PTEN Inhibition Facilitates Diabetic Corneal Epithelial Regeneration by Reactivating Akt Signaling Pathway

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Purpose: To investigate the contribution of phosphatase and tensin homologue (PTEN) on the delayed epithelial regeneration and impaired Akt activation in diabetic mice.

Methods: The expression of PTEN on cornea was compared between normal and diabetic mice. The corneal epithelial and nerve regeneration rate was evaluated in diabetic mice after the treatment with PTEN small interfering RNA (siRNA), PTEN inhibitors, or Akt inhibitor. The reactivation of epithelial regeneration-related signaling, including phosphorylated (p)-Akt, p-Stat3, Sirt1, and Parkin, were assessed with Western blot and immunofluorescence staining. The effects of PTEN inhibition on cellular proliferation and migration were further evaluated in cultured mouse corneal epithelial cells.

Results: PTEN messenger RNA and protein levels exhibited up-regulation in diabetic cornea. Upon central epithelial debridement, the epithelial regeneration rate was significantly promoted in diabetic mice with the treatment of PTEN inhibition than that of vehicle control (P < 0.05), which accompanied with the recovered levels of p-Akt, p-Stat3, Sirt1, and Parkin. However, the promotion of diabetic corneal epithelial regeneration rate and Akt reactivation was completely reversed by Akt inhibitor. In vitro, PTEN inhibition promoted their migration, but not the proliferation capacity. In addition, PTEN inhibitor treatment also improved the recovery of corneal nerve fiber density and sensitivity that was impaired in diabetic mice.

Conclusions: Elevated PTEN expression contributes to the impaired corneal epithelial regeneration and Akt activation in diabetic mice, which can be improved with PTEN inhibition.

Translational Relevance: Our study suggests that PTEN inhibition may serve as a new strategy for restoring the impaired corneal epithelial regeneration ability in patients with diabetes.

Introduction

Diabetes mellitus has been emerging as one of the most prevalent systemic diseases in the world with increasing prevalence. Approximately 70% of patients with diabetes suffer from corneal complications named as diabetic keratopathy, which includes corneal epithelial fragility, defects and recurrent erosions, ulcers, edema, superficial punctate keratitis, delayed and incomplete wound repair, endothelial changes, and neuropathy exemplified by decreased corneal sensitivity. The features were mainly caused by the changes of corneal epithelial basement membrane compositions, advanced glycation products deposition, corneal nerve ending damage, reduced tear secretion, and oxidative stress in the hyperglycemic conditions.

Phosphatase and tensin homologue (PTEN) is a potent tumor suppressor and contributes to the control of several important cellular signaling pathways. PTEN dephosphorylates phosphatidylinositol-3, 4, 5-phosphate (PIP3) and, therefore, represses the
activation of the phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway; thereby, it triggers a battery of downstream kinases such as glycogen synthase kinases, apoptotic factors such as BAD, and GTPases like Rac and Rho. Through controlling these pathways, PTEN plays an important role in the cell and governs a variety of biological processes, including maintenance of genomic stability, cell survival, migration, proliferation, and metabolism. In terms of wound healing, PTEN was identified as an essential factor to control electrotaxis for electrical signal–induced wound healing. Previous researches were mainly focused on the phosphatase function of PTEN to dephosphorylate PIP3 and negatively regulate the PI3K/Akt pathway. For the cornea, previous reports have demonstrated that injured corneal epithelium down-regulates the expression of PTEN at wound edges, allowing increased PI3K/Akt signaling, thereby contributing to a significant enhancement of cell migration and wound healing. However, there was no definite conclusion about the regulation of PTEN in diabetic keratopathy. Therefore, based on the characteristics of delayed epithelial wound healing and impaired Akt activation in the diabetic cornea, we hypothesized that PTEN may contribute to the pathogenesis and represent a potential target for the treatment of diabetic keratopathy. To address this hypothesis, we explored the role and mechanism of PTEN on corneal epithelial wound healing and nerve fiber regeneration by using type 1 diabetic mice and high glucose (HG)-treated corneal epithelial cells.

