Impaired Epithelial Wound Healing and EGFR Signaling Pathways in the Corneas of Diabetic Rats

Keping Xu[1,2] *and Fu-Shin X. Yu*[1,2]

Purpose. The purpose of the study was to investigate the effects of hyperglycemia on EGFR (epidermal growth factor receptor)-mediated wound response and signal transduction in the corneal epithelium of rats with type I diabetes mellitus (DM).

Methods. Corneal epithelia were removed from streptozotocin (STZ)- and weight-matched normal rats. Wound healing was monitored by fluorescein staining at 24 or 48 hours after epithelial debridement. Phosphorylation of EGFR, AKT, ERK, and BAD was determined by Western blot analysis. The distribution of phospho-AKT and proliferating cell nuclear antigen (PCNA) in rat corneas was examined by immunohistochemistry. Cell death was evaluated by TUNEL staining.

Results. A significant delay in corneal epithelial wound healing was observed 48 hours after wounding in the diabetic rats compared with the weight-matched control rats. In the DM rat corneas, epithelial cells demonstrated diminished responses to wounding, as assessed by the phosphorylation of EGFR and its downstream signaling molecules, AKT and ERK. Furthermore, although the distribution pattern of phospho-AKT suggested a role for AKT in epithelial migration and proliferation in the normoglycemic rat corneas, it was abrogated in the healing epithelia of the DM rats. Consistent with impaired AKT activity, the number of PCNA-stained cells was also greatly reduced in the healing corneas of the diabetic rats. Finally, decreases in pBAD (Ser^{136} and Ser^{112}) and increases in TUNEL-positive cells were observed in both the uninjured and healing corneal epithelia of the DM rats, but not of the control rats.

Conclusions. In the corneas of SZT rats, EGFR-PI3K-AKT and ERK, as well as their downstream BAD signaling pathways in migratory epithelium, were altered, resulting in increased apoptosis, decreased cell proliferation, and delayed wound closure. (*Invest Ophthalmol Vis Sci.* 2011;52:3301–3308) DOI: 10.1167/iovs.10-5670

With the rapid increase in the prevalence of diabetes mellitus (DM), its ocular complications have become a leading cause of blindness in the world.[1] In addition to abnormalities of the retina (diabetic retinopathy) and the lens (cataract), various types of corneal disorders are also relatively common in DM patients.[2] Abnormalities of the cornea include alterations in the epithelial basement membrane,[3–5] basal cell degeneration,[3,6] superficial punctate keratitis,[7] breakdown of barrier function,[8] and fragility,[9] depending on the duration of DM and on the serum concentration of glycated hemoglobin HbA_{1c}.[10,11] For many diabetic retinopathy patients undergoing vitrectomy, the removal of the epithelium is essential for corneal clarity. In postoperative patients, this procedure usually results in a considerable delay in corneal reepithelialization and often in several types of epithelial disorders, such as persistent epithelial defects and recurrent erosion.[2,12–15] Furthermore, delayed healing of the epithelial defect may be associated with sight-threatening complications, such as stromal opacity, surface irregularity, and microbial keratitis. Hence, facilitating epithelial healing would reduce the risk of these sight-threatening complications.[13,16] To date, although autologous serum,[13,16] topical insulin,[17,18] naltrexone (ligand for opioid growth factor receptor),[19] and gene therapy[20] have shown promise, a therapeutic modality for healing postsurgical and persistent corneal epithelial defects in diabetic patients is still lacking.[13] Hence, a better understanding of the mechanisms underlying delayed epithelial wound healing in diabetic corneas should lead to better management of the disease.

Although alterations of the underlying basement membrane,[21,22] the lack of sufficient innervation,[23,24] and/or low tear production[25,26] are likely contributing factors, prolonged hyperglycemia including elevated glucose in tears[27] may directly affect epithelial cells through elevated reactive oxygen species, resulting in epithelial defects and abnormalities. Using cultured human corneal epithelial cells (CECs), pig corneas, and human diabetic corneas, we recently showed that high glucose impairs epidermal growth factor receptor (EGFR) signaling and suppresses basal and wound-induced AKT phosphorylation, resulting in delayed wound healing in cultured porcine corneas in a ROS-related manner.[28] The AKT signaling pathway was also perturbed in the epithelia of human diabetic corneas, but not in the corneas of nondiabetic, age-matched donors, suggesting that hyperglycemia specifically targets the EGFR phosphatidylinositol 3′-kinase (PI3K)-AKT signaling pathway.[28] Hence, weakened EGFR signaling may contribute to the pathogenesis of diabetic keratopathy and epitheliopathy in diabetic patients. Of interest, it was recently reported that 4 weeks of hyperglycemia are sufficient to cause epithelial thinning and basal epithelial cell shape changes, and systematic administration of the EGFR inhibitor AG1478 attenuates these alterations.[29] Recent reports have shown the surprising result that cancer treatments with EGFR-targeting drugs such as cetuximab (an EGFR monoclonal antibody) and an EGFR kinase inhibitor, gefitinib, cause ocular abnormalities in some patients, including diffuse punctate keratitis and corneal erosion,[30,31] that have been observed frequently in diabetic corneas. Moreover, topical application of EGF is an effective therapy for persisting corneal erosion during cetuximab treatment,[32] suggesting a potential use for EGFR agonists for impaired corneal epithelial wound healing. To date, the question

From the Departments of [1]Ophthalmology and [2]Anatomy and Cell Biology, Wayne State University School of Medicine, Detroit, Michigan.

