

ONLINE FIRST

Recovery of Corneal Sensitivity, Calcitonin Gene-Related Peptide–Positive Nerves, and Increased Wound Healing Induced by Pigment Epithelial–Derived Factor Plus Docosahexaenoic Acid After Experimental Surgery

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Objective: To assess function of regenerated corneal nerves in correlation with epithelial wound healing after experimental nerve damage in rabbits treated with pigment epithelial–derived factor (PEDF) plus docosahexaenoic acid (DHA).

Methods: An 8-mm stromal dissection was performed in the right eyes of adult New Zealand rabbits. Treatment with PEDF+DHA was for 6 weeks. Corneal sensation was measured weekly by Cochet-Bonnet esthesiometer. After 8 weeks, immunofluorescence with β III-tubulin, calcitonin gene-related peptide, and substance P antibodies was performed to quantify nerves. Also, rabbits were treated with PEDF+DHA for 4 weeks after lamellar keratectomy, followed by 8-mm epithelial debridement and epithelial defect assessment. One week after surgery, corneas were stained with anti-Ki67 antibody to assess cell proliferation.

Results: Eight weeks after surgery, calcitonin gene-related peptide–positive nerve fibers in the PEDF+DHA group were similar to normal rabbit corneas but were decreased in the vehicle. Substance P was localized in the

subepithelial plexus but appeared in epithelial cells after nerve injury regardless of treatment. Five weeks after surgery, an increase in corneal sensitivity occurred in the PEDF+DHA group and reached normal values by 8 weeks. Pigment epithelial–derived factor plus DHA increased epithelial wound healing after lamellar keratectomy. One week after epithelial injury, Ki67-positive cells increased in the limbal area.

Conclusion: Pigment epithelial–derived factor plus DHA promotes regeneration of calcitonin gene-related peptide–positive corneal nerves, accelerating wound healing and return of corneal sensitivity.

Clinical Relevance: Pigment epithelial–derived factor plus DHA represents a new approach to regenerate nerves and a potential treatment for prevention of severe dry eye after surgery or diseases of the ocular surface.

Arch Ophthalmol. 2012;130(1):76-83.

Published online September 12, 2011.

doi:10.1001/archophthalmol.2011.287

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CORNEAL TISSUE IS HIGHLY innervated mostly by trigeminal sensory fibers that enter the corneal stroma from the limbal area. These fibers branch out to form the subepithelial plexus and terminate as fine fibers in the corneal epithelium.¹⁻³ Some sympathetic nerves are also present. Corneal sensitivity is essential for maintaining a healthy ocular surface, mainly because corneal nerves modulate cell proliferation and differentiation and wound healing.⁴⁻⁶ In addition, corneal nerves play a role in DNA synthesis, ion transport, and collagen expression.^{5,7-9} Recently, it has been shown that corneal de-

nerivation induces apoptosis of resident corneal cells.¹⁰ The mechanisms that mediate these effects are not completely understood. Neuropeptides and neurotransmitters have been detected in corneal nerves. The most studied neuropeptides in sensory nerves are calcitonin gene-related peptide (CGRP) and substance P (SP), which are both likely to play roles in the “trophic” efferent function of corneal sensory nerves.²

Damage to corneal nerves decreases sensitivity and causes decreased blink reflex and tear secretion, thereby increasing dry eye symptoms that can lead to neurotrophic keratitis. Dry eye secondary to corneal nerve damage has been observed

after laser vision correction with laser in situ keratomileusis (LASIK) and photorefractive keratectomy, long-standing contact lens use, herpes simplex virus infection, and diabetes mellitus.¹¹⁻¹⁴ Patients with Sjögren syndrome also have been shown to possess corneal nerve alterations that may contribute to ocular surface disease.¹⁵ Severe nerve damage, such as that observed after complete transection of nerves for penetrating keratoplasty or herpetic infections, can significantly impair epithelial wound healing. This can lead to more ominous complications, such as edema, ulceration, and corneal thinning. In light of this, the search for a treatment that could stimulate faster recovery of corneal nerves is vitally important to restoring normal physiological features of the cornea.

In a previous study, we showed that a combination of docosahexaenoic acid (DHA) and pigment epithelial-derived factor (PEDF) increases corneal nerve regeneration after injury during lamellar keratectomy.¹⁶ Docosahexaenoic acid is an ω -3 fatty acid that accumulates in the brain during synaptogenesis and dendrite formation.¹⁷ It is also the precursor of neuroprotectin D1 (NPD1), a stereoselective lipid mediator with potent anti-inflammatory and neuroprotective bioactivity¹⁸ that also promotes epithelial wound healing.¹⁹ Although corneas contain relatively low amounts of DHA,²⁰ mice fed with DHA-enriched diets have been shown to generate NPD1 in the cornea.¹⁹ Pigment epithelial-derived factor, on the other hand, is a broad-acting neurotrophic and neuroprotective factor involved in angiogenesis, neuronal cell survival, and cell differentiation.^{21,22} Pigment epithelial-derived factor strongly stimulates the synthesis of NPD1 from its precursor, DHA,²³ and corneas treated with PEDF and DHA show increased NPD1 synthesis.¹⁶

Although histological evidence indicates a significant increase in corneal nerve regeneration after PEDF+DHA treatment, it is unclear how well these regenerated corneal nerves function. Therefore, this study had a 2-fold purpose: (1) to investigate if corneal sensitivity and epithelial wound healing are restored when nerve regeneration is stimulated by treatment with PEDF+DHA and (2) to determine if sensory nerves expressing CGRP and SP are regenerated.

