Lack of Elevated Expression of TGFβ3 Contributes to the Delay of Epithelial Wound Healing in Diabetic Corneas

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Received: December 6, 2023
Accepted: March 12, 2024
Published: March 28, 2024

Citation: Gao N, Yu FS. Lack of elevated expression of TGFβ3 contributes to the delay of epithelial wound healing in diabetic corneas. *Invest Ophthalmol Vis Sci.* 2024;65(3):35. https://doi.org/10.1167/iovs.65.3.35

Purpose. To investigate the mechanisms underlying the differential roles of TGFβ1 and TGFβ3 in accelerating corneal epithelial wound healing (CEWH) in diabetic (DM) corneas, with normoglycemia (NL) corneas as the control.

Methods. Two types of diabetic mice, human corneal organ cultures, mouse corneal epithelial progenitor cell lines, and bone marrow–derived macrophages (BMDMs) were employed to assess the effects of TGFβ1 and TGFβ3 on CEWH, utilizing quantitative PCR, western blotting, ELISA, and whole-mount confocal microscopy.

Results. Epithelial debridement led to an increased expression of TGFβ1 and TGFβ3 in cultured human NL corneas, but only TGFβ1 in DM corneas. TGFβ1 and TGFβ3 inhibition was significantly impeded, but exogenous TGFβ1 and, more potently, TGFβ3 promoted CEWH in cultured TKE2 cells and in NL and DM C57BL6 mouse corneas. Wounding induced similar levels of p-SMAD2/SMAD3 in NL and DM corneas but weaker ERK1/2, Akt, and EGFR phosphorylation in DM corneas compared to NL corneas. Whereas TGFβ1 augmented SMAD2/SMAD3 phosphorylation, TGFβ3 preferentially activated ERK, PI3K, and EGFR in healing DM corneas. Furthermore, TGFβ1 and TGFβ3 differentially regulated the expression of S100a9, PAI-1, uPA/tPA, and CCL3 in healing NL and DM corneas. Finally, TGFβ1 induced the expression of M1 macrophage markers iNOS, CD86, and CTGF, whereas TGFβ3 promoted the expression of M2 markers CD206 and NGF in BMDMs from db/db or db/+ mice.

Conclusions. Hyperglycemia disrupts the balanced expression of TGFβ3/TGFβ1, resulting in delayed CEWH, including impaired sensory nerve regeneration in the cornea. Supplementing TGFβ3 in DM wounds may hold therapeutic potential for accelerating delayed wound healing in diabetic patients.

Keywords: corneal wound healing, TGFβ signaling, cornea, diabetic keratopathy, corneal nerve de-/regeneration, macrophages

With a rapid increase in the prevalence of diabetes mellitus (DM), projected to affect more than 600 million people by 2040, ocular complications have become a leading cause of blindness worldwide.[1,2] In addition to abnormalities of the retina (e.g., diabetic retinopathy)[3] and the lens (e.g., cataract),[4] up to 70% of people with diabetes also experience corneal problems, including keratopathy and neuropathy.[5,6] Corneal abnormalities include alterations in the epithelial basement membrane,[7] fewer hemidesmosomes,[8] the deposition of advanced glycation end products,[7,9] and a decrease in the density of corneal sensory nerve fibers and endings.[10] Hyperglycemia significantly alters the structure and function of corneal epithelial cells (CECs), resulting in basal cell degeneration,[11] decreased cell proliferation,[12,13] superficial punctate keratitis,[14] the breakdown of barrier function, fragility,[15,16] recurrent erosions, and persistent epithelial defects,[17] depending on the duration of DM and on the serum concentration of glycated hemoglobin HbA1c.[18] The epithelial abnormalities, termed keratopathy/epitheliopathy, are likely the result of these pathological changes and are resistant to conventional treatment regimens.[19] Hence, a better understanding of the pathogenesis of diabetic keratopathy should lead to better management of the disease.

Similar to other epithelial linings, the corneal epithelium is under constant physical, chemical, and biological insults, often resulting in tissue injury.[20] CECs respond rapidly to injury, initiating a healing process of cell migration as a sheet to cover the defect and to reestablish its barrier function.[10,21] Unlike diabetic cataract and retinopathy, diabetic keratopathy does not cause detectable clinical symptoms unless CECs are removed or the eye is injured. Epithelial wound healing is delayed in diabetic corneas and may be associated with sight-threatening complications such as stromal opacification, surface irregularity, and microbial keratitis.[19] Hyperglycemia is likely to execute its adverse effects on corneal wound healing by modifying the expression of a host of wound-response genes, including growth factors, cytokines/chemokines, and proteinases.[21–23] The altered expression of these factors, in turn, modifies the behavior of the injured cornea, leading to its further deterioration and delayed wound healing.

Among the multitude of cytokines and growth factors required for proper wound closure,[22] transforming growth factors, such as TGFβ, play a critical role in wound healing. TGFβ is a pleiotropic multifunctional growth factor/cytokine that regulates several essential cellular processes in the body. TGFs have profound effects on wound healing and tissue repair at different phases.[24] Three mammalian isoforms of TGFβ have been identified and are encoded by distinct genes under the control of different promoters.[25–29] In vitro, the three isoforms elicit similar responses.[30] In vivo, each isoform shows a unique expression pattern, suggesting that they each play a distinct function during development, wound repair, and fibrosis.[31] They also exhibit different physiological and pathological activities in certain cell types and tissues and have been the major target for drug development.[32–37] Our previous study demonstrated that TGFβ1 and TGFβ3 are markedly upregulated in response to wounding in rat corneas, but only TGFβ3 upregulation was dampened by hyperglycemia,[38] suggesting differential effects of hyperglycemia on the expression of TGFβ isoforms in response to wounding. Although TGFβ1, referred as TGFβ in many studies, and TGFβ3 have been studied individually, a side-by-side comparison of their roles in normal and diabetic corneas and during wound healing remains scarce.

In the current study, we used mouse model diabetes and CEC wound debridement to compare the effects of TGFβ1 and TGFβ3 in mediating corneal epithelial wound healing. We targeted TGFβ1 and TGFβ3 in normal (NL) corneas with neutralization antibodies and treated DM corneas with delayed wound healing with recombinant TGFβ1 and TGFβ3. We showed that TGFβ1 and TGFβ3 exhibited different effects on epithelial wound closure, post-wound sensory nerve regeneration, signaling pathways, and expression of wound-related genes. Targeting TGFβ isoforms and/or their associated signaling pathways may improve wound healing and suppress wound-associated fibrosis.

