VEGF-B selectively regenerates injured peripheral neurons and restores sensory and trophic functions

Victor H. Guaiquil^{1,2}, Zan Pan¹, Natalia Karagianni, Shima Fukuoka³, Gemstonn Alegre, and Mark I. Rosenblatt^{2,4}

Margaret M. Dyson Vision Research Institute, Department of Ophthalmology, Weill Cornell Medical College, New York, NY 10065

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VEGF-B primarily provides neuroprotection and improves survival in CNS-derived neurons. However, its actions on the peripheral nervous system have been less characterized. We examined whether VEGF-B mediates peripheral nerve repair. We found that VEGF-B induced extensive neurite growth and branching in trigeminal ganglia neurons in a manner that required selective activation of transmembrane receptors and was distinct from VEGF-A-induced neuronal growth. VEGF-B-induced neurite elongation required PI3K and Notch signaling. In vivo, VEGF-B is required for normal nerve regeneration: mice lacking VEGF-B showed impaired nerve repair with concomitant impaired trophic function. VEGF-B treatment increased nerve regeneration, sensation recovery, and trophic functions of injured corneal peripheral nerves in VEGF-B-deficient and wild-type animals, without affecting uninjured nerves. These selective effects of VEGF-B on injured nerves and its lack of angiogenic activity makes VEGF-B a suitable therapeutic target to treat nerve injury.

VEGF-B | nerve injury | neuronal growth | cornea

Nerves can be damaged either through trauma or disease. Nerves from the peripheral nervous system (PNS) have significantly greater capacity to regenerate and reinnervate their original targets after injury, compared with nerves from the CNS. The successful regeneration of PNS neurons requires a number of intrinsic and extrinsic factors, as well as a permissive micro-environment for axonal regrowth (1). Among the numerous growth factors able to induce nerve regeneration, the family of VEGFs has been implicated as a potent mediator of developmental neurogenesis and adult nerve regeneration (2–4). VEGF-A is a well-characterized and potent angiogenic factor but is also a strong inducer of nerve growth. Several studies have demonstrated that both VEGF-A and -B are expressed during peripheral nerve injury (2, 5). In the setting of injury, VEGF-B plays a role in cell survival, nerve protection, and growth (5, 6). The survival effect of VEGF-B on brain cortical neurons, retinal neurons, and motor neurons in the spinal cord is indicative of its pleiotropic role (5). VEGF-B treatment reduced stroke volume in a middle cerebral artery ligation model and increased survival of retinal ganglion cells in an optic nerve crush injury model (7), and VEGF-B knockout mice suffered severe strokes and exacerbated retinal ganglion cell death in both injury models (7–9). VEGF-B has also been used with promising results in Parkinson's disease (10) and amyotrophic lateral sclerosis models (11).

Given the ability of VEGF-B to regulate both vascular endothelial cells (angiogenesis) as well as axonal growth and survival after injury, it is unclear whether VEGF-B exerts its effects on nerve regeneration through the increase in blood supply or through direct effects on nerve tissue. Indeed, specific studies on its role on peripheral neurons independent of its vascular role are lacking. We have previously reported that VEGF-A can stimulate trigeminal neuronal cell growth and enhance cornea nerve regeneration, resulting in anatomical and functional recovery of peripheral injured nerves independently of its angiogenic effects (12). Here we studied the neuro-regenerative potential of VEGF-B in an avascular model of peripheral nerve injury in mice and the signaling elements involved in the induction of nerve growth. Our results demonstrated that (i) peripheral nerve regeneration is

impaired in mice lacking VEGF-B, (ii) VEGF-B can restore the anatomic and function innervation of target tissues by induction of nerve growth and nerve regeneration, (iii) the effects of VEGF-B are specific for injured nerves and are independent of any vascular effect, and (iv) the effects of VEGF-B on nerve regeneration are distinct from those observed for VEGF-A.