METHODS

SURGERY AND TREATMENT

Animals were treated according to the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic Research and the Institutional Animal Care and Use Committee at the Louisiana State University Health Sciences Center. Male New Zealand albino rabbits weighing 2 to 3 kg were used. Anesthesia was achieved with xylazine (10 mg/kg) and ketamine hydrochloride (50 mg/kg). Topical anesthesia in the form of tetracaine drops was also used. Lamellar keratectomy was performed in the right eye of each rabbit. The surgery consisted of an 8-mm stromal dissection through a 3-mm incision. No sutures were used. Topical moxifloxacin was used for prophylaxis postoperatively.

Treatment was administered immediately after surgery via a 72-hour collagen shield (Oasis, Glendora, California) soaked in the drug or vehicle. Pigment epithelial-derived factor was

obtained from BioProducts MD (Middletown, Maryland) with more than 90% purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Fifty nanograms of PEDF dissolved in phosphate-buffered saline and 400 μ g of DHA (Cayman Chemical, Ann Arbor, Michigan) complexed to albumin, 25%, were used for each shield. Tests performed in our laboratory showed that 25% of the DHA is absorbed into the collagen shield.²⁴ Collagen shields were changed twice a week for 6 weeks. A temporary tarsorrhaphy was performed and kept in place for the first 3 days after surgery. Animals were killed 8 weeks after surgery with an overdose of sodium pentobarbital via ear vein injection.

CORNEAL SENSITIVITY

Corneal sensitivity measurements of the central cornea inside the surgical area were performed weekly in each eye using a Cochet-Bonnet esthesiometer (Luneau Ophthalmologie, Chartres Cedex, France). 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.²⁵ The monofilament was touched to the cornea, making perpendicular contact with it until the flexibility of the filament was observed. The cornea was tested 4 times at each filament length until the response was 100% negative (no blink response). If the animal blinked 50% or more for the times tested, this was considered a positive response. Test times for each monofilament length were increased to 10, when the filament length was close to the threshold (50%), until the examiner felt the response was consistent.²⁵ If no blink response could be elicited at a monofilament length of 0.5 cm, corneal sensitivity was recorded as 0. The measurements were performed by an examiner who was masked to the treatments.

EPITHELIAL WOUND HEALING

To evaluate the effect of regenerated nerves on wound healing, an 8-mm epithelial defect was created with the use of an Algerbrush II (Alger Co Inc, Lago Vista, Texas) 4 weeks after lamellar keratectomy and treatment with PEDF+DHA or vehicle. This time was chosen because, according to prior results obtained in our laboratory,¹⁶ there are a significant amount of regenerated nerves 4 weeks after surgery; however, this is not close to the normal nerve area. As such, we considered that this time would give us the highest likelihood for finding the differences between these groups. Animals were examined every 24 hours and the epithelial defect was stained with methylene blue. The corneas were examined and photographed with a digital camera attached to a portable digital slitlamp (Optotek Medical). The images were analyzed with Image J (<http://rsb.info.nih.gov/ij/>) to calculate the injured area. Animals were killed 7 days after the epithelial defect was created.

IMMUNOHISTOCHEMICAL ANALYSIS

After rabbits were killed, whole corneas were excised and fixed with fresh paraformaldehyde, 2%, in 0.1M phosphate buffer (pH 7.4) for 2 hours at room temperature or overnight at 4°C. Corneas were then washed with phosphate-buffered saline 3 times for 5 minutes each and incubated with mouse monoclonal anti- β III-tubulin antibody (1:1000; Convection Antibody Services Inc, Berkeley, California), chicken anti-CGRP (1:1500; Chemicon International, Temecula, California), and anti-SP (1:1500; Biotechnology Inc, Santa Cruz, California) in goat normal serum, 1%, plus Triton X-100, 0.15%, in 0.1M phosphate-buffered saline for 24 hours at room temperature. After washing, the tissues were incubated with the corresponding secondary anti-

