Recovery of Corneal Sensitivity and Increase in Nerve Density and Wound Healing in Diabetic Mice After PEDF Plus DHA Treatment

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Diabetes 2017;66:2511–2520 | https://doi.org/10.2337/db17-0249

Diabetic keratopathy decreases corneal sensation and tear secretion and delays wound healing after injury. In the current study, we tested the effect of treatment with pigment epithelium-derived factor (PEDF) in combination with docosahexaenoic acid (DHA) on corneal nerve regeneration in a mouse model of diabetes with or without corneal injury. The study was performed in streptozotocin-induced diabetic mice (C57BL/6). Ten weeks after streptozotocin injection, diabetic mice showed significant decreases of corneal sensitivity, tear production, and epithelial subbasal nerve density when compared with age-matched normal mice. After diabetic mice were wounded in the right eye and treated in both eyes with PEDF+DHA for 2 weeks, there was a significant increase in corneal epithelial nerve regeneration and substance P–positive nerve density in both wounded and unwounded eyes compared with vehicle-treated corneas. There also was elevated corneal sensitivity and tear production in the treated corneas compared with vehicle. In addition, PEDF+DHA accelerated corneal wound healing, selectively recruited type 2 macrophages, and prevented neutrophil infiltration in diabetic wounded corneas. These results suggest that topical treatment with PEDF+DHA promotes corneal nerve regeneration and wound healing in diabetic mice and could potentially be exploited as a therapeutic option for the treatment of diabetic keratopathy.

Diabetes is the leading cause of blindness in developed countries (1). It affects multiple ocular structures and leads to several complications, such as diabetic retinopathy, cataracts, glaucoma, optic neuropathy, and dry eye (2). Studies have shown that 70% of patients with diabetes have corneal abnormalities, generally described as diabetic keratopathy (3–8). This condition produces a decrease in corneal sensation, punctate keratitis, and persistent epithelial defects. The consequences could result in increased corneal ulceration and, in some cases, perforation that leads to permanent vision loss.

Treatment for diabetic keratopathy currently remains a clinical challenge (5–8). Conventional therapies include lubricants and antibiotics, bandage contact lens, and tarsorrhaphy in an attempt to create a more favorable environment for wound healing (3,4,6). However, all of these methods are often inadequate for accelerating re-epithelialization because none of the present therapies can compensate for the underlying condition: impaired innervation. Therefore, it is essential that novel methods for treating this complication are devised, explored, and brought to clinical trial.

Studies conducted in our laboratory have shown that in rabbits, pigment epithelium-derived factor (PEDF), a neurotrophic and antiangiogenic factor belonging to the serpin family, in combination with docosahexaenoic acid (DHA), an n-3 fatty acid, stimulates nerve regeneration, restores sensitivity, and increases epithelial wound healing after experimental refractive surgery that damages the nerves (9–12). More recently, we have disclosed the anatomy of corneal innervation in the mouse, which shares many common features with human cornea, making the mouse an appropriate model to study pathologies involving corneal nerves (13). In the current study, we used a diabetic mouse model to investigate the effect of PEDF+DHA on sensitivity, tear secretion, wound healing, and nerve regeneration in corneas with or without injury.

RESEARCH DESIGN AND METHODS

Animals

Male C57BL/6 mice (8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and housed in

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Received 24 February 2017 and accepted 31 May 2017.

the Neuroscience Center of Excellence, Louisiana State University Health Sciences Center New Orleans (New Orleans, LA). The animals were handled in compliance with the guidelines of the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research, and the experimental protocol was approved by the Institutional Animal Care Committee for Animal Research of Louisiana State University Health Sciences Center New Orleans. Mice were induced to develop type 1 diabetes by a single intraperitoneal injection of streptozotocin (STZ; 200 mg/kg) in a 50 mmol/L sodium citrate buffer solution (pH 4.5, enzyme grade; Fisher) (14). Blood glucose levels and body weight were monitored weekly. The blood glucose levels were measured by a digital blood glucometer (Accu-Chek; Roche Diagnostics, Mannheim, Germany). Briefly, 10 μL blood collected from the mouse tail veins was applied to the test strip. The results were displayed on the meter in several seconds. Thirty-two mice that had high blood glucose levels (>250 mg/dL) for 10 weeks were used in the study with age-matched normal animals (n = 12 mice) as controls. In the wound-healing experiments, 16 diabetic mice were anesthetized with ketamine (200 mg/kg) and xylazine (10 mg/kg). The right eye was injured by removing the epithelium and one third of the anterior stroma of a 2-mm diameter central area of the cornea using a corneal rust ring remover, as previously described (15). After injury, the mice were randomly divided into two groups. In the treatment group, both eyes (including the unwounded left eye) were treated topically with PEDF (0.4 ng) plus DHA (80 ng) in 10 μL PBS containing 0.2% albumin three times per day for 2 weeks, whereas the animals in the control group received the vehicle (0.2% albumin free of fatty acids in PBS) the same way. The dose used in this study is based on previous experiments (9–12).

Antibodies and Other Materials

Rabbit monoclonal anti-PGP9.5 (EPR4118), rat monoclonal [7/4] anti-neutrophil (ab53457), and rabbit polyclonal anti–C-type mannose receptor 1 (CD206; ab64693) antibodies were purchased from Abcam (Cambridge, MA). Rat monoclonal (NC1/34HL) anti–substance P (SP) and anti-F4/80 (BM8) were purchased from Santa Cruz Biotechnology (Dallas, TX). Secondary antibodies Alexa Fluor 488 goat anti-rabbit Ig G (H+L), anti-rat Ig G (H+L), and Alexa Fluor 594 goat anti-rat Ig G (H+L) were purchased from Thermo Fisher Scientific (Waltham, MA). Optimal cutting temperature compound was from Sakura Finetek (Torrance, CA). STZ and DAPI were from Sigma-Aldrich (St. Louis, MO). PEDF was purchased from Bioproducts MD (Middletown, MD) and DHA from Cayman Chemical (Ann Arbor, MI). Human albumin was from Baxter (Westlake Village, CA). Accu-Chek Compact Plus was purchased from Roche Diagnostics (68298).

