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The Efficacy of Topical HGF on Corneal Fibrosis and Epithelial Healing after Scar-Producing PRK Injury in Rabbits

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Purpose: To determine the effect of topical hepatocyte growth factor (HGF) on myofibroblast development and corneal opacity after fibrosis-producing photorefractive keratectomy (PRK).

Methods: Twelve New Zealand rabbits had transepithelial PRK. Six rabbits received topical recombinant human HGF (rhHGF) (50 µL of 0.1 mg/mL) 3 times a day for 1 week beginning 6 hours prior surgery and until full closure of the epithelium, and 6 control rabbits received vehicle by the same schedule. Slit lamp photos were taken immediately and at 43 to 45 hours after surgery to determine the rate of epithelial healing. Slit lamp photographs and immunohistochemistry for α-smooth muscle actin were analyzed at 1 month in masked fashion.

Results: The rhHGF group tended to have slower re-epithelization when compared with the controls, but no statistically significant difference was noted ($P = 0.62$). There was no significant difference in the density of myofibroblasts in the central stroma ($P = 0.49$) or corneal opacity ($P = 0.84$) between the HGF and control groups at 1 month after PRK.

Conclusions: Topical rhHGF applied three times a day during the early postoperative period prior to epithelial closure did not significantly change the corneal epithelial healing rate, myofibroblast density, or opacity compared with vehicle after transepithelial -9.0 D PRK injury of the central cornea in rabbits.

Translational Relevance: HGF has been reported to decrease myofibroblast generation and fibrosis in many organs, but topical HGF applied to the cornea until epithelial healing had no effect on scarring fibrosis in rabbit corneas.

Introduction

Stromal fibrosis that occurs after trauma, surgery, or infection to the cornea is an important cause of vision loss, and current surgical treatments include surgeries, such as phototherapeutic keratectomy (PTK), lamellar keratoplasty, or penetrating keratoplasty. The current medical management for corneal scarring, including topical corticosteroids, have limited efficacy and new pharmacologic agents to limit fibrosis would be beneficial.

The cornea becomes scarred after injury as a result of the generation of large numbers of mature myofibroblasts from keratocyte-derived corneal fibroblasts and bone marrow–derived fibrocytes.[1,2] Myofibroblasts are themselves opaque[1] and produce large amounts of disorganized stromal extracellular matrix. The opacity of the myofibroblasts and the matrix they create combine to produce the stromal opacity in fibrotic corneas.[2] The development of myofibroblasts is stimulated by transforming growth factor β (TGF-β) and platelet-derived growth factor.[3,4]

Hepatocyte growth factor (HGF) has been reported to have an important role in inhibiting fibrosis in several organs, including the lung,[5,6] heart,[7] kidney,[8] and liver.[9,10] HGF and its receptor (c-Met) are expressed in the corneal epithelium, stromal cells, endothelium, and in the lacrimal gland.[11,12] A recent study reported that 7 days of topical HGF treatment prevented myofibroblast development and fibrosis in mouse corneas after lamellar keratectomy that included the limbus.[13]

The current masked study investigated the effect of topical HGF compared with vehicle on the development of α-smooth muscle actin (α-SMA)+ myofibroblasts and corneal opacity at 1 month after fibrosis-producing −9D photorefractive keratectomy (PRK) injury to the central cornea in rabbits. The rate of epithelial healing was also evaluated in the two groups.

Methods

Animals and Surgeries

All animals were treated in accordance with the tenets of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and the Animal Control Committee at the Cleveland Clinic Foundation approved this study.

Twelve 12- to 15-week-old female New Zealand white rabbits that weighed 2 to 3 kg each were included in this study. One eye of each rabbit was selected to have a circular 6.5-mm epithelial defect centered over the entrance pupil produced with transepithelial PTK using the VISX Star S4 IR excimer laser (Abbot Medical Optics, Irvine, CA) under dim lighting until the blue fluorescent pulses impacting the epithelium completely transitioned to black of the laser impacting the underlying stroma. A spherical -9D PRK ablation that was 6 mm in diameter was centered within the epithelial defect using the VISX Star S4 IR excimer laser.