Methods

Animals

Male C57BL/6 mice 6 to 8 weeks old were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China), and housed in a pathogen-free environment at the Shandong Eye Institute animal facility. All animal experiments were approved by the Ethics Committee of Shandong Eye Institute and carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The mice underwent induction of type 1 diabetes mellitus with intraperitoneal streptozotocin (50 mg/kg; Sigma-Aldrich, St. Louis, MO) injection as per our previous description. In the present study, diabetic mice were used 12 weeks after the final streptozotocin injection, with the blood glucose level above 16.7 mmol/L.

Corneal Epithelial Wound Healing

Mouse central corneal epithelium (diameter of 2.5 mm) was removed with Alger brush II corneal rust ring remover (Alger, Lago Vista, TX) in anesthetized normal and diabetic mice. For topical PTEN inhibitor application, dipotassium bisperoxovanadium dehydrate [bpv(Pic), 10 μM; Sigma-Aldrich], potassium bisperoxo oxovanadate hydrate [bpv(Phen), 10 μM; Sigma-Aldrich] and equal normal saline as the vehicle control were dropped onto the corneal surface by a 5-μL tip 6 times daily per eye for 3 days. For Akt inhibition, diabetic mice were injected subconjunctivally with Akt inhibitor tricirbine (0.65 μg/eye, Sigma-Aldrich) at 24 hours before and 0 hours, and 24 hours after the removal of corneal epithelium. Ofloxacin eye ointments were applied to all mice to avoid infection. After 24, 36, and 48 hours, corneal epithelial defects were visualized by staining with fluorescein sodium and photographed under slit lamp microscope (BQ900; Haag-Streit, Bern, Switzerland). The staining area was analyzed by using Image J (National Institutes of Health, Bethesda, MD) and calculated as the percentage of residual epithelial defect. Small interfering RNA (siRNA) targeting PTEN and control siRNA (20 μM, 5 μL/eye, Dharmacon, Lafayette, CO, USA) were injected twice (24 hours and 4 hours) before wounding, as described previously.

Corneal Sensitivity Measurement

Corneal esthesiometry was carried out as previous description by using a Cochet-Bonnet esthesiometer (Luneau Ophtalmologie, Chartres Cedex, France). The longest filament length with positive response was considered as threshold, which was verified at least three times.

In Vitro Wound Scratch Assay

The TKE2, which is a murine corneal epithelial stem/progenitor cell line, was presented by Dr Tetsuya Kawakita of Keio University (Tokyo, Japan). The cells were maintained in keratinocyte serum-free medium (Life Technologies, Shanghai, China) supplemented with 5 ng/mL human recombinant epidermal growth factor, 50 μg/mL bovine pituitary extract at 37°C in a humidified incubator with 5% carbon dioxide. The TKE2 cells were cultured in media containing 5 mM D-glucose (normal glucose), 25 mM D-glucose (HG), 200 nM bpv (Pic) (HG [HG + Pic]), 200 nM bpv (Phen) [HG (HG + Phen)], or 5 mmol/L...
D-glucose plus 20 mmol/L mannitol (high mannitol). For migration analysis, the cells were incubated with HG until confluence, subsequently wounded with a micropipette tip, and incubated with or without 200 nM bpv (Pic or Phen) for 24 hours. Cells were photographed, and the wound closure was measured by Image J (National Institutes of Health, Bethesda, MD). To determine the effect of bpv (Pic or Phen) on the proliferation of corneal epithelial cells, TKE2 cells were starved overnight in bovine pituitary extract-free keratinocyte serum-free medium and subsequently incubated with 200 nM bpv (Pic or Phen) for 24 hours. Cell proliferative ability was investigated by using cell counting kit-8 (Sevensea, Shanghai, China). Cells (1000 per well) were plated into 96-well plates with the corresponding medium, and cultured in a humidified incubator at 37°C for 3 days. Then cell counting kit-8 solution was added into the plate for 4 hours. The spectrometric absorbance was measured by microplate reader (Model 680; Bio-199 Rad, Hercules, CA) at 450nm subsequently.

