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Article

Angiogenesis

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Neurotrophins promote revascularization by local recruitment of TrkB⁺ endothelial cells and systemic mobilization of hematopoietic progenitors

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The neurotrophin brain-derived neurotrophic factor (BDNF) is required for the maintenance of cardiac vessel wall stability during embryonic development through direct angiogenic actions on endothelial cells expressing the tropomyosin receptor kinase B (TrkB). However, the role of BDNF and a related neurotrophin ligand, neurotrophin-4 (NT-4), in the regulation of revascularization of the adult tissues is unknown. To study the potential angiogenic capacity of BDNF in mediating the neovascularization of ischemic and non-ischemic adult mouse tissues, we utilized a hindlimb ischemia and a subcutaneous Matrigel model. Recruitment of endothelial cells and promotion of channel formation within the Matrigel plug by BDNF and NT-4 was comparable to that induced by VEGF-A. The introduction of BDNF into non-ischemic ears or ischemic limbs induced neoangiogenesis, with a 2-fold increase in the capillary density. Remarkably, treatment with BDNF progressively increased blood flow in the ischemic limb over 21 days, similar to treatment with VEGF-A. The mechanism by which BDNF enhances capillary formation is mediated in part through local activation of the TrkB receptor and also by recruitment of Sca-1⁺CD11b⁺ pro-angiogenic hematopoietic cells. BDNF induces a potent direct chemokinetic action on subsets of marrow-derived Sca-1⁺ hematopoietic cells co-expressing TrkB. These studies suggest that local regional delivery of BDNF may provide a novel mechanism for inducing neoangiogenesis through both direct actions on local TrkB-expressing endothelial cells in skeletal muscle and recruitment of specific subsets of TrkB⁺ bone marrow-derived hematopoietic cells to provide peri-endothelial support for the newly formed vessels.

Introduction

Numerous angiogenic factors, including VEGF-A, FGFs, and PDGFs, have been shown to induce neoangiogenesis in adult ischemic tissues. However, in most pre-clinical and clinical studies, the introduction of these factors as single agents has resulted in the formation of stabilized blood vessels for only a limited duration (1–3). Direct introduction of VEGF-A, FGF-1, or FGF-2 demonstrated some benefit in restoring vascularization of ischemic myocardium or limbs, but these effects were found to diminish over time. These observations have raised the possibility that long-lasting vessel stability may be mediated through the recruitment of other pro-angiogenic signaling pathways and that the delivery of these factors in combination may be essential for the assembly of long-lasting blood vessels. Indeed, it has been recently shown that delivery of a combination of FGF-2 and PDGF-BB can induce the formation of long-lasting blood vessels (4).

There is evidence that hematopoietic cells contribute to the revascularization of the ischemic or regenerating tissues by

releasing angiogenic factors, thereby supporting the assembly of new vessels (5–16). Several studies have demonstrated that the introduction of cytokines, including VEGF-A, can enhance co-mobilization of the endothelial progenitors and pro-angiogenic hematopoietic cells to ischemic limbs to promote the re-endothelialization process (5, 17). Although the contribution of endothelial progenitors to ischemic revascularization is less well defined (18, 19), several reports have shown that hematopoietic cells, mostly of the monocytic lineage, are recruited to the neoangiogenic niche and support revascularization by releasing angiogenic factors, including VEGF-A and metalloproteinases (20–23). In addition, accumulating evidence suggests that neuronal factors known for their roles in mediating axonal path finding, such as semaphorins, ephrins, netrins, and their receptors (24, 25), also play critical roles as angiogenic regulators.

Our laboratory has demonstrated that brain-derived neurotrophic factor (BDNF), through interaction with the BDNF receptor tyrosine kinase tropomyosin receptor kinase B (TrkB), can promote angiogenesis in the developing embryonic myocardium (26). Because BDNF and TrkB are selectively expressed by vessels in skeletal muscle and heart, we hypothesized that the BDNF/TrkB signaling pathway may also modulate angiogenesis in specific adult organs.

BDNF as well as the alternate TrkB ligand neurotrophin-4 (NT-4) are trophic factors best known for their differentiative and survival

Nonstandard abbreviations used: AdBDNF, recombinant adenovirus encoding rat BDNF together with the reporter protein GFP; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; NT-4, neurotrophin-4; PFA, paraformaldehyde; TrkB, tropomyosin receptor kinase B.

Conflict of interest: The authors have declared that no conflict of interest exists.

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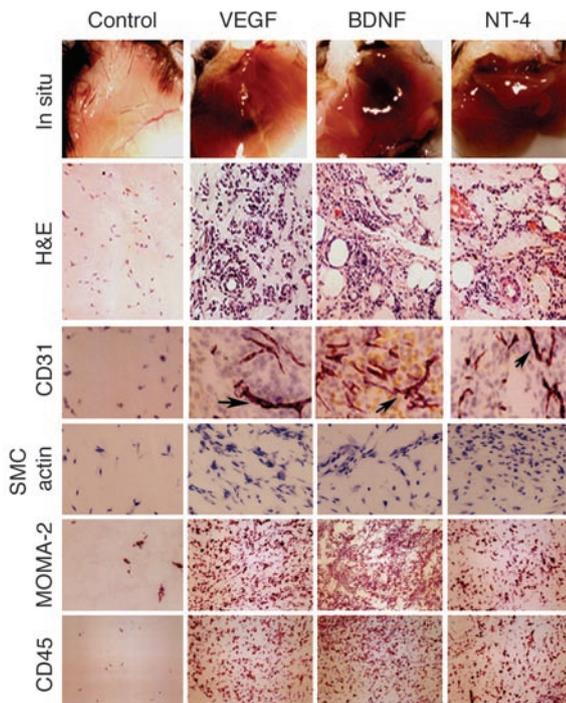


Figure 1

BDNF induces neoangiogenesis in an in vivo Matrigel assay. Young adult female mice were injected subcutaneously with 0.3 ml of growth factor-depleted Matrigel containing 64 U/ml heparin and recombinant human VEGF165 (30–50 ng/ml), recombinant human BDNF (50–100 ng/ml), NT-4 (50–100 ng/ml), or no growth factor addition (Control). After 14 days, the animals were sacrificed and the Matrigel plug was photographed (In situ) and harvested. Serial sections for each Matrigel plug were analyzed by investigators “blinded” to sample identity, and the degree of cellularity was quantified in central regions of the Matrigel (H&E). Immunoreactivity for CD31, smooth muscle α -actin (SMC actin), MOMA-2, and CD45 was assessed, followed by hematoxylin counterstaining. Matrigel plugs containing BDNF, NT-4, or VEGF-A showed an increase in the number of infiltrating endothelial-like cells arborizing (arrowheads) throughout the tissue compared with the control samples. Magnification, $\times 40$ (CD31) or $\times 20$ (H&E, smooth muscle α -actin, MOMA-2 and CD45).

action on neurons expressing the TrkB receptor tyrosine kinase. However, deficient expression of BDNF impairs the survival of TrkB-expressing endothelial cells in intramyocardial arteries and capillaries in the late gestational and early postnatal period, although the embryonic vasculature of the heart forms and can remodel into arteries, capillaries, and veins (26). Vascular hemorrhage in neonatal *BDNF*^{-/-} mice is restricted to cardiac vessels, probably reflecting the localized expression of BDNF and TrkB by mid-gestational capillaries and arterioles in cardiac and skeletal muscle. BDNF deficiency results in a reduction in endothelial cell-cell contacts and in endothelial cell apoptosis. Hemorrhage within the ventricular walls leads to hypocontractility of the heart and contributes to the perinatal death of BDNF-deficient animals. Conversely, BDNF overexpression in the mid-gestational mouse heart results in an increase in capillary density, establishing the essential role of BDNF in modulating cardiac microvascular endothelial cells during development (26).

The goal of the present study was to identify potential angiogenic pathways that are activated by BDNF in non-ischemic and ischemic tissues of adult mice. Here, we demonstrate that BDNF/TrkB functions as an alternate angiogenic pathway to modulate new vessel formation. The effects of recombinant protein or gene delivery were quantified for the induction of vascular channels and vessel morphology and were correlated with expression of the BDNF receptor TrkB on resident and migrating endothelial cells. We show that BDNF supports neoangiogenesis in part through direct effects on local TrkB-expressing endothelial cells in skeletal muscle and also by mobilization of pro-angiogenic Sca-1⁺CD11b⁺ hematopoietic cells. Thus, delivery of BDNF may provide a novel strategy to enhance organ-specific revascularization of ischemic tissues.

