Transgenic Overexpression of Vascular Endothelial Growth Factor-B Isoforms by Endothelial Cells Potentiates Postnatal Vessel Growth In Vivo and In Vitro

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Abstract—Vascular endothelial growth factors (VEGFs) play significant roles in endothelial growth, survival, and function, and their potential use as therapeutic agents to promote the revascularization of ischemic tissues in being avidly explored. VEGF-A has received most attention, as it is a potent stimulator of vascular growth. Results in clinical trials of VEGF-A as a therapeutic agent have fallen short of high expectations because of serious edematous side effects caused by its activity in promoting vascular permeability. VEGF-B, a related factor, binds some of the VEGF-A receptors but not to VEGF receptor 2, which is implicated in the vascular permeability promoting activity of VEGF-A. Despite little in vitro evidence to date for the ability of Vegf-B to directly promote angiogenesis, recent data indicate that it may promote postnatal vascular growth in mice, suggesting that it may have potential therapeutic application. We have specifically studied the effects of VEGF-B on vascular growth in vivo and on angiogenesis in vitro by analyzing transgenic mice in which individual isoforms (VEGFB<sup>167Tg</sup> and VEGFB<sup>186Tg</sup>) of VEGF-B are overexpressed in endothelial cells. VEGFB<sup>e60Tg</sup> and VEGFB<sup>186Tg</sup> mice displayed enhanced vascular growth in the Matrigel assay in vivo and during cutaneous wound healing. In the aortic explant assay, explants from VEGFB<sup>167Tg</sup> and VEGFB<sup>186Tg</sup> mice displayed elevated vascular growth, suggesting a direct effect of VEGF-B isoforms in potentiating angiogenesis. These data support the use of VEGF-B as a therapeutic agent to promote vascular growth, in part, by potentiating angiogenesis. Furthermore, the lack of vascular permeability activity associated with either transgenic overexpression of the VEGF-B gene in endothelial cells or application of VEGF-B protein to the skin of mice in the Miles assay indicates that use of VEGF-B as a therapy should not be associated with edematous side effects. (Circ Res. 2005;97:e60-e70.)

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endothelial cell proliferation and vascular permeability. Furthermore, transgenic overexpression of PIGF under the control of the K14 promoter leads to both vascular growth and edema in the skin of mice, which, in part, may be explained by the induction of expression of VEGF-A. This indicates that PIGF signaling is closely linked to that of VEGF-A, suggesting that, similar to VEGF-A, the use PIGF as a therapeutic agent to promote tissue revascularization may be associated with an edematous side effect.

VEGF-B promotes vascular growth in adult mice when administered either in the form of recombinant protein in Matrigel plugs or when expressed in situ by adenoviral vector at sites of experimentally induced tissue ischemia. This activity of VEGF-B was shown to be mediated by VEGFR-1, a receptor tyrosine kinase expressed by endothelial cells and a variety of other cell types, and which is induced in ischemic tissue via a hypoxia-inducible factor-1α–dependant mechanism. Signaling through VEGFR-1 has also been implicated in postnatal vessel growth in other model systems. There is no evidence to suggest that VEGF-B is either required for or can potentiate the activity of VEGF-A, and there have been no reports that VEGF-B can promote vascular permeability. On the contrary, we have previously reported that the ability of VEGF-A to induce vascular leakage in the Miles assay is not impaired in Vegfb gene knockout mice. Collectively, these data indicate that VEGF-B may be able to promote vascular growth without induction of vascular leakage and, as a therapeutic agent, may have a better side-effect profile than that predicted for VEGF-A and PIGF. However, not all reports support such therapeutic potential of using VEGF-B, as other studies found VEGF-B to be ineffectual at stimulating vessel growth in muscle and periventriculata when delivered by adnoviral vector. This may reflect tissue-specific differences in effectiveness of VEGF-B on vascular signaling and/or variations in the localized environment, as the presence of other growth factors. These conflicting results from studies investigating the ability of VEGF-B to promote vessels growth are confounded by a lack of in vitro evidence supporting a direct angiogenic activity of VEGF-B.

To clarify the function of VEGF-B isoforms in aspects of postnatal vessel growth, we have generated transgenic mice in which VEGF-Bα167 and VEGF-Bβ167 expression is driven by the endothelial cell–specific Tie2 promoter/enhancer. Analysis of these mice demonstrates a role for both isoforms of VEGF-B in the potentiation, rather than the initiation, of postnatal vessel growth in vivo and of angiogenesis in vitro. Further, the absence of edema in the skin of transgenic animals in combination with an inability of recombinant VEGF-B to induce vascular permeability in the Miles assay indicates that VEGF-B does not induce vascular leakage.

Materials and Methods

Recombinant VEGF-B Expression and Refolding

Recombinant VEGF-B isoforms were produced essentially as previously reported. The coding regions of the cDNA corresponding to amino acid residues 10 to 108 and 1 to 167 of both human and mouse VEGF-B were cloned into the pET-15b vector (EMD Biosciences, San Diego, Calif) and transformed into Escherichia coli strain BL21-CodonPlus-(DE3)-RP (Stratagene, La Jolla, Calif). Recombinant protein expression was induced mid-log phase in Superbroth (30% wt/vol tryptone, 15% wt/vol yeast extract, 0.5% wt/vol NaCl) with 10 mmol/L isopropyl β-D-thiogalactoside. Inclusion bodies were isolated and the material purified under reducing denaturing conditions (6 mol/L guanidine hydrochloride, 0.1 mol/L NaH2PO4, 10 mmol/L β-mercaptoethanol) by nickel metal affinity chromatography (MAC). The purified material was reduced and refolded by dialysis in redox solution as previously described. The refolded material was acidified by dialysis in 0.1 mol/L acetic acid and purified by reverse-phase high-performance liquid chromatography. The N-terminal His6 tag used for nickel MAC was subsequently removed by cleavage with Genenase I (New England Biolabs, Beverly, Mass).