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Corresponding author: Fu-Shin X. Yu, Kresge Eye Institute, Wayne State University School of Medicine, 4717 St. Antoine Street, Detroit, MI, 48201; fyu@med.wayne.edu.

of whether EGFR-mediated wound response in vivo is also compromised remains unanswered.

In this study, we used an epithelial debridement wound model in rats with type I diabetes to assess epithelial response and wound healing. Decreased EGFR signaling resulted in reduced phosphorylation of the proapoptotic protein BAD and consequently a decrease in proliferating cells and an increase in apoptotic cells in healing corneas of diabetic rats. Our results suggest that delayed epithelial wound healing may result, at least in part, from impaired EGFR signaling pathways in diabetic corneas.

Materials and Methods

Materials

Rabbit anti-EGFR, mouse anti-extracellular signal-regulated kinase (ERK2), phospho-(p)ERK1/2 and rabbit anti-proliferating cell nuclear antigen (PCNA) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-pEGFR (Tyr^{1068}), rabbit anti-AKT, pAKT (Ser^{473}), and pBAD (Ser^{136}, Ser^{112}) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Cellular apoptosis was evaluated by a fluorescein in situ apoptosis detection kit (ApopTag Plus TUNEL staining; Chemicon, Temecula, CA). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Induction and Maintenance of DM in Animals

All animal investigations were performed according to the regulations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the National Institutes of Health, and the guidelines of the Animal Investigation Committee of Wayne State University. Seventy male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). These rats (150 g) were divided into two groups. Thirty-five underwent induction of type I DM with an intraperitoneal injection of 55 mg/kg of STZ in ice-cold 0.01 M citrate buffer (pH 4.5),[33] with the controls injected with citrate buffer alone. A second dose of STZ was injected after 4 to 5 days in animals with serum glucose levels less than 200 mg/dL. This regimen produced insulin-deficient DM in 100% of the animals. The animals injected with citrate buffer were normoglycemic.

Serum glucose levels were monitored from rat tail vein with a glucometer (Contour; Bayer HealthCare LLC, Mishawaka, IN) three times a week, and the rats were weighted weekly after STZ was given. The serum glucose levels were targeted between 450 and 550 mg/dL which were considered the minimum level compatible with a stable nontoxic diabetic state.[34] If the levels were higher than 550 mg/dL, minimal insulin doses (Novolin N, NPH, 0.5–2 U) were administered subcutaneously. The STZ animals showed no sign of dehydration and urine ketones, but had gradual increases in body weight throughout the course. The normal and DM rats were housed in standard laboratory conditions.

Corneal Epithelial Debridement Wound

Six months after the inducement of the stable nontoxic diabetic state, the DM and weight-matched normal rats were anesthetized by an intraperitoneal injection of xylazine (7 mg/kg) and ketamine (70 mg/kg). The entire corneal epithelium of each cornea was then removed with a blunt scalpel blade under a dissecting microscope (Carl Zeiss Meditec, Oberkochen, Germany), equipped with a cold light source. To avoid infection of the injured corneas, we washed the conjunctival sac with sterile phosphate-buffered saline (PBS) before surgery, and applied eye drops (Ocuflox ofloxacin solution; Allergan, Inc., Irvine, CA) applied before and after surgery. Any animal that experienced inflammation or infection, edema, mineralization, and neovascularization was excluded from the study. Usually, one eye was wounded at a time in each animal.

Noninvasive Corneal Wound Assessment

Animals were anesthetized in an acrylic plastic chamber with isoflurane-wetted paper towels. The epithelial wound was visualized at 0, 24, 48, and 72 hours by instilling 3 μL of 0.25% fluorescein sodium and photographed under a dissecting microscope equipped with a digital camera (PowerShot A620; Canon, Tokyo, Japan) and a tungsten light source with cobalt blue filter (Welch-Allyn Inc., Skaneateles, NY). The photographs were analyzed for the area of the epithelial wound (Photoshop software; Adobe Systems, Mountain View, CA), and the data are presented as number of pixels in the fluorescent area.

Assessment of EGFR, AKT, and ERK Phosphorylation

To assess the effects of hyperglycemia on cell signaling, we scraped rat CECs off the healing and nonwounded contralateral eyes as the control at 48 hours after wounding, a time point at which wound closure had not yet occurred. The cells were therefore considered as migratory, and a sufficient amount of epithelial cells could be scraped off the injured corneas for biochemical analyses. The collected cells, pooled from four corneas for each condition, were lysed in RIPA buffer (150 mM NaCl, 100 mM Tris-HCl [pH 7.5], 1% deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 50 mM NaF, 100 mM sodium pyrophosphate, 3.5 mM sodium orthovanadate, proteinase inhibitor cocktail, and 0.1 mM phenylmethylsulfonyl fluoride), and used for Western blot analysis. A cell lysate of 10 to 30 μg protein was used to determine EGFR, AKT, and ERK activation by using antibodies against pEGFR (Tyr^{1068}), pAKT (Ser^{473}), and pERK1/2and BAD (Ser^{136} and Ser^{112}) with EGFR, AKT, and ERK2 levels, respectively, for equal protein loading.