Methods

Mice

Wild-type C57BL6 (B6) mice (8 weeks of age; 20–24 g body weight) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). B6 mice were induced to develop type 1 DM according to a low-dose streptozotocin (STZ; 50-mg STZ/kg mouse) induction mouse protocol, without fasting prior to STZ injections. Glucose levels and body weight were monitored weekly. Animals with blood sugar levels higher than 350 mg/dL were considered diabetic and were used (with age-matched animals as controls) 8 weeks after STZ injections.[39]

Corneal Epithelial Debridement Wound

Mice were anesthetized by intraperitoneal injection of ketamine–xylazine. The central corneal epithelium was then demarcated with a 2-mm trephine for mouse corneas or a 4-mm trephine for human corneas and then removed using a blade under a dissecting microscope. Care was taken to minimize injury to the epithelial basement membrane and stroma. While under anesthesia, ocular surfaces were protected from drying by topical administration of bacitracin ophthalmic ointment immediately after injury. The CECs that were scraped off the corneas during wounding and at the end of the experiment were collected with a blade, frozen immediately in liquid nitrogen, and stored at an Eppendorf tube at −80°C.

Assessment of wound closure was performed by fluorescein staining (0.1% sterile fluorescein solution in PBS) followed by rinsing of the ocular surface with PBS and photographing with a digital camera. The remaining denuded area was quantitated using Photoshop (Adobe, San Jose, CA, USA). The healing rate was calculated as follows: (original wound area − current wound area)/original wound area (in percent).

Western Blot

The CECs scraped off the corneas that served as controls during wounding and at the end of experiment were collected and frozen immediately in liquid nitrogen and stored at an Eppendorf tube at −80°C. For western blot, human and mouse CECs were lysed with radioimmunoprecipitation assay buffer. The lysates were centrifuged to obtain the supernatant. Protein concentration was determined by bicinchoninic acid (BCA) assay. The protein samples were separated by SDS-PAGE and electrically transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 3% BSA and subsequently incubated with primary and secondary antibodies. Signals were visualized using Thermo Scientific SuperSignal West Pico PLUS Chemiluminescent Substrate (#34580; Thermo Fisher Scientific, Waltham, MA, USA) using an Invitrogen iBright Imaging System (Thermo Fisher Scientific). Antibodies to phospho-Akt (p-Akt, #9271, 1:500 dilution), p-EGFR (#4470, 1:500 dilution), and p-SMAD2/SMAD3 (#8828, 1:500 dilution) were obtained from Cell Signaling Technology (Danvers, MA, USA); p-ERK antibody (#sc-7383, 1:1000 dilution) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and anti-β-actin antibody (#A1978, 1:10000 dilution), which served as the loading control, was obtained from Sigma-Aldrich (St. Louis, MO, USA.

Cell Culture and Reagents

The TKE2 mouse corneal epithelial progenitor *cell line* isolated from the basal layer of the limbal epithelium was derived from outbred CD-1 albino mice[40] and purchased from Sigma-Aldrich (#11033107). TKE2 cells were authenticated as having five out of nine authentication alleles and two repeat numbers, all of which are found in different mouse strains.[41] These cells may be induced to express PAX6 and keratin 12 mRNA (data not shown). TKE2 cells were cultured in keratinocyte serum-free medium (KSFM; Life Technologies, Carlsbad, CA, USA) supplemented with bovine pituitary extract and epidermal growth factor. To assess the effects of high glucose in culture media, TKE cells were cultured in either normal glucose (NG; 5-mM glucose + 20-mM mannitol) or high glucose (HG; 25-mM glucose) for three passages. At the fourth passage, TKE2 cells were cultured in six-well plates, wounded with a 20-µL *pipette tip*, and allowed to heal for 48 hours in KSFM. The scratch wounds were allowed to heal for 24 hours, and the same position was photographed 0 and 24 hours post-wounding.

Immunostaining of Whole-Mount Corneal Tissue

To semiquantitatively assess the extent of corneal innervation and to quantify macrophages (MΦ) in the corneas,

whole-mount confocal microscopy (WMCM) was used. Corneas excised at the indicated times were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) and stored at 4°C until further processing. Whole-mount staining of sensory nerves with anti–β-tubulin III Abs (clone TUJ1, 1:100 dilution; Covance, Princeton, NJ, USA) or MΦ with anti-F4/80 (#14-4801-82, 1:100 dilution; eBioscience, San Diego, CA, USA) was performed as previously described.[42] Corneal whole mounts were examined using an Eclipse 90i widefield fluorescent microscope (Nikon, Tokyo, Japan). WMCM images were obtained by scanning the whole corneas and were automatically assembled from the acquired scanning images, including residual conjunctival images. To quantify corneal innervation, manual labeling and measurements were used in ImageJ (National Institutes of Health, Bethesda, MD, USA). Innervation in a region was calculated as the percentage of area covered by β-tubulin III staining using ImageJ. To quantitate the number of F4/80-positive cells, images were analyzed by threshold-based automatic particle counting (http://imagej.net/Particle_Analysis) of the whole corneas.

Subconjunctival Injection of Molecular Reagents

The subconjunctival injection volume for mice was 5 µL at one site. Dosages for neutralizing antibodies for recombinant TGFβ1/TGFβ3 were determined according to the median ED_{50} values provided by the supplier, and the maximal concentrations were chosen. Anesthetized mice were injected with 5 µL cornea neutralizing antibodies (200 ng TGFβ1 or 500 ng TGFβ3) or recombinant TGFβ1/TGFβ3 (40 ng TGFβ1/10 ng TGFβ3) using a 34-gauge needle with a 0.01-mL Nanofil syringe 4 hours before wounding, with isoform-matched immunoglobulin G (IgG) or BSA as controls. At 24 hours post-wounding (hpw), the corneas were stained and photographed.

RNA Extraction and PCR Analyses

CECs were scraped off the cornea, and RNA was extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. cDNA was generated with an Invitrogen oligo(dT) primer followed by analysis using real-time PCR with PowerUp SYBR Green Master Mix for qPCR (Applied Biosystems, Waltham, MA, USA) based on the expression of β-actin. Table lists the primer pairs used.

Statistical Analysis

The statistical analyses were performed using Prism 6 (GraphPad, Boston, MA). Data are presented as mean ± SD. Experiments with two treatments and/or conditions were analyzed for statistical significance using a two-tailed Student's *t*-test. Experiments with more than two conditions were analyzed using one-way ANOVA. If more than two groups of mice were used, such as wounded and non-wounded diabetic and normal mice, a two-way ANOVA was used for analysis to determine overall differences. A Bonferroni post hoc test was performed to determine statistically significant differences. Significance was accepted at $P < 0.05$. Experiments were repeated at least twice to ensure reproducibility.