Human VEGFR-1 Chimeric Receptor

Cell-Based Assay

Development of the human VEGFR-1 cell–based assay has been previously reported. A cDNA encoding a chimeric receptor consisting of the extracellular domain of human VEGFR-1 fused to the transmembrane and intracellular domains of mouse erythropoietin receptor was generated by introducing a BglII site into the VEGFR-1 cDNA before the sequence encoding the transmembrane domain, followed by ligation of a BglII–NotI fragment consisting of the transmembrane and intracellular domains of erythropoietin receptor. The cDNA was subcloned into the pEF-BOS expression vector and transfected by electroporation into the interleukin (IL)-3–dependent pre-B cell line, Ba/F3, together with pCDNA3.1(+) neo vector (Invitrogen, Carlsbad, Calif) encoding Zeocin resistance. The transfected cells were selected in complete media (DMEM [Invitrogen]; 5% FCS, 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mmol/L GlutaMAX [Invitrogen]) supplemented with 250 μg/mL Zeocin (Invitrogen), and a Zeocin-resistant line able to proliferate in response to VEGF-A or VEGF-B in the absence of IL-3 was subsequently isolated and cloned by limit dilution. hVEGFR1/EpoR/BaF3 clones were routinely cultured in complete media supplemented with 5 ng/mL VEGF-Bα167.

For the assay of VEGFR-1 ligand activity, selected ligands were diluted into 96-well flat bottom tissue culture plates (TPP, Trasadingen, Switzerland). hVEGFR1/EpoR/BaF3 cells, washed 5 times in PBS, were plated at 6×104/well to give a final assay volume of 100 μL. For ligand neutralization assays, a fixed concentration of ligand was preincubated with antibody for 1 hour before the addition of hVEGFR1/EpoR/BaF3 cells. Cells were incubated for 72 hours at 37°C, 10% CO2, in a humidified container and proliferation assessed using a methane-thiosulfonate (MTS) dye reduction assay (Promega, Madison, Wis) with the colorimetric change measured at a wavelength of 450 nm (Wallach VICTOR microplate reader, PerkinElmer, Boston, Mass).

Generation of Transgenic Mice

All mice in this study were treated in accordance with the Australian National Health and Medical Research Council guidelines for the care and use of experimental animals. Transgenic mice (FVB/N-Tg[Tie2-VEGFα167]1Gfk [henceforth referred to as VEGFBα167/Tg] and FVB/N-Tg[Tie2-VEGFβ167]1Gfk [henceforth referred to as VEGFBβ167/Tg]) were generated by standard techniques in FVB/N strain mice using the expression constructs shown in Figure 1a. The constructs were designed to result in expression of each isoform of VEGF-B (GenBank Nos. U43368 and U43369) under the control of an altered Tie2 enhancer/promoter cassette. This enhancer/promoter combination has been reported to drive constitutive endothelial cell–specific transgene expression. Transgenic animals were genotyped by PCR amplification of tail-tip DNA using primers (F1 5’-ctc tct ccc age ctc atg cc-3’ and R1 5’-ctg ggt tga get cta age cc-3’) specific for the human VEGFB cDNA sequence. The lines of transgenic mice reported here were chosen for the studies after analysis by reverse transcription RT-PCR and immunohistochemistry to confirm that the transgene was being appropriately expressed (see below).

The number of transgene copies incorporated in each transgenic mouse line was estimated from genomic DNA preparations by semi-quantitative PCR (Figure 1b). The primers used to amplify the trans-
Transgene expression was scored by measuring band intensity of the PCR products at their threshold detection levels relative to amplification cycle in comparison to the endogenous diploid Vegfb gene sequence.

Transgene expression levels were compared in RNA isolated from lung tissue for the VEGFB167 tg and VEGFB186 tg mice by semiquantitative RT-PCR and referenced to the level of Hprt expression, as previously described.1 The cDNA and SV40 poly A signal sequence of either transgene was determined by PCR by comparison with the endogenous single-copy diploid Vegfb gene (shown at 24 cycles of amplification). c, RT-PCR analysis of transgene expression levels in VEGFB167tg and VEGFB186tg lung samples with reference to the level of Hprt expression (shown at 30 cycles of amplification). d, Expression and localization of human VEGF-B protein in VEGFB167tg and VEGFB186tg mice by immunofluorescence using anti-VEGF-B mAb on cryosections of heart. Note the specific staining in heart sections from wild-type mice by immunofluorescence using anti–VEGF-B mAb on cryosections of normal heart (Figure 1d) and by immunohistochemistry (IHC) on paraffin sections of healing cutaneous wounds (Figure 1c). e, Specific detection of transgene expressed VEGF-B protein in cryosections of heart using the anti–VEGF-B monoclonal antibody (mAb) (clone 2H10),26 which detects both human and mouse VEGF-B; transgenic mice were crossed with Vegfb+/− mice to generate VEGFB167tg;Vegfb+/− and VEGFB186tg;Vegfb+/− genotype animals. Similar sections from wild-type (C57BL/6J strain) and Vegfb+/− mice (crossed for 12 generations onto the C57BL/6J background) were used as controls. Sections prepared from cutaneous wounds were chosen because Tie-2 expression has been shown to be upregulated in newly formed vessels within the granulation tissue and existing vessels at wound edges. For IHC analysis using anti–human VEGF-B polyclonal Ab (N-19, Santa Cruz Biotechnology, Santa Cruz, Calif), full-depth cutaneous wound samples including surrounding tissue (at 5 days postwounding) were fixed by immersion in 4% paraformaldehyde (PFA) in PBS overnight at 4°C and paraffin embedded.

Matrigel Assay of Neovessel Growth in Vivo
A modified in vivo assay of neovessel growth described by Passanti et al33 was used. Briefly, 0.5 mL of ice-cold Matrigel matrix (BD Biosciences, Bedford, Mass) infused with 300 ng/mL of recombinant human basic fibroblast growth factor (FGF)34 (Invitrogen) was injected SC near the abdominal midline of 167tg, 167tg, 186tg, and wild-type littermate mice (8- to 10-week-old females). Soon after injection, the Matrigel solidified to form a semisolid plug. The mice were then maintained under normal conditions for 6 days, when the experiment was terminated and the angiogenic response studied. The mice were euthanized by CO2 asphyxiation and the Matrigel plugs with the supporting skin and peritoneal lining intact were dissected and frozen in OCT compound (Tissue-Tek, Sakura Finetek, Torrance, Calif). Vessels were identified by CD31 IHC on cryosections and neovessel growth quantified by measuring the density of vessels at the Matrigel/skin interface as described below. As macrophages also express CD31, single cells positive for CD31 were counted only if they surrounded a visible vessel lumen (ie, capillaries).