Results

BDNF and NT-4 promote neoangiogenesis in Matrigel plugs in vivo. Our prior studies indicated that BDNF overexpression in the gesta-

tional heart results in increased vessel density, with augmentation in capillary density but no increase in the number of vessels with smooth muscle cell ensheathment (26). To evaluate whether BDNF or the alternative TrkB ligand NT-4 could promote angiogenic activities in adult mice, we used the in vivo Matrigel implant model to assess angiogenesis in response to specific factors in a non-inflammatory setting. Mice were injected subcutaneously anterior to the abdominal rectus sheath with Matrigel alone or with Matrigel containing recombinant VEGF-A, BDNF, or NT-4 protein. Gross analysis of the Matrigel plugs in situ after 14 days showed bland control plugs, whereas those with incorporated VEGF-A, BDNF, or NT-4 were ruddy and vascular in appearance (Figure 1). Histochemical analysis of the Matrigel plugs revealed markedly increased cellularity induced by VEGF-A, BDNF, or NT-4, whereas control Matrigel plugs exhibited cellularity of less than 10% (Figure 1; quantification is presented in Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI200522655SDS1). Compared with control Matrigel, Matrigel containing VEGF-A, BDNF, or NT-4 showed extensive neovascularization with increased numbers of vascular channels containing erythrocytes (Figure 1). These vascular channels were confirmed to be lined by endothelial cells, as assessed by CD31 and von Willebrand factor immunoreactivity (Figure 1 and data not shown). However, few channels exhibited ensheathment by vascular smooth muscle cells, as assessed by smooth muscle α -actin immunoreactivity (Figure 1). Matrigel plugs containing VEGF-A at a concentration of 30 or 50 ng/ml yielded cellularity and vascular channel formation comparable to that observed with 50–100 ng/ml of BDNF or NT-4 (data not shown).

In order to further assess the cell types recruited to the center of the Matrigel plug, we performed immunohistochemistry using antibodies specific for leukocytes (CD45), macrophages/monocytes (MOMA-2), and lymphatic vessels (VEGFR-3). VEGF-A-, BDNF-, or NT-4-loaded Matrigel plugs were infiltrated with leukocytes and macrophages/monocytes expressing CD45 and MOMA-2 (Figure 1). Little immunoreactivity for VEGFR-3 was noted (data not shown), suggesting that BDNF and NT-4 do not recruit lymphatic vessels. A significant number of cells recruited to the center of BDNF Matrigel were TrkB positive cells (Supplemental Figure 1B).

BDNF fails to induce neoangiogenesis in corneal pocket assay. To test whether BDNF induces angiogenesis in a non-ischemic model, we used the corneal pocket assay, in which recombinant BDNF



or FGF-2 (bFGF) protein was incorporated in a hydon pellet and was implanted in a corneal pocket. Although bFGF induced an increase in corneal vessels (27), BDNF yielded no significant effects after implantation (Supplemental Figure 2A). To evaluate whether this lack of response reflected the absence of TrkB expression by local endothelial cells, we undertook immunohistochemical analysis. Indeed, endothelial cells of the anterior eye did not express TrkB, as assessed by double-label immunofluorescence microscopy (Supplemental Figure 2B). These results suggest that BDNF is ineffective in inducing an angiogenic response in organs in which TrkB expression in endothelial cells is low or absent.

Adenovirus-mediated expression of BDNF. Although the Matrigel model is useful in assessing endothelial cell recruitment and channel formation, the stability of recombinant proteins is limited and the complex process of vessel stabilization is incomplete. We thus utilized adenovirus vector-mediated gene delivery, which allows for the delivery of bioactive proteins for 2–3 weeks in immunocompetent mice. To this end, we generated recombinant adenovirus encoding rat BDNF together with the reporter protein GFP (AdBDNF) or encoding GFP alone (AdGFP) (28). After infection of 293 cells with this adenoviral construct, secreted BDNF protein could be isolated from the media (Figure 2A). To confirm the biological activity of secreted BDNF, we assessed the neuritogenic effect of BDNF on TrkB-expressing PC12 cells. Conditioned medium from 293 cells infected with AdBDNF induced robust neurite outgrowth of TrkB receptor-expressing PC12 cells, whereas no neuritogenesis was detected using conditioned medium from AdGFP-infected cells (Figure 2B).

To confirm that AdBDNF delivered to adult mice led to specific viral uptake and systemic expression of BDNF, we infected immunocompromised mice with AdBDNF or AdGFP by intravenous injection into the tail vein, which results in expression of adenoviral products primarily in the liver with subsequent release into the peripheral circulation (29). BDNF was detectable in the plasma after AdBDNF but not AdGFP injection (Figure 2C). As immunocompromised mice were used to minimize immune-mediated clearance of the recombinant adenovirus, prolonged expression of BDNF was observed and was well tolerated with only modest weight reduction (less than 10% reduction in weight, compared with AdGFP-infected animals), comparable to the effects of continuous administration of recombinant protein in prior studies (30, 31).

Administration of AdBDNF induces angiogenesis in the murine ear. As our previous studies suggested that BDNF/TrkB expression in the vasculature was necessary to induce neoangiogenesis, we postulated that local angiogenic responses to BDNF might depend upon the expression of TrkB by endothelial cells in the area of delivery. Immunostaining of normal ear skin using an antibody specific for TrkB documented punctate staining (Figure 3A), and double-fluorescence immunostaining utilizing antibodies specific for TrkB or CD31 used to detect endothelial cells demonstrated that approximately 5–10% of the dermal vascular channels co-expressed both proteins (Figure 3B).

To assess the effects of local BDNF overexpression, we injected AdBDNF, AdVEGF-A, or AdGFP subcutaneously in the base of the ear. Fourteen days after injection, the ear skin of mice injected with AdBDNF and AdVEGF-A was more ruddy in appearance compared with ears of mice injected with AdGFP. To quantify changes in vascular density, we assessed CD31 immunoreactivity in situ using whole-mount analysis of the ear skin (Figure 3C). Computer-assisted morphometric analysis confirmed that administration

of AdBDNF significantly increased the density of CD31⁺ vessels, as assessed by an increase in mean vessel length per unit volume, by 1.5-fold after 10, 14, or 28 days and a 2-fold increase on day 70 after injection compared with that of the littermates treated with AdGFP ($P < 0.001$) (Figure 3D). This induction in vascular density was comparable to that observed after AdVEGF-A injection.

Previous *in vitro* studies have documented that neurotrophins can induce vascular smooth muscle cell migration of TrkB-expressing smooth muscle cells (32). To determine whether BDNF delivery results in augmented vascular smooth muscle cell ensheathment, we performed double-immunofluorescence microscopy in whole-mount preparations to assess the proportion of vessels that exhibited immunoreactivity for both CD31 and smooth muscle α -actin (Supplemental Figure 4). The proportion of large-diameter, smooth muscle α -actin-positive vessels was comparable in mice treated with AdVEGF-A, AdBDNF, or AdGFP, suggesting that BDNF did not alter smooth muscle cell recruitment and