Table. Primers Used for Real-Time PCR

Gene	Forward Primer	Reverse Primer
β-actin	GACGGCCAGGTCATCACTATTG	AGGAAGGCTGGAAAAGAGCC
ccl3	AGATTCCACGCCAATTCATC	CTCAAGCCCCTGCTCTACAC
s1009	TGGGCTTCAACTGCTCTTACC	GGTTATGCTGCGCTCCATG
plat	GGCAGAACATACAGGGTGGT	CTGCAGTAATGCGATGTCGT
Plau	TTCAGAGCTTTCCACCGAAT	TCCTTTCTTAGAGGCCCGGT
serpine1	CTGGGTCAGTCGAAGGAGAG	ACGTCATACTCGAGCCCATC

Results

Differential Expression of TGFβ1 and TGFβ3 in Normal and Diabetic Human Corneal Epithelial Cells

In our previous study, we found that wounding induced the expression of both TGFβ1 and TGFβ3 in NL corneas but not TGFβ1 in DM corneas in diabetic rats and mice.[38,43] To confirm the pattern of TGFβ isoform expression, we used cultured human corneas from healthy individuals, normoglycemic patients, and diabetic patients and performed epithelium debridement wounding. Diabetic corneas were obtained from patients with retinopathy as an indicator of diabetic complications and were cultured in 25-mM glucose; normal corneas were cultured in 5-mM glucose. CECs collected during wounding were used as the control, and cells that migrated to the original wound bed were collected 48 hpw as wounded samples. In all samples, two bands of TGFβs were observed, likely representing latent and active forms of the proteins (Fig. 1). Similar to what was observed in diabetic rodent corneas, the basal levels of TGFβ1 and TGFβ3 detected in NL and DM corneas were similar. Wounding significantly increased the expression of both TGFβ1 and TGFβ3 in NL corneas, whereas TGFβ3 levels, compared to unwounded CECs, did not change after wounding in ex vivo human DM corneas. Hence, TGFβ1 and

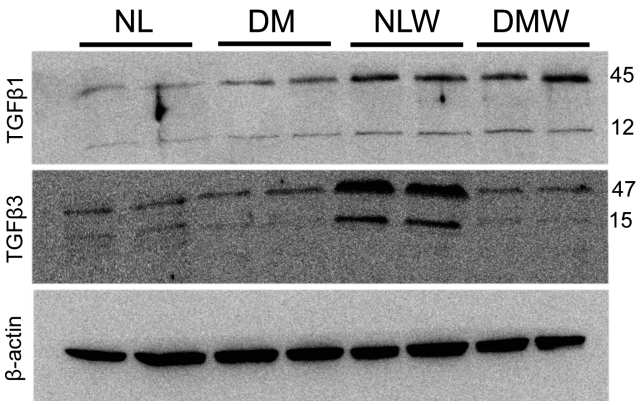

Figure 1. TGFβ1 and TGFβ3 expression in organ-cultured human NL and DM corneas with or without wounding. Human corneas, obtained from Eversight Eye Bank, were processed for organ culture and subjected to epithelial debridement to create a 4-mm-diameter wound. The unwounded and wounded corneas from nondiabetic (NL) patients were cultured in 5-mM glucose + 20-mM mannitol, and those from diabetic (DM) patients in high glucose (25-mM glucose). After being cultured for 48 hours post-wounding, the epithelial cells migrated to the original wound bed in wounded corneas (NLW for nondiabetic wounded and DMW for diabetic wounded) or remained at the center in unwounded corneas. These were then collected and processed for western blot analysis. β-actin was used as the internal control for equal protein loading. The numbers on the *right side* indicate the molecular weight of corresponding bands in kilodaltons (kDa).