Assessment of Neovessel Growth in Healing Wounds
VEGFB167tg, VEGFB186tg, and wild-type littermate mice (8- to 10-week-old males and females) were anesthetized by IP injection of anesthetic (0.8 mg/kg fentanyl citrate, 25 mg/kg fluanisone, 12.5 mg/kg midazolam hydrochloride) and 2 full-depth cutaneous wounds were made on the backs using a 6-mm Keyes dermal punch. Wounds were left uncovered, and after 5 or 10 days, the mice were euthanized by CO2 asphyxiation for wound analysis. Wounds were dissected with the surrounding skin intact and fixed by immersion in 4% PFA in PBS overnight at 4°C. Wounds were bisected at the widest point and embedded in paraffin. Vessels were identified on paraffin sections by CD31 IHC and vessel density determined by counting CD31-positive structures and single positive cells surrounding a
visible vessel lumen (capillaries) per microscopic field within the granulation tissue.

**Aortic Explant Assay**

A modified version of the mouse aortic ring assay (Masson et al.) was used to assess angiogenesis in aortic explants from VEGFB<sub>167Tg</sub> and VEGFB<sub>186Tg</sub> mice. Essentially, the descending aorta from VEGFB<sub>167Tg</sub>, VEGFB<sub>186Tg</sub>, and wild-type littermate mice (C57BL/6J×FVB/N strain, F1 progeny) were dissected and placed into ice-cold DMEM. Aortas were then placed in a Petri dish containing DMEM and the periarteriofibroadipose tissue removed under a stereo dissection microscope using fine-tipped forceps and microdissection scissors. The aortas were then longitudinally bisected and cut into 1-mm squares. After washing in fresh DMEM, individual squares of aorta tissue were embedded in a drop (25 μL) of rat tail collagen solution that was placed centrally in the bottom of a well of a 24-well flat bottom tissue culture plate (BD Labware, Franklin Lakes, NJ). The collagen solution was polymerized by placing the tissue culture plate in a humid incubator for 10 minutes at 37°C.

Assay medium (300 mL of MCDB131 [Invitrogen] supplemented with 2.5% serum from FVB/N strain mice, as previously described) was added to the assay medium to a final concentration of 50 μg/mL. In other experiments, assay explants from VEGFB<sub>167Tg</sub>, VEGFB<sub>186Tg</sub>, and wild-type littermate mice were cocultured in the same collagen gel with aortic explants from VEGFB<sup>-/-</sup> mice (C57BL/6J strain). Cultures were incubated in a humid incubator at 37°C/5% CO<sub>2</sub> for 6 to 8 days for optimal endothelial vessel–like growth. For analysis, collagen gels containing the aortic explants were fixed in situ with 2% PFA in PBS (20 minutes at room temperature [RT]) and endothelial vessel–like outgrowth identification using fluorescent isothiocyanate (FITC)-conjugated BS-1 lectin (10 μg/mL in 10 mmol/L HEPES-buffered saline, pH 7.5; Sigma–Aldrich) or by inverted light microscopy. Angiogenesis was assessed by counting the number of vessel-like sprouts and the number of vessel-like branches per aortic explant.

**Endothelial Progenitor Cell Assay**

The levels of circulating endothelial progenitors (EPCs) in VEGFB<sub>167Tg</sub>, VEGFB<sub>186Tg</sub>, and wild-type littermate mice were determined from 500 μL of heparinized blood samples obtained by heart puncture as previously described.

**Miles Assay of Vascular Permeability**

The vascular permeability at sites of intradermal injections of recombinant human VEGF-B<sub>10</sub> (0.1 to 100 ng in 20 μL of 0.01% BSA/PBS) were compared with a single dose of recombinant human VEGF-A<sub>165</sub> (10 ng/site; R&D Systems, Minneapolis, Minn) using an adaptation of the Miles assay (10 ng/site; R&D Systems, Minneapolis, Minn) using an adaptation of the Miles assay (10 ng/site; R&D Systems, Minneapolis, Minn) using an adaptation of the Miles assay in FVB/N strain mice, as previously described.

**Antibodies and Immunohistochemistry**

Endothelial cell proliferation in adult tissues was assessed in paraffin sections using MCM-7 IHC. Briefly, paraffin sections were dewaxed in xylene and hydrated through a graded series of alcohol. Antigen epitope was heat retrieved in 10 mmol/L citrate buffer, pH 6.0, and the sections permeabilized by incubation in 0.1% Triton X-100/PBS for 10 minutes at RT. Endogenous peroxidase activity and nonspecific protein-binding sites were sequentially blocked by incubation with 3% hydrogen peroxide/1% NaN<sub>3</sub> in PBS for 30 minutes and 1% BSA/PBS for 20 minutes at RT. Sections were then incubated with 1 mg/mL of biotinylated MCM7 Ab2 (Clone 47DC141;NeoMarkers, Fremont, Calif) at RT for 90 minutes followed by incubation with streptavidin-conjugated horseradish peroxidase (1/6000 dilution; DAKO Cytomation, Glostrup, Denmark) at RT for 30 minutes. Peroxidase activity was visualized using diaminobenzidine (DAB) and the slides lightly counterstained with hematoxylin. Endothelial cells and macrophages in tissue sections were identified by CD31 and F4/80 IHC, CD31 and F4/80 IHC were performed on paraffin sections of adult tissue and wound-healing samples and on cryosections of Matrigel samples. For paraffin-embedded sections, samples were dewaxed in xylene and hydrated through a graded series of alcohol. Antigen epitopes were retrieved by incubating the sections with 1 μg/mL proteinase K in PBS at RT for 10 minutes or with 0.1% trypsin in PBS for 15 minutes at 37°C for CD31 and F4/80, respectively. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide/1% NaN<sub>3</sub> in PBS for 30 minutes at RT. To block nonspecific protein binding and tertiary antibody binding sites, the slides were sequentially incubated in 4% skim milk powder in PBS and 10% normal goat serum in PBS at RT for 15 and 30 minutes, respectively. Samples were then incubated overnight at 4°C with anti-mouse CD31 (1/800 dilution, clone MEC 13.3, rat IgG<sub>2a</sub>), Pharmingen, San Jose, Calif) or anti-mouse macrophage F4/80 (1/50 dilution of culture supernatant clone AC-1, rat IgG<sub>2a</sub>). Primary antibodies were detected by sequential incubation with polyclonal rabbit anti-rat IgG (1/750 dilution; Sigma–Aldrich, St Louis, Mo) and DAKO EnVision+ anti-rabbit peroxidase system (DAKO Cytomation) at RT for 30 minutes. Peroxidase activity was visualized using DAB and the slides lightly counterstained with hematoxylin. Cryosections (7 μm) were air dried overnight, fixed with ice cold acetone for 5 minutes at RT, and air dried again. After rehydration in PBS, IHC for CD31 and F4/80 were performed essentially as described above for paraffin sections without the antigen retrieval.