Immunohistochemistry

Normal and DM rat eyes were embedded in OCT (Ted Pella, Inc. Redding, CA) 24 hours or 48 hours after wounding, snap frozen on dry ice, and stored at −80°C. Cryostat sections were used for immunofluorescence staining. The whole corneal sections, usually two to three on each slide, were stained with rabbit antibodies against pAKT (Ser^{473}) or PCNA, and subsequently with anti-rabbit fluorescence–conjugated secondary antibodies (Jackson ImmunoResearch Laboratory, West Grove, PA). The slides were then washed in PBS and mounted in medium containing 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI, Vectashield; Vector Laboratories, Burlingame, CA) for nuclear staining and examined with a fluorescence microscope equipped with a digital camera (Axioplan 2 with Apotome; Carl Zeiss Meditec). Negative controls included substitution of rabbit or mouse IgG isotypes for the first antibody, and they exhibited no fluorescence (data not shown).

In Situ Cellular Apoptosis

To detect apoptotic cells, we fixed rat corneal cryostat sections in 2% paraformaldehyde and stained them with a fluorescein in situ apoptosis-detection kit (ApopTag plus TUNEL staining, Chemicon). The stained slides were examined with a fluorescence microscope, as described in the immunohistochemical studies.

Statistical Analysis

The digital images of fluorescein staining were analyzed, to assess the reepithelialization rate. All data are presented as the mean ± SD. Significant differences between two groups were evaluated by Student's *t*-test, and the differences were considered significant at $P < 0.05$.

Results

Delayed Growth and Development in Diabetic Rats

As a first step in assessing pathologic changes and to delineate the pathways leading to delayed epithelial wound healing in

diabetic corneas, we used a well-characterized rat model of human type I diabetes induced by STZ. Rats injected with citrate buffer had average levels of blood glucose of approximately 110 mg/dL when measured in a random-feeding state, whereas most of the STZ-treated rats had blood glucose levels of 450 to 550 mg/dL. The DM rats were maintained for 6 months after they were given STZ (Fig. 1B). Figure 1A shows that the growth of the DM rats was delayed when compared to that of their normal counterparts.

Attenuated Corneal Epithelial Wound Healing in Diabetic Rats

To assess the effects of hyperglycemia on epithelial wound healing, we chose weight-matched rats (~500 g; 7.5 months old for the DM rats, 4.5 for the normal rats) for similar eye size and surgically scraped epithelial cells from limbus to limbus in diabetic and control rats (Fig. 2A). At 24 hours after wounding, the remaining wound, as assessed with fluorescein staining, was approximately 46% of the size of the original wound in DM rats, whereas the control group had wounds at 39% of the size of the original wound. By 48 hours, corneal epithelial wound closure in the DM group was significantly delayed with 19.3% of the wound area remaining, compared with 7.4% observed in normal rat corneas ($P < 0.01$, Student's *t*-test; Fig. 2B). By 72 hours, no fluorescein staining was observed in the corneas of either group (data not shown), indicating the closure of the debridement wound.

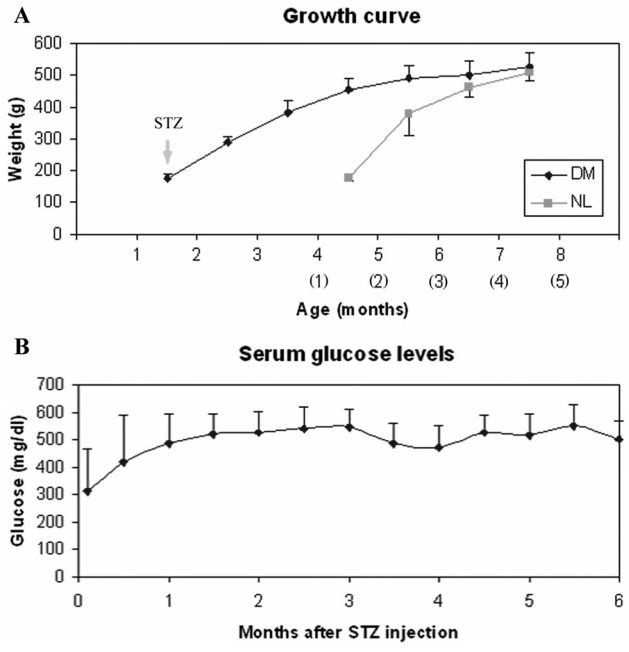

FIGURE 1. Delayed growth and development and sustained hyperglycemia in rats rendered diabetic with streptozotocin (STZ). (**A**) Growth curve of diabetes mellitus (DM, $n = 35$) or normal control rats (NL, $n = 35$) during the course of the study. The NL rats were born 3 months later (age in months is shown in parenthesis) than the DM ones. (**B**) Dynamic monitoring of serum glucose levels in the DM rats. The levels were recorded three times per week, starting from day 4 of STZ administration. Any rats with 500 mg/dL or higher were treated with minimal units of insulin. Random serum glucose levels in normal rats averaged 100 ± 14.1 mg/dL. Data are expressed as the mean ± SD.