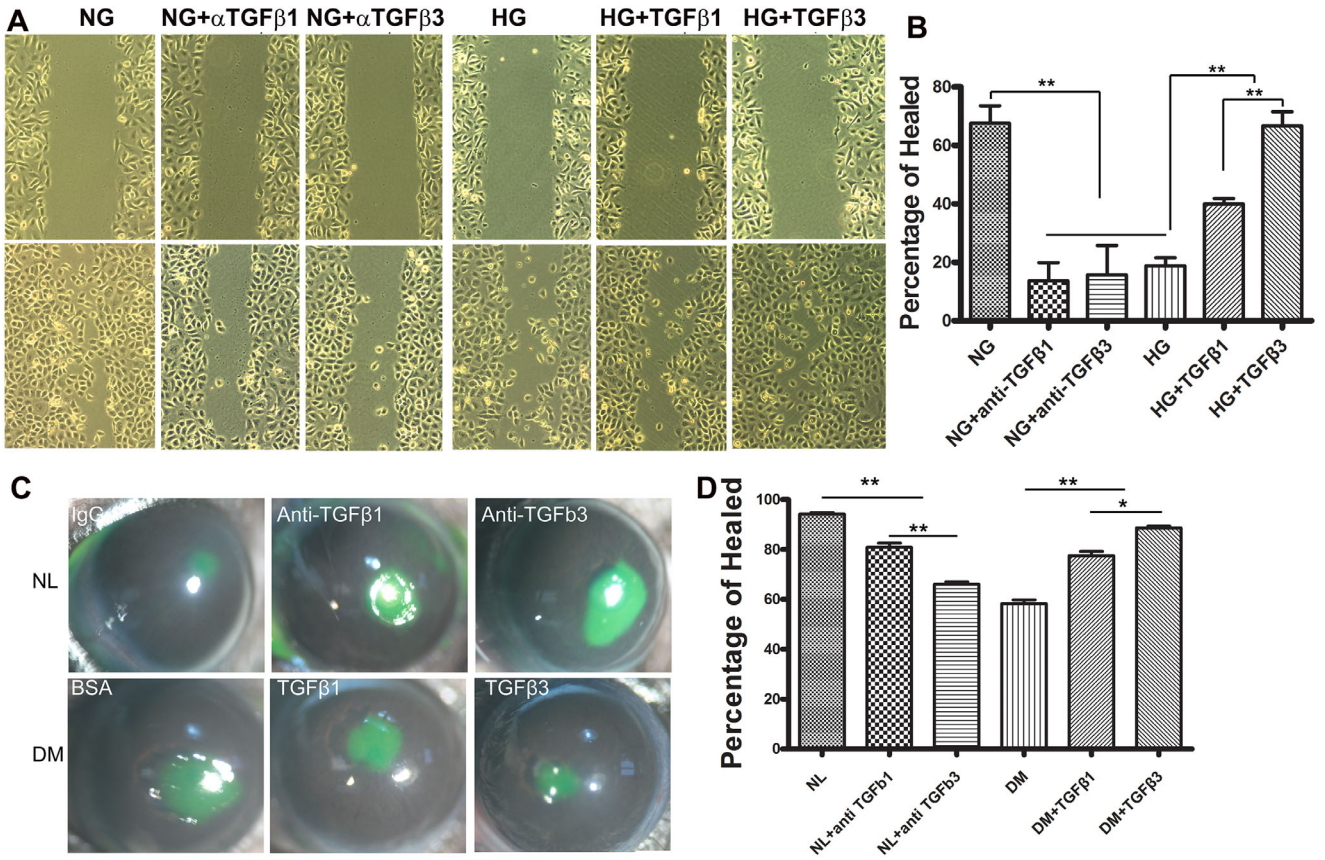

Figure 2. Effects of altering TGFβ signaling on epithelial wound closure of NL or DM mouse corneas in vitro and in vivo. (**A**) TKE2 cells were cultured in 5-mM glucose + 20-mM mannitol and/or in high glucose (25-mM glucose) for three passages. At the fourth passage, TKE2 cells were cultured in six-well plates. Upon reaching confluence, two scratch wounds crossing each other at a 90° angle were made and allowed to heal in KBM media containing anti-TGFβ1/TGFβ3 or recombinant TGFβ1/TGFβ3. The same sites were photographed at 0 and 48 hours post-wounding. (**B**) The wound areas were measured, and the percentage of the healing area relative to the original wound was calculated. The results are presented as a percentage of healed ($n = 5$). (**C**) NL and DM corneas were pretreated with BSA (control) or recombinant TGFβ1 and TGFβ3 4 hours prior to epithelial debridement. The corneas were wounded by 2-mm-diameter epithelium debridement and treated with the same dosage of TGFβ immediately after wounding (0 h). The wounds were allowed to heal in vivo. At 24 hours post-wounding, injured corneas were stained with fluorescein to photograph the remaining wound area. (**D**) The wound sizes were calculated using Adobe Photoshop and are presented as a percentage of healed ($n = 3$). $^{*}P < 0.05$, $^{***}P < 0.001$ (two-way ANOVA). Two independent experiments were performed.

TGFβ3 exhibited similar expression patterns in human and rodent CECs in response to wounding.

Differential Effects of TGFβ1 and TGFβ3 on Corneal Epithelial Wound Healing

After observing the expression of TGFβ1 and TGFβ3 during homeostasis and wound healing in human corneas, we investigated the roles of TGFβ1 and TGFβ3 in corneal epithelial wound healing using two complementary approaches: neutralizing monoclonal anti-TGFβ1 or TGFβ3 antibodies and exogenous TGFβ1/TGFβ3 administration in cultured TKE2 cells and NL and DM B6 mice. To create a hyperglycemia environment, TKE2 cells were cultured in either normal glucose (NG; 5-mM D-glucose with 20-mM mannitol) or high glucose (HG; 5-mM D-glucose) for three passages. In the fourth passage of TKE2 cells cultured in NG, neutralization of TGFβ1 or TGFβ3 markedly delayed wound closure compared to the control. On the other hand, exogenous TGFβ3 (and to a lesser extent TGFβ1) increased the rate of wound closure in HG-cultured TKE cells (Figs. 2A, 2B). In vivo, mice were injected with 5 µL/cornea neutralizing antibodies or recombinant TGFβ1/TGFβ3 using a Hamilton syringe 4 hours before wounding, with isoform-matched IgG as the controls. Epithelial wounds were photographed at 0 and 24 hpw (Fig. 2C), and the percentage of wound closure was quantified (Fig. 2D). Subconjunctival injection of both TGFβ1 and TGFβ3 antibodies delayed epithelial wound closure, with TGFβ3 neutralization exhibiting significantly higher inhibitory effects than TGFβ1 neutralization in B6 mouse corneas. On the other hand, recombinant TGFβ3 accelerated wound healing more effectively than TGFβ1 in the corneas of STZ DM mice. Hence, these results demonstrate that, although TGFβ1 and TGFβ3 are both required for proper epithelial wound healing, TGFβ3 is the more effective isoform in promoting re-epithelialization in NL corneas and, more importantly, in DM corneas, suggesting that TGFβ3 may be a promising therapeutic target for promoting corneal wound healing in diabetic patients.

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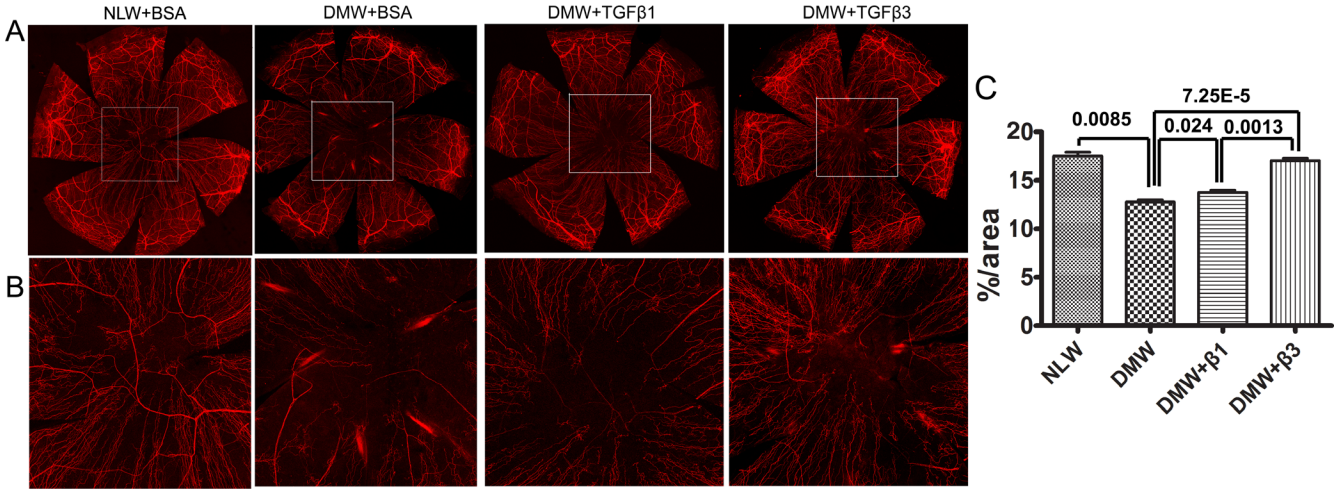

Figure 3. Effects of exogenous TGFβ isoforms on sensory nerve regeneration in healing DM corneas. (**A**) Diabetic corneas, with normal (nondiabetic) corneas as the control, were pretreated and wounded as described in Figure 2C. DM corneas (DMW, diabetic wounded) and NL (nondiabetic) corneas treated with BSA as the control were harvested 3 days post-wounding (dpw). An additional subconjunctival injection of BSA or recombinant TGFβ isoforms was administered at 1 dpw. The corneas were stained for β-tubulin III, and images of the entire cornea were captured (*top panels*). (**B**) High-magnification images are shown in the *bottom panels*. (**C**) To quantify corneal innervation, manual labeling and measurements were used in ImageJ. Innervation in a region was calculated as the percentage of area covered by β-tubulin III staining using ImageJ and are presented as percentages (mean ± SD, $n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test). Two independent experiments were performed.