VEGF-B protein expression was detected in heart tissue by IF. Cryosections were air dried overnight and fixed by immersion in ice-cold 50% acetone/50% methanol for 10 minutes. Endogenous biotin was blocked using DAKO Biotin Blocking System (DAKO Cytomation). Nonspecific protein-binding sites were blocked using 1% cold-water fish gelatin (Sigma–Aldrich) in PBS for 30 minutes at RT. Sections were then incubated with biotinylated anti–VEGF-B mAb (clone 2HI10, 1 μg/mL) in PBS overnight at 4°C and bound antibody detected by incubation with Rhodamine streptavidin conjugate (1/500 dilution in PBS) for 30 minutes at RT. IHC detection of human VEGF-B protein was confirmed on paraffin sections of cutaneous wounds, as previously described, using anti-human VEGF-B polyclonal peptide Ab (10 μg/mL, N-19, Santa Cruz Biotechnology) with heat retrieval of antigen epitopes using citrate buffer, pH 6.0.

VEGF-R1 expression was detected by IHC on paraffin-tissue sections from VEGFB<sub>167Tg</sub>, VEGFB<sub>186Tg</sub>, and wild-type littermate mice. Following blocking of endogenous peroxidase activity and nonspecific protein-binding sites by sequential incubation with 3% H<sub>2</sub>O<sub>2</sub>/1% NaN<sub>3</sub> in PBS and 4% skim milk power in PBS, dewaxed sections were incubated for 2 hours at RT with anti–VEGF-R1 rabbit polyclonal peptide Ab (4 μg/mL,C-17, Santa Cruz Biotechnology). Sections were then washed in PBS and the bound antibody detected by incubation with DAKO EnVision+ anti-rabbit peroxidase system (DAKO Cytomation) at RT for 30 minutes. Peroxidase activity was visualized using DAB and the slides lightly counterstained with hematoxylin. As a negative control for all IF and IHC, primary antibody was omitted.

**Results**

**Expression and Biological Activity of Human and Mouse VEGF-B Isoforms**

Recombinant human (rh) and mouse VEGF-B<sub>10</sub> to 108 and VEGF-B<sub>167</sub> were expressed as N-terminal His6-tagged proteins in Escherichia coli. The monomeric proteins were purified by MAC and refolded by redox dila. The dimeric protein was separated from the monomeric material by reverse-phase high-performance liquid chromatography. SDS-PAGE analysis of the human and mouse isoforms is shown in Figure 2a.

Human and mouse VEGF-B isoforms were assessed for biological activity in the hVEGFR1/EpoR/BaF3 cell–based assay. Both human and mouse VEGF-B<sub>10</sub> to 108 and VEGF-B<sub>167</sub> were shown to be active (Figure 2b), with human and mouse...
VEGF-B10 to 108 showing activity similar to that of human VEGF-A165 (EC50 = 0.1 nmol/L). In contrast, full-length human and mouse VEGF-B167 had 10- and 30-fold less activity, with EC50 = 1 and 3 nmol/L respectively.

**Characterization of a Species Cross-Reactive VEGF-B–Specific Neutralizing mAB**

The generation and preliminary characterization of the human VEGF-B neutralizing mouse mAb 2H10 has been described previously.26 To determine whether mAbs raised against human VEGF-B were cross-reactive with mouse VEGF-B and, thus, potentially of value in the analysis of VEGF-B activity in mouse models, the binding of 2H10 to the human and mouse VEGF-B was assessed by Western blot analysis (Figure 2c). Results presented in Figure 2c demonstrate that mAb 2H10 was able to bind to the human and mouse VEGF-B10 to 108 and VEGF-B167 isoforms. This interaction was confirmed using a variety of other approaches including ELISA and Biacore analysis (results not shown). 2H10 did not bind to reduced human and mouse VEGF-B, indicating that the 2H10 epitope is structural.

In addition to binding to both human and mouse VEGF-B, mAb 2H10 was shown to be a potent antagonist of both of these proteins. Results presented in Figure 2d demonstrate that mAb 2H10, but not the isotype control C44 antibody, inhibited human and mouse VEGF-B–mediated proliferation of hVEGFR1/EpoR/BaF3 cells. The mAb appeared to be slightly more potent against human VEGF-B (IC50 = 1 nmol/L) than against mouse VEGF-B (IC50 = 3 nmol/L).

**Evaluation of Transgene Expression in VEGFB167Tg and VEGFB186Tg Mice**

A small number of viable individual transgenic founder animals with incorporation of the VEGFB transgenes were identified by PCR amplification of genomic DNA from tail-tip biopsies. More frequent transgene positive individuals were identified when mid-gestation embryonic stages were genotyped (data not shown), suggesting that high levels of expression of the transgene might be detrimental to development. Similarly, attempts to generate viable transgenic mice with ubiquitous overexpression of either VEGFB167 or VEGFB186 under the control of the HmgCoA gene promoter were unsuccessful, despite the identification of transgene positive mid-gestation embryos (data not shown). Of the viable transgenic mouse lines successfully derived, only 1 line each of VEGFB167Tg and VEGFB186Tg mice had detectable levels of transgene mRNA, as assessed by RT-PCR analysis of lung samples. We estimated by PCR amplification of the VEGFB transgenes in comparison with diploid endogenous Vegfb gene that approximately 16 copies of the
VEGFB<sup>167</sup> and 2 copies of the VEGFB<sup>186</sup> transgenes were incorporated in the genome of these mouse lines (Figure 1b). Transgene expression levels, as estimated by RT-PCR from total RNA derived from lung tissue, showed that expression was approximately 12.7% and 1.5% of the Hprt expression level in the VEGFB<sup>167</sup>Tg and VEGFB<sup>186</sup>Tg mice, respectively (Figure 1c).