HGF and Control Solutions, Masking, and Treatment

All HGF and vehicle solutions were prepared in an outside lab, such that a fresh masked aliquot of HGF or vehicle was provided and thawed to 4°C for each animal for each day of treatment. Thus investigators treating, or directing treatment, of the rabbits did not know whether each rabbit received HGF or vehicle until all of the data for epithelial healing, myofibroblast density, and corneal opacity was finalized, and the key was provided. There were six rabbits in the HGF group, and six rabbits in the vehicle control group. Recombinant human HGF (rhHGF) was obtained from R&D Systems (Cat. #294-HG, Minneapolis, MN) and was frozen in aliquots at 0.1 mg/mL rhHGF in 0.1% bovine serum albumin (BSA) (R&D Systems; BSA was used as a carrier to prevent the rhHGF from being absorbed to siliconized tubes or pipette tips) in phosphate-buffered saline (PBS). The vehicle was 0.1% BSA in PBS. A new tube of masked rhHGF or vehicle solution was thawed to 4°C for each animal each day to provide the three doses per day. Prior studies showed that human HGF activates c-Met in rabbit cells (Wilson SE, unpublished data, 1994).[14]

Each animal received 50 µL rhHGF solution or 50 µL of vehicle solution (numbered vials so the investigators were masked) in the eye to be treated every 2 hours beginning 6 hours prior to surgery. Three more 50 µL doses were applied in each treated eye that day beginning immediately after transepithelial PRK and then 2 and 4 hours later. Rabbits continued to receive masked 50 µL of rhHGF or vehicle control in the treated eye 3 times per day for 1 week at approximately at 9 AM, 1 PM, and 5 PM (the corneal epithelium of all eyes healed between 4 and 6 days after the transepithelial PRK). One drop of 0.3% ciprofloxacin hydrochloride (Ciloxan; Alcon Laboratories, Inc., Ft. Worth, TX) was also applied after PRK twice a day until the corneal epithelium was healed in the treated eye.

General anesthesia for transepithelial PRK, slit lamp photos or 24 hours prior to the euthanasia was performed by intramuscular injection of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg). Topical 1% proparacaine hydrochloride (Alcon Laboratories, Inc.) was also applied to each treated eye prior to surgery. Tylenol (Johnson and Johnson, Skillman, NJ, acetaminophen) in drinking water at a concentration of 2 mg/mL was provided to all rabbits beginning 24 hours prior the procedures and was maintained postsurgery until the corneal epithelium healed.