TGFβ3 Promoted Post-Wound Sensory Nerve Regeneration in DM Corneas of B6 Mice

We previously reported that DM decreases not only the density of nerve endings in corneas but also sensory nerve regeneration during wound healing.[39,42] We reasoned that the reduced TGFβ3 may contribute to these abnormalities in sensory nerve regenerations in DM corneas. Whole-mount confocal microscopy showed that hyperglycemia resulted in delayed sensory nerve regeneration, as identified by the β-tubulin III antibody (Fig. 3). Exogenously added TGFβ1 accelerated sensory nerve regeneration in wounded DM (DMW) corneas compared to control DMW eyes but less effectively than TGFβ3-treated DMW corneas, suggesting that TGFβ signaling may directly or indirectly mediate sensory nerve regeneration in the corneas (Figs. 3A, 3B). The nerve pixel area in normal corneas was 17.50% ± 0.56% of the central area (Fig. 3, bottom panels), compared to 12.76% ± 0.44% in DM corneas treated with TGFβ1 (13.76% ± 0.31%) or TGFβ3 (17.03% ± 0.46%) (Fig. 3C).

TGFβ1 and TGFβ3 Differentially Mediated Cell Signaling Pathways in DM B6 Mice

Having shown that TGFβ1 and TGFβ3 promote corneal wound healing in DM mice, we then investigated how TGFβ1 and TGFβ3 differentially signal during corneal wound healing by determining TGFβ1- and TGFβ3-mediated cell signaling pathways (Fig. 4). Western blotting revealed that wounding induced activation (phosphorylation) of EGFR, Akt, ERK1/2, and SMAD2/SMAD3 in wounded NL (NLW) corneas. Compared to NL corneas, wounding induced similar levels of SMAD2/SMAD3 phosphorylation, but the staining intensities of p-EGFR, p-Akt, and p-ERK were weaker in the DM corneas. Although exogenous TGFβ1 markedly upregulated p-SMAD2/SMAD3 expression, TGFβ3 further upregulated the expressions of p-EGFR, p-ERK, and p-Akt but not p-SMAD2/SMAD3. This suggests that TGFβ3 may potentially prolong EGFR transactivation and signaling[42] but TGFβ1 may enhance SMAD2/SMAD3 signaling in response to wounding in DM mouse corneas.

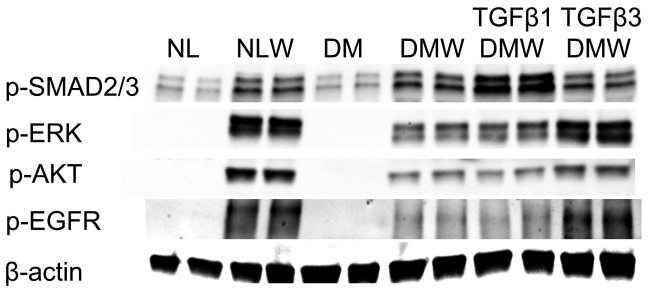

Figure 4. Effects of exogenous TGFβ isoforms on the activation of canonical and noncanonical signaling pathways in healing DM corneas. Diabetic corneas were pretreated and wounded as described in Figure 2C. The epithelial cells that migrated to the original wound bed in diabetic wounded (DMW) corneas and nondiabetic wounded (NLW) corneas were collected for analysis. Western blot analysis was conducted to assess the activation of canonical signaling (p-SMAD2/SMAD3) and noncanonical signaling pathways (p-Akt, p-ERK), as well as p-EGFR activation, with β-actin serving as the internal control for equal protein loading. The analysis focused on unwounded and wounded diabetic corneas harvested at 4 hours post-wounding. An additional subconjunctival injection of BSA or recombinant TGFβ isoforms was given at 1 day post-wounding.

Differential Effects of TGFβ1 and TGFβ3 on the Expression of Wound-Induced Genes

Having identified that TGFβ1 and TGFβ3 promote wound healing in normoglycemic (NL) and diabetic (DM) corneas and activate different signaling pathways, we next investigated the genes differentially regulated by these two isoforms in normal and DM corneas using qPCR