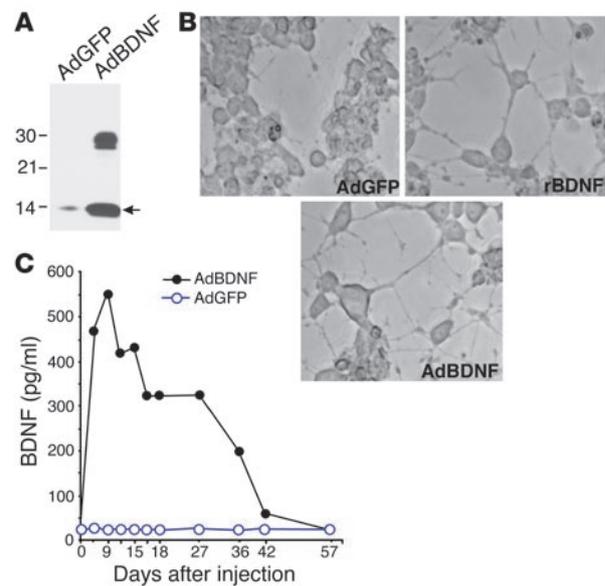


Figure 2

Expression of biologically active BDNF protein after adenovirus-mediated gene delivery. (A) 293 cells were infected with AdBDNF or AdGFP at an MOI of 100, and media was harvested 48 hours after infection. Secretion of BDNF was confirmed by Western blot analysis after protein separation by SDS-PAGE. The 13-kDa immunoreactive band (arrow) is consistent with mature BDNF, and the 29-kDa and 17-kDa species correspond to incompletely processed proforms that are released when the gene is expressed at high levels. (B) The biological activity of BDNF expressed by adenovirus vector was evaluated using PC12 cells stably expressing TrkB. Media from 293 cells infected with AdBDNF, AdGFP, or rBDNF were added to cells, and neuritogenesis (the presence of neurite processes greater than one cell body in length) was assessed 24 hours after treatment using Normasky imaging; neurite outgrowths were visualized with a 20 \times objective. (C) *In vivo* expression of BDNF following injection of AdBDNF in the tail veins was measured by ELISA using a commercially available kit and a recombinant BDNF standard (BDNF E_{max} ImmunoAssay System). The BDNF E_{max} kit has a minimum sensitivity of 7.8 pg/ml. The ELISA for BDNF was carried out on plasma samples of SCID mice injected in the tail vein with 1×10^9 PFU of AdBDNF. Blood was collected every 2–3 days after adenovirus vector administration. Absorbance was measured at 450 nm using a microplate reader and the BDNF concentration was normalized to that of recombinant protein.

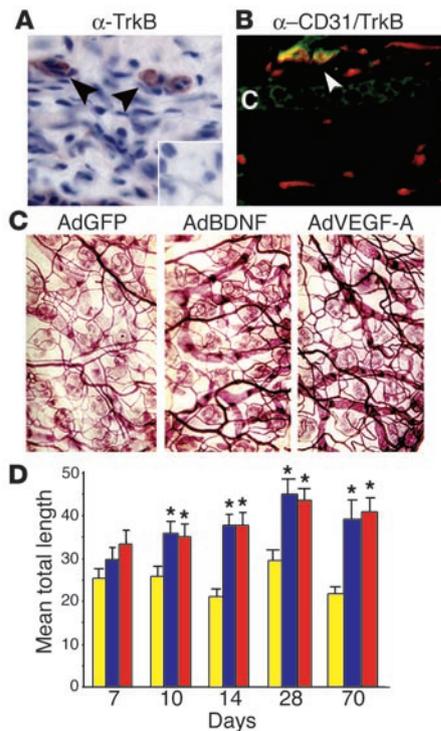


Figure 3

AdBDNF promotes neoangiogenesis in the mouse ear model. (A) Immunohistochemical analysis of expression of TrkB in a section from the base of the ear pinna of a wild-type mouse. The black arrowheads show blood vessels positive for TrkB. α -, anti-. Magnification, $\times 40$. (B) Section from the base of the ear pinna of an untreated mouse was examined for expression of TrkB and CD31 using double-immunofluorescence microscopy. Section was incubated with biotinylated anti-CD31, followed by rhodamine-avidin, and with TrkB antisera, detected with fluorescein-conjugated secondary antibody. The white arrowhead shows the colocalization of CD31 and TrkB. C, cartilage. Magnification, $\times 40$. (C) Whole-mount immunostaining with anti-CD31 of ear skin of mice treated with AdGFP, AdVEGF-A, or AdBDNF ($n = 3/\text{group}$). One week to 10 weeks after injection, ears were removed and the skin was separated from cartilage. Skin was permeabilized with Triton and was incubated with antisera against CD31 for whole-mount immunohistochemistry. VIP-based immunodetection yielded a red reaction product. Magnification, $\times 10$. (D) Quantitative analysis of the total vessel length from 4 peripheral fields of tissues obtained at 7, 10, and 14 days and 4 and 10 weeks after injection was performed by investigators, who were “blinded” to sample identity, using NIH Image. $*P < 0.001$. Yellow, AdGFP; blue, AdVEGF-A; red, AdBDNF.

ensheathment. These results suggest that overexpression of BDNF in the ear significantly increased vessel number and maintained a durable increase in vascular density.

In order to determine whether BDNF mediates its actions via modulation of VEGF-A/VEGFR2 signaling to promote new vessel formation, we also investigated the effect of inhibition of VEGFR-2 by using a neutralizing monoclonal antibody (mAb) to VEGFR-2 in combination with AdBDNF in the ear model. Computer-assisted morphometric analysis confirmed that administration of AdBDNF significantly increased the density of CD31⁺ vessels by a 1.5-fold increase in mean vessel length per unit volume at 14 days after injection compared with that of mice treated with AdGFP. However, inhibition of VEGFR-2 (at day 0 and day 7) or IgG as a control did not impair the increase in mean vessel length elicited by AdBDNF (data not shown). These results suggest that BDNF-mediated blood vessel formation is not dependent on VEGF-A/VEGFR-2 signaling.

BDNF expression is induced in ischemic muscle. To determine whether BDNF expression is induced locally after muscle ischemia, we assessed the expression of endogenous BDNF protein by Western blot analysis and immunohistochemistry of tissue sections. BDNF protein expression was induced at 5 and 7 days after femoral artery ligation in a rat model (Figure 4A). Immunohistochemistry of tissue sections showed an increase in expression of BDNF by blood vessels and myocytes of the ischemic limb at 7 days after ligation (Figure 4B). We also assessed the expression of TrkB receptor in control and ischemic limb. Although the level of protein expression of TrkB was not different in the control compared with the ischemic limb, the phosphorylation level of the TrkB receptor was increased in the ischemic limb (Figure 4C). These data suggest that induction of endogenous BDNF protein and phosphorylation of the TrkB receptor contribute to the revascularization of hypoxic musculature.

BDNF enhances ischemic revascularization after femoral artery ligation. In order to assess the potential angiogenic activity of BDNF in

ischemic conditions, we subjected C57BL/6 mice to femoral artery ligation followed by the administration of AdBDNF, AdVEGF-A, AdNull, or PBS. In initial dose-response studies, AdBDNF (1×10^7 , 1×10^8 , or 1×10^9 PFU) or AdNull, as the negative control, was administered to rats in the ligated skeletal muscle. Hemodynamic data were obtained before (day 0) and after (days 5, 9, 13, 16, and 21) ligation, and results are summarized in Supplemental Figure 5. At day 0 (before ligation), the blood flow ratio (ischemic/normal limb) was 1, and after ligation the flow ratio demonstrated significant ischemia (a ratio of 0.25). At day 5, blood flow to the ischemic limb increased in all the groups due to the expression of endogenous angiogenic growth factors. However, at days 9–21, animals injected with a dose of 1×10^7 or 1×10^8 PFU AdBDNF had improved blood flow (a ratio of 0.6–0.8) compared with that of the group treated with higher doses, in which a delay in the blood flow recovery was noted ($P < 0.05$) (Supplemental Figure 5). These results demonstrated that the blood flow ratio increased in a dose-dependent manner and was significantly higher at the dose of 1×10^8 PFU.