Results

VEGF-B Promotes Trigeminal Ganglion Neuron Growth and Survival in Vitro. Peripheral nerves such as those that innervate the cornea have their origin far from the site they innervate at the trigeminal ganglia (TG). To evaluate the role of VEGF-B on promoting peripheral nerve growth we treated isolated TG neuronal cells with different concentrations of VEGF-B and found that strong formation and elongation of neurites is induced when cells were incubated with 50 ng/mL VEGF-B; at this concentration the length of neurites is almost fourfold greater than in untreated controls (Fig. 1*A, B*, and *G*). The effect of VEGF-B on inducing neurite growth is dose dependent, as shown in Fig. S1. The VEGF-B signaling leading to neurite growth was inhibited by soluble VEGF receptor 1 (VEGFR1) (sFlt1) (Fig. 1*C*), antibodies against VEGFR1 (Fig. 1*D*), or neuropilin 1 (NRP1) (Fig. 1*F*), but not by antibodies against VEGFR2 (Fig. 1*E*). VEGFR1 and NRP1 are

Significance

Peripheral nerve injury is a major neurological disorder that can cause multiple motor and sensory disturbances. In this study we found that VEGF-B can be used as a previously unidentified therapeutic for treating peripheral nerve injury. We demonstrated that VEGF-B stimulated nerve regeneration and enhanced the recovery of both tissue sensation and the ability of nerves to enhance healing of innervated tissue. The physiologic relevance of VEGF-B is demonstrated by our findings showing that mice lacking VEGF-B have impaired nerve regeneration and that nerve injury resulted in increased endogenous expression of VEGF-B. We discover that VEGF-B induces strong elongation and branching of neurons and requires specific transmembrane receptors as well as activation of a complex intracellular signaling.

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Conflict of interest statement: V.H.G., Z.P., N.K., and M.I.R. have disclosed intellectual property related to the use of vascular endothelial growth factor in peripheral nerve repair.

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¹V.H.G. and Z.P. contributed equally to this work.

²Present address: Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, Chicago, IL 60612.

³Present address: Department of Ophthalmology, Tokyo Kyosai Hospital, Tokyo 153-8934, Japan.

⁴To whom correspondence should be addressed. Email: mrosenbl@uic.edu.

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Fig. 1. VEGF-B-induced neurite growth and survival on TG neurons via VEGFR1 and NRP1. Neurons from TG of thy1-YFP mice were isolated and cultured as indicated in *Methods*. (A) After 3 d in culture, few neurites grew in neurons cultured only in medium. (B) Addition of VEGF-B immediately after plating the cells induced long and extensive branching of neurites. (C) The neurite growth was competitively inhibited by soluble VEGFR1 at an equimolar dose or (D) by antibodies against VEGFR1 or (F) neuropilin 1, (E) but not by antibodies against VEGFR2. (G) Quantification analysis of neurite length demonstrated that VEGF-B-induced growth requires activation of VEGFR1 and NRP1. (H, *Inset*) The effect of VEGF-B on neuronal survival, analyzed as described in *Methods*, showed that there was a time-dependent cell death of TG neurons after 3 d in culture, and only 25% of neurons were found viable at this time point. (H) However, neuronal viability increased to 50% when cells were treated with a single dose of 50 ng/mL of VEGF-B. Data represent the mean $\pm$ SEM of three independent experiments, and a P value < 0.05 was considered statistically significant between treatments. (Scale bar, 50 μ m.)

expressed in TG neurons (Fig. S2), and quantification of the VEGF-B-induced neurite length demonstrated that both VEGFR1 and NRP1 are required for nerve growth and can be inhibited in a dose-dependent manner by specific antibodies (Fig. 1G and Fig. S3). However, VEGF-B action does not require VEGFR2 activation because inhibition of the receptor via antibodies or blocking its tyrosine kinase domains with the specific inhibitor SU 1498 and Ki 8751 does not block the VEGF-B-induced neurite growth (Fig. 1G and Fig. S4). TG neurons cultured without serum died in a time-dependent manner, and only 25% of cells survived after 3 d in culture (Fig. 1H, *Inset*). However, addition of 50 ng/mL VEGF-B as a single dose during the first day of culture was protective against neuronal death, with 50% of cells surviving at days 2 and 3 (Fig. 1H). Thus, our results show that VEGF-B not only promotes neurite growth but is also neuroprotective in vitro.

VEGF-B Induced Extensive Neurite Elongation and Branching. The effect of VEGF-B on neurite growth was significantly different from that observed previously for VEGF-A (12). VEGF-B induced extensive elongation and branching of the neurites, whereas VEGF-A induces mainly neurite elongation, with few branching nodes (Fig. 2A). The VEGF-B-induced neurite elongation was fivefold higher than in untreated controls and more than twofold higher than that induced by VEGF-A treatment (Fig. 2B). Additionally, VEGF-B significantly induced neurite branching in TG neurons: the number of branching

nodes was sevenfold higher than in untreated controls and more than fourfold higher than in VEGF-A-treated neurons (Fig. 2B).