**Immunofluorescence and Corneal Whole Mount Staining**

Frozen sections (7 μm) were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 5% bovine serum albumin at room temperature. The samples were incubated with primary antibodies overnight at 4°C and subsequently with corresponding secondary antibodies incubate for 1 hour at room temperature. All samples were counterstained with 4′,6-diamidino-2-Phenylindole (Sigma-Aldrich), and images were captured with an Eclipse TE2000-U microscope (Nikon, Tokyo, Japan). Corneal whole-mount immunofluorescence staining was performed as previously described. Mouse eyeballs were collected and fixed in Zamboni’s fixative for 1 hour, then the cornea was dissected around the scleral–limbal region and blocked by phosphate-buffered saline with 0.1% Triton X-100, 2% goat serum, and 2% bovine serum albumin for 2 hours, and subsequently incubated in the same incubation buffer with Alexa Flou 488 conjugated neuronal class III β-tubulin mouse monoclonal antibody (Merck-Millipore, Darmstadt, Germany) overnight at 4°C. After washing for five times, the flat mounts were imaged on an LSM880 Zeiss inverted microscope (Carl Zeiss Meditec, Jena, Germany). The quantification of corneal innervation was calculated as the percentage of area positive for β-tubulin staining as previously described.

**Reverse Transcription (RT) Quantitative-Polymerase Chain Reaction (qPCR)**

For RT qPCR, total RNA was extracted from the whole cornea or corneal epithelium, using Nucleospin RNA kits (Macherey-Nagel, Düren, Germany) according to the instructions supplied by the manufacturer. Cornea was collected under the microscope after the mice were humanely killed. Complementary DNAs were synthesized using the PrimeScript First-Strand cDNA Synthesis kit (TaKaRa, Dalian, China). Real-time PCR was performed using SYBR Green PCR reagents and the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). The primer sequences for mouse PTEN were CCAGAGACAAAAAGGGAGTCACA (forward) and TTCCGCCACTGAACATTGG (reverse). The cycling conditions were 10 seconds at 95°C followed by 45 two-step cycles (15 seconds at 95°C and 1 minute at 60°C). The quantified data were analyzed with Sequence Detection System software (Applied Biosystems) by using β-actin as the internal control.

**Western Blot Analysis**

Total protein was extracted from lysed samples of the mouse corneal epithelium (four eyes per group) in RIPA buffer. Samples were run on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and then transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The blots were blocked with 5% bovine serum albumin for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies, including p-Akt, Akt, p-Stat3, Stat3, Sirt1, and Parkin, and subsequently with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ) for 1 hour at room temperature. Finally, the blots were visualized via enzyme-linked chemiluminescence by using the ECL kit (Millipore, Billerica, MA) and quantified by using Image J software (National Institutes of Health).

**Statistical Analysis**

Data in this study were representative of at least three different experiments and were presented as the means ± standard deviation. Statistical analysis was performed using SPSS 22.0 software (SPSS, Chicago, IL), the Student t-test, and one-way analysis.
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Figure 1. Mouse model of diabetic keratoplasty. Hyperglycemia was induced with intraperitoneal streptozotocin injection in adult C57BL/6 mice. After 4 months of final injection, the blood glucose (A; n = 10 per group), corneal sensation (B; n = 10 per group), and sub-basal nerve fiber density (C; n = 5 per group) were measured and compared with age-matched normal mice. Representative images of corneal nerve fibers were whole-stained with anti-β III-tubulin antibody (D). * P < 0.05.

Results

Corneal Characteristics of Type 1 Diabetic Mice

To investigate the corneal characteristics in hyperglycemia conditions, a streptozotocin-induced type 1 diabetic mouse model was used according to our previous reports. Blood glucose level, corneal sensitivity, and corneal sub-basal nerve fiber density were detected at 4 months after the final streptozotocin injection (n = 10 per group). The blood glucose was maintained at more than 25.0 mmol/L for 4 months (Fig. 1A), and the corneal sensitivity of diabetic mice was exhibited as a significant impairment accompanied by prolonged duration of hyperglycemia compared with that of age-matched normal mice (Fig. 1B). Moreover, the whole-mount corneal staining showed that the density of the sub-basal nerve plexus was significantly decreased in diabetic mice than that of normal mice (Figs. 1C, D).