To directly compare the efficacy of BDNF and VEGF-A, we applied the optimal dose to the mouse model of hindlimb ischemia, in which extensive immunohistochemical analysis could be undertaken. Hemodynamic measurements in each group confirmed a statistically significant increase in the blood flow in the group of animals treated with either AdBDNF or AdVEGF-A, compared with that of control groups treated with AdNull or PBS ($P < 0.001$) (Figure 4D). Immunohistochemical analysis of ischemic skeletal muscle after treatment of animals with AdBDNF and AdVEGF-A treatment demonstrated extensive cell infiltration (Figure 4E). Immunohistochemistry using antibodies specific for MOMA-2 or CD45 (Figure 4E) revealed that the majority of these cells represented MOMA-2⁺ monocytes/macrophages. Importantly, a 2-fold increase in CD31⁺ cells was detected in animals receiving AdBDNF or AdVEGF-A compared with that of animals

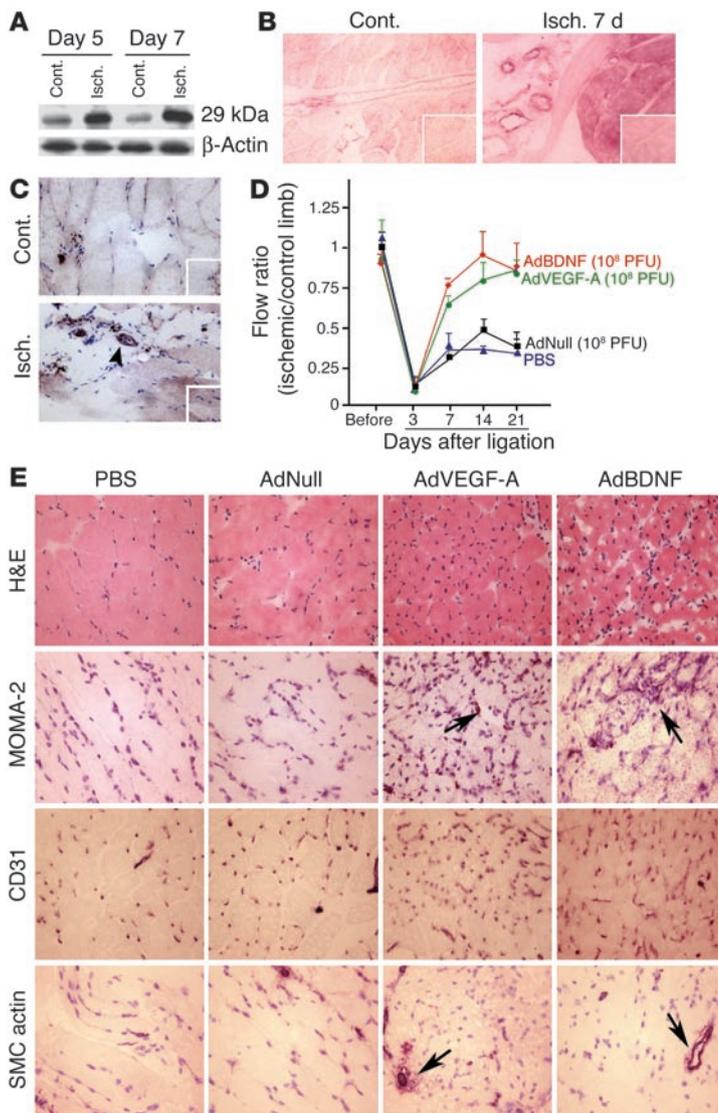


Figure 4

BDNF accelerates the revascularization of the ischemic limbs. (A and B) Western blot analysis of tissue lysates at 5 and 7 days after ligation using polyclonal anti-BDNF (Santa Cruz Biotechnology) (A), followed by a chemiluminescence-based detection, ECL and immunohistochemical detection of tissue sections to assess BDNF expression (B) in ischemic (Isch.) or non-ischemic (Cont.) limb at day 7 after ligation. BDNF expression was detected using frozen sections, anti-BDNF, and a VIP substrate, resulting in a red-purple reaction product. Magnification, $\times 10$. (C) Immunohistochemical detection of phosphorylated TrkB in mouse muscle sections, in ischemic (Isch.) or non-ischemic (Cont.) limb, 21 days after vessel ligation. The boxes in the lower right corners of B and C represent the negative controls. (D) Hindlimb blood flow monitored serially for 3–21 days after ligation in mice receiving 1×10^8 PFU AdBDNF (red), AdVEGF-A (green), AdNull (black), or PBS (blue). Blood flow is calculated as the ratio of flow in the ischemic limb to that in the non-ischemic limb. Values are expressed as the mean \pm SEM for 5 animals per condition. (E) Immunohistochemical analysis, 21 days after ligation, of quadriceps muscle sections from animals receiving the indicated treatment (above columns). Endothelial cells, smooth muscle cells, and monocytes/macrophages were identified using antibodies against CD31, smooth muscle cell α -actin (arrows), and MOMA-2 (arrows), respectively. Magnification, $\times 40$. Sections were analyzed and photographed by investigators “blinded” to sample identity and are representative of tissue obtained from 5 animals evaluated per group.

receiving AdNull (Figure 4E and Supplemental Figure 3). However, immunohistochemical staining demonstrated no difference in the number of cells positive for smooth muscle α -actin (Figure 4E) and analysis of histological sections confirmed that the increase in vessel density reflected changes in small-caliber, rather than large-caliber, smooth muscle cell–ensheathed vessels.

To directly test whether the TrkB receptor mediates the angiogenic actions of BDNF in an ischemic hindlimb in a mouse model, we utilized a genetic approach. Although *TrkB*^{-/-} animals die perinatally, *TrkB*^{+/-} mice on a C57BL/6 background survive to adulthood and have receptor levels that are half of those of the wild-type littermates (33). *TrkB*^{+/-} and *TrkB*^{-/-} littermates were subjected to femoral artery ligation followed by the administration of 1×10^8 PFU of AdBDNF, AdVEGF-A, or AdNull ($n = 3$ in each group). Hemodynamic data were obtained before (day 0) and after (days 6, 9, 13, and 16) ligation (Figure 5A). After ligation, the blood flow ratio decreased significantly, demonstrating severe ischemia. At days 9–16, *TrkB*^{+/-} mice injected with AdVEGF-A or AdBDNF exhibited improved blood flow compared with that of animals injected with AdNull, with results comparable to

those obtained with wild-type C57BL/6 mice (Figure 4D). However, *TrkB*^{+/-} mice injected with AdBDNF exhibited a delay in the recovery of blood flow compared with that of wild-type mice, most notable at days 9 and 13. AdVEGF-A treatment did not produce a significant difference in *TrkB*^{+/-} versus *TrkB*^{-/-} animals, although a trend toward a delay in blood flow at days 6–9 was noted, which may reflect impaired responsiveness to endogenous rises in BDNF (Figure 5A). Immunohistochemical analysis using antibody against CD31 demonstrated a decrease in blood vessel density in *TrkB*^{+/-} mice injected with AdBDNF compared with the respective wild-type animals (Figure 5, B and C). In addition, a decrease in MOMA-2 immunoreactivity was detected in *TrkB*^{+/-} compared with wild-type animals. These results suggest that BDNF is able to promote blood vessel development in ischemic conditions comparable to that elicited by VEGF-A and that this angiogenic action of BDNF is mediated by activation of the TrkB receptor.

AdBDNF induces mobilization of a subset of CD11b⁺ hematopoietic cells. To assess the potential chemotactic activity of BDNF as a means of recruitment of pro-angiogenic bone marrow cells to the peripheral blood and ischemic tissue, we examined potential chemotactic actions of BDNF on bone marrow cells in an in vitro transwell migration assay. BDNF (at 200 ng/ml) induced a 2.5-fold increase in the migration of bone marrow cells from the upper chamber to the lower chamber (Figure 6A). This migratory effect of BDNF was comparable to that of VEGF-A, a previously established potent chemotactic factor for subsets of the hematopoietic cells (17). BDNF was chemotactic for bone marrow–derived cells in vitro, and this effect was dose dependent. A significant number of bone marrow cells that migrated in response to BDNF were MOMA-2⁺ and Sca-1⁺ cells, as characterized by the use of immunofluorescence