Corneal Sensitivity

Corneal sensitivity within the central area was measured under a surgical loupe with a Cochet-Bonnet esthesiometer, as previously described (9–12). Briefly, the length of the monofilament was varied from 6.0 to 0.5 cm in 0.5-cm fractions until the corneal touch threshold was found. The central cornea was tested four times at each filament length. The response was considered negative when no blink was elicited by the monofilament touch. A positive response was considered when the animal blinked more than or equal to 50% the number of times tested. If no blink response could be elicited at a monofilament length of 0.5 cm, corneal sensitivity was recorded as 0. Sensitivity was measured after 10 weeks of diabetes and at 3, 7, and 12 days after treatment with PEDF+DHA or vehicle in the wounded and unwounded diabetic corneas and in nondiabetic mice. Both eyes were measured by an examiner (T.L.P.) who was blinded to the treatment.

Measurement of Tear Volume (Schirmer's Test)

Tear volume, without systemic and topical anesthesia, was assessed as previously described (16) with a phenol red–soaked cotton thread (Zone-Quick; Menicon America, San Mateo, CA) and applied using forceps in the lateral canthus for 15 s. The wetting length of the thread was read by the examiner in a masked fashion under a microscope by using a ruler offered by the manufacturer. Tear volume was measured in mice at 10 weeks after STZ injection and on days 4, 8, and 12 after corneal wounding and treatment with PEDF+DHA or vehicle.

Corneal Wound Healing Evaluation

At days 1 and 2 after injury, 16 mice treated with PEDF+DHA or vehicle were euthanized and the eyes immediately enucleated. The corneas were stained with 0.5% methylene blue for 1 min and then washed with PBS for 2 min; the area of the cornea that was not covered by the epithelium was stained in blue. Photographs were taken with a dissecting microscope (SMZ-1500; Nikon) through an attached digital camera (DXM 1200; Nikon), and the images were analyzed using Photoshop software (Adobe Systems) (15).

Immunofluorescence Staining and Imaging

Two weeks after treatment, 16 diabetic mice that were injured in the right eye were euthanized, and the eyes were enucleated and fixed with Zamboni's fixative (American MasterTech Scientific, Lodi, CA) for 15 min. Then the corneas were carefully excised along the sclerocorneal rim and fixed for an additional 45 min, followed by three washes with PBS. To block nonspecific binding, corneas were incubated with 10% normal goat serum plus 0.5% Triton X-100 solution in PBS for 60 min at room temperature. Tissue was then incubated with primary monoclonal rabbit anti-PGP9.5 (1:500) or rat anti-SP (1:100) antibody in PBS containing 5% goat serum plus 0.5% Triton X-100 for 24 h at room temperature and constantly shaken. After washing with PBS (three times for 10 min each), the corneas were incubated with the corresponding secondary antibodies Alexa Fluor 488 goat anti-rabbit Ig G (H+L) or Alexa Fluor 594 goat anti-rat Ig G (H+L) for 24 h at 4°C and

then washed thoroughly with PBS. Images were taken as described previously (13,17). Briefly, four radial cuts were performed on each cornea, and the tissue was mounted flat on a slide with the endothelium side up. Images were acquired with an IX71 fluorescent microscope (Olympus). The images at the same layer as those recorded at the subbasal layer were merged together to build an entire view of the corneal epithelial subbasal nerves.

To study cell infiltration, eyes obtained after 1 and 2 days of injury were fixed in 2% fresh prepared paraformaldehyde and embedded in optimal cutting temperature compound. Serial 6-μm cryostat sections were cut, mounted on microscope slides, air dried, and stored at −20°C until use. For immunostaining, the sections were washed in PBS and blocked with 5% goat serum and 0.3% Triton X-100 in PBS for 30 min at room temperature and then incubated overnight at 4°C with the following antibodies: rat anti-F4/80 monoclonal (1:100), rabbit anti–C-type mannose receptor 1 (known also as CD206) polyclonal (1:500), and rat anti-neutrophil monoclonal (1:500) antibodies. After three washings with PBS, the sections were incubated with the corresponding secondary antibodies (1:1,000) Alexa Fluor 488 goat anti-rat or Alexa Fluor 594 goat anti-rabbit for 1 h at room temperature. Negative controls were incubated with serum IgG and the appropriate secondary antibodies. DAPI was used to counterstain the nuclei. The sections were examined with an IX71 fluorescent microscope (Olympus) with a ×20 magnification objective lens.

Data Analysis

The nerve fiber densities within the central area (~3.14 mm^2 per cornea) were assessed as the percentage