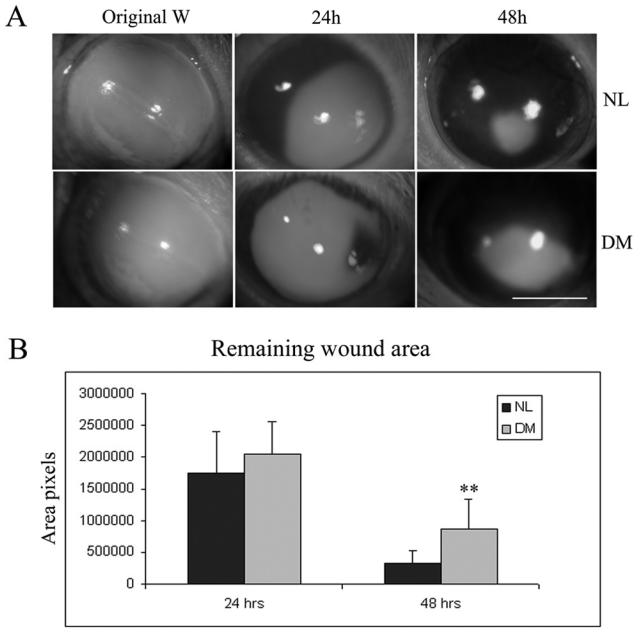

FIGURE 2. Impaired corneal epithelial wound closure in diabetic rats. Corneal wounds were made by scraping epithelia from limbus to limbus, and the wounds were allowed to heal for 72 hours. The wounded corneas were stained with 0.25% fluorescein sodium to monitor the remaining wound area at 24 or 48 hours pw. (**A**) Fluorescein staining of corneal epithelial defects immediately after wounding (original W) or at the indicated times after epithelial wounding. The images are representative of four corneas per condition from five independent experiments. Scale bar, 3 mm. (**B**) Changes in the remaining wound areas in pixels (mean ± SD) from 10 to 12 eyes in normal (NL) or DM corneas were evaluated by image-analysis software (Student's *t*-test, $^{**}P < 0.01$).

Impaired EGFR-Mediated PI3K-AKT and ERK Pathways and Their Dependent BAD Phosphorylation in Corneal Epithelial Cells of Diabetic Rats

Our previous studies showed a correlation between impaired EGFR signaling and hyperglycemia in human CECs.[28] To understand the effects of chronic hyperglycemia on cell responses to wounding, we assessed EGFR-mediated signaling in rat CECs collected from corneas 48 hours after wounding with cells collected during epithelial debridement as the unwounded control. At 48 hours after wounding, there were wounds remaining in both normal and diabetic rat corneas; thus, the cells from these corneas can be characterized as healing or migratory epithelia. Although total EGFR was detected in all samples, with varying levels among animals, phosphorylation of EGFR at Tyr^{1068} was detected in the cell lysate of normal rats, but not in that of diabetic rats with either wounded or nonwounded eyes (Fig. 3A). Consistent with reduced phospho-EGFR, the levels of phospho-AKT detected with site-specific antibodies (Ser^{473}) were also greatly reduced in the CECs of uninjured corneas and to a lesser extent, the corneas, at 48 hours after (post) wounding (pw) (Fig. 3B). Similarly, the phosphorylation of ERK, another major downstream effector of EGFR, was reduced in the CECs of nonwounded and wounded diabetic corneas compared with the normal ones. Moreover, there appeared to be more phospho-ERK in the epithelia of wounded corneas when compared to the uninjured corneas of diabetic rats (Fig. 3C).

AKT and ERK are known to phosphorylate the proapoptotic protein BAD. Hence, AKT and ERK-specific phosphorylation of

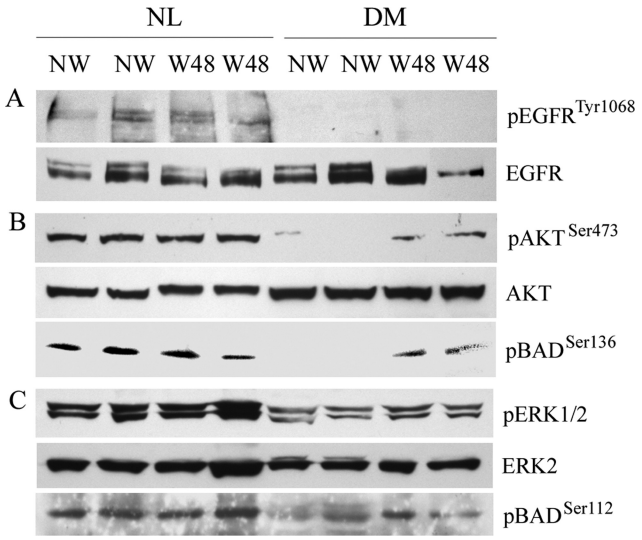

FIGURE 3. Weakening of the EGFR-PI3K-AKT and ERK signaling pathways in diabetic rat corneal epithelial cells. Rat corneal epithelial cells, pooled from four corneas for each sample, were collected 48 hours after wounding (W48) with nonwounded (NW) as the control in normal (NL) or DM corneas, lysed in RIPA buffer, and processed for Western blot analysis. Equal amounts of protein (30 μg) were subjected to immunoblot using antibodies against phospho-EGFR (Tyr^{1068}), pAKT (Ser^{473}), pERK, or pBAD (Ser^{136} and Ser^{112}). After they were stripped, the same membranes were reprobed with an antibody against the corresponding total protein. The blots were grouped as (**A**) EGFR, (**B**) PI3K-AKT, and (**C**) MAPK-ERK signaling pathways.