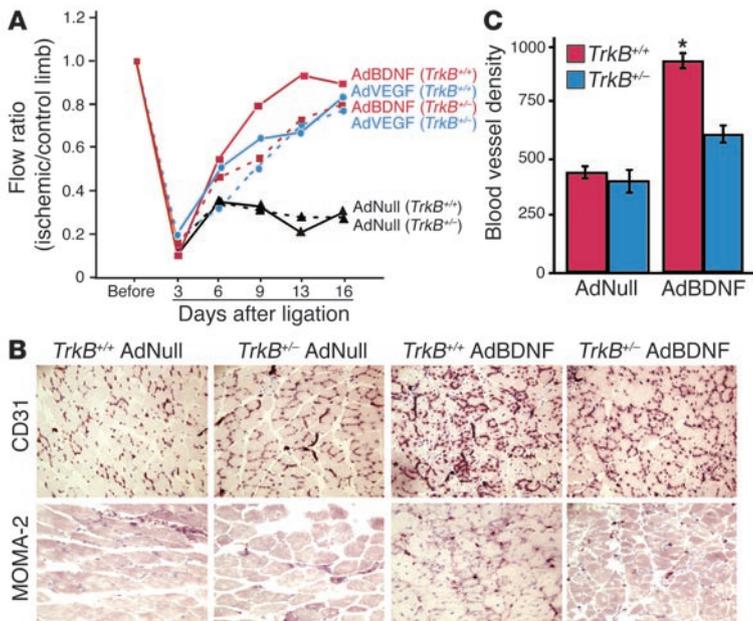


Figure 5

Assessment of the potential role of TrkB receptor in mediating the angiogenic effects of BDNF in the ischemic hindlimb model. (A) Hindlimb blood flow monitored serially in *TrkB*^{+/+} (solid line) and *TrkB*^{-/-} (dashed line) mice receiving 1×10^8 PFU of AdBDNF, AdVEGF-A, or AdNull. Hindlimb blood flow was monitored serially before and after ligation for 3–16 days. An increase of blood flow is noted in wild-type animals treated with AdBDNF or AdVEGF-A. However, a delay in blood flow recovery is detected in *TrkB*^{-/-} mice treated with AdBDNF compared with that of the respective wild-type mice. (B) Immunohistochemical analysis, 21 days after ligation, of quadriceps muscle sections from animals receiving the indicated treatment (above columns). Endothelial cells and monocytes/macrophages were identified using antibodies against CD31 and/or MOMA-2, respectively. Magnification, $\times 40$. Sections were analyzed and photographed by investigators “blinded” to sample identity and are representative of tissue obtained from 3 animals evaluated per group. (C) Quantitative morphometric analysis of the total blood vessel density from 6 sections/condition from tissues obtained at 21 days after ligation, by investigators “blinded” to sample identity, disclosed 2-fold increase in CD31⁺ vessels.

detection of these markers (Figure 6B). These data suggest that BDNF has a chemotactic action on a subset of bone marrow cells.

To evaluate whether BDNF can promote chemotaxis of bone marrow components in vivo, we assessed the mobilization of hematopoietic cells using C57BL/6 mice that were injected intravenously with AdBDNF, AdVEGF-A, or AdNull (1×10^8 PFU for each group), with the last two serving as positive and negative controls, respectively. This approach results in sustained elevation of the cytokines in the peripheral blood for 10 days until immune cells clear adenoviral vectors (34) (Figure 2C), and leukocytes, platelets, and hematopoietic stem cells from peripheral blood were quantified. Six to 11 days after AdBDNF delivery, there was no change in the platelet count in treated animals (data not shown). However, a significant increase in total leukocyte number in peripheral blood was observed on day 6 after treatment in animals injected with AdBDNF (1.6-fold) or AdVEGF-A (1.3-fold) compared with that of the control group treated with AdNull (Figure 7A). The increase in leukocytes was more pronounced on day 11 after treatment, with a 3.4-fold-increase in animals treated with AdBDNF and a 3.3-fold increase in the group treated with AdVEGF-A compared with that of mice injected with AdNull.

To assess whether BDNF mediated mobilization of bone marrow cells into the peripheral blood was mediated indirectly via concomitant induction of VEGF-A, we subjected C57BL/6 mice to tail vein injection of AdBDNF and assessed plasma of injected animals for VEGF-A protein using a murine VEGF enzyme-linked immunosorbent assay (ELISA) kit. Remarkably, no VEGF-A protein in the plasma was detected after AdBDNF administration (<3 pg/ml).

To identify the subset of hematopoietic cells mobilized in the peripheral blood as a result of plasma elevation in BDNF, we assessed their phenotype by flow cytometry. Six days after AdBDNF introduction, a significant increase in the total number of mobilized Sca-1⁺CD11b⁺ hematopoietic cells was detected. By day 11, a 3.1-fold increase in CD11b⁺ cells was noted, suggesting that sustained expression of BDNF resulted in mobilization of more mature hematopoietic cells (Figure 7, B and C). Immunohistochemical analysis demonstrated that a significant

number of mobilized cells expressed TrkB receptor (Figure 6C). We further purified the Sca-1⁺ population from bone marrow of wild-type C57BL/6, and immunocytochemical analysis using antibody against TrkB, demonstrated that a significant number of Sca-1⁺ cell population express TrkB receptors (Figure 6D).

We have also examined the mobilization of VEGFR1⁺ and VEGFR2⁺ cell populations in the peripheral blood of mice injected with AdBDNF, AdVEGF-A, or AdNull. AdVEGF-A induced mobilization of both VEGFR1⁺ and VEGFR2⁺ cells. Compared with control AdNull treatment, AdBDNF delivery induced the mobilization of a significant number of VEGFR2⁺ cells and, to a lesser degree, VEGFR1⁺ cells. However, neutralizing mAb to VEGFR2 did not influence the AdBDNF-mediated mobilization of Sca-1⁺CD11b⁺ cell populations (Figure 7D). These data suggest that BDNF can exert a direct chemotactic action on subsets of bone marrow-derived Sca-1⁺CD11b⁺ cells expressing TrkB, promoting their mobilization from the marrow to the peripheral circulation, where they may finally incorporate into the vasculature in areas of tissue ischemia.

Discussion

Revascularization of adult tissues is a complex process and is modulated by the collaboration of known as well as unrecognized angiogenic factors. In this manuscript, we have provided evidence that BDNF/TrkB acts as a novel inducible ligand/receptor system to promote neovascularization in vivo. BDNF induces vessel sprouting and supports a long-lasting augmentation in vessel integrity and stability. Although best characterized by their survival, differentiative, and synaptic effects on neurons, the neurotrophins are emerging as ligands capable of regulating several components of the vascular bed, exhibiting direct effects upon both endothelial (26) and vascular smooth muscle cells (32, 35).

While most prior studies have examined the effects of neurotrophins or their receptors during vascular development through analysis of gene-targeted animals (26), our studies suggest that delivery of BDNF to adult mice can induce the recruitment of endothelial cells and the formation of rudimentary vascular chan-

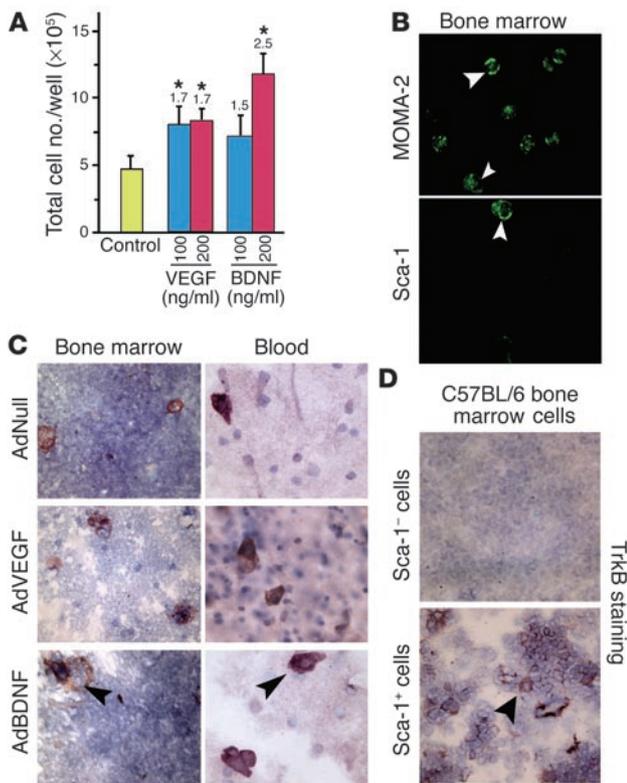


Figure 6

Chemotactic effect of BDNF on bone marrow–derived cells. **(A)** Bone marrow was harvested from femurs and chemotactic assays were performed using Transwell plates. After 6 hours of exposure to 100 or 200 ng/ml of recombinant VEGF-A or BDNF, cells were counted using Trypan Blue exclusion by an observer “blinded” to experimental conditions. * $P < 0.001$. The numbers above the bars indicate the fold increase in migrated cells. This experiment is representative of 2 experiments, with 4 different animals for each condition. **(B)** Immunofluorescence microscopy was performed on migrating cells in the presence of BDNF to detect MOMA-2 and Sca-1 immunoreactivity using fluorescein-avidin detection. MOMA-2⁺ and Sca-1⁺ cells are indicated by white arrowheads. Magnification, $\times 100$. **(C)** Immunohistochemical analysis of TrkB expression by nucleated cells from the bone marrow (left) or peripheral blood (right) of C57BL/6 mice injected in the tail vein with AdNull, AdVEGF-A, or AdBDNF (arrowheads show TrkB⁺ cells). **(D)** Immunohistochemical analysis of TrkB expression in a Sca-1⁺ population isolated from the bone marrow of wild-type C57BL/6 mice. Sca-1⁺TrkB⁺ cells are indicated by the black arrowhead. Magnification, $\times 40$.