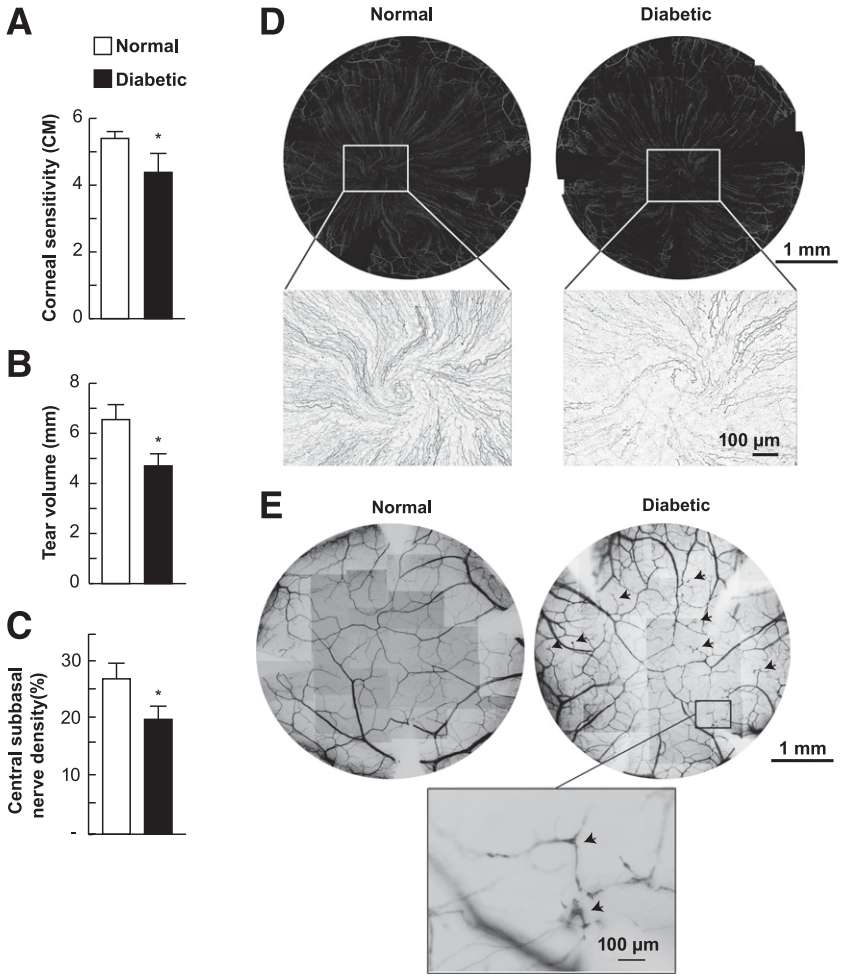

Figure 1—Changes in sensitivity, tear production, and epithelial and stromal nerve architecture in STZ-induced diabetic mice for 10 weeks. Corneal sensitivity (*A*) measured by a Cochet-Bonnet esthesiometer and tear volume (*B*) by Schrimer's test were significantly lower in diabetic mice (n = 32) as opposed to normal mice (n = 12). Data are expressed as mean ± SD. *P < 0.001. *C*: Immunofluorescence of the entire corneal whole mounts labeled with PGP 9.5 antibody shows that mice with hyperglycemia for 10 weeks had a significant decrease in the density of corneal central subbasal nerves compared with age-matched controls. Data are expressed as mean ± SD (*P < 0.001; n = 10 corneas/group). *D*: Representative whole mounts show entire corneal subbasal nerve architecture. An amplified figure of the inset shows in more detail the differences in innervation in the vortex area between corneas in normal mice and in mice after 10 weeks of STZ injection. *E*: Whole mount of stromal nerves. Many neuropathies (arrows) were present in the stromal nerve branches of diabetic corneas. Highlighted image in the inset shows more detail of a neuropathy.

of whole-mount images. To get a better contrast, the fluorescent images were changed to grayscale mode and placed against a white background using Photoshop imaging software. The subbasal nerve fibers in each image were carefully drawn with four-pixel lines following the course of each fiber by using the brush tool in Photoshop imaging software. The nerve area and the total area of the image were obtained by using the histogram tool. The percentage of total nerve area was quantified for each image as described previously (13,17).

To quantify macrophage and neutrophil cells, positive-stained cells were counted in a masked fashion from four randomly selected microscope fields per cornea (two sections per cornea) and averaged. Four corneas were counted per condition.

Differences in corneal nerve densities (including PGP9.5- and SP-positive nerves), sensitivity, wound healing, and inflammatory cells were expressed as means ± SD, and Student *t* test was performed. A *P* value <0.05 was considered a statistically significant difference between the two groups.

RESULTS

Hyperglycemia Alters Tear Production, Corneal Sensitivity, and Damage to Corneal Innervation

After 10 weeks of STZ injection, the blood glucose levels in diabetic mice were significantly higher (425 ± 89 mg/dL; n = 32) than the age-matched normal mice (134 ± 22 mg/dL; n = 12) ($P < 0.001$), whereas the weight gain was significantly lower (18.4 ± 1.3 in diabetic mice vs. 26 ± 3.1 g in nondiabetic mice) ($P < 0.001$). The diabetic mice exhibited a significant reduction of corneal sensitivity from 5.4 ± 0.21 cm in the normal mice to 4.4 ± 0.61 cm ($P < 0.001$) (Fig. 1*A*) and a significant reduction in tear production (6.6 ± 0.6 mm in normal mice vs. 4.7 ± 0.48 mm in diabetic mice) ($P < 0.001$) (Fig. 1*B*).

Hyperglycemia also damages both corneal epithelial and stromal nerves. Based on the analysis of corneal whole mounts, the density of central epithelial subbasal nerves in the normal mice was 27.2 ± 2.7% and was significantly decreased in diabetic mice to 20.1 ± 2.3% ($P < 0.001$; n = 10 corneas/group) (Fig. 1*C* and *D*). Figure 1*E* shows the whole-mount view of the entire stromal nerve