BAD was also assessed. In normoglycemic rats phospho-BAD at both Ser^{136} (AKT specific) and Ser^{112} (ERK specific) was detected in CECs, with no apparent increase in healing corneas. In hyperglycemic rats, phosphorylation at Ser^{136} was undetectable in uninjured CECs and was observed in healing CECs. The levels of phospho-BAD at Ser^{112} in the CECs of diabetic rats were lower than those in normoglycemic rats and were similar between wounded and nonwounded corneas.

Altered Phospho-AKT Distribution in Corneal Epithelia of Diabetic Rats

The alterations in phospho-AKT in diabetic corneas were also examined by immunohistochemistry (Fig. 4). Phospho-AKT staining was strong, continuous, and associated with the cell surface in all basal cells in the normal rat corneas. In the uninjured diabetic rat corneas, a few cells at the basal layer were phospho-AKT–positive and the staining was much weaker. The distribution patterns of phospho-AKT in uninjured rat corneas were very similar to those observed in the human corneas.[28] At 24 hours pw, there was a clearly defined leading edge, and cells at the leading edge of normal rat cornea were pAKT positive, with strong staining in the region toward the wound center. However, the staining intensity in the leading edge of the DM corneas was weak and obscure. Away from the leading edge, the epithelia were stratified and a strong staining of phospho-AKT was seen in the basal layer of the normoglycemic corneas. In an intriguing finding in the diabetic rat corneas, the staining of phospho-AKT was usually seen in the wing but not in the basal cell layer(s). At 48 hours pw, several continuous layers of epithelial cells, including basal epithelial cells, were pAKT positive and associated with the cell surface in the normal corneal epithelia, whereas nonbasal and isolated pAKT-positive cells were observed in the DM corneal epithelia. We were unable to stain the rat corneas with phospho-EGFR or -ERK antibodies.

Decreases in Proliferation Potential and Increases in Apoptosis in Corneal Epithelia of Diabetic Rats

Having shown that there were decreases in the activation of AKT and in phosphorylation of BAD, we next investigated epithelial proliferation and apoptosis in diabetic rat corneas. Figure 5 showed immunostaining of PCNA. In uninjured corneas, PCNA-positive cells were observed in isolated basal epithelial cells in both normal and diabetic rats, consistent with the limited number of proliferating cells in the corneas.[35] In the wounded corneas, most cells at the leading edge of the normoglycemic corneas were PCNA positive, whereas no staining was observed in the diabetic ones. In the region behind the leading edge, where migrating epithelia were still single layered, there were PCNA-positive cells in the diabetic corneas, yet the density of the positive cells was less than in the normal controls. More PCNA-positive cells were also observed in the normal corneas when compared to the diabetic ones at 48 hours pw.

Consistent with decreases in phospho-BAD, there was increased TUNEL staining of the epithelium of diabetic rat corneas (Fig. 6). The TUNEL-positive cells were observed in the apical epithelial layer of nonwounded and 48-hour pw corneas in the DM rats. No TUNEL-positive cells were observed in the epithelia of the uninjured or healing corneas in normal rats.

DISCUSSION

In this study, we investigated the effects of chronic hyperglycemia on the corneal epithelial wound response and healing in a rat model of type 2 diabetes. Consistent with previous findings in other laboratories, we observed that the closure of a corneal epithelial debridement wound in DM rats maintained in a hyperglycemia state for 6 months was significantly delayed compared with that in weight-matched normoglycemic controls. The epithelial cells derived from both nonwounded and healing corneas of hyperglycemic rats had much reduced EGFR phosphorylation as well as activation of its downstream effectors, PI3K-AKT, and ERK, compared with controls. Of note, we