To further investigate the mechanisms by which VEGF-B-stimulated nerve growth differs from that of VEGF-A, we examined the changes in the transcriptome of isolated trigeminal neurons. The gene expression profile in response to the application of VEGF-A or -B showed two- to eightfold differences (90% confidence) on approximately 200 genes (Fig. S5A), and most of these genes were up-regulated compared with untreated TG neurons (Fig. S5B). We found that VEGF-A induced higher expression of VEGF receptors 1, 2, and 3 compared with untreated or VEGF-B-treated neurons, and both VEGF-A and -B induced higher expression of NRP1 compared with controls (Fig. S6A). Although the expression levels of common signaling elements implicated in the VEGF pathway were similar (Fig. S6B), we found significant changes in gene expression in important mediators of the Notch, Wnt, and Semaphorin/Plexin signaling pathways, as well as genes involved in cellular sprouting and neuroprotection (Fig. S7). Target validation using a γ secretase inhibitor demonstrated that activation of the Notch pathway is required for VEGF-induced neurite elongation (Fig. S8), demonstrating the complexity of VEGF-induced neuronal growth signaling.

The PI3K-Akt pathway has been implicated in VEGF signaling (13, 14). To identify potential mechanisms for VEGF-B activity on peripheral nerves, we used a specific PI3K inhibitor and demonstrated that the PI3K pathway is required for VEGF-induced neurite elongation (Fig. S9). We examined whether other ligands that activate this pathway could induce neuronal growth. We treated TG neurons with placenta growth factor 2 (PlGF2) (which binds to VEGFR1) and heparin binding-EGF (HB-EGF) (which activates Akt) and analyzed the induction of neurite growth after 4 d incubation. Both ligands induced potent neurite growth (Fig. S10). However, the neurons were less responsive to PlGF2 or HB-EGF than when treated with VEGF-B.

Peripheral Nerve Injury Induces Endogenous VEGF-B Expression.

Previous studies have indicated that VEGF-B plays a minimal role under physiologic conditions but may have more profound functions in disease (2, 11). We aimed to determine whether the expression levels of VEGF-B change in sensory nerve innervated

Fig. 2. VEGF-B induces greater neurite elongation and branching than VEGF-A. Isolated trigeminal neurons were treated with either VEGF-A or VEGF-B at 50 ng/mL and neurite growth analyzed after 3 d in culture using NeuroLucida software. (A) VEGF-A induced neurite growth with limited branching, whereas VEGF-B induced more extensive elongation and branching. (B) Quantification of the total neurite length demonstrated that VEGF-B induces strong growth with a greater branching area than VEGF-A. Data represent the mean $\pm$ SEM of five independent experiments. $*P \leq 0.01$. (Scale bar, 50 μ m.)

tissues after peripheral nerve injury. We used a model of PNS injury in which the subbasal corneal epithelial plexus is removed via epithelial debridement to damage the superficial dense nerve endings. C57BL/6 wild-type mice were subjected to corneal epithelial debridement, and epithelial and stromal cells were collected immediately after the procedure and at 1, 3, and 7 d later to determine protein expression. VEGF-B protein levels analyzed by ELISA showed a concomitant time-dependent increase, with highest amounts of VEGF-B on day 7. After 1 wk the VEGF-B protein expression was sixfold higher in epithelium and threefold higher in the stroma compared with levels observed at the time of debridement (Fig. 3*A*). These data were corroborated by Western blot that also showed a significant increase of VEGF-B in both epithelium and stroma after 1 wk of cornea injury (Fig. 3*B*).