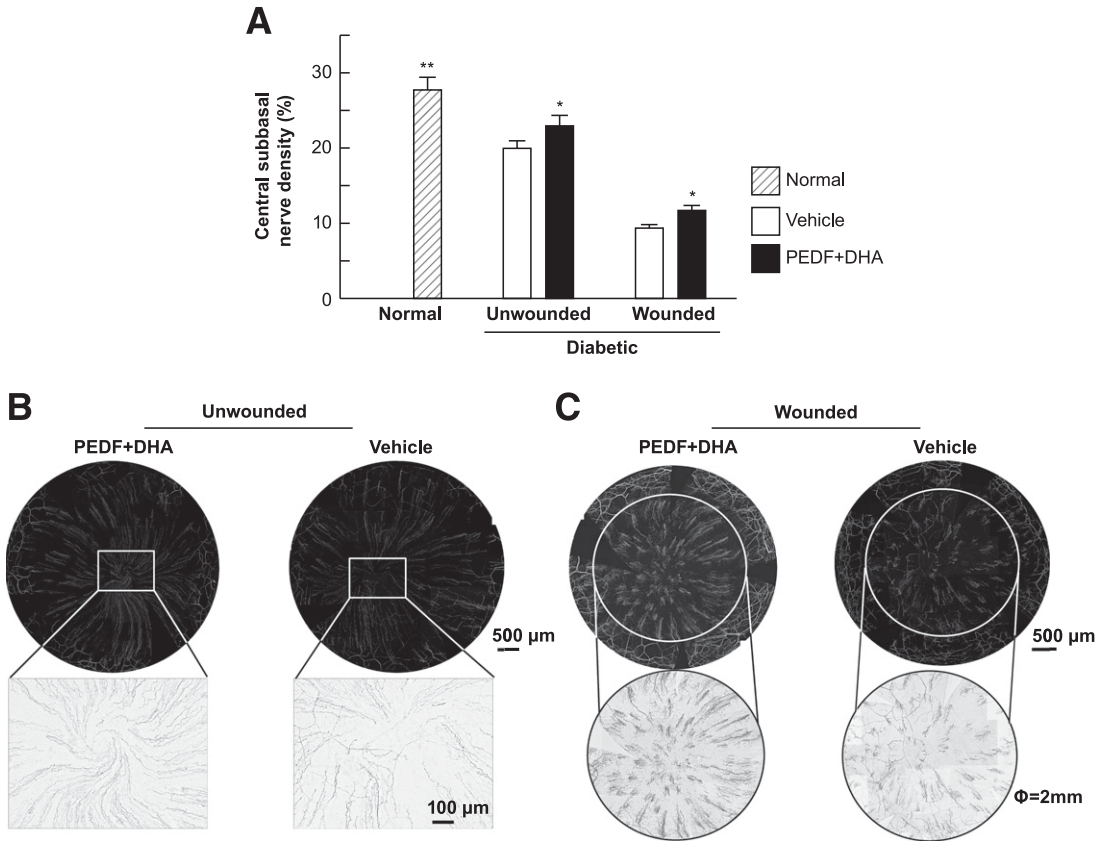

Figure 2—Topical application of PEDF+DHA promotes nerve regeneration in wounded and unwounded diabetic mouse corneas. The right corneas of 16 diabetic mice were injured and the left eye unwounded, and then corneas were treated as described in RESEARCH DESIGN AND METHODS. *A*: Treatment for 2 weeks with PEDF+DHA significantly increased corneal epithelial nerve density in both wounded (right eye) and unwounded (left eye) diabetic mouse corneas. Data are expressed as mean ± SD (*$P < 0.05$; **$P < 0.01$; n = 8 mice/group). Representative whole mounts show the entire corneal epithelial nerves stained with PGP 9.5 antibody of an unwounded left cornea (*B*) and wounded cornea (*C*). The inset in *B* shows the vortex area in the unwounded cornea. Nerve density was calculated within the injured area (diameter 2 mm) as marked in circles. The bottom two images in *C* show the nerves traced with a four-pixel brush by using Photoshop imaging software.

architecture of a normal and diabetic cornea. Several neuropathies (Fig. 1*E*, arrows) were detected only in stromal nerve branches of the diabetic eyes.

PEDF+DHA Promotes Corneal Nerve Regeneration in Diabetic Mice

After diabetic mice were wounded in the right eye and treated in both eyes with PEDF+DHA for only 2 weeks, there was a significant increase in corneal epithelial nerve regeneration in both wounded and unwounded eyes compared with vehicle-treated corneas (Fig. 2*A*). In the unwounded left corneas (n = 8 corneas/group), the central subbasal epithelial nerve density was 23 ± 1.4% in the PEDF+DHA-treated group versus 20 ± 1.8% in the vehicle-treated group ($P < 0.0\ 01$). Note in Fig. 2*B* the difference in the anatomy of the nerves between the two treatments and the nerve density in the vortex in the amplification of the inset. In the wounded right corneas (n = 8 corneas/group), the newly regenerating epithelial nerve density was 11.7 ± 0.7% in the PEDF+DHA group versus 9.4 ± 1% in the vehicle-treated group (Fig. 2*A*) ($P < 0.001$). Figure 2*C* shows a representative architecture of the total subbasal nerves in the wounded area after 2 weeks of PEDF+DHA or vehicle treatment. Hyperglycemia decreased SP nerve density in unwounded and wounded corneas (Fig. 3*A*). The normal mouse cornea contained 15.8 ± 1.8% SP-positive nerves (10). There was a significant decrease in SP nerve density in the diabetic corneas, regardless of treatment. However, in comparison with the vehicle-treated group, the PEDF+DHA-treated corneas showed a significantly higher density of SP-positive nerves in both the wounded and unwounded eyes (Fig. 3*A*). In the unwounded diabetic corneas, 2 weeks of treatment with PEDF+DHA produced a 68% recovery of SP-positive nerves. In the wounded corneas, the recovery after treatment was 33% of the total nerves in nondiabetic mouse corneas. Figure 3*B* shows representative images of SP-positive nerves of a 2-mm diameter demarked area in normal and diabetic unwounded and wounded corneas.

PEDF+DHA Enhances Corneal Sensitivity and Tear Production in Diabetic Mice

Along with nerve regeneration, PEDF+DHA also enhanced recovery of corneal sensitivity in diabetic mice. Three days after wounding, the diabetic corneas showed very little sensitivity, regardless of treatment. By days 7 and 12, there was an increase in corneal sensitivity in both groups (Fig. 4*A*), and PEDF+DHA treatment induced a significant increase when compared with the vehicle-treated group ($P < 0.05$). In the diabetic unwounded corneas, the vehicle group showed a progressive decrease in corneal sensitivity from day 3 to day 12, whereas the unwounded left corneas treated with PEDF+DHA showed a gradual and significant increase compared with the vehicle ($P < 0.05$).