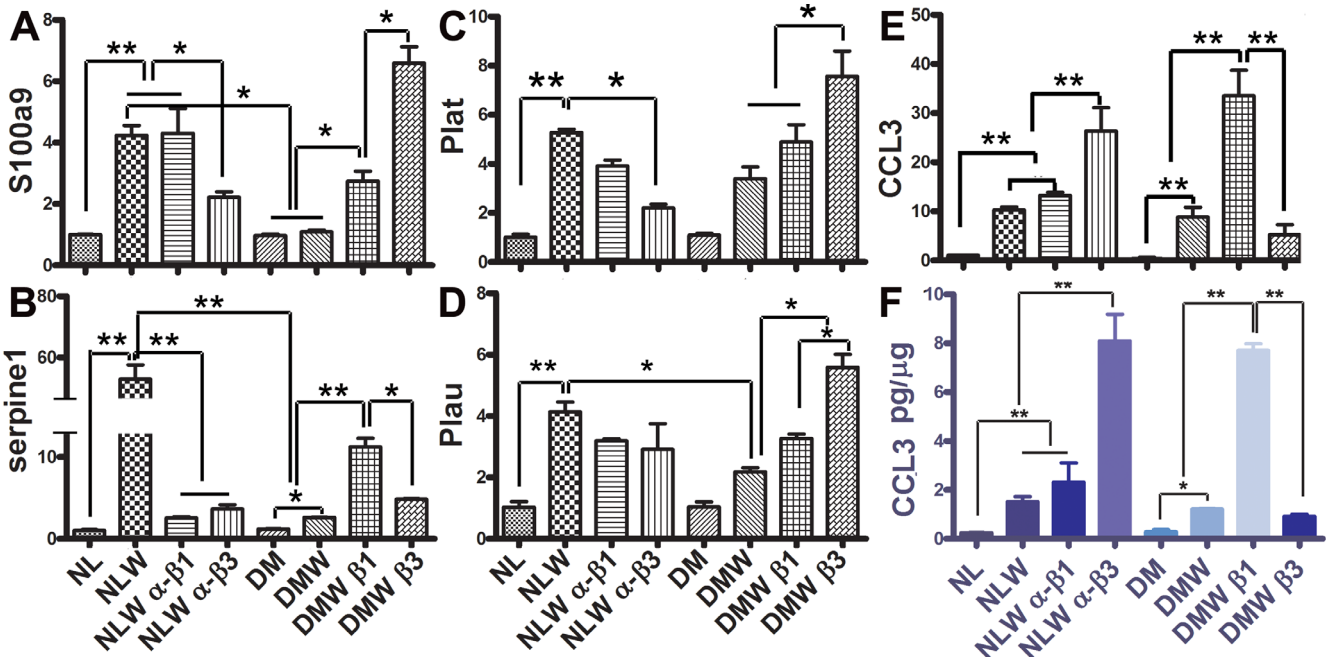

Figure 5. Effects of altering TGFβ signaling on the expression of wound healing–related genes in NL and DM corneas. NL and DM mice were pretreated with TGFβ neutralizing antibodies (α-β1 or α-β3) and exogenous TGFβ isoforms (TGFβ1 or TGFβ3), respectively, and then wounded as described in Figure 2C. At 24 hours post-wounding, cells that had migrated into the original wounds were scraped off the corneas and collected as wounded corneal epithelial cells (CECs). The collected CECs were subjected to RNA isolation for quantitative reverse transcription PCR (qRT-PCR) or ELISA. The qRT-PCR results for S100a9 (**A**), SERPINE1 (**B**), PLAT (**C**), PLAU (**D**), and CCL3 (**E**) in CECs are presented as fold increases (mean ± SD) over the nonwounded NL CECs, which were set as a baseline value of 1 ($n = 3$). (**F**) ELISA results for CCL3 are presented as picograms per microgram of CEC lysates ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (two-way ANOVA). Two independent experiments were performed. NLW, nondiabetic wounded; DMW, diabetic wounded.

(Figs. 5A–5E) and/or ELISA (Fig. 5F). S100a9, a member of the S100 family of proteins containing two EF-hand calcium-binding motifs, may form a heterodimer with S100a8, termed calprotectin; it is an antimicrobial peptide involved in wound healing and acts as a damage-associated molecular pattern molecule or alarmin.[43,44] In NL corneas, injury-induced S100a9 expression was suppressed by TGFβ3 but not TGFβ1 neutralization, whereas in DM corneas wound-induced S100a9 expression was totally dampened. The regressed S100a9 expression was partially restored by exogenous TGFβ1, but exogenous TGFβ3 markedly augmented S100a9 expression to a level higher than that in NL corneas.

Plasminogen is activated to plasmin by either tissue-type or urokinase-type plasminogen activator, and this system is also involved in wound healing.[45–47] Our qPCR revealed that, in healing NL CECs, wounding induced the expression of SERPINE1 (plasminogen activator inhibitor 1, PAI-1), tissue plasminogen activator (tPA), and urokinase plasminogen activator (uPA), were dampened in the DM corneas. The expression of PAI-1 is restored by TGFβ1, whereas tPA and uPA were restored by TGFβ3 in DM healing CECs (Figs. 5B–5D). PAI-1 is a serine protease inhibitor that functions as the principal inhibitor of tPA and uPA.[48] Overall, wound-induced upregulation of PAI-1, tPA, and uPA was totally retarded by hyperglycemia, while TGFβ3 promotes plasminogen activation through the suppression of PAI1 and the induction of tPA and uPA.

CCL3 is a chemokine expressed by corneal epithelial cells and recruits macrophages (MΦ) to the site of injury.[49] Wound-induced CCL3 expression was observed at both mRNA and protein levels, and this upregulation was elevated by TGFβ3 but not by TGFβ1 neutralization in NL corneas (Figs. 5E, 5F). In DM corneas, significant induction of CCL3 was observed in healing CECs, and this increase was markedly amplified by TGFβ1, but not TGFβ3 (Figs. 5E, 5F), suggesting that CCL3 is a downstream gene of TGFβ1, whereas TGFβ3 acts as a negative regulator of CCL3 expression in both NL and DM corneas.

TGFβ3 Promoted Macrophage Infiltration in Healing Diabetic Corneas

CCL3 is a potent chemokine of MΦ, and our previous study showed that, although neutrophil infiltration was greatly increased, the infiltration of MΦ in wounded DM corneas was decreased.[10,50] MΦ have been shown to participate in regulating corneal wound healing by balancing the inflammatory response.[51] To determine whether MΦ infiltration is influenced by TGFβ isoforms, wounded DM corneas were stained with F4/80 and visualized using WMCM with antibody F4/80 (Fig. 6). There were 2833 ± 942.57 F4/80-positive cells in untreated DM corneas. The number of MΦ was increased in the presence of TGFβ1 (4978 ± 173.24), and there were markedly more in the presence of TGFβ3 (8835 ± 997.73) in wounded DM corneas (Figs. 6A–6C).

Differential Gene Expression in Cultured Bone Marrow–Derived Macrophages Challenged With TGFβ1 and TGFβ3

Functionally, MΦ can be characterized as inflammatory type 1 (M1) and pro-repair type 2 (M2), which are