Slit Lamp Analysis and Cornea Cryofixation

Immediately after surgery, when the epithelial defect was exactly a 6.5-mm diameter circle (Fig. 1) (because the excimer laser in PTK mode precisely ablated the epithelium), each anesthetized rabbit eye had 1% fluorescein solution dropped on the cornea, and a standardized cobalt blue slit lamp photo was taken. Then at 43 to 45 hours after surgery (Fig. 1), while the epithelium was in the midst of healing, each anesthetized rabbit eye again had 1% fluorescein solution dropped on the cornea, and another standardized cobalt blue slit lamp photo was taken. The baseline epithelial image and the 43 to 45 hour epithelial healing image for each eye were analyzed with Image-Pro Plus v7.0.1.658 software (Media Cybernetics, Rockville,
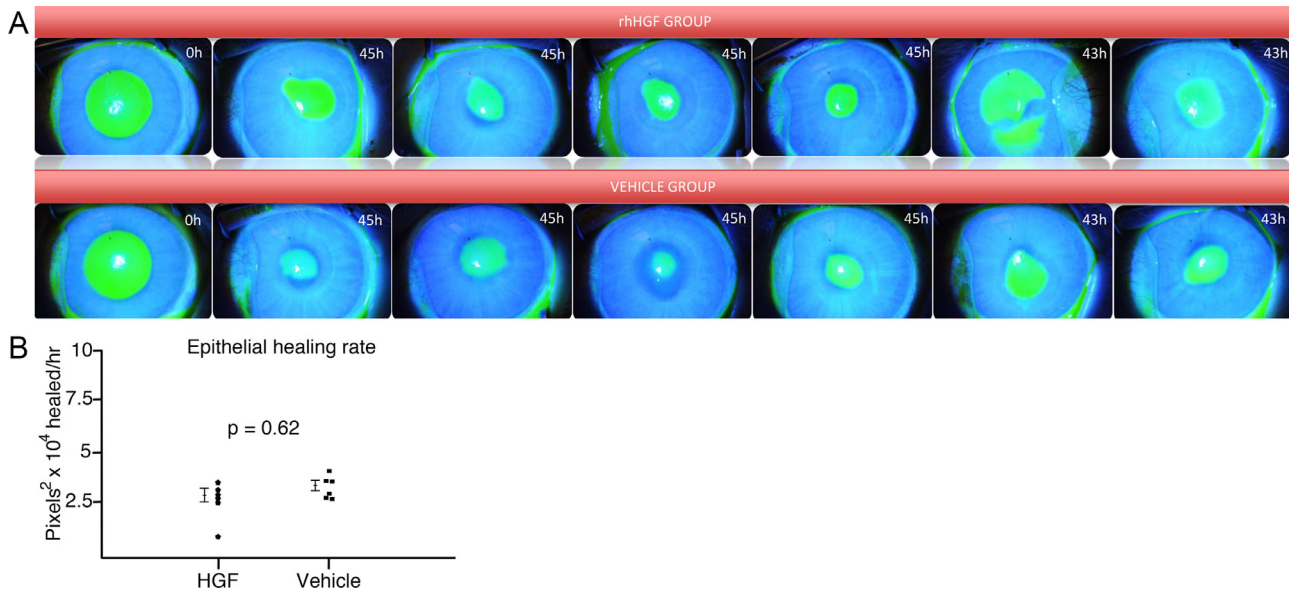
Figure 1. (A) Fluorescein staining of epithelial defects in the vehicle-treated and HGF-treated groups. All corneas had identical 6.5-mm epithelial defects (0 hour) immediately after transepithelial PRK surgery and one example cornea is shown for each group. The remaining epithelial defect is noted for each vehicle-treated and HGF-treated cornea, along with the time after surgery the defect was photographed. These epithelial defects were analyzed with Image-Pro Plus to provide data for the graph in B. (B) The pixels^2 healed per hour was calculated from the epithelial defect at time 0 immediately after surgery to the 43 to 45 hour time point after surgery. There was no difference in the rate of healing in the two groups.

MD), and the difference between the two time points was used to calculate the rate of epithelial closure per hour for each eye. Epithelial healing rates were measured in pixels squared of area healed per hour (px^2/h).

At 1 month after PRK surgery (which is the peak of corneal fibrosis development in rabbits after PRK), the rabbits were anesthetized and had standardized slit lamp photographs of the corneas to document opacity (Fig. 2). These images in Figure 2A were analyzed with open source FIJI ImageJ v1.52 software (https://imagej.net/Fiji) to acquire the images and calculate the pixels^2 of corneal opacity in the central 6 mm of the cornea where the PRK ablation was performed using previously described methods.[15]

Rabbits were then euthanized with 100 mg/kg intravenous injection of Beuthanasia (Merck, Kenilworth, NJ), and the corneal-scleral rims were removed with sharp Westcott scissors and 0.12-mm forceps without touching the cornea, and embedded in liquid optimal cutting temperature compound (Sakura Fine Tek, Torrance, CA) within a 24 mm × 24 mm × 5 mm mold (Electron Microscopy Sciences, Hatfield, PA) and snap frozen. The frozen tissue blocks were maintained at –85°C until sectioning. Tissue sections (8 μm in thickness) were cut with a cryostat (HM525; Thermo Fisher Scientific, Waltham, MA) and were placed on

25 mm × 75 mm × 1 mm microscope slides (Superfrost Plus; Thermo Fisher Scientific) and maintained frozen at –20°C until immunohistochemistry (IHC) was performed.