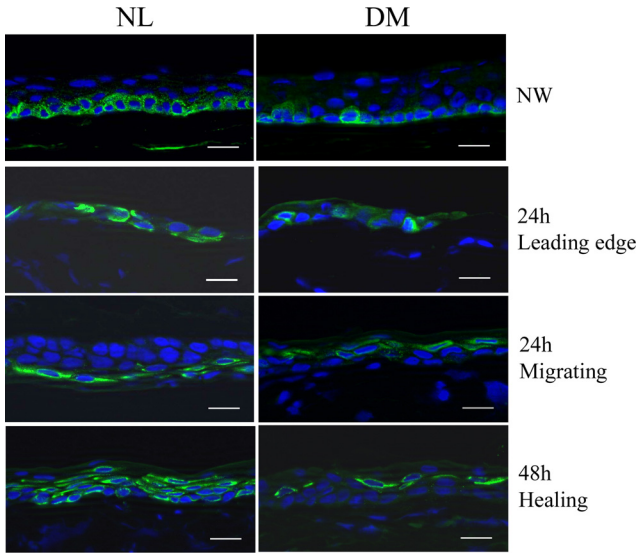

FIGURE 4. Phospho-AKT staining in healing corneal epithelia of diabetic rats. Cryostat sections of normal (NL) or DM rat corneas were immunostained with antibody against p-AKT (Ser^{473}) in corneal epithelium 24 or 48 hours after wounding, with nonwounded (NW) corneas as the control. The merged images of immunofluorescence of pAKT and nuclear staining of DAPI are representative of five corneas per condition from two independent experiments. Scale bar, 20 μm.

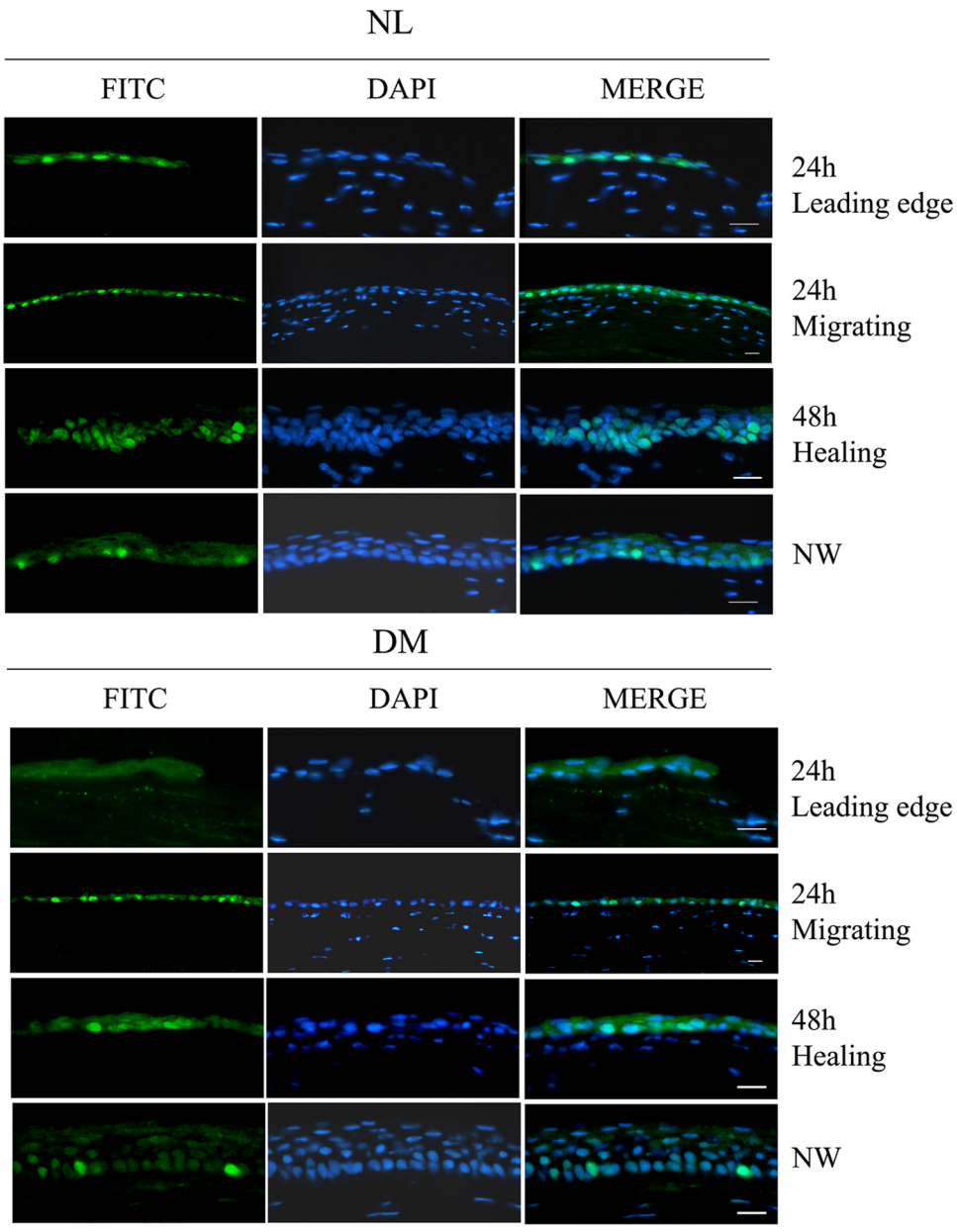

FIGURE 5. Immunohistochemistry of PCNA in healing corneal epithelia of diabetic rats. Cryostat sections of normal (NL) or DM rat corneas were immunostained with an antibody against PCNA in the corneal epithelium, 24 or 48 hours after wounding, with nonwounded (NW) corneas as the control. Merged images of nuclear immunoreactivity of PCNA and nuclear staining of DAPI in the corneal epithelium are representative of five corneas per condition from two independent experiments. Scale bar, 20 μm.