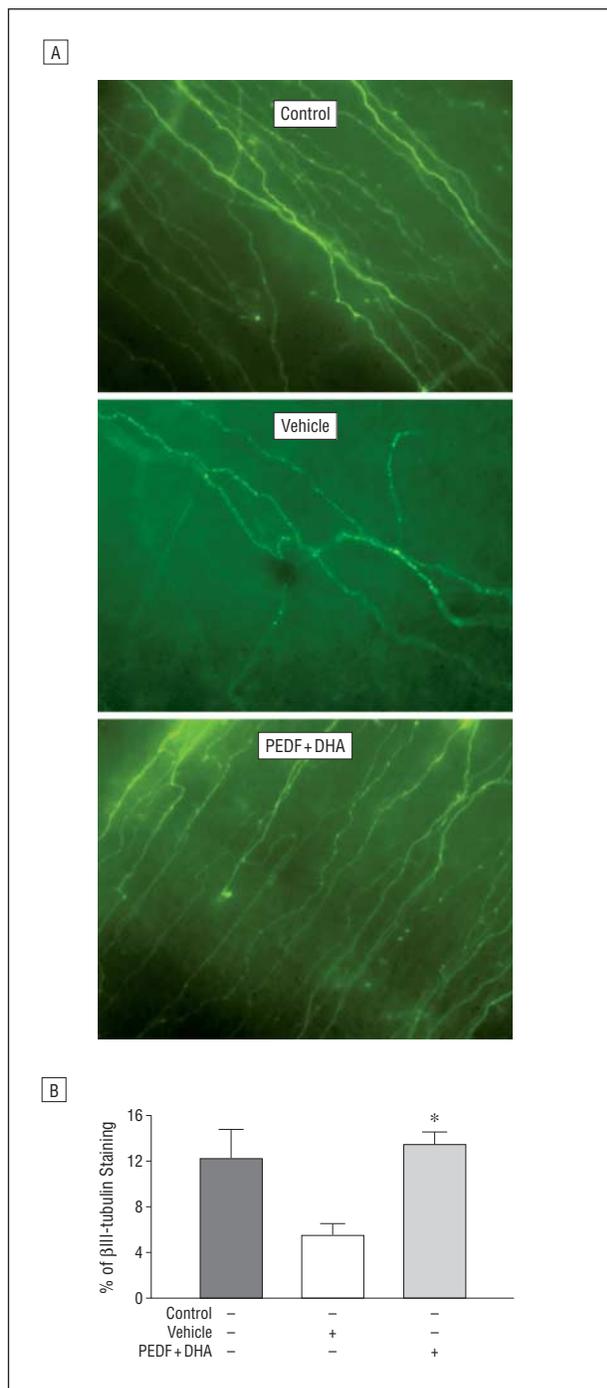


Figure 1. Effect of pigment epithelial–derived factor (PEDF) plus docosahexaenoic acid (DHA) on nerve density 8 weeks after surgery. A, Immunohistochemical analysis of the subepithelial nerve plexus of rabbit corneal whole mounts stained with anti- β III-tubulin antibody (original magnification $\times 20$ [objective lens]). B, Quantification of β III-tubulin–stained subepithelial nerves in the PEDF+DHA–treated group compared with the vehicle-treated group and controls (no surgery). There were 4 rabbits in each group. *Significant difference with respect to untreated animals.

bodies. For double staining, we first added the primary antibodies chicken anti-CGRP for 72 hours or rat anti-SP for 1 week and the secondary antibodies Alexa Fluor 488 donkey antirat IgG or antichick IgG (Invitrogen, Eugene, Oregon) overnight. After being fixed with fresh paraformaldehyde, 2%, for 30 minutes followed by thorough washing, the same tissues were double stained with monoclonal anti- β III-tubulin antibody as the pri-

mary antibody and Alexa Fluor 594 goat antimouse IgG (Invitrogen) as the secondary antibody.

The injured area was viewed and photographed with a fluorescence microscope (Eclipse TE200; Nikon, Tokyo, Japan) equipped with a Nikon camera (DXM1200) using MetaVue imaging software (Molecular Devices, Sunnyvale, California). The β III-tubulin–positive tissue area at the subepithelial nerve plexus was calculated and compared with the total area using an image analysis program. Only the nerve fibers that were in sharp focus for each image were traced to calculate the nerve area. The investigators obtaining the images and calculating the nerve area were masked to the conditions of each specimen. Corneal whole mounts allowed for visualization of the total nerve area stained with β III-tubulin.^{1,16} From the same area, CGRP- and SP-positive areas were calculated and compared with the β III-tubulin–positive area. Ten different images of different areas within the injured zone were analyzed per rabbit.

Frozen sections of the corneal limbal area were obtained from animals that underwent epithelial removal after lamellar keratectomy. Sections were stained with anti-Ki67 antibody (1:2000; Sigma, St Louis, Missouri). Ki67 is a prototypic cell cycle–related nuclear protein that is expressed by cells in all phases of active cell cycle (G1, S, G2, and M), and it is used to establish the cell growing fraction. The number of positive cells was counted in 10 images per rabbit, which were averaged to calculate the proliferating fraction.

STATISTICAL ANALYSIS

All data are expressed as mean (SD). The number of rabbits analyzed in each experiment is described in the Figure legends. Statistical comparison between the PEDF+DHA and vehicle groups was performed by *t* test. *P* values $< .05$ were considered significant.

RESULTS

TREATMENT WITH TOPICAL PEDF+DHA INCREASES CORNEAL NERVE SURFACE AREA AFTER SURGERY

In agreement with what we found in our previous work,¹⁶ immunohistochemical evaluation of rabbit corneas treated with PEDF+DHA for 6 weeks and analyzed at 8 weeks after surgical injury of the sensory nerves showed increased corneal nerve area when compared with untreated controls (**Figure 1**). Whole mounts stained with β III-tubulin showed a mean (SD) nerve area of 13.6% (2.2%) in the control group, 5.6% (0.9%) in the vehicle group, and 12.2% (2.6%) in the PEDF+DHA group. This represents a statistically significant difference between the PEDF+DHA–treated and vehicle groups ($P = .004$). The difference between the control group and the PEDF+DHA group was not statistically significant.

