Abstract—Vascular endothelial growth factors (VEGFs) and their receptors have emerged as central regulators of the angiogenic process. However, involvement of VEGF-B, one of these factors, in angiogenesis remains obscure. Mice received subcutaneous injection of Matrigel alone or Matrigel with human recombinant protein rhVEGF-B$_{167}$ or with rhVEGF-A$_{165}$. After 14 days, cell ingrowth in the Matrigel plug was increased by 2.0- and 2.5-fold in rhVEGF-B$_{167}$-treated and rhVEGF-A$_{165}$-treated mice, respectively ($P<0.01$), in association with a raise in phospho-Akt/Akt (1.8-fold, $P<0.01$) and endothelial NO synthase (eNOS) (1.80- and 1.60-fold, respectively; $P<0.05$) protein levels measured by Western blot. VEGF-B–induced cell ingrowth was impaired by treatment with NOS inhibitor (N$^\omega$-nitro-L-arginine methyl ester; L-NAME, 10 mg/kg per day). Treatment with neutralizing antibody directed against the VEGF-B receptor VEGF-R1 (anti-VEGFR1, 10 $\mu$g) completely abrogated VEGF-B–related effects. Proangiogenic effect of VEGF-B was confirmed in a mouse model of surgically induced hindlimb ischemia. Plasmids containing human form of VEGF-A (phVEGF-A$_{165}$) or VEGF-B (phVEGF-B$_{167}$ or phVEGF-B$_{186}$) were administered by in vivo electrotransfer. Angiographic score at day 28 showed significant improvement in ischemic/nonischemic leg ratio by 1.4- and 1.5-fold in mice treated with phVEGF-B$_{165}$ and phVEGF-B$_{186}$, respectively ($P<0.05$). Laser Doppler perfusion data also evidenced a 1.5-fold increase in phVEGF-B$_{167}$–treated and phVEGF-B$_{186}$–treated mice ($P<0.05$). Such an effect was associated with an upregulation of phospho-Akt/Akt and eNOS protein levels in the ischemic legs and was hampered by treatment with anti-VEGFR1. This study demonstrates for the first time that VEGF-B, in part through its receptor VEGF-R1, promotes angiogenesis in association with an activation of Akt and eNOS-related pathways. (Circ Res. 2003;93:667–674.)

Key Words: vascular endothelial growth factor-B □ angiogenesis □ ischemia □ endothelial nitric oxide

Neovascularization of ischemic cardiac or skeletal muscle may be sufficient to preserve tissue integrity or function and may thus be considered as a therapeutic goal. The specific mechanisms regulating angiogenesis are not fully understood, but several potential regulators of this process have been described. Among the known angiogenic factors, vascular endothelial growth factor-A (VEGF-A) has emerged as a central regulator of the angiogenic process under both physiological and pathological conditions. VEGF exerts its effect through two endothelial receptors, tyrosine kinase VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), and may induce vessel growth by activating several proangiogenic pathways. VEGF-A increases the expression of both endothelial NO synthase (eNOS) and inducible NO synthase (iNOS). In mice ischemic hindlimb, the angiogenic response to VEGF-A requires the activation of eNOS gene. Similarly, in ischemic heart, the induction of coronary collateralization by VEGF-A involves the production of NO. VEGF-A–induced vessel growth may also imply activation of the serine/threonine kinase Akt-related endothelial pathway. Enhanced Akt signaling in the endothelium promotes angiogenesis in rabbit ischemic limb. Interestingly, VEGF-A–induced endothelial cell survival and migration requires the phosphorylation of Akt, suggesting that Akt is a downstream effector of VEGF-A signaling. Akt may contribute to the angiogenic reaction by phosphorylating eNOS, leading to a persistent calcium-independent enzyme activation, or by inhibiting the apoptotic process. Finally, VEGF-A may modulate new vessel formation by regulating matrix metalloproteinase (MMP) production and activation. MMP is an important effector mechanism in angiogenesis. Two members of the MMP endopeptidase family, MMP-9 (92 kDa) and MMP-2 (72 kDa) are able to degrade the extracellular matrix components of the basement membrane. VEGF-A has been shown to activate the MMP gene transcription, which has been reported to affect the angiogenic process.