Treatment with PEDF+DHA also increased tear production in diabetic mice in both wounded and unwounded conditions. As shown in Fig. 4*B*, a significant increase was observed at day 8 after treatment in the unwounded eyes

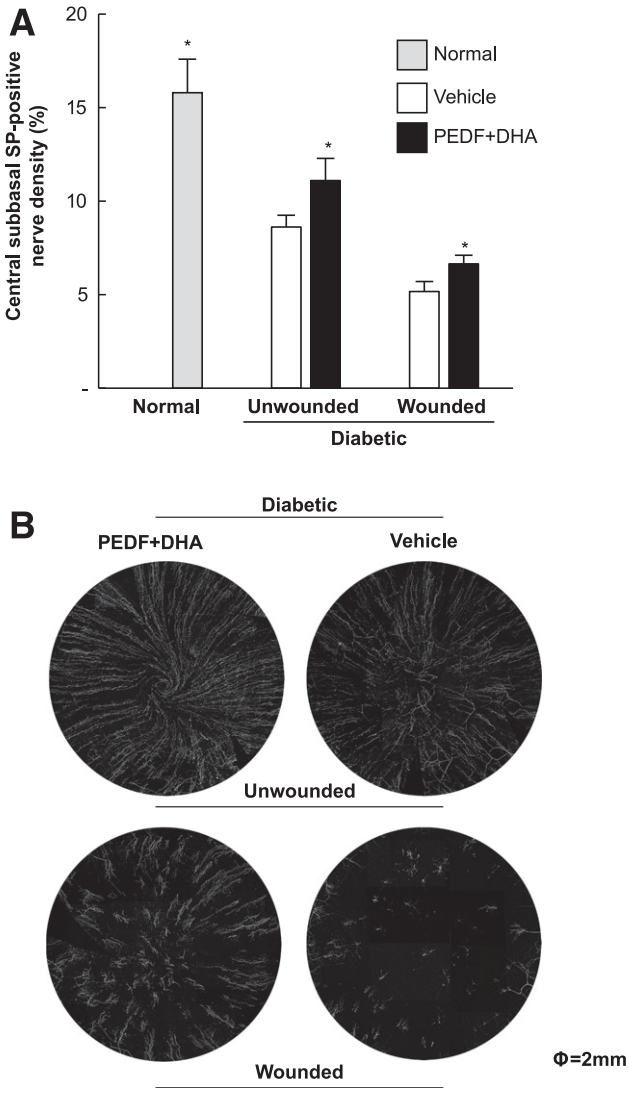

Figure 3—PEDF+DHA treatment increased SP-positive epithelial nerve regeneration in wounded and unwounded diabetic mouse corneas. *A*: The PEDF+DHA group showed a higher density of SP-positive nerves than that of the vehicle group in both the wounded and unwounded eyes. Data are expressed as mean ± SD (*$P < 0.01$; n = 6 mice/group). *B*: Representative images show the whole mounts of SP-positive epithelial nerve fibers in the central corneas (diameter 2 mm) of diabetic mice of both treated groups with or without injury.

($P < 0.01$) and at day 12 in both the wounded ($P < 0.01$) and unwounded ($P < 0.05$) corneas.

PEDF+DHA Accelerates Corneal Wound Healing and Modulates Inflammatory Response in Diabetic Mice

Epithelial wound closure was evaluated by corneal staining with methylene blue as described (15). Treatment with PEDF+DHA significantly promoted wound healing on day 1 after injury, with >50% reduction of the wounded area compared with vehicle-treated corneas (Fig. 5*A* and *B*). On day 2 after injury, the wounded area was much smaller but still showed a significant decrease in the PEDF+DHA-treated corneas ($P < 0.05$). The time of complete closure of epithelial defects was ~48.67 ± 3.93 h (mean ± SD;

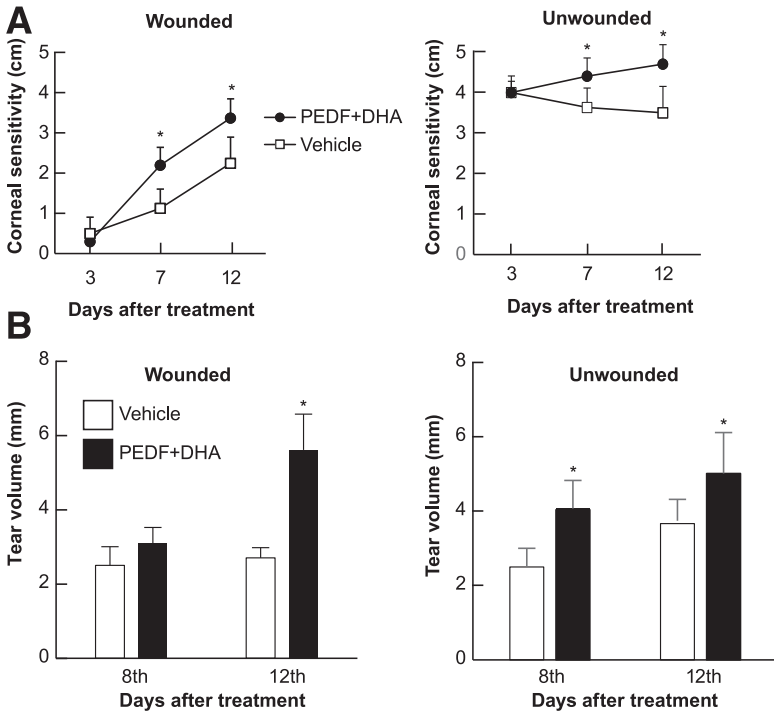

Figure 4—Effect of PEDF+DHA on corneal sensitivity and tear production of wounded and unwounded diabetic mice. *A*: Corneal sensitivity in wounded and unwounded corneas was measured at 3, 7, and 12 days after PEDF+DHA or vehicle treatment. The values correspond to mean ± SD (*$P < 0.05$; $n = 5$ mice/group). *B*: Tear volume was measured at 8 and 12 days after treatment. Data are expressed as mean ± SD (*$P < 0.05$, $n = 5$ mice/group).

