 However, even though these myofibroblasts were in proximity to the nascent EBM (Figs. 3K, 3M), the EBM perlecan that appeared to be within the nascent regenerating EBM up to 4 days after −9-D PRK (Fig. 3G) became disorganized and undetectable immediately beneath the epithelium at 14 and 30 days after −9-D PRK (Figs. 3K, 3M), in contrast to corneas that had −4.5-D PRK at these same time points after surgery (Figs. 3L, 3N). Corneal fibroblasts do not produce perlecan in vitro.9 These results further support the hypothesis that direct participation of keratocytes is necessary for normal regeneration of the EBM.1,2,4–8–10

Importantly, the results in Figures 1 and 2 relate to individual effects of growth factors and cytokines on cultured keratocytes. Figure 3, on the other hand, relates to the more complex in situ situation where the response is regulated by not only the individual cytokines and growth factors tested in Figures 1 and 2, but also likely other cytokine and growth factors and cellular-matrix interactions at play in the organ after injury.

Experiments, such as those in Figure 4, had not been possible until now because there was no antibody available for IHC for keratocan in rabbit corneas in situ after injury.44 Figure 4A shows the uniform distribution of keratocan in the keratocytes and surrounding stroma of the unwounded rabbit cornea. At 1 month after high correction −9-D PRK that caused

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**Figure 5.** TEM of EBM ultrastructure and the anterior stroma at 1 month after −9- or −4.5-D PRK, or in control unwounded, rabbit corneas. (A) In an unwounded cornea, lamina lucida, and lamina densa (arrowheads) are present beneath the epithelium (e). S is stroma in all panels. (B) At 1 month after −4.5-D PRK, the normal lamina lucida and lamina densa (arrowheads) of the EBM are regenerated beneath the epithelium (e). There are no cells seen in the subepithelial stroma with high levels of rough endoplasmic reticulum suggestive of myofibroblasts. In panels (A) and (B), note the uniform diameter of collagen fibrils throughout the stroma, with some seen longitudinally and others cut transversely. (C) At 1 month after −9-D PRK, normal lamina lucida and lamina densa cannot be detected (arrowheads) beneath the epithelium (e) and the subepithelial stromal is filled with layered myofibroblasts (arrows, and same cells seen in IHC for α-SMA shown in (C)) with large amounts of rough endoplasmic reticulum. Note the relative disorganization of the collagen fibrils in the extracellular matrix (‘) between the myofibro-

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*Magnification bars are 1 µm in each panel.*
severe scarring (fibrosis), the anterior cornea beneath the epithelium was populated by α-SMA+ myofibroblasts (Fig. 4B), which could also be noted in multiple layers beneath the epithelium when the cornea was imaged with TEM (Fig. 5C). The distribution of keratocan in the posterior stroma of the fibrotic cornea versus the normal cornea was also less homogeneous (Fig. 4B). Interestingly, between the α-SMA+ myofibroblasts and the keratocan+ kerocytes, there was a band of α-SMA– keratocan– cells. Figure 4C demonstrates that many of the cells in that band were vimentin+. Figure 4E shows that in this cornea 25% of the cells in the α-SMA– keratocan– band were also CD45+. Cells in this band that were vimentin+ CD45+ were likely fibrocytes or their progeny that were being driven by TGF-β from the overlying epithelium (in the absence of a mature EBM; Fig. 5C) to develop into myofibroblasts.41 Only by TGF-β were likely fibrocytes or their progeny that were being driven that this same epithelium-derived TGF-β and (2) promoting the development of mature myofibroblasts acts at least at two levels to promote fibrosis in this high-pathophysiology of disease. 

Studies have suggested that defective EBM regeneration in the higher injury –9-D PRK (in contrast to lower injury –4.5-D PRK) allows for continued epithelium-derived TGF-β1 penetration into the stroma that drives myofibroblast development from precursors.11–14,45 It appears from the current study that this same epithelium-derived TGF-β1 could participate in inhibiting mature BM regeneration by downregulating perlecan and nidogen production by kerocytes. Thus, the TGF-β1 acts at least at two levels to promote fibrosis in this high-injury PRK model, by (1) inhibiting regeneration of mature EBM, the lack of which would tend to facilitate ongoing injury PRK model, by (1) inhibiting regeneration of mature EBM, the lack of which would tend to facilitate ongoing injury PRK) allows for continued epithelium-derived TGF-β1 into the stroma, and (2) promoting the development of mature myofibroblasts from both keratocyte-derived and bone marrow-derived precursor cells.45 Conversely, IL-1α or -1β trigger myofibroblast apoptosis when these cells are in a low TGF-β milieu.46 By stimulating perlecan and nidogen production by kerocytes, IL-1 presumably tends to promote EBM regeneration that inhibits stromal myofibroblast development and promotes apoptosis of any mature myofibroblasts that develop. The multiple and complex interactions between TGF-β and IL-1, as well as those of other growth factors and cytokines, that orchestrate the response to corneal injury seem to be at the heart of the regenerative versus fibrotic corneal repair pathways, and further investigation is needed to understand these important interplays.

The present work demonstrates that IL-1 and TGF-β, critical regulators of the overall wound healing response in the cornea, have important roles in modulating the production of perlecan and nidogens in the cornea during the wound healing response to injury that determines whether the EBM regenerates normally, and, therefore, whether the cornea heals with transparency or fibrosis. It seems likely these master regulators play similar roles in other organs where regenerative versus fibrotic repair is important in the pathophysiology of disease.

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