Impaired Nerve Regeneration in *Vegf-b*^{-/-} Mice Is Ameliorated by VEGF-B Treatment. Because peripheral nerve injury induces increasing expression of VEGF-B we aimed to determine if VEGF-B is required for nerve regeneration. *Vegf-b*^{-/-} mice have no evident neurological deficit and are developmentally healthy (5, 11). First, we analyzed by immunostaining the corneal nerve density and distribution in *Vegf-b*^{-/-} and wild-type mice and found no differences when both strains were compared (Fig. 4*A* and *B, Top*). We then used the PNS corneal injury model described above to remove via epithelial debridement the trigeminal nerve endings, and after 1 wk we measured the rate of nerve regeneration back into this damaged area. Greater numbers of growing nerve endings into the damaged area were seen in wild-type mice compared with *Vegf-b*^{-/-}, especially in the peripheral cornea that presented a smaller area without nerve growth (yellow dotted line). We found that *Vegf-b*^{-/-} mice showed significantly impaired nerve regrowth into the injured zone both at the central and peripheral areas of the cornea (Fig. 4*A* and *B, Middle*). We then examined the ability of exogenous VEGF-B to rescue the impaired corneal nerve regeneration seen in *Vegf-b*^{-/-} by injecting a single bolus of recombinant VEGF-B in the adjacent subconjunctival space immediately after the time of epithelial debridement. By 1 wk after injury the exogenous VEGF-B induced greater nerve regeneration in both wild-type and *Vegf-b*^{-/-} mice compared with untreated mice (Fig. 4*A* and *B, Bottom*). The quantification of the newly regenerated nerves growing into the injured zone demonstrated that the application of VEGF-B to wild-type mice enhanced nerve regeneration in both

Fig. 3. Endogenous VEGF-B expression increases after peripheral nerve injury. The peripheral nerve endings that innervate the cornea were injured in C57BL/6 mice by central corneal epithelial debridement. Epithelial and stroma samples were collected at the time of debridement or after 1, 3, and 7 d. (A) The ELISA data showed that the VEGF-B protein levels steadily increased in both epithelium and stroma during all days analyzed, with higher level observed in the epithelium at day 7. (B) Western blot analysis from these samples corroborated that the levels of VEGF-B significantly increased after day 7 postinjury in both the epithelium and stroma. Data are expressed as mean ± SEM of two independent experiments (n = 6). *P < 0.05 vs. control epithelium at day 0, **vs. control stroma at day 0.

Fig. 4. VEGF-B is required for anatomical and functional nerve regeneration. C57BL/6 and *Vegf-b*^{-/-} mice were left untreated or subjected to corneal epithelial debridement. Mice were injected subconjunctivally with 0.5 ng of recombinant mouse (rm) VEGF-B or an equal volume of 0.1% BSA as control. After 1 wk corneas were harvested, flat mounted, and stained with β3-tubulin antibody. Composite images were obtained using an automated microscope stage and Axiovision software (Mosaic module, Zeiss). Nerve density was obtained by analyzing composite images of the central and peripheral cornea using Neurolucida software. (A and B, Top) Normal central and peripheral nerve density and distribution was observed in both wild-type and *Vegf-b*^{-/-} untreated mice. (B, Middle) In the debrided corneas, the nerves regenerated in the peripheral area in wild-type but not in *Vegf-b*^{-/-} mice, as indicated by the yellow dashed lines that zoned the area without superficial nerves, and (A, Middle) scarce nerve regeneration was seen in the central cornea in both types of mice. (A and B, Bottom) The administration of VEGF-B induced rapid nerve regeneration in the central and peripheral cornea in wild-type mice but only in the peripheral cornea in *Vegf-b*^{-/-} mice. (C and D) Nerve tracing analysis shows that *Vegf-b*^{-/-} mice have a delay in nerve growth compared with wild type, and (C) VEGF-B induced a significant increase in central nerve regeneration only in wild-type mice but (D) significantly increased peripheral nerve regeneration in both types of mice. The functional sensation of the regenerated nerves in the ocular surface was evaluated by the von Frey Hair technique (Methods) in both wild-type and *Vegf-b*^{-/-} mice. (E) After 1 wk postinjury, corneal sensation was similarly decreased in wild-type and *Vegf-b*^{-/-} mice. (F) However, after 2 wk postinjury there was a significant recovery of sensation in mice treated with VEGF-B. In wild-type mice the regenerated nerves reached the same sensation level as normal untreated controls, whereas *Vegf-b*^{-/-} mice demonstrated a delay in recovering complete sensation. Data are expressed as mean ± SEM of three independent experiments (n = 8). *P < 0.05. (Scale bar, 20 μm.)

the central and peripheral corneas, whereas the neuroregenerative effects of VEGF-B on *Vegf-b*^{-/-} mice seemed confined to the peripheral cornea at the 7-d time point (Fig. 4*C* and *D*).

VEGF-B Improves Functional Sensation of Injured Nerves. The cornea is a highly sensitive tissue, and we next investigated whether the

not only restores sensation but also restores the critical trophic function of PNS sensory nerves.