VEGFB<sup>167</sup>Tg and VEGFB<sup>186</sup>Tg mice are viable, fully fertile and appeared healthy. Aged-matched mice VEGFB<sup>167</sup>Tg and VEGFB<sup>186</sup>Tg mice (6- to 8-weeks old) displayed similar vessel densities in normal tissues to wild-type littermates, as assessed by platelet-endothelial cell–adhesion molecule (PECAM)-1 IHC on section of various organs (ie, liver, heart, and kidney; data not shown). Endothelial cell proliferation in vessels of the heart, liver, and kidney was also comparable between VEGFB<sup>167</sup>Tg, VEGFB<sup>186</sup>Tg, and their wild-type littermates, as determined by MCM-7 IHC (data not shown). IF staining for VEGF-B protein on cryosections of heart from VEGFB<sup>167</sup>Tg;Vegfb<sup>−/−</sup> and VEGFB<sup>186</sup>Tg;Vegfb<sup>−/−</sup> mice using anti–VEGF-B mAb (2H10) confirmed transgene protein expression. Cryosections of heart from wild-type (C57BL6J strain) and Vegfb<sup>−/−</sup> mice were used as positive and negative controls, respectively. Positive staining was observed in wild-type heart sections, and no staining was observed in Vegfb<sup>−/−</sup> heart sections, verifying specific VEGF-B protein staining (Figure 1d). Broad vascular staining was observed in the hearts of both VEGFB<sup>167</sup>Tg;Vegfb<sup>−/−</sup> and VEGFB<sup>186</sup>Tg;Vegfb<sup>−/−</sup>, consistent with Tie2 promoter/enhancer–driven transgene expression in endothelial cells (Figure 1d). IHC using anti-human VEGF-B (rabbit polyclonal, N19; Santa Cruz Biotechnology) on paraffin sections of various tissues including heart, kidney, and liver and on 5-day-old, full-depth cutaneous wounds from both VEGFB<sup>167</sup>Tg and VEGFB<sup>186</sup>Tg mice was also performed as previously described<sup>39</sup> but was less sensitive in detecting the transgene expression. Staining was only observed in the endothelium in blood vessels at wound edges (Figure 1e) and occasional newly formed vessels in the granulation tissue of VEGFB<sup>167</sup>Tg and VEGFB<sup>186</sup>Tg wounds. This indicates that transgene expression was increased in the vascular endothelium of vessels at wound edges and in neovessels during wound healing, which is consistent with previous reports showing upregulation of Tie-2 expression in this region in healing cutaneous wounds in mice.<sup>32</sup> No staining of the vascular endothelium was observed in similar wound sections from wild-type mice (Figure 1e).

To determine whether the VEGFB transgenes altered expression of VEGFR-1, expression of the receptor was compared by IHC on paraffin sections of normal tissues from VEGFB<sup>167</sup>Tg and VEGFB<sup>186</sup>Tg mice with similar sections from wild-type littermates. In all genotypes, positive staining for VEGFR-1 was observed in endothelial cells in a restricted number of vessel types, predominantly small to medium veins and arteries. VEGFR-1 expression was not detected in capillary endothelium in any of the organs tested, including the heart, 1 of the sites where VEGFB transgene expression was detected (see Figure 1d). The intensity of endothelial staining and the types of vessels showing positive staining of endothelial cells were similar between the genotypes indicating that VEGFR-1 expression was not altered by VEGFB transgene expression (data not shown).

**Assessment of Neovascular Growth in VEGFB<sup>167</sup>Tg and VEGFB<sup>186</sup>Tg Mice in the Matrigel Plug Assay and During Cutaneous Wound Healing**

Vessel density at the Matrigel/skin interface of Matrigel plugs infused with 300 ng/mL bFGF was measured after 6 days in VEGFB<sup>167</sup>Tg, VEGFB<sup>186</sup>Tg, and wild-type littermate mice. Histological examination of H&E-stained cryosections of these plugs revealed an encapsulation of the Matrigel plug by granulation tissue and significant cellular invasion into the Matrigel matrix, largely consisting of macrophages as identified by F4/80 IHC, which was most concentrated at the periphery of the plug. Cellular invasion into the Matrigel matrix was associated with substantial angiogenesis as evidenced by in growth of PECAM-1–positive vessel structures into the Matrigel matrix from the surrounding granulation tissue (Figure 3a). Vessel density at the Matrigel/skin interface was significantly increased in Matrigel plugs from VEGFB<sup>167</sup>Tg (75% increase, P = 0.0054 versus wild-type littermates) and VEGFB<sup>186</sup>Tg mice (52% increase, P = 0.0068 versus wild-type littermates) when compared with vessel density in the same region of Matrigel plugs from wild-type littermates (Figure 3b). Levels of macrophages surrounding and invading the Matrigel plugs appeared similar in all genotypes (data not shown).

Apart from reproductive tissues, wound healing is 1 of the few nonpathological processes where neovessel growth is observed in the adult. During wound healing, neovessel growth is associated with the proliferative phase of wound healing and may facilitate the formation and, in later stages, the resolution of the granulation tissue. Based on previously published studies showing the kinetics of Tie-2 expression in wounds in mice,<sup>32</sup> we compared angiogenic responses in granulation tissue at 5 and 10 days following full-depth cutaneous wounding among VEGFB<sup>167</sup>Tg, VEGFB<sup>186</sup>Tg, and wild-type littermates by measuring vessel density. The vessel density in the granulation tissue was significantly increased in both VEGFB<sup>167</sup>Tg and VEGFB<sup>186</sup>Tg compared with wild-type littermates at both 5 and 10 days postwounding (Figure 3c). The level of increase in vessel density at 10 days postwounding between the transgenic animals and the wild-type littermates was comparable to that observed between the same transgenic mice and wild-type littermates in the Matrigel plug assay (66% and 41% increase over wild-type vessel density for VEGFB<sup>167</sup>Tg and VEGFB<sup>186</sup>Tg, respectively; Figure 3c). The granulation tissue at 5 and 10 days postwound consisted primarily of macrophages and vascular tissue, as assessed by F4/80 (Figure 3d) and PECAM-1 (Figure 3e) IHC, respectively, and the levels of F4/80 positive cells in the granulation tissue appeared similar among genotypes when wounds from the various mouse genotypes were compared at the same time point (data not shown).