α-SMA Immunohistochemistry

IHC was performed on all corneas to study expression of α-SMA. Slides containing three sections of each eye were washed twice in 1x PBS for 5 minutes and then fixed using 4% paraformaldehyde for only 5 minutes. Slides were washed twice more in 1x PBS for 5 minutes and sections were blocked using 5% normal donkey serum (#017000121, The Jackson Laboratory, Bar Harbor, ME) in 1x PBS for 1 hour at room temperature. Primary antibody goat anti-α-SMA (NB300-978, Novus Biologicals, Centennial, CO) diluted 1:100 in 5% normal donkey serum in 1x PBS were placed on the slides and incubated at room temperature for 1 hour. Slides were then washed twice in 1x PBS for 5 minutes. Secondary antibody donkey anti-goat Alexa Fluor 568 (A-11057; Thermo Fisher Scientific, Bedford, OH) diluted 1:200 in 5% normal donkey serum in 1x PBS was added to the sections for 1 hour at room temperature. Slides were finally washed three times in 1x PBS, air dried, and mounted with Vectashield mounting media containing DAPI (H-1200; Vector Laboratories, Burlingame,
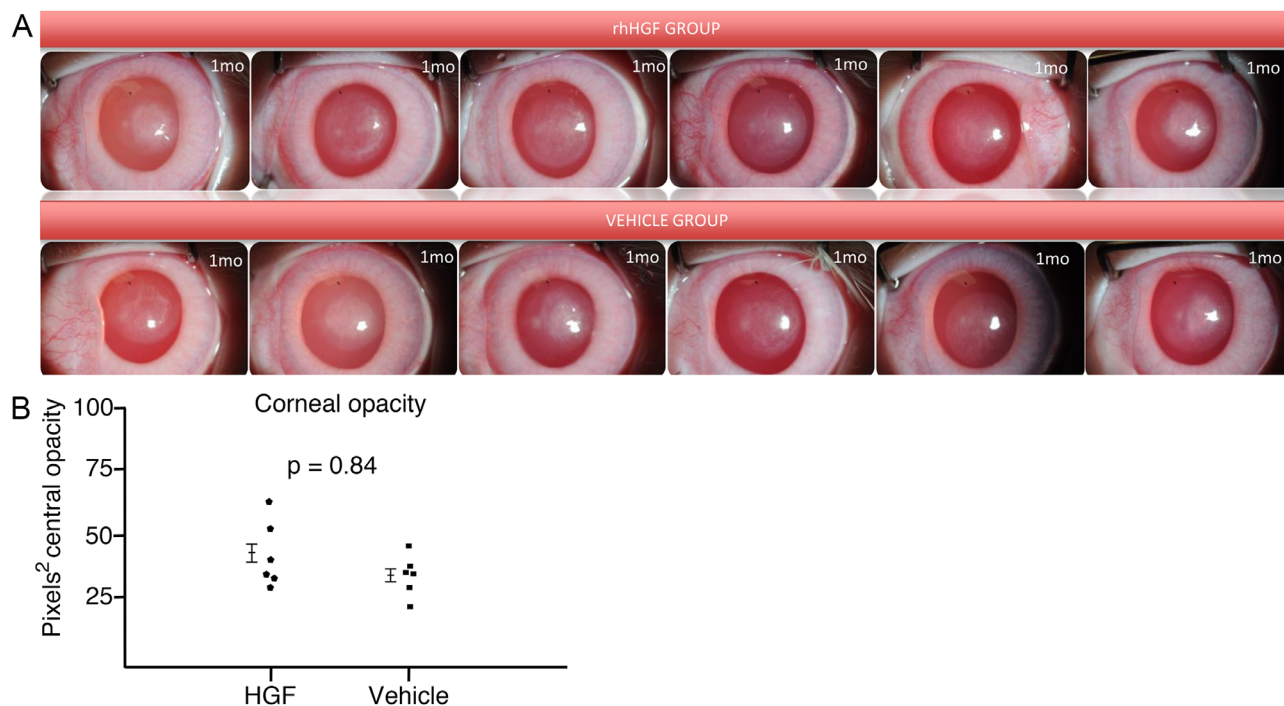
Figure 2. (A) Slit lamp photos of each of the corneas in the HGF-treated and vehicle-treated groups at 1 month after transepithelial −9D PRK. Dense corneal opacity was noted in all corneas. (B) Imaging analysis was performed to determine the average level of opacity in pixels^2 of the central 6 mm of each cornea in the HGF-treated and vehicle-treated groups. There was no statistically significant difference between the two groups.