nels in the Matrigel model system, as well as promote increased vascular density consisting of both large- and small-diameter vessels, after delivery to the ear skin of adult mice. Localized expression of the BDNF receptor TrkB on a subset of endothelial cells present in the uninjured dermis suggests that BDNF may act directly upon this resident population of cells, although the possibility of recruitment of blood borne vascular precursors to the dermis cannot be excluded. Indeed, the cellular accumulation of the Matrigel plugs in response to BDNF as well as the well studied angiogenic factor VEGF-A is likely to reflect a significant component of blood-borne cells that are capable of differentiating in situ into vascular channels. Our results suggest that both VEGF-A and BDNF are most effective in recruiting endothelial cells, as compared to vascular smooth muscle cells, into the Matrigel matrix, as few vascular channels became ensheathed with vascular smooth muscle cells in response to either ligand. In addition, the morphological characteristics of the increased vascularity of VEGF-A- or BDNF-treated adult dermis is quite similar, with evidence of both large-vessel engorgement and increased density of tortuous small-caliber vessels. These results suggest that in this short-term overexpression system, BDNF exerts its effects mostly on endothelial cells rather than vascular smooth muscle cells.

Unlike the well established effects of VEGF-A on promoting vascular permeability (36, 37), we observed no histological evidence of vascular hemorrhage or perivascular edema after BDNF administration. Indeed, the focal red skin lesions that appeared at 2 weeks after AdVEGF-A administration, similar to those observed upon transgenic delivery of VEGF-A to mouse skin (37), were not detectable in AdBDNF-treated animals. This observation is consistent with our prior observations that BDNF overexpression in the developing heart results in increased cardiac capillary density, with no evidence of vascular hemorrhage, suggesting distinct effects of

VEGF-A and BDNF on interendothelial cell junction formation and stabilization.

BDNF may exert its angiogenic action by 2 distinct mechanisms. Our observation that BDNF can induce new vessels in organs in which a subpopulation of endothelial cells express TrkB (ear skin), but is ineffective in tissues lacking a TrkB endogenous vascular component (corneal), suggests that BDNF can recruit TrkB⁺ endothelial cells to promote the assembly of new vessels. These observations are further strengthened by the observations that BDNF-mediated angiogenesis is delayed in *TrkB*^{-/-} animals compared with wild-type littermates. Administration of AdBDNF into an ischemic limb of the *TrkB*^{-/-} mouse resulted in a delay in blood flow recovery, which is in accordance with a lack of increase in blood vessel density. In addition, BDNF can induce the mobilization and recruitment of TrkB⁺Sca1⁺CD11b⁺ bone marrow progenitors that home to sites of vascular injury and contribute to vessel formation by releasing angiogenic factors. These data suggest that neurotrophic factors may marshal neoangiogenesis, in parallel with neuroprotective effects, to support innervation and blood supply to ischemic limbs.

Overexpression of BDNF protein after tail vein delivery of AdBDNF induces significant recruitment of bone marrow–derived cells. We postulated that local delivery of AdBDNF can promote the recruitment of bone marrow–derived cells to the site of injury. This activity of BDNF is similar to that of the VEGF-A and FGF family of growth factors, which can activate both chemotactic signaling pathways as well as local survival, thereby providing for a full complement of activities necessary for neoangiogenesis. The mechanism by which BDNF promotes the mobilization of TrkB⁺ pro-angiogenic hematopoietic cells from bone marrow is a complex process and may be mediated through direct actions. Direct expression of BDNF in the blood of C57BL/6 mice did not induce upregulation of VEGF-A protein in the plasma of the injected animals. Furthermore, administration of AdBDNF in conjunction with DC101 antibody (a neutralizing antibody against VEGFR-2) did not block the mobilization of Sca-1⁺CD11b⁺ cells induced by overexpression of BDNF. These results together suggest that mobilization of Sca-1⁺CD11b⁺ cells to the peripheral blood is directly mediated through the receptor TrkB. Neurotrophins such as nerve growth factor (NGF), through the activation of TrkA tyrosine kinase, induce the migra-

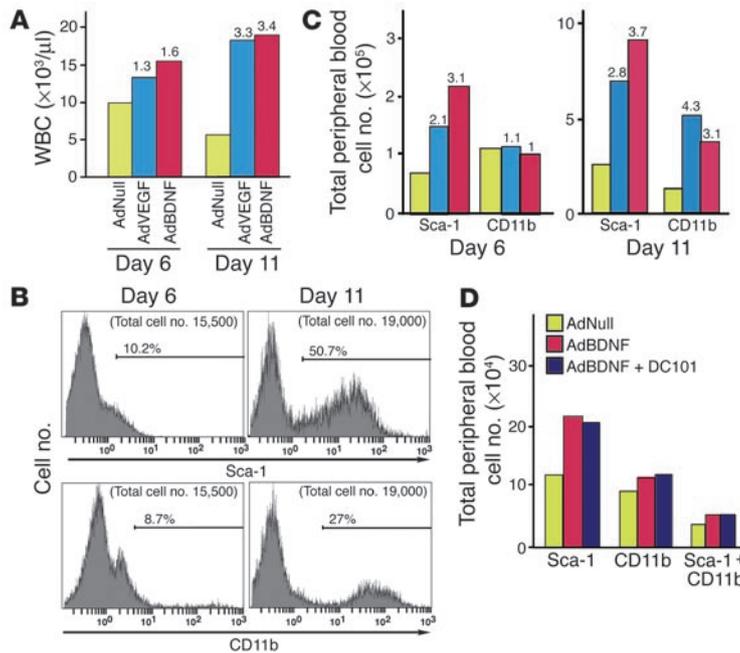


Figure 7 AdBDNF induces mobilization of hematopoietic cells. (A) Total white blood cells (WBCs) in peripheral blood were quantified at 6 or 11 days after the injection of AdNull (yellow), AdVEGF-A (blue), or AdBDNF (red) into the tail veins of C57BL/6 mice. (B) Flow cytometry analysis of peripheral blood from animals treated with AdBDNF. CD11b⁺ and Sca-1⁺ cells are mobilized by AdBDNF. Percentages indicate percentage of positive cells detected by flow cytometry. (C) Flow cytometry analysis detected a 3.1-fold increase for Sca-1 at day 6 and 3.7 and 3.1-fold increases for Sca-1 and CD11b, respectively, 11 days after injection, in animals treated with AdBDNF. The bar graph illustrates the calculated cell number obtained by multiplying the percentage of positive cells, as measured by flow cytometry, by total WBC count. This experiment was done with 2 animals in each group treated. The numbers above the bars indicate the fold increase in WBC count. (D) Flow cytometry analysis of the peripheral blood in animals injected with AdNull (yellow), AdBDNF (red), or AdBDNF in combination with DC101 antibody (blue), 6 days after injection. Treatment with the DC101 antibody had no effect on the mobilization of Sca-1⁺ cells in animals treated with AdBDNF. The bar graph illustrates the calculated cell number obtained by multiplying the percentage of positive cells, as measured by flow cytometry, by total WBC count. This experiment was performed with 3 animals in each group treated.

tion of smooth muscle cells (35) and also induce expression of MMP-9 by vascular smooth muscle cells. BDNF may also activate proteases such as MMP-9 and MMP-2 to promote the mobilization of c-Kit⁺ hematopoietic cells (38). Direct activation of MMP-9 by BDNF may also result in the release of cytokines and chemokines that activate stem cells, including soluble Kit ligand, driving the mobilization of cKit⁺Sca-1⁺ hematopoietic progenitors from the bone marrow (15).