Taken together, our data demonstrate that VEGF-B is required for proper nerve regeneration in the cornea. The increased endogenous VEGF-B expression in response to injury or the application of exogenous VEGF-B promotes nerve regeneration without affecting undamaged nerves or inducing unwanted neovascularization. VEGF-B-mediated nerve regeneration restored the sensation and healing responses of PNS innervated tissues. The relative inactivity of VEGF-B on intact neurons, its lack of significant angiogenic activity, and its potent action in restoring PNS function to damaged tissues make VEGF-B an important therapeutic target for treating injured peripheral nerves.

Methods

Animals. All experiments were performed according to the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision. C57BL/6 and neurofluorescent thy1-YFP mice were purchased from Jackson Laboratories, and the VEGF-B knockout (*Vegf-b*^{-/-}) mice were a generous gift from David A. Greenberg (Buck Institute for Research on Aging, Novato, CA).

TG Neuronal Growth Assay. TG neuronal cells were obtained and cultured as described previously (12). To evaluate the effect of VEGF-B on neuronal growth, TG neuronal cells were treated with 10–100 ng/mL recombinant mouse VEGF-B¹⁸⁶ (R&D 767-VE-010). The VEGF-B treatments were replenished every other day. Neurite formation and growth were followed up to 3 d. Inhibitions studies to determine VEGF-B–receptor interactions were carried out as follow: VEGF-B availability was competitively inhibited by treating the cells at the same time with 50 ng/mL VEGF-B and an equal molar concentration (2.6 nM) of recombinant human-soluble VEGFR1 (sVEGFR1/sFlt1, CellSciences CRF103). VEGF receptors were inhibited by using neutralizing antibodies against VEGFR1 (R&D AF471), VEGFR2 (R&D MAB4431), and NRP1 (R&D MAB566). TG cells were incubated in a dose-dependent manner with anti-VEGFR1, anti-VEGFR2, or anti-NRP1 for 1 h before the addition of 50 ng/mL VEGF-B. The tyrosine kinase domains of VEGFR2 were inhibited with specific inhibitors by incubating TG neuronal cells with 10 μ M SU 1498 (Sigma T4192) or 5 nM Ki 8751 (Sigma K1265) for 1 h before adding 50 ng/mL VEGF-B.

Corneal Micropocket Assay. A sucralfate/hydron pellet impregnated with VEGF-B (100 ng per pellet) or vehicle was implanted into a corneal micropocket in anesthetized thy1-YFP mice subjected to a partial lamellar incision parallel to the limbus (32). Mice were allowed to recover for 1 d, after which a subset received corneal debridement that removed a circular 2-mm region of central epithelium and the underlying subbasal nerves. The corneas were harvested 3 d later and processed as previously described (12).

Subconjunctival Injection of VEGF. Wild-type C57BL/6 and *Vegf-b*^{-/-} mice were subject to corneal epithelial debridement as described above or left intact. A 5- μ L volume containing 0.5 ng VEGF-B¹⁸⁶ in PBS was injected subconjunctivally, at the lateral temporal bulbar conjunctiva, using a 10- μ L microsyringe (Hamilton Co.). An equal volume of 0.1% BSA was used in control mice. The recovery of the corneal epithelial injury was imaged at 24, 40, and 48 h by applying 1% fluorescein solution to the corneal surface and wound closure evaluated as previously described (12). Corneas were harvested after 1 wk for immunodetection of nerves. Possible growth of blood vessel into the cornea was monitored by slit lamp examination.

Corneal Whole Mount and Immunofluorescence. Harvested corneas from thy1-YFP mice were fixed in 4% (vol/vol) paraformaldehyde (PFA) for 1 h and then stained with DAPI to label nuclei before mounting. The YFP fluorescent nerves were imaged with a fluorescence microscope at the site of pellet implantation and at a site 180° opposite to the pellet. Images were analyzed using Neurolucida. C57BL/6 wild-type and *Vegf-b*^{-/-} mouse corneas were flat mounted and fixed as previously described (12). Samples were stained with rabbit anti- β 3 tubulin antibody (Abcam 18207) overnight at 4 °C, followed by Alexa Fluor 568-conjugated goat anti-rabbit IgG (Life Technologies A11011) at room temperature for 1 h and counterstained with DAPI.

Statistics. Data are presented as mean $\pm$ SEM. The significance of differences was evaluated using ANOVA followed by post hoc test for multiple comparisons, with $P < 0.05$ considered statistically significant unless otherwise stated.

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