showed for the first time, that the phosphorylation of the proapoptotic protein BAD, indicative of its inactivation, was also impaired in uninjured and to a lesser extent, in the healing corneal epithelia of diabetic rats. Consistent with decreases in AKT activation and BAD phosphorylation, there were decreases in PCNA-positive cells, especially in the wound's leading edge, and increases in TUNEL-positive cells in the diabetic rat corneas. To our knowledge, this is the first report directly linking the decrease in cell proliferation and increase in apoptosis to delayed epithelial wound healing in diabetic cornea. Taken together, we conclude that hyperglycemia perturbs the EGFR-PI3K-AKT and ERK signaling pathways in normal and healing corneas and that increased cellular apoptosis and decreased cell proliferation may be the contributing factors in the impairment of corneal epithelial wound healing in diabetic corneas.

In the literature, age-matched animals were used in most studies of diabetic wound healing.[6,36–38] In this study, we maintained the STZ rats in a hyperglycemic state (~500 mg/dL) for ~6 months (age, 7.5 months) and chose weight-matched normoglycemic SD rats (age, 4.5 months) as the controls for wound-healing experiments. Because of delayed growth of STZ rats (Fig. 1), choosing a proper control for a corneal wound-healing study was problematic. On the one hand, aging is known to affect wound healing. On the other hand, eye size may be related to body mass; hence, the size of sublimbal corneal epithelial debridement is likely to be smaller in diabetic rats than in age-matched control rats. Given that rats age approximately 35 times faster than humans, neither age group is considered to be of advanced age. Hence, we chose weight-matched normal and DM rats for this study with diabetic rats at 7.5 months of age. We created limbus-to-limbus epithelial debridement wounds in the cornea. Similar to other studies using age-matched STZ rats,[6,36] we observed delayed wound closure within the first 24 hours; however, the difference at this time point did not reach statistical significance ($P > 0.5$). At 48 hours pw, the average remaining wound area in the diabetic rats was significantly larger than those in the normoglycemic rats. Forty-eight hours was chosen for the biochemical studies, because at that time point most whole corneal wounds were

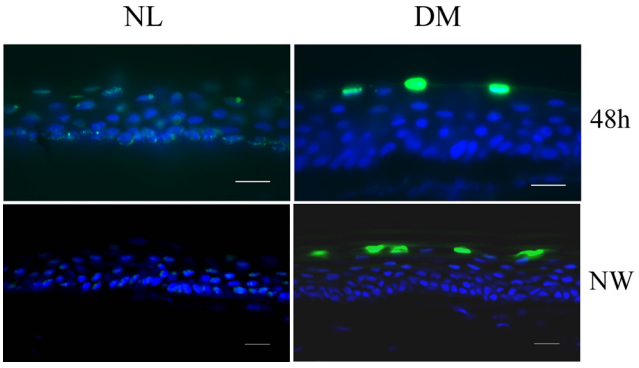

FIGURE 6. Increased cellular apoptosis in diabetic rat corneal epithelium assessed by TUNEL staining. Rat corneal cryostat sections of normal (NL) or DM corneas 48 hours after wounding were evaluated by fluorescein in situ apoptosis detection (TUNEL staining) along with nonwounded (NW) corneas. The merged images of TUNEL and nuclear staining of DAPI are representative of three to five corneas per condition from two independent experiments. Scale bar, 20 μm.

nearly healed, or delayed healing was observed. We conclude that the healing of limbus-to-limbus corneal epithelial debridement wounds was impaired in SZT-induced diabetic rats. Our results, similar to those in other studies,[6,37–39] indicated that chronic hyperglycemia leads to diabetic complications, including poor wound healing, in animal models of human type I DM.

Diabetic keratopathy has been linked to defective basement membrane assembly,[40–42] alterations of the corneal extracellular matrix, elevated matrix metalloproteinase,[43] and, most important, defects in innervation due to diabetic neuropathy (neurotrophic keratopathy).[2,44] Although animal models, primarily rat and mice, of human DM have been the subject of many studies,[3,6,36–38,45–48] few studies have gone beyond histochemical analysis, because of the limitation of sample size (the amount of cells collected from the cornea). In our laboratory, we have established an effective Western blot analysis system that allows detection of the activation of multiple signaling molecules simultaneously. In this study, we used an animal model of type I diabetes and epithelial debridement wounding. We observed that although the levels of phospho-EGFR-PI3K-AKT and -ERK in DM were lower than that observed in normal rats, the healing epithelial cells, unlike those isolated from the unwounded corneas, had clearly detectable phospho-AKT in DM rats. This suggests that the epithelial response to wounding was impaired but still somewhat functional and that defects in EGFR signaling may be a cause for the delayed epithelial wound closure. This result is consistent with a pig organ culture study that showed impairment of EGFR signaling in corneas cultured in high glucose. The mechanisms underlying the impairment of EGFR signaling in diabetic corneas, including the defects in the expression and/or the release of EGFR ligands which are tightly regulated in normal corneas,[49–52] are currently under investigation in the laboratory.