**Decreased EPCs in the Circulation of VEGFB<sup>167</sup>Tg Mice**

The levels of EPCs in the blood were significantly reduced in VEGFB<sup>167</sup>Tg mice (P = 0.004) when compared with levels of these cells in the blood of wild-type littermates (Figure 4). The levels of EPCs in the blood of the VEGFB<sup>186</sup>Tg mice were not significantly different from the levels of these cells in the blood of wild-type littermates.
VEGFB<sub>167Tg</sub> and VEGFB<sub>186Tg</sub> Mice Display Increased Endothelial Vessel–Like Sprouting in the Aortic Explant Assay

The dynamics of endothelial vessel–like outgrowth (angiogenesis) from cultured aortic explants was similar to that previously described. Vessel-like endothelial outgrowth was preceded by the migration of fibroblast-like cells from aortic explants into the surrounding collagen matrix, which was visible within the first 24 hours of culture (data not shown). Vessel-like endothelial outgrowths (identified by fluorescence microscopy after staining with FITC-conjugated BS-1 lectin; Figure 5a) from the aortic explants were first observed after 4 days in culture and maximal vessel growth was observed 4 days later, as assessed by both the number of vessels sprouts and the complexity of vessel branching (as measured as a ratio of the number of branches to the number of vessel sprouts). VEGFB<sub>167Tg</sub> and VEGFB<sub>186Tg</sub> explants displayed significantly greater numbers of vessel sprouts when compared with explants from wild-type littermates (Figure 5b). Branching complexity was comparable among the different mouse genotypes (wild-type littermates: 1.44±0.14, VEGFB<sub>167Tg</sub> = 1.56±0.07, and VEGFB<sub>186Tg</sub> = 1.70±0.09 branches/vessel like sprout). The addition of anti–VEGF-B mAb (clone 2H10) to the culture medium significantly reduced the number of vessel sprouts in VEGFB<sub>167Tg</sub> and VEGFB<sub>186Tg</sub> aortic explant cultures when compared with their respective isotype mAb (clone C44)–treated control explant cultures (Figure 5c). In the presence of anti–VEGF-B antibody, the number of vessel sprouts in wild-type littermates was significantly increased in 5- and 10-day-old cutaneous wounds from VEGFB<sub>167Tg</sub> and VEGFB<sub>186Tg</sub> mice when compared with age-matched cutaneous wounds from wild-type littermates. Endothelial cells and macrophages in cutaneous wound samples were identified by PECAM-1 (d) and F4/80 (e) immunohistochemistry, respectively, on paraffin sections (pictured are photomicrographs of sequential paraffin sections of a 5-day-old cutaneous wound from a wild-type mouse. Scale bars=100 µm). Data represent mean vessels/mm skin-Matrigel interface ±SEM of 15 sections in total/genotype (3 different sample depths each of Matrigel plug/mouse×5 mice/genotype) (b) and mean vessels/field (magnification, ×200) in the granulation tissue ±SEM of 6 wounds/genotype at each time point (c). Differences between means were considered significant in P<0.05. b, *P<0.0054 and **P<0.0068 when compared with wild-type littermates. c, On 5-day-old wounds, *P<0.0084 and **P<0.0005 when compared with wild-type littermates; on 10-day-old wounds, *P<0.0074 and **P<0.0011 when compared with wild-type littermates.
The levels of EPCs in the blood of VEGFB<sup>167Tg</sup> and VEGFB<sup>186Tg</sup> mice. The levels of EPCs in the blood of mice were determined by culturing the mononuclear cells isolated from 500 μL of blood for 4 days and then measuring the number of differentiated endothelial cells, as identified by FITC BS-1–positive staining. The endothelial lineage of these cells was confirmed by double fluorescent staining with FITC BS-1 and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine–low-density lipoprotein. Significantly fewer differentiated endothelial cells were present in the 4-day-old cultures of mononuclear cells isolated from the blood VEGFB<sup>167Tg</sup> mice but not in 4 day old cultures of mononuclear cells isolated from the blood VEGFB<sup>186Tg</sup> mice, when compared similar cultures of mononuclear cells isolated from the blood of wild-type littermates. Data represents mean FITC BS-1–positive cells/field (magnification, ×200) ± SEM of 16 fields/genotype (4 fields/culture/mouse ×4 mice/genotype). Differences in means were considered significant if P<0.05. *P=0.004 when compared with wild-type littermate cultures. Photomicrographs depict representative fields of view of FITC BS-1 lectin–stained cells in 4-day-old mononuclear cell cultures isolated from the blood of wild-type littermate, VEGFB<sup>167Tg</sup> and VEGFB<sup>186Tg</sup> mice. Scale bars—50 μm.

Figure 4. The levels of EPCs in the blood of VEGFB<sup>167Tg</sup> and VEGFB<sup>186Tg</sup> mice. The levels of EPCs in the blood of mice were determined by culturing the mononuclear cells isolated from 500 μL of blood for 4 days and then measuring the number of differentiated endothelial cells, as identified by FITC BS-1–positive staining. The endothelial lineage of these cells was confirmed by double fluorescent staining with FITC BS-1 and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine–low-density lipoprotein. Significantly fewer differentiated endothelial cells were present in the 4-day-old cultures of mononuclear cells isolated from the blood VEGFB<sup>167Tg</sup> mice but not in 4 day old cultures of mononuclear cells isolated from the blood VEGFB<sup>186Tg</sup> mice, when compared similar cultures of mononuclear cells isolated from the blood of wild-type littermates. Data represents mean FITC BS-1–positive cells/field (magnification, ×200) ± SEM of 16 fields/genotype (4 fields/culture/mouse ×4 mice/genotype). Differences in means were considered significant if P<0.05. *P=0.004 when compared with wild-type littermate cultures. Photomicrographs depict representative fields of view of FITC BS-1 lectin–stained cells in 4-day-old mononuclear cell cultures isolated from the blood of wild-type littermate, VEGFB<sup>167Tg</sup> and VEGFB<sup>186Tg</sup> mice. Scale bars—50 μm.