CGRP IS PRESENT IN REGENERATED CORNEAL NERVES IN SIMILAR PERCENTAGE AS IN THE NORMAL RABBIT CORNEA

Immunohistochemical analysis was performed to identify different types of corneal sensory nerves. Quantification of CGRP-positive nerves against total nerve area showed that in normal rabbit corneas the mean (SD) percentage of CGRP-positive neurons was 44.2% (0.1%).

Eight weeks after lamellar dissection and damage to the corneal nerves, CGRP-positive nerves represented only mean (SD) 24.9% (0.04%) of the total nerve area stained with β III-tubulin in animals receiving vehicle; the percentage of these nerves increased to mean (SD) 45.4% (0.07%) in animals treated with PEDF+DHA (**Figure 2**). These data show that treatment with PEDF+DHA enhances recovery of sensory nerves, which express the neuropeptide CGRP in the same proportion as the normal cornea, thus suggesting that the regenerated corneal nerves are functional. The lower percentage of CGRP-positive nerve fibers in the vehicle group may be due to their delayed process of regeneration, suggesting that a smaller proportion of the fibers present at 2 months are able to express or release this neuropeptide.

SP LOCALIZES TO THE CORNEAL EPITHELIAL CELLS AFTER NERVE INJURY

Immunostaining showed that SP-positive nerves in the normal rabbit corneas represented 7.85% of the total nerve area. However, after injury, SP was no longer found in the epithelial or subepithelial nerves, but it was present in the epithelial cells. This phenomenon was observed in both the treated as well as the untreated group (**Figure 3**). We found no difference in the amount of SP between the 2 groups.

TREATMENT WITH PEDF+DHA ENHANCES RECOVERY OF CORNEAL SENSITIVITY AFTER NERVE INJURY

Measurements of corneal nerve sensitivity using a Cochet-Bonnet esthesiometer showed that sensitivity in the uninjured rabbit corneas was in the range of 3 to 4 cm (**Figure 4**, table). Central corneal sensitivity was absent 1 week after surgery and remained low for the first 4 weeks. By the fifth week, there was a significant increase in corneal sensitivity in the PEDF+DHA-treated group. This difference was statistically significant when compared with the untreated group and remained significant until the end of the 8 weeks ($P = .008$), when sensitivity almost reached normal levels. In the vehicle group, there was a slow but progressive increase in corneal sensitivity until the end of the 8 weeks, but this increase remained less than 50% of the sensitivity in control eyes.

TREATMENT WITH PEDF+DHA ACCELERATES EPITHELIAL WOUND HEALING AFTER NERVE DAMAGE

As an indirect measure of corneal nerve functionality, epithelial wound healing was assessed in PEDF+DHA-treated and untreated animals 4 weeks after undergoing lamellar corneal dissection and damage to sensory nerves. An 8-mm epithelial debridement was performed as described in the "Methods" section, and the defect was stained with methylene blue and measured every 24 hours until complete closure was observed. The PEDF+DHA-treated animals had consistently smaller epithelial defects than vehicle-treated animals at 24 and 48 hours after epithelial wounds were created. The difference was not statistically significant at 72 hours, when