Up-Regulated PTEN Expression in Diabetic Corneal Epithelium

To examine the expression of PTEN, mouse corneas were collected and analyzed by using RT-qPCR, Western blot, and immunofluorescence staining. In diabetic mice, the messenger RNA transcripts of PTEN were up-regulated by 2.4-fold (Fig. 2A) when compared with that of control mice. Correspondingly, the protein level of PTEN in diabetic cornea was increased by 2.0-fold compared with the control mice (Fig. 2B). The immunofluorescence staining in normal and diabetic mice corneal sections further confirmed the positive and intense expression of PTEN in diabetic corneal epithelium, compared with the control mice.
Figure 2. Hyperglycemia up-regulated PTEN expression of corneal epithelium. Cornea were collected from diabetic mice and age-matched normal mice. PTEN expression was measured and compared with RT-qPCR (A; n = 3 per group), Western blot (B; n = 5 per group). and immunofluorescence staining (C). *P < 0.05.

Knockdown of PTEN Promotes Diabetic Corneal Epithelial Wound Healing

Given the up-regulation of PTEN in diabetic epithelium, we tested whether PTEN played a role in diabetic corneal wound healing using a complementary approach with siRNA knockdown. PTEN siRNA was injected before epithelial debridement, and RT-qPCR and Western blot analysis revealed significant down-regulation of PTEN at the RNA and protein levels (Figs. 3A, B). After 24 hours of epithelial debridement, the diabetic mice under treatment with PTEN siRNA showed rapid epithelial regeneration compared with the treatment of control nonspecific siRNA (24.54% ± 2.04% vs. 44.40% ± 7.08%; P < 0.05). Finally, the diabetic corneal epithelium was completely recovered at 48 hours after PTEN siRNA treatment, while the mice with control siRNA treatment still assumed significant epithelial defect (Figs. 3C, D).

Topical Application of PTEN Inhibitor Improves Diabetic Corneal Epithelial and Nerve Regeneration

To investigate the effects of PTEN inhibitor on diabetic corneal epithelial wound healing, the entire corneal epithelium was scraped in diabetic mice and their age-matched control mice. The diabetic mice were subsequently treated with topical applications of PTEN inhibitor. From 24 hours of epithelial debridement, the corneal epithelial regeneration rate showed significant differentiation between the two groups of PTEN inhibitor treatment and vehicle control treatment in diabetic mice (Figs. 4A, B). Although the normal mice completed the epithelial regeneration at 36 hours, the diabetic mice with PTEN inhibitor treatment exhibited complete epithelial recovery at 48 hours, while the diabetic mice with vehicle control treatment still assumed significant epithelial defect. Furthermore, the effects of PTEN inhibitor on diabetic corneal nerve regeneration were also examined at 3 days after epithelial debridement. Similar to our previous descriptions, diabetic mice showed significantly delayed nerve fiber regeneration after epithelial injury, while PTEN inhibitor supplement exhibited faster sub-basal nerve fiber regeneration than vehicle control treatment, both at the central and peripheral areas of the cornea (Figs. 4C, D). In consistent with the nerve regeneration, PTEN inhibitor bpv(Pic) significantly improved the corneal sensation recovery in diabetic mice than vehicle control mice (4.85 ± 0.24 mm vs. 3.90 ± 0.24 mm, P < 0.05) after 14 days of epithelial debridement (Fig. 4E).