VEGFB<sup>167Tg</sup> and VEGFB<sup>186Tg</sup> mice. a, Black and white photomicrograph of a 6-day-old wild-type aortic explant culture stained with FITC-BS-1 lectin, showing vessel-like endothelial outgrowth from explant into the surrounding collagen gel. Scale bar=500 μm. b, Aortic explants from VEGFB<sup>167Tg</sup> and VEGFB<sup>186Tg</sup> mice displayed significantly greater numbers of vessel sprouts/explant when compared with explants from wild-type littermates. c, Addition of neutralizing anti-VEGF-B antibody significantly reduced the level of vessel sprouting in both VEGFB<sup>167Tg</sup> and VEGFB<sup>186Tg</sup> explant cultures compared with that observed in wild-type aortic explant cultures treated with isotype control mAb, confirming a direct action of both VEGFB transgenes on vessel growth. Addition of neutralizing anti-VEGF-B antibody to wild-type explant cultures was without effect on vessel sprouting when compared with isotype control mAb–treated explants. d, Coculture of aortic explants from Vegfb<sup>−/−</sup> mice with aortic explants from VEGFB<sup>167Tg</sup> and VEGFB<sup>186Tg</sup> mice, but not from wild-type littermates or Vegfb<sup>−/−</sup> mice, significantly increased the levels of vessel sprouts in the Vegfb<sup>−/−</sup> aortic explants. Data represent mean vessel sprouts/explant ± SEM of 16 (b) and 8 (c and d) explants/genotype. Differences in means were considered significant if P<0.05. *P=0.0001 and **P=0.01 when compared with explants from wild-type littermates. c, **P=0.0001 and **P=0.0004 when compared with explants with isotype control mAb–treated explants of the same genotype. d, **P=0.019 and **P=0.02 when compared with Vegfb<sup>−/−</sup> explants cocultured with explants from wild-type littermates.

Figure 5. Increased vessel sprouting in aortic explants from VEGFB<sup>167Tg</sup> and VEGFB<sup>186Tg</sup> mice. a, Black and white photomicrograph of a 6-day-old wild-type aortic explant culture stained with FITC-BS-1 lectin, showing vessel-like endothelial outgrowth from explant into the surrounding collagen gel. Scale bar=500 μm. b, Aortic explants from VEGFB<sup>167Tg</sup> and VEGFB<sup>186Tg</sup> mice displayed significantly greater numbers of vessel sprouts/explant when compared with explants from wild-type littermates. c, Addition of neutralizing anti-VEGF-B antibody significantly reduced the level of vessel sprouting in both VEGFB<sup>167Tg</sup> and VEGFB<sup>186Tg</sup> explant cultures compared with that observed in wild-type aortic explant cultures treated with isotype control mAb, confirming a direct action of both VEGFB transgenes on vessel growth. Addition of neutralizing anti-VEGF-B antibody to wild-type explant cultures was without effect on vessel sprouting when compared with isotype control mAb–treated explants. d, Coculture of aortic explants from Vegfb<sup>−/−</sup> mice with aortic explants from VEGFB<sup>167Tg</sup> and VEGFB<sup>186Tg</sup> mice, but not from wild-type littermates or Vegfb<sup>−/−</sup> mice, significantly increased the levels of vessel sprouts in the Vegfb<sup>−/−</sup> aortic explants. Data represent mean vessel sprouts/explant ± SEM of 16 (b) and 8 (c and d) explants/genotype. Differences in means were considered significant if P<0.05. *P=0.0001 and **P=0.01 when compared with explants from wild-type littermates. c, **P=0.0001 and **P=0.0004 when compared with isotype control mAb–treated explants of the same genotype. d, **P=0.019 and **P=0.02 when compared with Vegfb<sup>−/−</sup> explants cocultured with explants from wild-type littermates.

Discussion

VEGF-B has been reported to promote postnatal angiogenesis in vivo. Although postnatal blood vessel growth is commonly assumed to be solely governed by angiogenesis, recent data indicate that it also occurs through the recruitment of bone marrow–derived EPCs in a process akin to embryonic vasculogenesis. The VEGF-B receptor VEGFR-1 has recently been implicated in mobilization of EPC from the bone marrow, suggesting that VEGF-B may be able to promote this process. This raises the question of whether VEGF-B is truly angiogenic, as suggested in previous in vivo studies, or whether VEGF-B may have promoted vessel growth by vasculogenesis through mobilization/recruitment of EPCs. The true mode of action of VEGF-B in promoting blood vessel growth (angiogenesis,
VEGFB wild-type littermates. Neovessel growth was also increased in the Matrigel assay. Intradermal injection of 10 ng of VEGF-A165 induced vascular leakage above control vehicle, as determined by a significant increase in the accumulation of Evans Blue dye at injection sites. In contrast, intradermal injections of rhVEGF-B167 at doses ranging from 0.1 to 100 ng/injection site were without effect on vascular permeability when compared with control vehicle alone (ie, 0 ng/site). Data represent mean absorbance units (620 nm) ± SEM of 4 replicates/dose for rhVEGF-B167 and 16 replicates of each control vehicle and VEGF-A at 10 ng/site. Differences in means were considered significant if \( P \leq 0.05, \ P < 0.0001 \) when compared with control vehicle alone (0 ng/site).

Figure 6. VEGF-B does not induce vascular leakage in the Miles assay. Intradermal injection of 10 ng of VEGF-A165 induced vascular leakage above control vehicle, as determined by a significant increase in the accumulation of Evans Blue dye at injection sites. In contrast, intradermal injections of rhVEGF-B167 at doses ranging from 0.1 to 100 ng/injection site were without effect on vascular permeability when compared with control vehicle alone (ie, 0 ng/site). Data represent mean absorbance units (620 nm) ± SEM of 4 replicates/dose for rhVEGF-B167 and 16 replicates of each control vehicle and VEGF-A at 10 ng/site. Differences in means were considered significant if \( P \leq 0.05, \ P < 0.0001 \) when compared with control vehicle alone (0 ng/site).