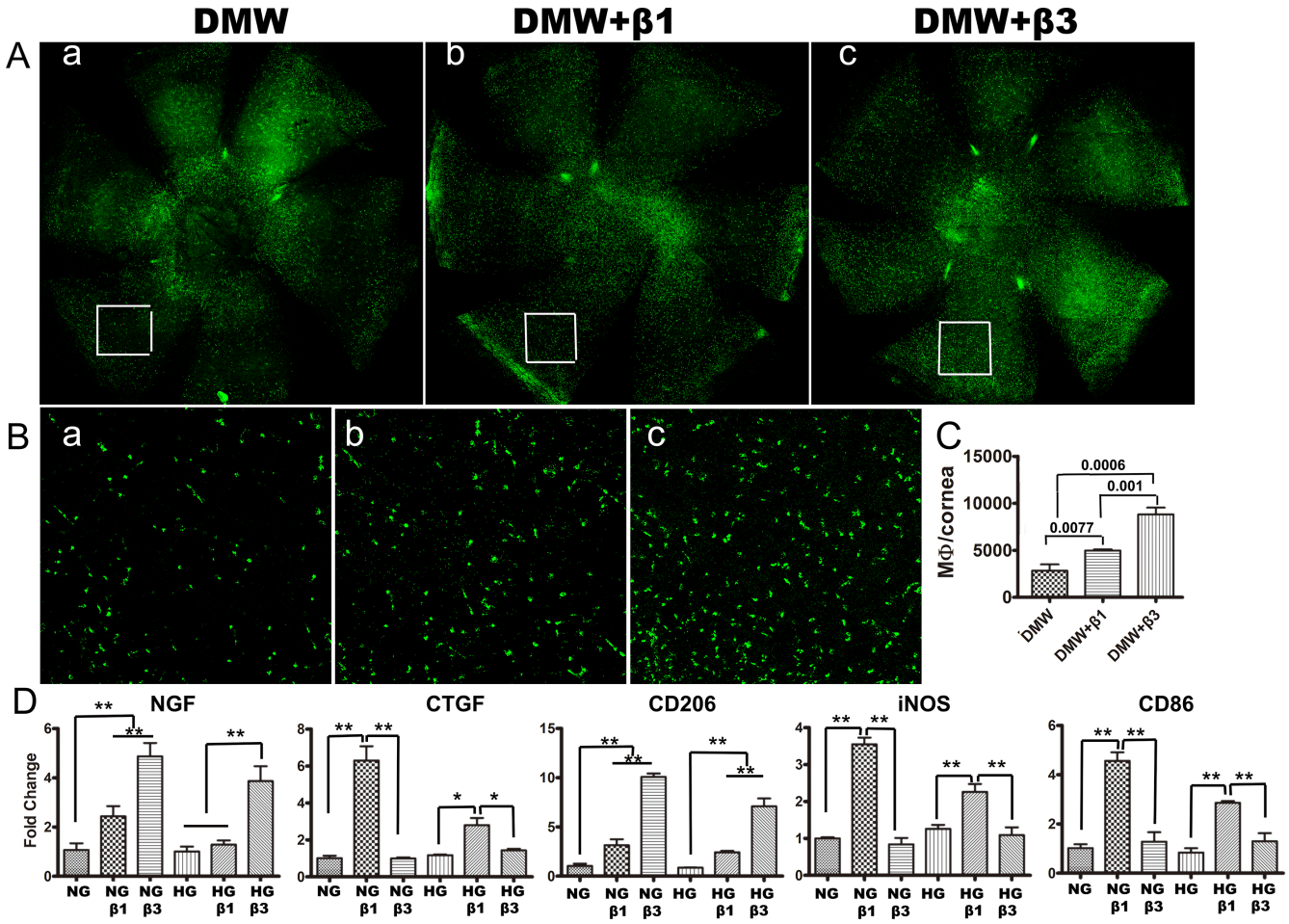

Figure 6. Effects of TGFβ1 and TGFβ3 on macrophage infiltration in vivo and gene expression in vitro. (**A**) Diabetic (DM) corneas, with nondiabetic (NL) corneas as the control, were wounded and allowed to heal for 24 hours. Wounded corneas were stained with F4/80 and visualized using WMCM. The WMCM images, acquired with region of interest (ROI) scanning of a defined area, were automatically assembled to display whole corneas from B6 mice. (**B**) A selected area in **A**, marked with a rectangle, was amplified to show the density of macrophages. *Scale bars*: 125 µm (*top panel*) and 58 µm (*bottom panel*). (**C**) To quantitate the number of F4/80-positive cells, images were analyzed by threshold-based automatic particle counting (http://imagej.net/Particle_Analysis) of the whole corneas, and the results are presented as mean ± SD ($n = 3$). Two independent experiments were performed. *$P < 0.05$ (one-way ANOVA). (**D**) Bone marrow cells derived from normal and diabetic mice were cultured in normal and high-glucose media containing CSF-1 and IL-3 for 3 days. Mature macrophages, generated by proteolytic digestion of the nonadherent cells, were cultured in the presence or absence of TGFβ1 or TGFβ3 for specified durations. The cells were lysed and subjected to qPCR analysis of macrophage markers (iNOS, CD86, CD206), NGF, and profibrosis (CTGF) genes. Results are representative of two independent experiments ($n = 3$ each). *$P < 0.05$, **$P < 0.01$ (two-tailed, unpaired Student's *t*-test).

promoted by TGFβ3 through miR-494.[52] To test the role of TGFβ1 and TGFβ3 in macrophage polarization, we isolated bone marrow cells from db/db type 2 diabetes mellitus (T2DM) mice with db/+ as the control and induced macrophage differentiation in vitro with high glucose for db/db and normal glucose for db/+ bone marrow–derived macrophages (BMDMs). To test the underlying mechanism of TGFβ1/TGFβ3 promoting nerve reservation via MΦ, BMMΦ were treated with TGFβ1 or TGFβ3 for 2 hours and processed for qPCR. We observed upregulation of iNOS, CD86 (markers for the M1 phenotype), and connective tissue growth factor (CTGF), a fibrosis-related gene,[53] by TGFβ1, whereas TGFβ3 induced CD206 (M2 marker) and nerve growth factor (NGF) in both NG and HG cultured BMMΦ (Fig. 6D). TGFβ1 also stimulated CD206 in NG and HG and NGF in NG cultured BMDM at much less extent than TGFβ3.

Discussion

Among the three mammalian isoforms of TGFβ isoforms, TGFβ1 is perhaps the most important ligand in the pathogenesis of fibrotic diseases in the eye, including corneal and conjunctival scarring, secondary cataracts, and proliferative vitreoretinopathy.[54–57] Scarless wound healing may be influenced by the relative ratio of TGFβ1 and TGFβ3.[58,59] In this study, we demonstrated that TGFβ3 accelerates delayed wound healing and sensory nerve regeneration more effectively than TGFβ1. The discovery that TGFβ1 and TGFβ3 each has a unique role in mediating corneal epithelial wound healing is of great significance. Selective targeting of an isoform or its specific pathway can avoid the undesired consequences of non-selective alteration of TGFβ signaling.