It remains to be determined whether BDNF will promote long-lasting stable vessels. Preliminary analysis suggests that the vessels newly formed after the introduction of BDNF can remain patent for at least 3 months after ischemic revascularization. This stabilization may be mediated mainly through the recruitment of TrkB⁺ hematopoietic cells, as we did not detect any increase in the recruitment of smooth muscle α -actin-immunoreactive pericytes. This result is surprising, as we have previously shown that vascular smooth muscle cells express TrkB. It is possible that the simultaneous upregulation of VEGF-A or angiopoietin-2 after ischemic

injury may deter the recruitment of pericytes until there is complete reversal of the hypoxic microenvironment.

Although all vascular beds are responsive to VEGF-A, due to the expression of low levels of VEGFR-2 on endothelial cells, prior studies have suggested that TrkB receptors are selectively expressed, predominantly by endothelial cells lining the arteries and capillaries of cardiac and skeletal muscle (26), and by vascular smooth muscle cells (32, 35) of large arteries. However, our results indicate that BDNF can induce angiogenic actions in organs in which only a minority of resident endothelial cells are expressing TrkB, suggesting that expansion and recruitment of local endothelial cell populations or chemotactic actions on blood-borne vascular precursors could account for the observed increases in vessel density that persisted for many weeks. Indeed, recent studies examining the angiogenic actions of the related neurotrophin NGF in skeletal muscle suggest that high doses of recombinant protein are capable of promoting vessel growth, although the target cells or receptors responsible for these actions have not been characterized (39–41). Thus, the studies using NGF (39–41) and our results with BDNF provide new evidence for neurotrophins as growth factors with potential angiogenic actions that may be relevant for the treatment of ischemic vascular disease in future studies. In addition, the major benefit of the selective angiogenic activity of BDNF, which is restricted due to its more limited receptor expression and its lack of secondary side effects, including a lack of effect in the induction of vascular permeability, could lead to the development of specific angiogenesis therapy. Collectively, our data provide a platform for studying the role of neurotrophins in ischemic vasculopathies.

Methods

Adenovirus vectors. A replication-deficient recombinant adenovirus vector based on human adenovirus serotype 5 encoding native rat BDNF was used under the control of constitutive cytomegalovirus promoter (28). AdGFP (28) and AdVEGF-A (16) were used as negative and positive controls, respectively. All adenovirus vectors were propagated in 293 cells, purified by CsCl density purification, dialyzed, and stored at -80°C as previously described (28). The titer of each viral stock and absence of replication-competent virus was determined by plaque assay in 293 and A549 cells, respectively. The expression and secretion of BDNF was confirmed by infection of 293 cells for 2 hours at 37°C by AdBDNF or AdGFP at an MOI of 100. Conditioned media were collected at 48 hours, subjected to SDS-PAGE (12.5% polyacrylamide gel), and blotted onto a PVDF membrane (Immobilon P; Millipore Corporation). BDNF protein was detected using polyclonal antibodies against mature human BDNF (Santa Cruz Biotechnology), followed by a chemiluminescence-based detection method (ECL; Amersham Pharmacia Biotech).

BDNF-induced neuritogenesis of PC12 cells expressing TrkB. The bioactivity of BDNF secreted by adenovirus vector-infected cells was assessed by adding the conditioned media of 293 cells infected either with AdBDNF or AdGFP to PC12 cells stably expressing TrkB (generously provided by Pantelis Tsoulfas, University of Miami School of Medicine, Miami, Florida). Neuritogenesis, as



evidenced by the extension of neurite processes greater than 2 cell bodies in length at 24 hours after treatment, was evaluated using Normasky imaging.

BDNF expression in vivo. SCID mice (BALB/c background) were purchased from the Jackson laboratory and were maintained in Thorensten units with filtered, germ-free air. Experimental protocols involving animals were reviewed and approved by the Weill Medical College Institutional Animal Care and Use Committee (IACUC) (New York, New York). BDNF expression after adenoviral delivery to the tail vein was measured in SCID mouse plasma by ELISA using a commercially available kit and recombinant human BDNF as a standard (BDNF E_{max}, ImmunoAssay System; Promega). The BDNF immunoassay has a minimum sensitivity of 7.8 pg/ml and ELISA for BDNF was carried out on plasma samples of SCID mice injected with 1×10^9 PFU of AdBDNF or AdGFP in the tail vein, at 2- to 3-day intervals after adenovirus vector administration.

The ELISA for VEGF-A (Quantikine M-ELISA kit; R&D Systems) was carried out on plasma of C57BL/6 mice injected with 1×10^8 PFU of AdBDNF or AdNull in the tail vein, at 2- to 3-day intervals after adenovirus vector administration. The VEGF immunoassay has a minimum sensitivity of 3 pg/ml. Absorbance was measured at 450 nm using a microplate reader, and VEGF-A or BDNF concentrations were normalized using the recombinant protein as recommended by the manufacturer.

Immunohistochemistry of TrkB and CD31 expression in the skin of adult mice. To detect endothelial cell expression of the TrkB receptor, we harvested ears from euthanized BALB/c mice (Jackson laboratory) and incubated them in 30% sucrose at 4°C prior to cryoprotection in OCT/sucrose (1:1 vol/vol). Cryostat sections (10 µm) were stained with rabbit anti-TrkB (H181 at a dilution of 1:20; Santa Cruz) or were co-stained with this antibody and biotinylated anti-mouse CD31 (MEC13.3, PharMingen). For single staining, sections were treated with 0.1% hydrogen peroxide followed by the primary antibody and signal amplification utilizing the avidin-biotinylated horseradish peroxidase complex method (ABC Vectastain; Vector Labs). For the co-staining experiment, immunoreactivity was detected using FITC-conjugated goat anti-rabbit and rhodamine-conjugated anti-biotin secondary antibodies (Vector Laboratories Inc.), respectively, and was visualized using an immunofluorescence microscope.

In vivo Matrigel studies. Recombinant proteins and heparin sulfate were incorporated in Matrigel (Collaborative Biomedical Products) at the concentrations indicated in Results. Female C57BL/6 mice (6 weeks of age; Jackson Laboratories) were injected subcutaneously above the rectus abdominus with 0.3 ml of Matrigel, either combined with VEGF (murine VEGF-A; R&D Systems Inc.), BDNF, or NT-4 (Promega) or alone as a negative control. Mice were sacrificed after 2 weeks and the Matrigel plugs were dissected. The dissected specimens were divided in half: 1 sample was fixed for 4 h at 4°C in 3% paraformaldehyde (PFA) in PBS, followed by dehydration and paraffin embedding; the other sample was frozen in OCT/sucrose (1:1 vol/vol) and was sectioned using a cryostat.

For histological analysis, paraffin-embedded sections were processed and stained using hematoxylin and eosin (H&E). Ten central sections at least 5 mm from the edge of the Matrigel plug were photographed by investigators “blinded” to sample identity. Cellularity was assessed on a scale of 0–4, with ‘0’ indicating an acellular matrix and ‘4’ representing cellular crowding, and with ‘1’ reflecting 10–20% cellularity, ‘2’ reflecting 30–50% cellularity, and ‘3’ reflecting 60–80% cellularity. Photographs were assigned scores

by 2 individuals “blinded” to sample identity, with mean grading concordant in greater than 80% of the samples and differing by one numerical score in the remainder.

Immunohistochemical analysis was performed on frozen sections with biotinylated antisera against CD31 (MEC 13.3, 5 mg/ml; PharMingen) or smooth muscle α -actin (clone 1A4, 95 ng/ml; Dako). Sections were treated with 0.1% hydrogen peroxide, followed by the primary antibody and signal amplification utilizing the avidin-biotinylated horseradish peroxidase complex method (ABC Vectastain, Vector Labs). Quantification of vessel density was performed on 6 central sections for each sample, using a gridded eyepiece, by investigators “blinded” to sample conditions. Immunohistochemical analysis was performed using antibodies against CD45 (to detect leukocytes; clone 30-F11 at a dilution of 1:50; BD PharMingen), MOMA-2 (to detect monocytes/macrophages; at a dilution of 1:100; Chemicon International), or TrkB receptor (H181 at a dilution of 1:20; Santa Cruz).