CA) to allow visualization of all nuclei. Negative controls were included with each experiment in which sections were incubated without primary antibody. The sections were analyzed and photographed with a Leica DM5000 microscope (Leica, Buffalo Grove, IL) equipped with Q-imaging Retiga 4000RV camera (Surrey, BC, Canada) and Image-Pro software (Media Cybernetics, Inc.).

The density of myofibroblasts in a 400X microscopic field of the central cornea bisected by epithelial basement membrane were counted at the microscope so individual myofibroblasts could be distinguished by up- and down-focusing. Cells that stained α-SMA-positive were manually counted in three nonoverlapping adjacent fields per section. Thus the average of nine counts of α-SMA-positive cells per 400X field were used to determine the myofibroblast density per 400X field for each eye.

Statistical Analysis

Variations were expressed by the mean and standard error of mean. Data were analyzed with a two-tailed Mann–Whitney U test. A P value <0.05 was considered significant.

Results

Effect of HGF and Vehicle on the Rate of Corneal Epithelial Healing

The epithelial defects immediately after transepithelial PRK was exactly a 6.5-mm circle in all 12 eyes in this study. One eye is shown from each group at time 0 in Figure 1A to demonstrate the precision of epithelial defect generation using this method. The epithelial defect of each cornea in the study at 43 to 45 hours after transepithelial −9D PRK is also shown in Figure 1A. The rate of epithelial healing was $27,419 \pm 3259$ pixels^2 per hour and $33,668 \pm 1746$ pixels^2 per hour in the HGF- and vehicle-treated groups, respectively (Fig. 1B), and was not significantly different between the two groups ($P = 0.62$).