Consistent with the Western blot analysis results, immunohistochemistry also revealed a decrease in the staining intensity of phospho-AKT in diabetes, compared with normoglycemic corneas. The staining patterns observed in uninjured corneas were very similar to those in human corneas.[28] Recently, Saghizadeh et al.[53] reported that the overexpression of matrix metalloproteinase-10 and cathepsin F, two proteinases with elevated expression in human diabetic corneas,[54] resulted in a decrease in phosphorylated AKT and delayed epithelial wound closure in cultured, normal human corneas, suggesting that defects in cell–matrix interaction may contribute to impaired EGFR-PI3K-AKT signaling in diabetic corneas. It is of interest to note that two factors, insulin and HGF, which were shown to accelerate delayed diabetic corneal healing, are also known to stimulate EGFR transactivation in vitro.[55,56] Although no increases in AKT and ERK phosphorylation have been observed in human diabetic corneas overexpressing c-Met, our preliminary study showed topical insulin accelerated epithelial wound healing without altering EGFR-AKT signaling (Yu F-SX, unpublished results, 2009). Further studies to assess cross-talk of these trophic factors in mediating cell signaling and wound healing are warranted.

In addition to changes in activities, we showed alterations in phospho-AKT distribution in healing diabetic cornea. In the nondiabetic corneas, phospho-AKT staining was strong in epithelial cells at the leading edge. The active protein was mostly in the front half of the cells and/or near the sites of cell–matrix adhesion. In the diabetic rat corneas, the staining of phospho-AKT is much weaker, with no clear concentration of phospho-AKT within the cells at the leading edge. Another intriguing alteration in phospho-AKT staining is the nonbasal distribution of phospho-AKT in the stratified epithelium of the diabetic corneas. In the normoglycemic rats, phospho-AKT was primarily found, either in the basal cells near the leading edge, or in basal and wing cells of the stratified epithelia. In diabetic healing corneas, the phospho-AKT staining was weak in intensity and found in nonbasal cells of stratified epithelia. Hence, although abnormalities in the basement membrane are likely to influence EGFR-PI3K-AKT activation, the lack of active AKT in the basal cells in healing diabetic corneas may in turn retard the disassembly and reassembly of the basement membrane, which probably plays a role in the healing of large-diameter debridement wounds.[57] Taken together, these results shed new light on the role of PI3K-AKT in the cornea: regulating and assembling the cell migration apparatus that drives cell–matrix interaction and forward movement. Defects in both AKT activation and/or cellular localization may contribute to the delayed epithelial wound healing in the diabetic cornea.

PI3K/AKT has been shown to control diverse cellular activities, including cell survival, growth, proliferation, metabolism, and migration.[58] Similarly, the Ras/Raf/MEK/ERK pathway is involved in the regulation of cell cycle progression and apoptosis.[59] Downstream effectors of EGFR, AKT, and ERK promote cell survival by phosphorylating proapoptotic molecules such as Bad and FoxO3a.[60] BAD phosphorylation is a major mechanism by which trophic factors inactivate the apoptotic machinery.[61,62] Consistent with phospho-AKT levels in rat CECs, AKT-specific site phosphorylation of BAD was observed in normoglycemic CECs. However, the site-specific phosphorylation was not detectable in uninjured epithelia and was reduced in the healing epithelial cells of diabetic rats. Parallel to the AKT pathway, the Ras/Raf/MEK/ERK signal-transduction pathway regulates cell cycle progression and cell survival in diverse types of cells. Decreases in pERK activities were found in DM corneal epithelia with low levels of pBAD (Ser^{112}). These novel findings reveal that BAD functions as a sentinel for select apoptotic signals in the cornea. Hyperglycemia may tip the balance of BAD phosphorylation, resulting in CEC apoptosis and delayed wound healing.

To determine whether epithelial abnormalities, including basal cell degeneration[3] and altered cell proliferation[6,38] can be found in a rat model of diabetes, we identified TUNEL-positive cells in diabetic corneas at the apical layer, even in healing corneas, where cell proliferation, required for cell repopulation, is expected. Similarly, TUNEL-positive cells were found in human diabetic, but not age-matched control corneas (Xu K and Yu F-SX, unpublished data, 2010). Detected increases in TUNEL-positive cells suggest that hyperglycemia increases epithelial apoptosis in the cornea, in line with decreases in BAD phosphorylation. We also used PCNA staining as a marker for proliferating cells. We found that wounding greatly increased

the number of PCNA-positive cells, and that hyperglycemia significantly decreased the number of PCNA-positive cells in healing epithelia of rat corneas. Decreases in PCNA-positive cells suggest an impaired proliferating capacity in the diabetic corneas in response to wounding. Hence, hyperglycemia appears to impair cell proliferation and to induce epithelial apoptosis in the cornea in an EGFR-PI3K-AKT– and ERK-related manner. To date, the direct link between dysfunctional EGFR signaling and the diabetic pathophysiological condition remains to be established. Using STZ diabetic rats, we recently observed that combination of two EGFR ligands, HB-EGF and TGF-α, applied topically accelerates delayed corneal epithelial wound healing, suggesting that restoring aberrant EGFR-signaling pathways may represent an effective therapeutic strategy for treating diabetic keratopathy and delayed corneal wound healing in the cornea.

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