$n = 6$ mice) in the PEDF+DHA-treated eyes and 61.33 ± 4.32 h in the vehicle-treated eyes ($n = 6$ mice). In a normal mouse with a similar injury, the wound will be closed between 46 and 52 h (15). This shows that treatment with PEDF+DHA stimulates wound closure at the same speed as that of nondiabetic mice. Previous studies have shown that PEDF+DHA treatment reduces the inflammatory response in rabbit corneas after lamellar keratectomy and herpes simplex virus-1 infection (11,12,18). To test whether the treatment would have a similar effect on the diabetic mouse corneas, we evaluated neutrophil and macrophage infiltration in the corneas after injury. Higher cell infiltration occurred for neutrophils in the wounded corneas on day 1 as opposed to day 2, which is when the epithelial wound was much smaller. Compared with the vehicle-treated eyes, on day 1, the corneas treated with PEDF+DHA showed a significant decrease in neutrophil infiltration, from 13.6 ± 1.52 to 9.36 ± 0.64 neutrophils/field ($P < 0.05$). By day 2, there was again a decrease in infiltration of neutrophils in the PEDF+DHA-treated corneas (Fig. 6*B*). In contrast, on day 1 after injury, the number of F4/80$^+$ macrophages was higher in the PEDF+DHA-treated corneas (21.2 ± 2.7) as opposed to those in the vehicle-treated corneas (10.9 ± 0.9) (Fig. 6*C*). When the sections were double labeled with a monoclonal rat anti-F4/80 (a pan-marker for macrophages) and a polyclonal rabbit CD206 antibody (a type 2 macrophage marker) (Fig. 6*C* and *D*), it was shown that 83 ± 3% of the F4/80-positive cells were also stained for CD206 at day 1 after PEDF+DHA treatment, whereas in the vehicle, 63 ± 6% of the total cells were CD206 positive ($P < 0.05$). On day 2, the percentage of type 2 macrophages increased to 95 ± 2.6% in the corneas treated with PEDF+DHA and only 71 ± 8% in the vehicle-treated corneas, with a significant difference of $P < 0.01$ (Fig. 6*C*).

DISCUSSION

Corneal innervation provides protective and trophic functions to tissue. It is well documented that diabetes causes damage to corneal nerve fibers that causes the decrease in corneal sensation, tear secretion, and corneal epithelial repair after injury (6–8,19–22). Our recent study of human corneas from donors with type 1 diabetes showed that decreased epithelial nerve density was not related to age but was instead significantly affected by the duration of diabetes, and pathological examination showed that there were many neuropathies present in the stromal nerves (22). In agreement with these findings in humans, we now show that hyperglycemia for 10 weeks in mice also produces a significant decrease in epithelial nerve density and that there were many stromal nerve neuropathies, suggesting that the mouse model used in this study is appropriate for investigating corneal pathologies produced by diabetic complications.

An interesting finding in the human diabetic cornea was the appearance of a few regenerated nerves, which were found in all of the examined diabetic corneas regardless of

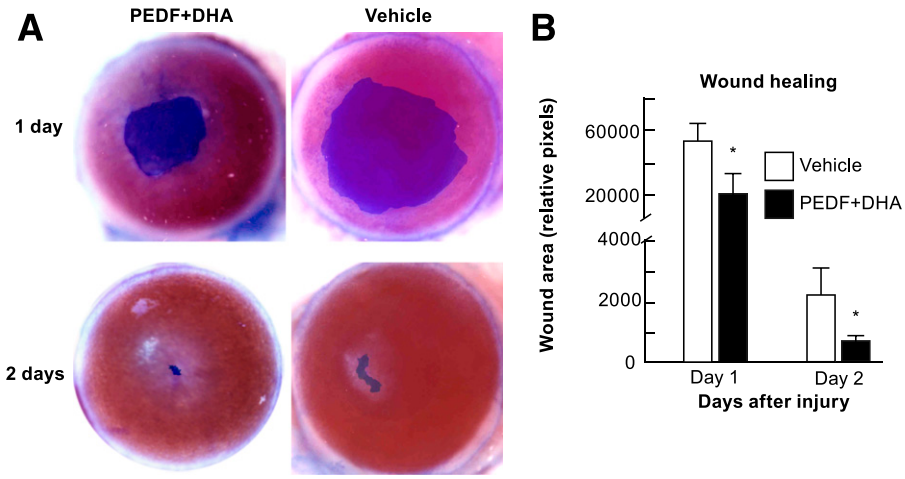

Figure 5—Effect of PEDF+DHA treatment on wound healing in diabetic corneas. The right eyes of 16 mice with hyperglycemia for 10 weeks were injured and divided randomly into two groups and treated for 1 or 2 days with PEDF+DHA or vehicle. *A*: The wounded corneas were stained with 0.5% methylene blue and photographed with a surgical microscopy through an attached digital camera. *B*: Wounded area. Data are expressed as mean ± SD (*$P < 0.05$; $n = 4$ mice/group).

the severity or duration of diabetes (22). The coexistence of nerve regeneration with neuropathy implies that the balance between nerve damage and repair may play a critical role in the development of keratopathy during diabetes. Therefore, preventing corneal damage by optimal glycemic control and promoting nerve regeneration by new therapeutic approaches should be helpful in treating the effects of the disease in the cornea. In recognition of the importance of corneal nerves in diabetes, several recent clinical studies have used in vivo confocal microscopy to observe alterations in the corneal nerves that serve as markers for early detection of diabetic neuropathy (23,24). However, new treatment modalities for diabetic keratopathy remain limited. Topical application of the aldose reductase inhibitors naltrexone (opioid antagonist) and nicergoline (ergoline derivates) have been reported to significantly promote corneal wound healing in diabetic rats (25–27), but their effect on corneal innervation is unknown. Treatment of diabetic rats with ilepatril (Sanofi), a vasopeptidase inhibitor that protects neuropeptide degradation and increases corneal sensitivity and innervation (28). Most recently, vascular endothelial growth factor (VEGF)-B, a member of the VEGF family, has been shown to enhance both corneal epithelial wound healing and the regeneration of injured corneal nerves, yet its effect on diabetic keratopathy has not been investigated (29).