Early studies have shown that in fetal skin, where injury will not result in scar formation, only TGFβ3 expres-

sion increases while TGFβ1 levels remain steady, in clear contrast to what is found in adults.[60] Moreover, in mice and rats, oral mucosal wounds heal faster with minimal scarring in comparison with skin wounds[61]; an increased TGFβ3/TGFβ1 ratio is recognized as an underlying cause for faster healing and less scarring in the oral mucosa during wound healing.[62–64] In the cornea, limbal stem cell deficiency (LSCD) can be treated with transplantable autologous oral mucosal epithelial cells. Their presence was found to promote healing of protected LSCD corneas from scar formation after repeated scrapings.[65] Biomaterial-free cultured oral mucosal epithelial cell sheets have been used successfully in ocular reconstruction for subjects with total LSCD.[66,67] Our study revealed that, in the human cornea, similar to mice and rats, wounding induced upregulation of both TGFβ1 and TGFβ3 expression in healing epithelia in normoglycemia corneas, whereas hyperglycemia suppressed the expression of TGFβ3 but not TGFβ1, delaying epithelial wound closure. Importantly, human keratoconus cells expressed less TGFβ3 but not TGFβ1 compared to normal human corneal fibroblasts. Exogenous TGFβ3 downregulated the expression of the key profibrotic receptor, TGFβRII.[68,69] Hence, we postulated that unbalanced expressions of TGFβ1 and TGFβ3 may be a common mechanism for delayed wound healing and fibrosis in the corneas under pathological conditions, such as diabetic keratopathy, stromal injury, and keratoconus.

In addition to being more potent in promoting impaired epithelial wound healing, exogenous TGFβ3 is more effective compared to TGFβ1 in inducing sensory nerve regeneration in post-wounding diabetic corneas. To date, the effects of TGFβ isoforms on neurite outgrowth are controversial.[70] Although TGFβ1 was shown to significantly increase the length of neurites extended from differentiated retinal ganglion (RGC-5) cells,[71] other studies suggested that only TGFβ2 increased neurite length and branching pattern in cultured myenteric neurons.[72–74] Several studies have also suggested that TGFβ inhibits neurite outgrowth, as evidenced by the application of TGFβ1 suppressing neurite outgrowth in primary culture of cerebellar granule neurons.[75] To our knowledge, our study is the first to demonstrate the role of TGFβ3 in promoting sensory nerve regeneration in diseased corneas; the therapeutic potential of TGFβ3 in neuronal regeneration has not been explored yet and warrants further investigation.

Under physiological conditions, TGFβ is critical in regulating tissue homeostasis and renewal whereas under pathological conditions, TGFβ signaling plays an important role in regulating inflammatory progression and wound healing.[76–78] TGFβ signals through SMAD2/SMAD3-dependent canonical and noncanonical signaling pathways.[79] The non-SMAD signaling pathways include ERKs, JNK, p38 MAPK, PI3K/Akt, NF-κB, and Rho family GTPases.[80] We postulated that TGFβ isoforms might be involved in balancing SMAD-dependent and -independent pathways via their receptors. Indeed, we showed that in DM corneas with delayed wound healing, exogenous TGFβ1 stimulated strong SMAD2/SMAD3 phosphorylation (activation), but TGFβ3 exhibited stronger effects on ERK and PI3K/Akt activation. Thus, transactivating noncanonical signaling pathways by TGFβ3 may accelerate delayed epithelial wound closure in DM corneas.

We used qPCR to assess the expression of S100a9, plasminogen activator inhibitor-1 (PAI-1)/tissue-type PA (tPA)/urokinase-type PA (uPA), and CCL3 in NL and DM corneas in response to epithelial wounding. S100a9 is an antimicrobial peptide and is known to be involved in wound healing.[81,82] Our data show that, whereas TGFβ1 exhibited some effects on S100a9 expression in DM corneas, TGFβ3 neutralization retarded and exogenous TGFβ3 markedly upregulated S100a9 expression, suggesting that TGFβ3 plays a role in mediating S100a9 expression.

Plasminogen activation is known to play an important role in cell migration and wound healing.[83,84] Our study of PAI-1 revealed that its defects in this pathway contribute to delayed wound healing in DM corneas.[85] In the current study, TGFβ1 and TGFβ3 were shown to be required for wound-induced SERPINE1 expression in NL corneas. In DM corneas, SERPINE1 expression was greatly suppressed and somewhat augmented by TGFβ1. On the other hand, TGFβ3 significantly upregulated the expression of uPA and tPA, which were suppressed by hyperglycemia, to a level higher than that in NL corneas. Hence, the overall effects of TGFβ1 are inhibitory, whereas TGFβ3 promotes plasminogen activation, consistent with their pro- and antifibrotic roles during wound healing and tissue repair.

TGFβ1 was shown to suppress the expression of CCL3, a chemokine involved in the recruitment of inflammatory cells, through the ERK signaling pathway.[86] Our study showed that, in both NL and DM corneas, wound-induced CCL3 was upregulated by TGFβ1 and suppressed by TGFβ3. Because elevated expression of CCL3 was suggested as a common mechanism for macrophage accumulation in tissues such as the lung and the liver under pathological conditions and during fibrogenesis,[87–89] a strategy utilizing TGFβ3 to downregulate CCL3 might be used for treating these inflammatory and fibrotic diseases.

Macrophages have been shown to participate in regulating corneal wound healing by balancing the inflammatory response.[51] We demonstrated that TGFβ3, compared to TGFβ1, significantly increased the infiltration of BMDMs in DM mouse corneas. Macrophages can be polarized and acquire specific phenotypes such as M1 and M2.[90,91] M1 activity inhibits cell proliferation and causes tissue damage, but M2 activity promotes cell proliferation and tissue repair.[92] Our data suggest that TGFβ1 induced M1 macrophage polarization (iNOS and CD86 upregulation), and TGFβ3 induced M2 macrophage polarization (CD206 upregulation). Hence, the ability of TGFβ3 to promote macrophage M2 polarization may be an underlying mechanism for its strong effects on corneal epithelial wound healing in diabetic corneas. Moreover, the wound-induced profibrosis gene CTGF and antifibrosis gene NGF were further augmented by TGFβ1 and TGFβ3, respectively, consistent with their pro- and antifibrotic roles during wound healing and tissue repair.

Our results increase our understanding of the pathogenesis of diabetic neurotrophic keratopathy, delayed epithelial wound healing, and sensory nerve regeneration, which are common features of DM. In this study, TGFβ3 was shown to play a unique role compared to TGFβ1 in mediating epithelial wound healing. The impaired expression of TGFβ3 may contribute to delayed wound healing in DM corneas; hence, recombinant TGFβ3 may be used for treating diabetic neuropathy and keratopathy.

Acknowledgments

Supported by grants from the National Eye Institute, National Institutes of Health (R01-EY010869, R01-EY017960,

R01EY035785, P30-EY04068) and Research to Prevent Blindness.

Disclosure: **N. Gao**, None; **F.-S. Yu**, None

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