PTEN Inhibitor Reactivates Epithelial Regeneration-Related Signaling Pathways

To elucidate the mechanism underlying the promotion of PTEN inhibitor on diabetic corneal epithelial wound healing, we investigated the effects of bpv(Pic or Phen) on the activation of Akt, Stat3 and Sirt1 that altered in diabetic corneal epithelium by using immunofluorescence staining and Western blot. Immunofluorescence staining revealed the expression levels of p-Akt, p-Stat3 and Sirt1 were significantly up-regulated in diabetic corneal epithelium after topical PTEN inhibitor application for 3 days (Fig. 5A). Consistently, the Western blot revealed significant upregulation of p-Akt, p-Stat3 and Sirt1 expression in PTEN inhibitor-treated corneas (Fig. 5B). The results suggest that PTEN inhibitor application in diabetic mice reactivates Akt, Stat3 and Sirt1 that are impaired by hyperglycemia. In addition, mitophagy is involved in diabetic corneal epithelial repair, so we detected Parkin and got a positive result in immunofluorescence staining and Western blot.
Figure 3. Local application of PTEN siRNA promotes epithelial wound healing in diabetic mice. Diabetic mice were pretreated with the nonspecific control (Ctrl si) or PTEN-specific siRNA (PTEN si) 24 and 4 hours before epithelial debridement. Corneal epithelial samples were collected and subjected to the analysis of RT-qPCR (A; n = 3 per group) and Western blot (B; n = 3 per group). Mouse corneas were stained with fluorescein sodium (C) and the residual epithelial defects were analyzed as the percentage of original wound area (D; n = 6 per group). *P < 0.05.

Promotion of PTEN Inhibition Was Mediated through Reactivating AKT Signaling

To evaluate the role of Akt reactivation on the promotion of PTEN inhibition in diabetic corneal epithelial wound healing, Akt inhibitor tricirbine was pre-injected subconjunctivally at 24 hours before, 0 hour, and 24 hours after epithelial debridement and followed with topical application of PTEN inhibitor bpv(Pic). After 48 hours of epithelial debridement, mouse corneas were stained with fluorescein sodium staining for the measurement of epithelial defects and collected for Western blot to measure p-Akt protein levels. As shown in Figures 6A and B, the pretreatment of Akt inhibitor fully reversed the promotion of PTEN inhibitor on epithelial regeneration rate in diabetic mice, similar to the mice with Akt inhibitor treatment. Western blot analysis revealed that p-Akt expression resulted in a significant difference in Pic (1.4-fold), Pic + Akt inhibitor (0.5-fold), and Akt inhibitor (0.1-fold) in diabetic mice compared with the diabetic control mice (Fig. 6C). The results suggest that promotion of PTEN inhibitor in diabetic corneal epithelial regeneration mainly mediated through the reactivation of Akt signaling impaired by hyperglycemia.

PTEN Inhibitor Promotes the Migration, But Not Proliferation, of HG-Treated Corneal Epithelial Cells

To assess the effect of bpv(Pic or Phen) on corneal epithelial wound healing in vitro, mouse corneal epithelial cells were incubated with HG, with an equal concentration of mannose as osmotic control. To evaluate the proliferation of PTEN inhibitor, 1000 cells were inoculated and treated with HG medium in the absence or presence of 200 nM bpv(Pic or Phen). After 3 days, we used for cell counting kit-8 assay and the results indicated that the effect of bpv(Pic or Phen)
on improving the corneal epithelial cells proliferation was not significant (Fig. 7A). To detect the migration of TKE-2 cells incubated with HG, the corneal epithelial monolayer was wounded and incubated with or without 200 nM bpv(Pic or Phen) for 24 hours. The results showed that corneal epithelial wound closure was significantly delayed by HG treatment, whereas bpv(Pic or Phen) improved the migration capacity of HG-treated cells to the same level of normal cells (Figs. 7B, C).

**Discussion**

In diabetes mellitus, the corneal epithelium is exposed to high levels of glucose. Patients with diabetes mellitus are at increased risk of developing ocular surface disorders such as impaired corneal sensitivity, persistent epithelial defects, and recurrent epithelial erosions. In comparison with normal mice, the corneal sensitivity, nerve fiber density, and epithelial wound healing is significantly decreased in diabetic mice. Furthermore, in the present study, we found that PTEN expression was significantly increased in both messenger RNA and protein levels in the cornea of diabetic mice when compared with normal mice. The application of PTEN inhibitor significantly promoted corneal epithelial wound healing in type 1 diabetic mice.