Ear model. The angiogenic response to the administration of adenovirus expressing BDNF or VEGF genes was investigated using a mouse non-ischemic model. All protocols and care of animals were performed in accordance with institutional guidelines. Female BALB/c mice (6–8 weeks of age) were purchased from Jackson Laboratory and were injected in the base of the ear on day 0 with 1×10^8 PFU of adenovirus vector expressing human VEGF-A, rat BDNF, or GFP, in a volume of 50 µl. Each group consisted of 3 to 5 animals, and injections were performed bilaterally. Animals were inspected bi-weekly and were sacrificed at 7–70 days after injection.

Vessel density analysis in the whole mount ear skin. Animals were sacrificed at 1, 2, 4, or 10 weeks after injection. Ears were removed and fixed in 3% PFA at 4°C overnight. The skin was separated from the cartilage and was permeabilized with 0.3% Triton X-100 (J.T. Baker Inc.). Ear skin was incubated in 3% hydrogen peroxide to quench endogenous peroxidase, followed by incubation in 3% rat serum for 30 minutes at room temperature. For whole-mount analysis, samples were incubated with antibody against CD31 (MEC 13.3; PharMingen) followed by the VIP Substrate Kit reaction (Vector Laboratories). Samples were dehydrated through 90% and 100% alcohol and were cleared in xylene and mounted with VectaMount (Vector Laboratories). Immunoreactivity staining was visualized using an Axioskop microscope. Immunofluorescence staining for CD31 and smooth muscle α -actin in whole ear mounts was performed as described above. We used monoclonal antibody against mouse CD31 (MEC 13.3; PharMingen) and monoclonal antibody against smooth muscle α -actin (clone 1A4; Zymed Laboratories Inc.). Immunofluorescence staining was detected using FITC-conjugated goat anti-mouse and rhodamine-conjugated anti-biotin secondary antibody (Vector Laboratories) for each antibody, respectively, and was visualized with an Olympus BX51 fluorescent microscope using an UplanFl 20 \times objective. The ear skin was photographed in a minimum of 5 regions at the distal periphery by investigators “blinded” to sample identity. Blood vessels were quantified using Photoshop Image analysis by measurement of the total length of vessels lined by CD31⁺ endothelial cells per µm². All analyses were performed by investigators “blinded” to sample identity.

Corneal pocket assay. The mouse corneal assay was performed according to standard procedure. In brief, hydrop/sucralfate pellets were formulated and were implanted into the avascular



corneas of 6- to 8-week-old male C57BL/6 mice after mice were sedated by anesthesia. Ingrowth of new vessels after 5 days was considered a positive response. The pellets contained 50 ng bFGF (used as a positive control) or 50 ng BDNF (Promega).

Mouse ischemic hind limb model. The potential angiogenic action of BDNF was assessed in a mouse ischemic hindlimb model. All experiments were performed according to protocols approved by the IACUC of Weill Medical College of Cornell University (New York, New York). Eight-week-old male C57BL/6 mice ($n = 5$ per group; Jackson Laboratories) or rats (Sprague Dawley; Taconic) were used. Animals were anesthetized by intraperitoneal injection of a mixture of 0.3 ml ketamine (Fort Dodge Animal Health) and xylazine (Bayer) (10 mg xylazine per kg body weight and 100 mg ketamine per kg body weight), and the abdomen and legs were shaved and sterilized with alcohol. A midline incision through abdominal skin was made and an extraperitoneal dissection laterally along the tissue planes was carried out to expose the femoral artery in the upper part of the left limb. The artery was then ligated both proximally and distally using silk suture (Black Surgalloy, CV-1 Taper 3/8, 9 mm; United States Surgical) and the intervening 6-mm section was excised. The midline incision was then closed using 4-polysorb suture (United States Surgical). Serial Scanning Laser Doppler measurements (Lisca PIM-II Laser Doppler; Perimed) were obtained in fully anesthetized mice pre-operatively and post operatively and every 7 days for a period of 2 weeks. At 21 days, the mice were sacrificed by CO₂ inhalation and tissues were harvested for hindlimb skeletal muscle histology. For dose-response experiments, AdBDNF was injected into the ischemic limb in a rat model at a dose of 1×10^7 , 1×10^8 , or 1×10^9 PFU and with AdNull as the control. Virus was injected at 4 sites (25 μ l per site) into the underlying quadriceps and hamstring in an ischemic hindlimb. Hemodynamic measurements were made as described above. The blood flow ratio represents the ratio of blood flow of ischemic limb to the control limb.

Immunohistochemistry of the skeletal muscle in hindlimb ischemia. After mice or rats were sacrificed, the hindlimb muscles were dissected and frozen in OCT/sucrose (1:1). Histochemical and immunohistochemical analyses were assessed using frozen sections of cryopreserved samples obtained 21 days after injection. Cryosections 10 μ m in thickness were incubated with antibodies against CD31 (for blood vessel density analysis), smooth muscle α -actin (clone 1A4; Dako), or MOMA-2 (for cell type determination; Chemicon International), and the reaction product was developed with VIP. Immunohistochemical analysis was performed with a minimum of 5 regions of the skeletal muscle for 3–5 animals per condition; samples were photographed and images were subjected to NIH Image analysis by investigators “blinded” to experimental conditions.

In vitro chemotactic assay. Bone marrow was harvested from the femurs of C57BL/6 mice by flushing with 1 ml of X-VIVO 20 medium (Bio-Whittaker). Red blood cells were lysed using lysis buffer (Roche Diagnostic Corporation) and cells were counted using Trypan blue. Chemotactic assays were performed using 8- μ m Transwell plates (Costar, Corning Inc.) in which 2×10^6 bone marrow cells were placed in the upper chamber and potential chemotactic agents were added to the lower. A 24-well plate was prepared by transfer of 1 ml of X-VIVO-20 medium to each bottom well containing the cytokines, VEGF-A (R&D Systems Inc), or recombinant human BDNF (Promega) at concentrations of 100 and 200 ng/ml. After 6 hours of incubation at 37°C with

CO₂, cells migrated to the lower chamber and were harvested and counted using Trypan blue. Migrating cells were centrifuged onto superfrost plus slides at 72 g for 8 min. Cytospin specimens were fixed with methanol/acetone (1:1), and immunofluorescence staining for Sca-1 (to detect hematopoietic stem cells; at a dilution 1:100; rat anti-mouse; BD-Biosciences) and MOMA-2 (to detect monocytes/macrophages; at a dilution of 1:100; rat anti-mouse; Chemicon International) was performed. Immunofluorescence staining was detected using biotinylated rabbit anti-rat (mouse absorbed) followed by FITC-conjugated avidin (Vector Laboratories) for each antibody and was visualized with an Olympus BX51 fluorescent microscope using an UplanFl 100 \times objective.

Peripheral blood analysis. Mice were injected with AdBDNF, AdVEGF-A, AdNull, or DC101 antibody (42) (ImClone Systems), and retro-orbital blood was collected, using capillary pipettes (Unopette; Fisher Scientific), 6 or 11 days after injection. Total leukocytes and platelets were counted using a hemacytometer. For immunostaining analysis of bone marrow cells, femurs of C57BL/6 were flushed as described above. The Sca-1⁺ cell population was separated using anti-Sca-1 MicroBeads and magnetic-activated cell sorting with an MS Column (Miltenyi Biotec) and was centrifuged onto Superfrost Plus slides at 72 g for 8 min, using Cytospin 3 (Shandon). Immunostaining for TrkB receptor on Sca-1⁺ cells was performed as described above.

Flow cytometry analysis. Peripheral blood of mice treated with AdBDNF, AdVEGF-A, or AdNull were collected retro-orbitally using a capillary pipette (Fisher Scientific) in presence of heparin (American Pharmaceutical Partner Inc.). Red blood cells were lysed using red blood cell lysis buffer (Roche). Cells were incubated for 30 minutes at 4°C with the following PE-conjugated monoclonal antibodies: anti-mouse CD11b (Immunotech) and anti-mouse Sca-1 (PharMingen). The cells were analyzed by color flow cytometry using a flow cytometer (Coulter Elite flow cytometer) (36). Total blood cells were spun onto Superfrost Plus slides at 72 g for 8 min, using cytospin 3 for immunohistology analysis.

Statistical analysis. All results are expressed as mean \pm SEM. The statistical significance of differences between groups was analyzed by Student's *t* test. Differences were considered significant at a *P* value of less than 0.05, and each experiment was performed at least twice.

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