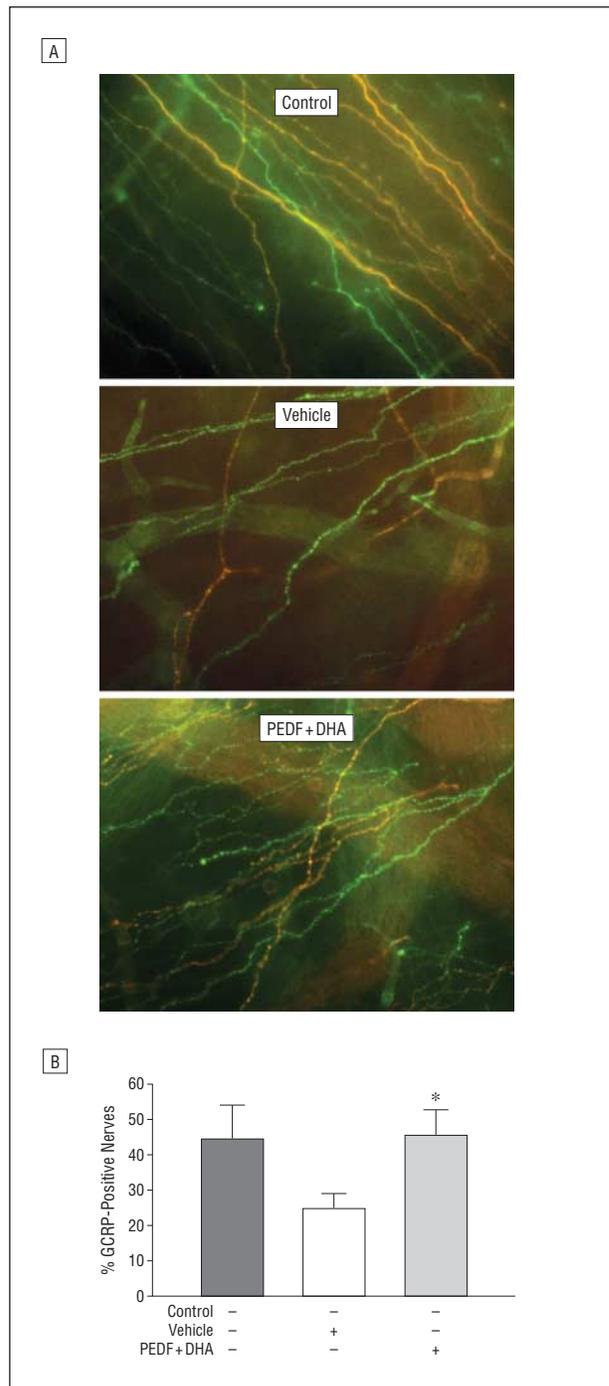


Figure 2. Eight weeks after lamellar dissection and damage to the corneal nerves. A, Immunohistochemical analysis of rabbit corneal whole mounts with anti- β III-tubulin (green) and anti-calcitonin gene-related peptide (CGRP) (red) antibodies 8 weeks after surgery. Merged images show the CGRP-positive nerves in orange for the 3 groups (original magnification $\times 20$ [objective lens]). B, Quantification of CGRP-positive nerves against the total nerve area for each group. There were 4 rabbits in each group. *Significant difference with respect to vehicle-treated animals. PEDF+DHA indicates pigment epithelial-derived factor plus docosahexaenoic acid.

the majority of the epithelial defects had already closed (**Figure 5**). The trend observed was suggestive of faster closure in the PEDF+DHA group, not only when compared with the vehicle group, but also when compared with animals with no previous lamellar keratectomy.

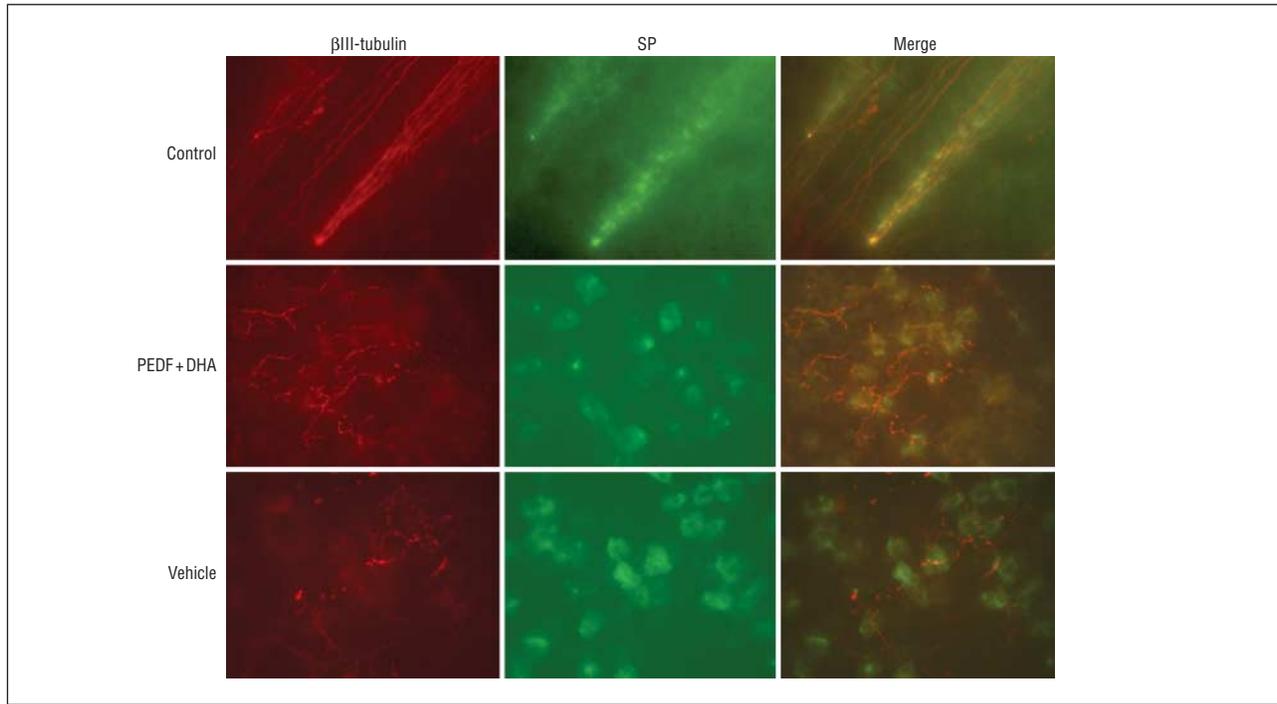


Figure 3. Immunohistochemical analysis of rabbit corneal whole mounts stained with anti- β III-tubulin (green) and anti-substance P (SP) antibodies (red) 8 weeks after surgery. The control group shows SP localized in the subepithelial nerve plexus (top row). After injury, SP is present in the corneal epithelium (second and bottom rows). There were 4 rabbits in each group (original magnification $\times 20$ [objective lens]).