Consistent with our previous studies (9–12), we demonstrated that topical application of PEDF+DHA for 2 weeks significantly increased regeneration of corneal sensory nerves in both wounded and unwounded diabetic corneas and showed that the nerves were functional, as demonstrated by the restoration of corneal sensitivity, increase in tear volume, and upregulated expression of the sensory neuropeptide SP.

Although the cellular mechanisms underlying this treatment have not been investigated in the current study, based on previous studies from our group and others, we can suggest possible mechanisms involved in the action of PEDF+DHA in this diabetic model. One of the main mechanisms could be the neuroprotective and antioxidative actions mediated by PEDF+DHA. Oxidative stress has been proposed as a primary pathogenic factor responsible for the development and progression of diabetic peripheral neuropathy in which mitochondrial dysfunction, induced by chronic hyperglycemia, leads to axonal regenerative failure (30–32). In the eye, PEDF-mediated mechanisms have been reported to protect the retina against reactive oxygen species damage in diabetic retinopathy and neuropathy (33,34). PEDF attenuates caspase-3 activity by improving the ratio of Bcl2/Bax in advanced glycation end product–exposed pericytes and reduces reactive oxygen species generation by downregulating the membrane components of NAPDH oxidase, $p22^{PHOX}$, and $gp91^{PHOX}$, thus suppressing NADPH oxidase activity in advanced glycation end product–exposed endothelial cells (35,36). Furthermore, PEDF and a 44-mer PEDF peptide recently have been shown to accelerate corneal wound healing and promote limbal stem cell self-renewal (37).

DHA belongs to the n-3 family of fatty acids and is concentrated in synapse and cellular membranes of the brain and retina, playing an important role in aging, memory formation, synaptic membrane function, and neuroprotection (38). PEDF+DHA, through a signaling involving neuroprotectin D1 (NPD1) synthesis, promotes the survival of photoreceptor and retinal pigment epithelial cells from degeneration induced by oxidative stress (39,40). PEDF, per se, can activate antioxidant-responsive elements expression in retinal pigment epithelial cells, whereas PEDF+DHA potentiates this antioxidant-responsive element upregulation (41). The cornea expresses both PEDF and its receptor and contains very low amounts of DHA. Our previous studies have shown that