The levels of EPCs were decreased in the blood of VEGF167Tg mice when compared with wild-type littermates, implicating VEGF-B in aspects of EPC biology. At this stage we are not able to definitively demonstrate the cause of the reduced numbers of circulating EPCs in the VEGF167Tg mouse but suggest that this may not reflect negative regulation of EPC mobilization from the bone marrow. Vascular growth in the granulation tissue of healing wounds involves the recruitment of EPCs.45 We found that neovessel growth was equally increased in the Matrigel and wound-healing models in the VEGF167Tg and VEGF186Tg. We propose that this observation is inconsistent with a negative effect of VEGF-B on EPC mobilization and suggest that the reduced levels of EPCs in the blood of the VEGF167Tg mouse may be attributable to increased tissue recruitment or endothelial adherence of EPCs to the vascular endothelium in these animals. Unlike VEGF-B186, which is freely secreted, VEGF-B167 remains cell associated, where it potentially may exhibit an adhesion molecule-like action,46 and when expressed by endothelial cells as in the VEGF167Tg mouse may result in increased adherence of EPCs to the vasculature, thus removing them from the circulation. Tie-2 expression is upregulated in neovessels in healing wounds,32 and, accordingly, VEGF167Tg mice and VEGF186Tg mice displayed increased transgene expression in neovessel endothelium (compared with resting-state skin). This is associated with a significant increase in vessel density in granulation tissue of VEGF167Tg and VEGF186Tg wounds in comparison to wild-type littermate wounds. During wound healing, localized transgene expression may be sufficient to promote preferential recruitment and/or differentiation of EPCs from the circulation to the site of neovessel growth. In our wound healing and Matrigel studies, as in previous studies,13 the ability of VEGF-B to promote vascular growth in vivo may, therefore, represent effects on angiogenesis and/or vasculogenesis.

To determine the direct effects of VEGF-B on angiogenesis without the contribution of circulating EPCs to the vascular growth, we specifically selected the aortic explant assay in preference to other in vitro endothelial cell–based angiogenesis assays because it more closely resembles the physiological angiogenesis process. Vessel growth in this system using rat...
aortic explants has been shown to directly involve angiogenesis, although the contribution of tissue resident EPCs in vessel growth in this assay has not been explored and should not be excluded. Similar to the results obtained in both the Matrigel assay and wound-healing studies, aortic explants from VEGFB167Tg and VEGFB186Tg mice exhibited increased vessel-like endothelial outgrowth in comparison to wild-type littermate aortic explants. Increased vessel growth in both VEGFB167Tg mice and VEGFB186Tg mice was caused by greater vessel-like endothelial sprouting rather than increased vessel branching. This result is in contrast to a previous study in a similar system using embryonic quail heart explants reporting a role for VEGF-B in vessel branching rather than vessel sprouting. In that study, the role of VEGF-B was determined by the addition of an anti–VEGF-B polyclonal antibody (C19; Santa Cruz Biotechnology). However, these data should be interpreted with caution because the ability of this antibody to neutralize VEGF-B signaling through its receptor has not, to our knowledge, been specifically assessed. Furthermore, this antibody was designed against a peptide region mapping to the carboxyl terminus of VEGF-B and, therefore, can only bind to a single isoform of VEGF-B, which we have determined to be VEGF-B167 by immunoblotting of recombinant VEGF-B isoforms (A.M., M.C., G.K., unpublished observation, 2003). In our studies, the action of both VEGF-B transgenes on potentiation of vessel growth was completely blocked in the presence of anti–VEGF-B mAb (clone 2H10), which is shown to neutralize VEGF-B signaling through VEGFR-1, confirming a direct effect of both VEGF-B isoforms in the potentiation of angiogenesis rather than inherent differences in the endothelial responsiveness of aortic explants between wild-type littermates and transgenic animals. Although the contribution of tissue-resident EPCs to vascular growth in this assay remains undetermined, we conclude that the differences in vessel growth between transgenic and wild-type explants cannot be solely attributable to increased levels of tissue-resident EPCs in the transgenic explants because VEGF-B activity was required during the assay to potentiate vessel growth. Support for a direct action of both VEGF-B transgenes on potentiation of angiogenesis comes from the evidence of a diffusible factor in the potentiation of angiogenesis from VEGFB167Tg and VEGFB186Tg aortic explants to Vgfb−/− explants in a coculture system. From the early in vitro characterization of the VEGF-B isoforms, we had predicted that only the VEGF-B186 should be able to signal to the remote Vgfb−/− explants because VEGF-B167 has been shown to be retained by the secreting cell. It is possible that the VEGF-B transgene effect involves a second messenger, but this is outside the scope of the present study.

The vascular permeability activity of VEGF-A presents a problem for the use of VEGF-A as a therapeutic agent for promoting tissue revascularization because it also promotes edema. This is illustrated by studies where overexpression of VEGF-A in the skin of mice induced vascular leakage that was evident by disruption of skin architecture. In contrast, there was no evidence of edema in the skin of VEGFB167Tg and VEGFB186Tg mice by histological examination. Injections of recombinant VEGF-B167 protein failed to stimulate vascular leakage in the Miles assay when used at concentration up to 10-fold excess of a concentration of VEGF-A, which potently induced vascular leakage in the same animal (wild-type mice, FVB/N strain). This indicates that, unlike VEGF-A, VEGF-B does not promote vascular leakage and indicates that VEGF-B may provide a more favorable therapy for promoting/potentiating tissue revascularization, as it should not have edematous side effects.

In conclusion, our data demonstrate that both isoforms of VEGF-B potentiate, rather than initiate, postnatal vessel growth in vivo. We also present in vitro evidence to show that VEGF-B can potentiate angiogenesis. Because VEGF-B does not appear to promote vascular leakage, its use as a therapeutic agent to promote vascular growth should not be associated with edematous side effects, unlike related factors VEGF-A and PIGF. These data suggest that VEGF-B may provide an effective therapy to promote/enhance vascular growth in diseases associated with impaired revascularization and ischemia, such as ischemic heart disease.

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Transgenic Overexpression of Vascular Endothelial Growth Factor-B Isoforms by Endothelial Cells Potentiates Postnatal Vessel Growth In Vivo and In Vitro

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In an article by Mould et al (Circ Res. 2005;97:e60–e70), the authors omitted a statement from the Acknowledgement section. The corrected Acknowledgment section should read:

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