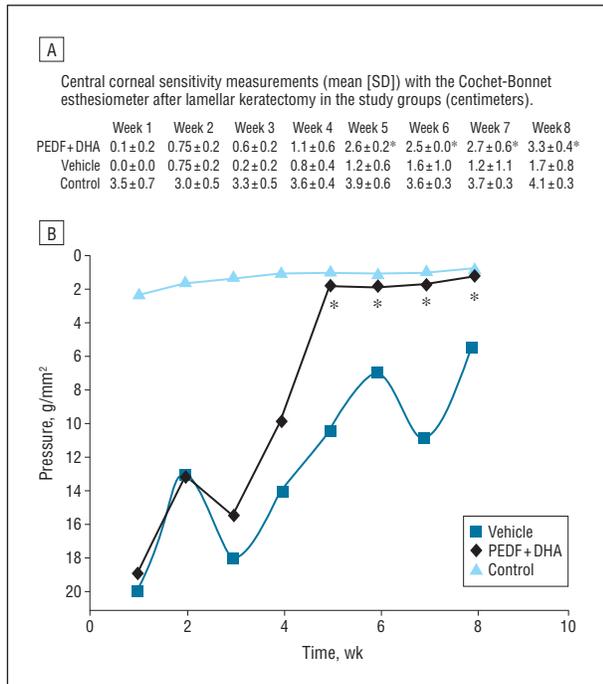


Figure 4. The graph shows the values expressed as pressure in grams per millimeter squared to indicate mechanical force. There was an increase in corneal sensitivity in the pigment epithelial-derived factor (PEDF) plus docosahexaenoic acid (DHA)-treated group reaching almost normal levels at 8 weeks after surgery. There was a statistically significant difference ($*P < .05$) between the PEDF+DHA group vs the vehicle group beginning 5 weeks after surgery that persisted up to 8 weeks after surgery. There were 4 rabbits in each group.

PEDF+DHA INCREASES LIMBAL CELL PROLIFERATION DURING WOUND HEALING

To further investigate the effect of treatment during epithelial wound healing, anti-Ki67 antibody was used to stain the proliferating fraction of the corneal epithelium (**Figure 6**). At the limbal area, we found that cell proliferation was increased in the PEDF+DHA-treated group

compared with the vehicle-treated group. The mean (SD) proliferating fraction of cells in the limbal area in the vehicle group was 2.2% (0.3%) and in the PEDF+DHA-treated group was 9% (0.3%), with a P value of 8.5513×10^{-5} . This represents more than a 4-fold increase in cell proliferation at the limbal area in the PEDF+DHA-treated group. Positive staining was rarely detected in the normal corneas.