Effect of HGF and Vehicle on Central Corneal Opacity at 1 Month after PRK

At 1 month after transepithelial −9.0 PRK in rabbits, the central corneal opacity (Fig. 2A) was 42 ± 4 pixels^2 and 34 ± 3 pixels^2 in the HGF- and vehicle-treated groups, respectively (Fig. 2B). Although there
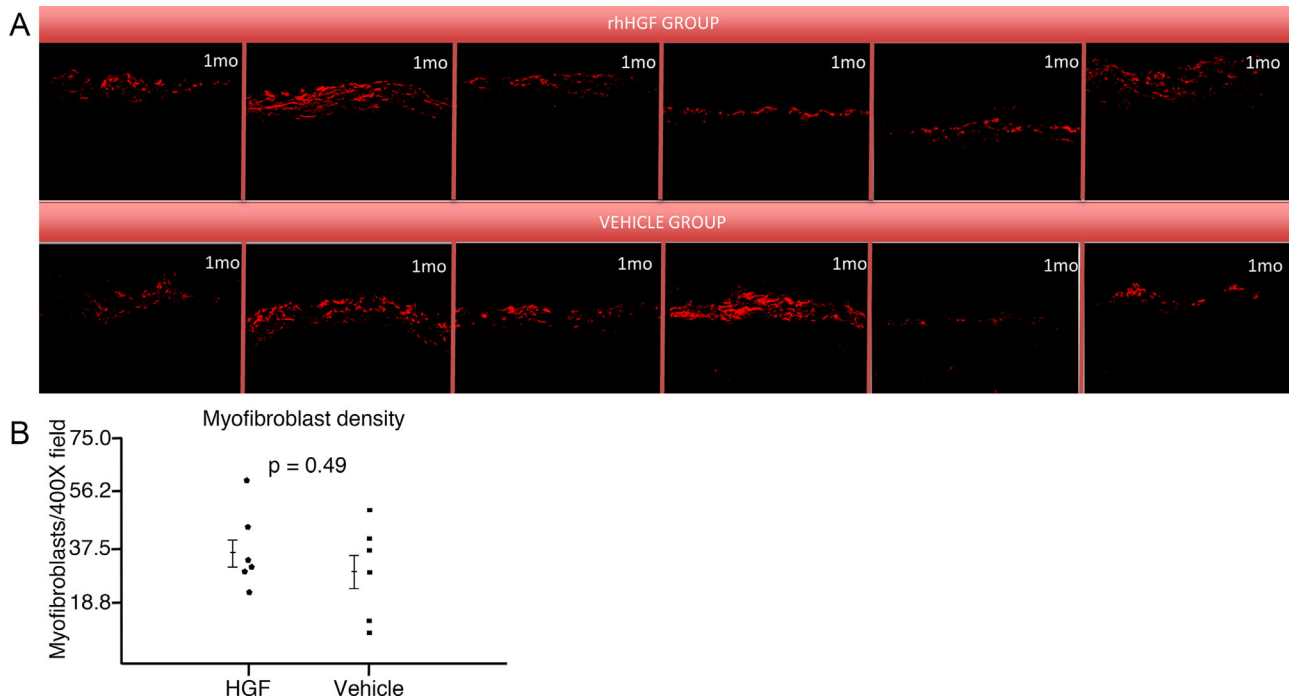
Figure 3. (A) IHC for myofibroblast marker α-SMA in rabbit corneas at 1 month after -9D transepithelial PRK. Representative staining for a section from each cornea in the vehicle-treated and HGF-treated groups is shown. Control IHC was performed without primary antibody and no staining was detected (not shown). Unwounded control corneas had no α-SMA+ cells (not shown). 100X mag. Note the myofibroblast counting at the microscope was performed at 400X, in which individual myofibroblasts could be discerned for Fig. 2B. (B) The myofibroblast density for each cornea at 1 month after -9D PRK was determined from three 400X central corneal fields bisecting the epithelial basement membrane in each of three corneal sections for each cornea in the HGF-treated and vehicle-treated groups. There was no difference in the mean myofibroblast density in the central cornea of the two groups.

was a small trend toward an increase in opacity in the HGF-treated group, the difference was not statistically significantly different ($P = 0.84$).

Effect of HGF and Vehicle on Myofibroblast Density at 1 Month after PRK

At 1 month after transepithelial -9.0 PRK in rabbits, the anterior stromal α-SMA-positive myofibroblast density (Fig. 3A) was 37 ± 5 cells/400X field and 30 ± 6 cells/400X field in the HGF- and vehicle-treated groups, respectively (Fig. 3B), and was not significantly different between the two groups ($P = 0.49$).

Discussion

HGF is a glycoprotein growth factor produced by mesenchymal cells (keratocytes and corneal fibroblasts in the cornea) that is best known for its role in stromal-epithelial interactions in modulating the functions of epithelial cells via the c-Met HGF receptor.[11] Thus in the cornea, HGF is produced by keratocytes and corneal fibroblasts to modulate the proliferation, migration, differentiation, and apoptosis of corneal epithelial cells.[16] HGF is also produced by the lacrimal gland and secreted into tears to modulate corneal and conjunctival epithelial cells.[12,17]

More recently, HGF has been reported to have a role in inhibiting fibrosis in organs such as lung,[5,6] heart,[7] kidney,[8] and liver.[9,10] Mittal et al.[13] reported that mesenchymal stem cells can ameliorate myofibroblast-mediated fibrosis in mouse corneas that have anterior lamellar keratectomies by secreting HGF. They also showed that topical HGF could decrease corneal opacity produced by lamellar keratectomy injury by modulating myofibroblast differentiation. There are possible reasons why the results in the present study did not correspond to the results in another species (mice) after lamellar keratectomy injury reported by Mittal et al.[13] First, the type of injury produced in the mouse model was very different from the one used in the present rabbit study. Mittal et al.[13] performed a