In diabetic keratopathy, one of the most know characteristics is delayed corneal epithelium regeneration caused by the impaired activation of the PI3K/Akt signaling pathway. Many growth factors and their cognate receptors regulate many overlapping...
Figure 5. PTEN inhibitor improved the impaired epithelial regeneration-related signaling pathways in diabetic mice. Diabetic mice were topically applied with 10 μM bpv (Pic) 24 hours before epithelial debridement. After 48 hours of injury, mouse corneas were harvested for immunofluorescence staining (A) and Western blot (B; n = 3 per group) of p-Akt, p-Stat3, Sirt1, and Parkin. *P < 0.05.

pathways and impact many common downstream cellular functions, such as epidermal growth factor receptor, insulin receptor, and insulin-like growth factor-1 receptor induce autophosphorylation and trigger the downstream signaling cascades include MAPK, PI3K/Akt, and Jak/Stat.27–30 The sensory innervation of the cornea is a major determinant of epithelial health and healing capacity.5,31 Akt is a key enzyme in insulin signaling that regulates glycometabolism and, because PTEN is the main phosphatase that negatively regulates the PI3K/Akt pathway, an effective strategy to address the inhibition of PTEN in diabetes is to regulate the downstream pathway that converts blood glucose to muscle glycogen.32 The inhibition of PTEN is being considered as a conceivable drug target to treat diabetes. In addition, studies have reported Sirt3 can regulate mitophagy level to promote diabetic corneal epithelial wound healing33; therefore, mitophagy has also become an entry point for diabetic keratopathy.

About diabetic keratopathy treatment, insulin seems to be an obvious drug to use against diabetes and its complications. Naltrexone, an opioid antagonist, was shown to facilitate corneal epithelial wound healing and seems to be ready for clinical use owing to its safety and efficacy.34,35 Down-regulation of signaling through the axis PI3K/Akt by neuronal PTEN restrains axon outgrowth and nerve regeneration in peripheral and central nervous systems. Many studies support the negative role of PTEN in nerve growth and regeneration, which makes PTEN inhibitor as a feasible approach to revert neurologic damage under pathologic conditions.36–38 The PTEN inhibitor bpv(Pic) was reported to promote neurite outgrowth,39 bpv(Pic)
Figure 6. Akt inhibitor reverses the promotion of PTEN inhibition on diabetic epithelial wound healing. Diabetic mice were injected subconjunctivally with Akt inhibitor and followed with topical application of PTEN inhibitor bpv (Pic). Mouse corneas were stained with fluorescein sodium (A) and the resident epithelial defects were analyzed (B; n = 6 per group) at 48 hours after epithelial debridement. The samples of corneal epithelium were subjected to Western blot analysis for the comparison of p-Akt levels (C; n = 3 per group). *P < 0.05.

Figure 7. PTEN inhibitor improves the migration capacity of corneal epithelial cells impaired by HG. Mouse corneal epithelial cells were treated with 200 nM PTEN inhibitor in the presence of 30 mM glucose or mannose as osmotic control. Cell proliferation was measured by cell counting kit-8 (A; n = 6 per group). Cellular migration was measured with the wounded confluent monolayer and represented as the percentage of primary wounding area (B). Cell migration was observed with or without 200 nM bpv(Pic or Phen) treatment for 24 hours (C; n = 3 per group). *P < 0.05, n.s.: not significant.
provides neuroprotection against oxidative stress and neurotoxicity in Alzheimer’s disease,40 and bpv(Pic) and bpv(Phen) have protective effects on oxidative stress-induced cardiomyocyte injury.41 In addition, PTEN inhibition not only functions as a neuroprotective factor in the nervous system, but also has many functions in wound repair, such as regulating the migration of a variety of epithelial cells. PTEN inhibitors were associated with wound healing in many models, PTEN inhibition improves wound healing in lung epithelia or corneal epithelium injury.18,42 bpv (Pic) promotes proliferation and alleviates senescence to stimulate skin renewal,43 and bpv (Phen) reverses HG-induced apoptosis in human umbilical vascular endothelial cells.44

In conclusion, our study demonstrates that PTEN inhibitor promotes diabetic corneal epithelial wound healing by triggering the reactivation of the Akt signaling. The multiple function of PTEN inhibition provides a novel potential therapy for treatment of persistent corneal epithelial defects in patients with diabetic mellitus.

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