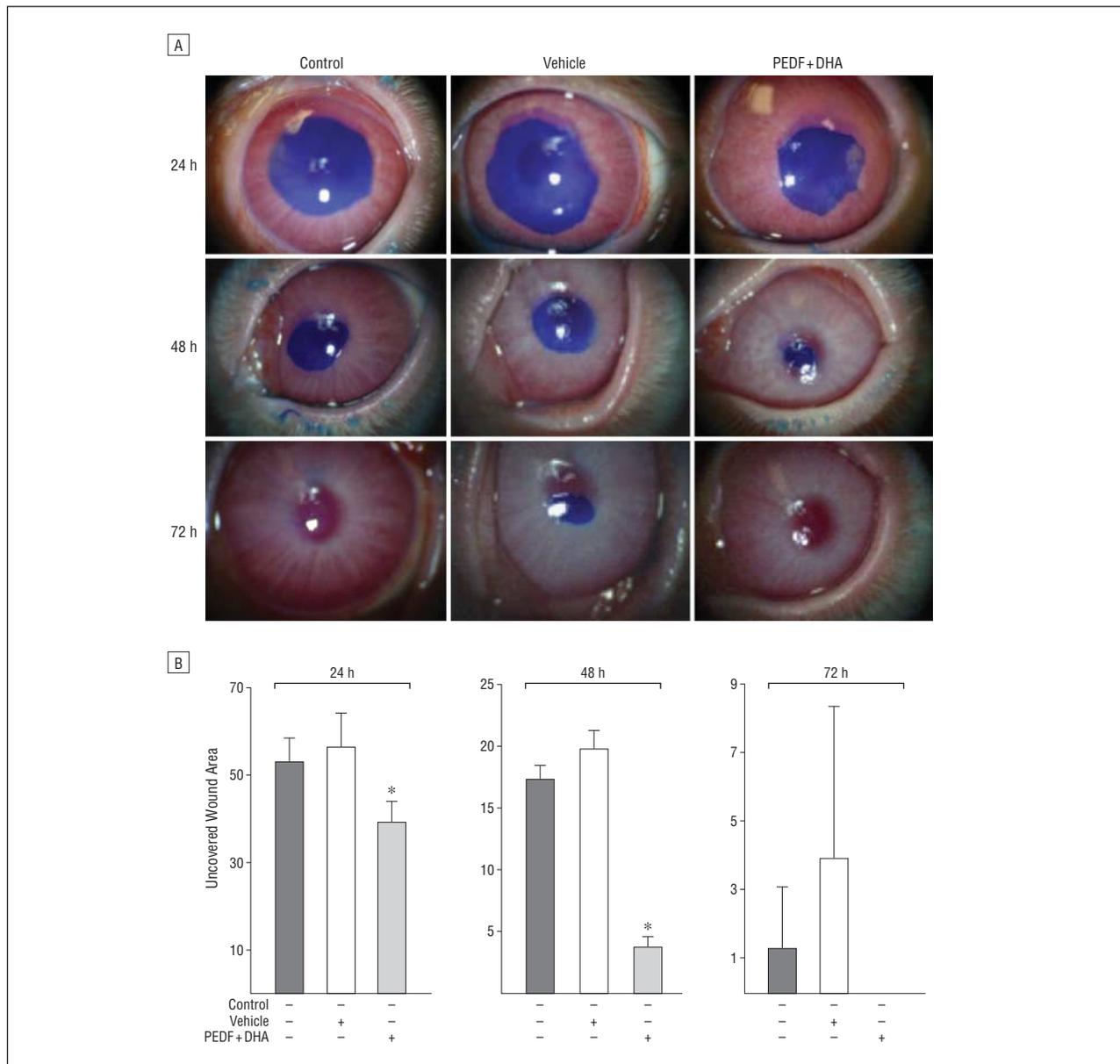


Figure 5. Four weeks after lamellar keratectomy, an 8-mm epithelial debridement was made to rabbits treated with pigment epithelial–derived factor (PEDF) plus docosahexaenoic acid (DHA) and vehicle. Controls were corneas without previous surgery. A, Epithelial defect stained with methylene blue at 24, 48, and 72 hours after epithelial removal. B, The area of absent epithelium was calculated for each rabbit at the different points and then compared with the initial injury (8 mm) to yield the healed area. The PEDF+DHA–treated animals had consistently significantly smaller epithelial defects (*) when compared with the vehicle group and control group at 24 and 48 hours. The difference did not reach statistical significance at 72 hours. There were 4 rabbits in each group.

COMMENT

Clinical and experimental studies have shown that corneal nerve integrity is critical for maintaining a healthy ocular surface and that damage alters metabolism and vitality of the corneal epithelium, impairs epithelial wound healing, and leads to trophic ulceration. During lamellar keratectomy, a stromal dissection is performed in the midstroma, severing all corneal nerves. This is similar to the nerve damage that occurs after creating a LASIK flap. It is known that corneal sensitivity is reduced after LASIK and that the number of stromal nerve fiber bundles remains at less than 50% 1 year after surgery.²⁶

After lamellar keratectomy, nerve fibers disappear by the first week and corneal sensitivity is absent; then fibers slowly begin to regenerate and sensitivity gradually returns. Our studies go up to 8 weeks and show that nerve area and corneal sensitivity remain significantly lower than prior to surgery in vehicle-treated animals.

Our data show convincing evidence that treatment with PEDF+DHA enhances regeneration of corneal sensory nerves and that these nerves are functional, as demonstrated by (1) their ability to express CGRP in the same proportion as normal rabbit corneas, (2) the return of corneal sensation to almost normal levels at 8 weeks, and (3) the increase in epithelial wound healing. The fact that epithelial wound healing is faster in treated corneas com-

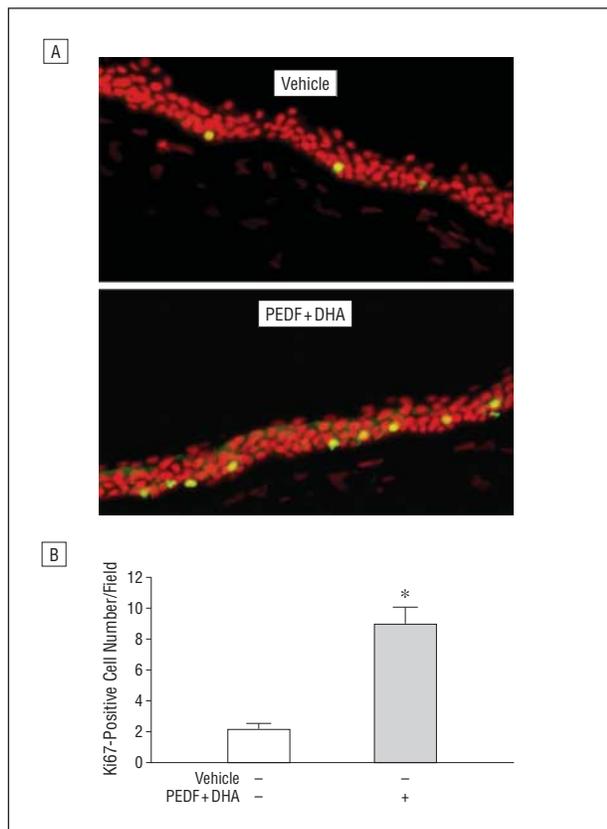


Figure 6. Limbal cell proliferation 4 weeks after lamellar keratectomy and 1 week after epithelial debridement in the pigment epithelial–derived factor (PEDF) plus docosahexaenoic acid (DHA)–treated group vs the vehicle group (original magnification $\times 40$). A, Immunohistochemical analysis of rabbit corneal sections with anti-Ki67 antibody. Images were taken at the limbal area. B, Quantification of the growing fraction for the PEDF+DHA–treated group was 9.06% and for the vehicle group, 2.2%. *Significant difference. There were 4 rabbits in each group.

pared with controls may suggest some additional direct effects of the treatment to the epithelium.