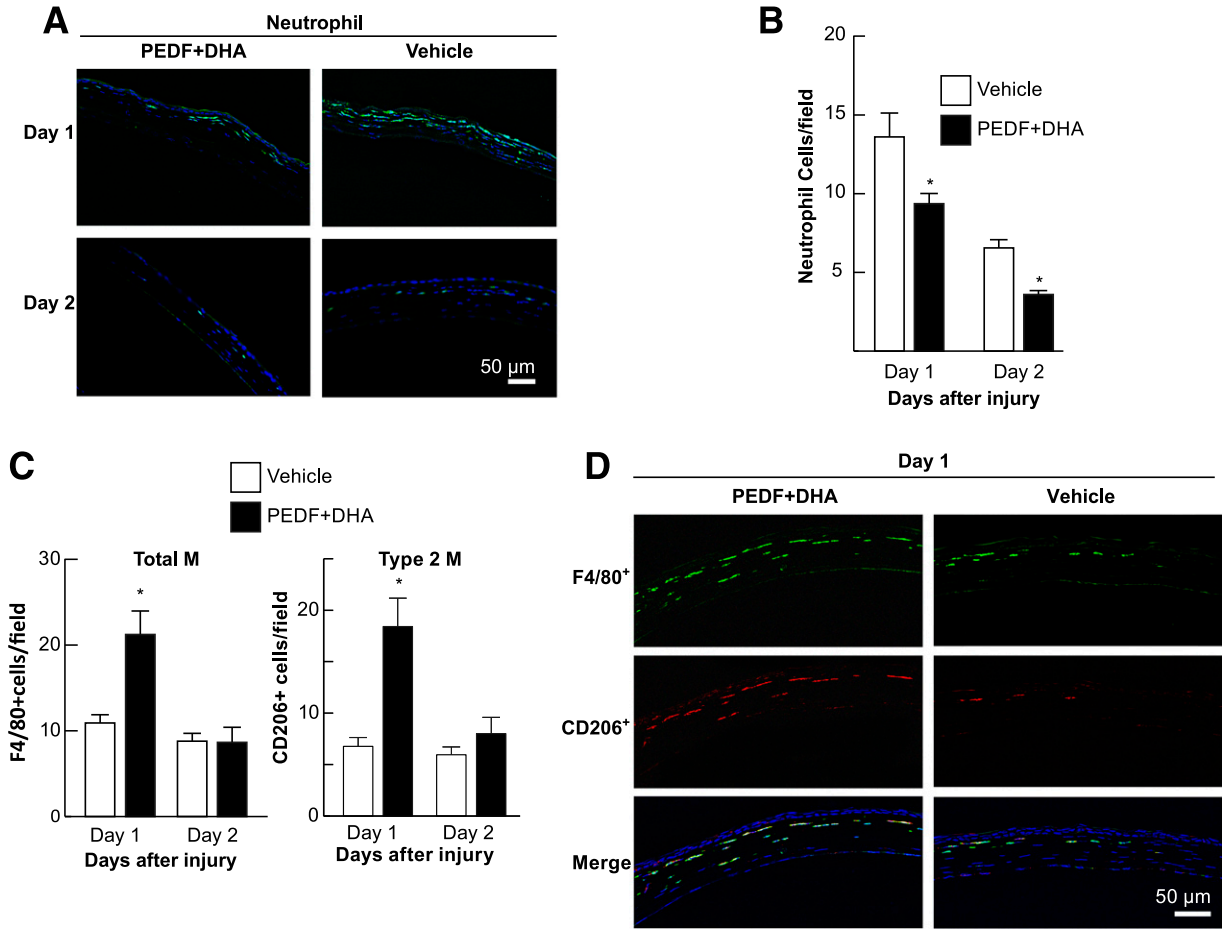

Figure 6—Changes in neutrophils and macrophages (M) after injury in diabetic corneas. The mice were treated as described in Fig. 5 and then processed for immunofluorescence by using antibodies against neutrophils and macrophages. *A*: Representative immunofluorescence image of an injury cornea showing staining with anti-neutrophil (green) and DAPI (blue). *B*: Neutrophils were counted as explained in RESEARCH DESIGN AND METHODS, and values represent the average of four corneas per condition ± SD. *$P < 0.05$. *C*: F4/80- and CD206-positive cells were counted in four fields per slide, two slides per cornea. Values represent the means ± SD of four corneas per condition. *$P < 0.01$. *D*: Representative immunofluorescence of injured corneas stained with F4/80 and CD206 antibodies on day 1. In the merged figure, double staining is shown, with an increase in M2 macrophages when the corneas were treated with PEDF+DHA.

treatment with PEDF+DHA increases corneal NPD1 synthesis and promote nerve regeneration following experimental surgery, whereas PEDF or DHA treatment alone are not able to stimulate nerve regeneration in a significant way (9). Therefore, it is very likely that the effect of neuroprotection observed in the current study is through a similar mechanism. In a recent study, Coppey et al. (42) found that in feeding diabetic rats with a diet containing menhaden oil, a source of n-3 fatty acids, there was improvement in corneal nerve density and sensitivity. The action could be through NPD1 and other docosanoids synthesized from menhaden.

Another mechanism may be attributed to the neurotrophic function of this treatment. It is well known that the interactions between the corneal nerves and resident cells play an important role in maintaining a healthy ocular surface (43,44). Hyperglycemia not only impairs the corneal cellular metabolisms but also damages corneal innervation and reduces neuropeptide nerve fibers. As a result, the homeostasis between corneal cells and nerves is disrupted. Neuropeptides, including calcitonin gene-related peptide and SP, released from the sensory nerve terminals, have been shown to induce epithelial cell proliferation, migration, and adhesion, facilitate corneal wound healing, and play a role in the regulation of tear production and mucus secretion from goblet cells (45–47). In turn, neurotrophins and growth factors secreted by corneal cells, such as nerve growth factor (NGF), brain-derived nerve growth factor, glial cell–derived nerve growth factor, VEGF, and other regeneration-related growth factors, support nerve outgrowth and survival (48–50). In the current study, treatment with PEDF+DHA increased the SP-positive nerve density in diabetic corneas and accelerated wound healing after injury, suggesting that PEDF+DHA through their neurotrophic activities could serve as a new therapeutic approach in the treatment of corneal injuries and ulcers produced by diabetes.

A third mechanism involves the anti-inflammatory action of this treatment. Our previous studies have shown

that PEDF+DHA attenuates the inflammatory response produced by injury or herpes simplex virus-1 infection (9–12,18). A new finding shows that treated corneas display an increased number of type 2 (M2) macrophages, as characterized by the increased expression of mannose receptor CD206. It is well known that, beyond increasing inflammation and stimulating the immune system, macrophages also play an important anti-inflammatory role and can decrease immune reactions through the release of cytokines and growth factors (51). M1 macrophages stimulate inflammation, whereas M2 macrophages decrease inflammation and help in the repair of axons after injury (51). Diabetes is known to have a compromised macrophage function, and macrophage dysfunction impairs resolution of inflammation, leading to a delayed wound healing in diabetic mice (52). In this study, we show that treatment with PEDF+DHA increases the number of macrophages, especially M2, in the wounding area, suggesting that the treatment can stimulate macrophage function that contributes to enhanced wound healing.

As an additional mechanism, the treatment could remedy diabetes-induced DHA metabolic deficiencies in ocular tissues. Diabetes decreases retinal DHA production because of a decrease in the expression of fatty acid elongases (53), and a DHA-rich diet can fully prevent diabetes-induced retinal vascular pathology (54). The meibomian gland also expresses long-chain fatty acids and the elongase ELOVL4 that can synthesize very long fatty acids (55). Diabetes causes significant morphological changes and dysfunction of the meibomian glands (56,57), leading to tear lipid deficiency and dry eye. Tear film contains DHA, and there is a decrease in the ratio of n-3 fatty acids in patients with dry eye (58). In the current study, we did not investigate the levels of DHA contents in tear film; however, the elevated tear production and corneal sensitivity strongly suggest that topical application of PEDF+DHA could ameliorate the diabetes-induced DHA deficiency, thus maintaining a healthy ocular surface.

In summary, we used a mouse model to study the action of PEDF combined with DHA on diabetic keratopathy. Our results show that the treatment for 2 weeks significantly increased the density of corneal epithelial nerves and SP-positive nerve fibers along with an increase in the return of corneal sensitivity and tear volume. In addition, this treatment also enhanced corneal wound healing and modulated the inflammatory response triggered by injury by increasing the repair of M2 macrophages. Taken together, this study suggests that PEDF+DHA, with their neuroprotective, antioxidative, neurotrophic, and anti-inflammatory properties, could potentially be considered as a therapeutic option for the treatment of diabetic keratopathy.

Funding. This work was supported by National Institutes of Health grant R01-EY-19465 and National Institutes of Health COBRE Phase III Neuroscience Research Pilot Project Program 149750141G (parent grant GM-103340) and in part by an unrestricted departmental grant from Research to Prevent Blindness, Inc. (New York, NY).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. J.H. and H.E.P.B. contributed to designing the experiments. J.H. contributed to the acquisition and analysis of data and drafting and critical review of the manuscript. T.L.P. contributed to acquisition and analysis of data and review of the manuscript. A.K. helped with animal examination for sensitivity and tear secretion, quantifying inflammatory cells, and reviewing the manuscript. H.E.P.B. supervised the study and wrote and reviewed the manuscript. H.E.P.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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