3-mm diameter lamellar keratectomy similar to that first reported by Hutcheon et al.[18]; however, Hutcheon et al. used a 2-mm lamellar keratectomy, which confined the injury to the cornea, similar to the transepithelial PRK injury in the present study. Because C57Bl/6 mice have a corneal diameter of only 2.6 ± 0.2 mm,[19] the Mittal et al.[13] injury would have involved most, if not all, of the limbus in each mouse cornea. Thus the healing response to this injury would likely be very different from a central corneal injury, such as PRK, penetrating keratoplasty, or microbial keratitis, including conjunctivalization of the healing epithelium.[20,21] Also, Mittal et al.[13] found myofibroblasts present in the superficial corneal stroma after the lamellar keratectomy involving the limbus after only a few days and ended their study at 7 days, whereas prior studies of central corneal injuries in C57Bl/6 mice found that, although small numbers of α-SMA-positive cells may be seen in the cornea the first few days after injury, many more of these myofibroblasts developed at 2 to 4 weeks after injury.[22] Mittal et al.[13] also only applied topical HGF two times per day. Thus major differences in the injuries to the corneas and HGF application could explain the differences in results. Miyagi et al.[23] also demonstrated that HGF can suppress the myofibroblast phenotype promoted by TGF-β1 in human corneal stromal cells in vitro, and suggested HGF had the potential for use as a therapeutic agent to decrease corneal fibrosis after corneal injury.

The present study was designed to examine the effects of topical HGF in modulating myofibroblast-mediated fibrosis after a highly reproducible, fibrosis-producing, central corneal injury (transepithelial PRK) in rabbits. It also examined the effect of high-dose topical HGF in modulating epithelial healing after the PRK procedure. RhHGF was used in this study because it has been previously shown to modulate functions in rabbit cells (Wilson SE, unpublished data, 1994).[14] This study found no effect of topical 0.1 mg per mL HGF started prior to surgery and continued three times a day for 1 week (when the epithelial defect was closed in all eyes and no further HGF was likely to penetrate into the stroma) in preventing myofibroblast development or corneal opacity after PRK. Also, no effect of this high dose of HGF on corneal epithelial healing was noted, although the high dose was primarily selected to facilitate entry into the stroma to potentially modulate myofibroblast development.

It is likely HGF expression by keratocytes and cornea fibroblasts modulates myofibroblast development in the cornea given the work of Mittal et al.[13] and studies demonstrating that HGF production by cells in other organs regulates myofibroblast development and fibrosis.[5,7] This active role of keratocytes and corneal fibroblasts controlling myofibroblast development, on the one hand, and serving as precursors of myofibroblasts, on the other hand, deserves further investigation.

Thus it is possible that other modalities for delivering HGF to the central stroma could have better regulated fibrosis after central corneal, profibrotic injury. Thus the use of a vector-producing prolonged, continuous expression of HGF could have more effectively suppressed myofibroblast development, as has been reported in other systems.[24,25] Alternatively, a small molecule agonist of the c-Met receptor that could pass through the corneal epithelium, and therefore could be given topically for a longer period of time following surgery, could potentially have a greater effect on myofibroblast development than a large growth factor like HGF that is likely unable to pass through the intact epithelium. Thus based on the findings of HGF modulating fibrosis in the cornea with a corneo-limbal injury model[13] and in other organs,[5–10] further investigation into the activation of c-Met and resulting inhibition of myofibroblast development is warranted.

It is interesting that HGF applied topically did not accelerate epithelial healing in the present study. It is possible that the high dose of HGF used to facilitate stromal penetration actually is detrimental to epithelial healing (which was the trend in this study (Fig. 1B), as was reported in a prior bovine study.[26] Alternatively, it is possible that exogenous HGF would provide no benefit to regenerate the epithelium in a cornea where keratocytes, corneal fibroblasts, and tears are already providing adequate HGF.[11,12,16]

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