The number of nerve fibers positive for CGRP and SP vary according to different species. However, in general, CGRP fibers are more abundant than SP fibers.² We found an average of 44% CGRP immunoreactive fibers in the normal rabbit corneas and approximately 8% SP-positive fibers. The finding that PEDF+DHA regenerates sensory fibers in rabbit corneas that are able to produce or release CGRP in the same proportion as normal corneas suggests that these nerves are fully functional. Currently, the role of CGRP in the cornea is not known. It has been implicated in epithelial differentiation⁵ and in the initiation of acute inflammation as a response to injury.²⁷ The percentage of the total nerve area in the CGRP-positive vehicle-treated group is reduced, suggesting that some of the nerves present are not yet able to express CGRP.

An interesting finding was the strong positive staining of SP in the epithelium after injury. It is known that SP is released in peripheral terminals after activation of nociceptive fibers.²⁸ We found no differences in epithelial staining after treatment with PEDF+DHA. One possibility is that after injury there is a rapid and strong release of SP that is maintained for a long time and does

not correlate with the degree of nerve regeneration. It is also possible that part of SP is synthesized in stimulated epithelial cells. In support of this possibility, it has been reported that inflammation stimulates SP expression.²⁹ We found that human epithelial cells in culture produce a weak expression of SP (data not shown); these data are in agreement with a previous report that detected endogenous SP in human corneal epithelial cells.³⁰ Substance P stimulates epithelial cell proliferation and, in combination with insulinlike growth factor 1, synergistically stimulates epithelial migration and accelerates wound healing.^{5,31}

Electrophysiological studies show that corneal sensory fibers respond selectively to different stimuli, such as temperature, chemical irritants, and mechanical forces.²⁸ These fibers also respond to endogenous mediators released by corneal cells after injury.²⁸ In addition, neurons that innervate the cornea can be classified as thin myelinated A-delta axons and unmyelinated C axons, both of which transmit signals to the trigeminal nerve at different speeds. It will be important in the future to determine if PEDF+DHA treatment promotes functional regeneration of all subtypes of sensory nerves. A recent report shows that patients diagnosed with unilateral herpes simplex virus keratitis have decreased sensitivity to mechanical insult and heat in the affected cornea, suggesting that herpes simplex virus infection affects preferentially neurons that express receptors activated by these stimuli.³²

Some growth factors also have been implicated in corneal nerve regeneration. Regeneration of corneal sub-basal nerves was observed in mice treated with vascular endothelial growth factor, and blockage of vascular endothelial growth factor reduces growth of cultured neurons.³³ Nerve growth factor has been shown to increase corneal sensitivity after LASIK.³⁴ Prior studies in our laboratory show that nerve growth factor plus DHA topical treatment increases corneal nerve regeneration in rabbits after photorefractive keratectomy.²⁴ Based on our observations, however, the response obtained with nerve growth factor plus DHA was not as significant as when PEDF+DHA was used. We do not know the underlying mechanisms by which PEDF+DHA treatment exerts its effect in corneal nerve regeneration, but our working hypothesis involves the synthesis of NPD1 from DHA. Because PEDF is 10 times more potent than any other growth factor tested (including nerve growth factor) in inducing the synthesis of NPD1 from DHA,²³ we speculate that this is the reason why the effect we observed with PEDF was greater. Further studies are needed to support this hypothesis.

Different approaches for treating chronic epithelial defects caused by neurotrophic keratopathy may involve the use of topical preparations from a patient's serum. Autologous serum application has been reported to improve recovery after neurotrophic keratopathy,³⁵ and it is thought that neurotrophic factors present in the serum are responsible for this effect. Substance P and insulinlike growth factor have been used with success in this setting as well.³⁶ Another option is the use of amniotic membrane transplant.^{37,38} Our present data show that PEDF+DHA application can be a new approach for re-

storing functional corneal nerves and stimulating epithelial wound healing in neurotrophic keratitis.

Submitted for Publication: March 4, 2011; final revision received July 7, 2011; accepted July 16, 2011.

Published Online: September 12, 2011. doi:10.1001/archophthalmol.2011.287

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Author Contributions: Dr H. E. P. Bazan 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.

Financial Disclosure: None reported.

Funding/Support: This work was funded by National Institutes of Health National Eye Institute grant R01 EY019465.

Role of the Sponsors: This funding agency had no role in the design and conduct of the study; in the collection, analysis, and interpretation of the data; and in the preparation, review, or approval of the manuscript.

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