The VEGF family of growth factors presently comprises the following five members: VEGF-A, placenta growth
factor, VEGF-B, VEGF-C, and VEGF-D. VEGF-B exists as two protein isoforms, VEGF-B<sub>167</sub> and VEGF-B<sub>186</sub>, resulting from alternatively spliced mRNA. VEGF-B binds specifically to VEGFR-1. However, VEGF-B forms heterodimers with VEGF-A, a property likely to alter its receptor specificity and biological effects. VEGF-B is widely expressed in heart, skeletal muscle, and vascular cells. Nevertheless, VEGF-B biological function remains obscure at present. Several lines of evidence indicate that VEGF-B may modulate cell proliferation and vessel growth. Conditioned medium from transfected cells expressing VEGF-B stimulates DNA synthesis in endothelial cells. VEGF-B levels increase both throughout development and after birth, closely correlating with the progression of cardiac angiogenesis. In dilated ischemic cardiomyopathy, the downregulation of VEGF-B levels is correlated with the decrease in capillary density.

We therefore hypothesized that VEGF-B might affect the angiogenic process. To test this hypothesis, we analyzed the angiogenic effect of VEGF-B in two different models of angiogenesis, i.e., the in vivo model of Matrigel and a mouse model of surgically induced hindlimb ischemia. Finally, we attempted to investigate the molecular events associated with VEGF-B effects by investigating changes in phospho-Akt and eNOS protein levels and MMP activity.

Materials and Methods

All experiments and analyses were performed in a randomized and blinded fashion. Angiogenesis Assay Using the Matrigel Model

Sexually mature 8-week-old C57Bl/6 female mice (Iffa Credo, Lyon, France) received a 0.5-ml subcutaneous injection of Matrigel alone (Becton Dickinson) or Matrigel with human recombinant protein rhVEGF-B<sub>145</sub> (500 ng/mL of Matrigel), rhVEGF-A<sub>165</sub> (500 ng/mL of Matrigel) (Sigma), rhVEGF-B<sub>167</sub> + Anti-R1 (neutralizing antibody against mouse VEGF-R1, 10 μg, AF471, R&D Systems), or rhVEGF-B<sub>145</sub> + Anti-R2 (neutralizing antibody against mouse VEGF-R, 10 μg, AF644, R&D Systems). The specific role of NO in VEGF-B–induced neovascularization was assessed by treatment with phVEGF-B<sub>167</sub> + NO synthase inhibitor (L-NAME, 10 mg/kg per day in the drinking water, Sigma). In a preliminary set of experiments, rhVEGF-B<sub>145</sub> and rhVEGF-A<sub>165</sub> at a dose of 500 ng/mL of Matrigel have been shown to induce a maximal angiogenic response (data not shown). After the injection, the Matrigel formed rapidly a subcutaneous plug that was left for 14 days before removal.

Matrigel Preparation

On day 14, the mice were euthanized and the skin of the mouse was easily pulled back to expose the Matrigel, as previously described. Plugs were then removed and fixed with 3.7% formaldehyde at 4°C for 12 hours, embedded in paraffin, sectioned, and stained with Masson trichrome. Three successive sections (5 μm) were then examined (×20 and ×40 magnification, Olympus BH-2, Leica).

Quantification of Angiogenesis

Histological Score

The histological views were used for semiquantitative evaluation of angiogenesis, as previously described. Three different scores were defined as follows: score 1, no colonization by cells or formation of a slight peripheral coat around the plug with a disorganized infiltration of cells; score 2, a cell coat thickened with a marked structural infiltration in the Matrigel; and score 3, a deep and massive infiltration with erythrocyte presence. Such scores were performed in a double-blind fashion.

Cellular Density

The histological analysis was completed with cellular density measurement using Cyquant Cell Proliferation Assay kit (Molecular Probes), as previously described.

Identification of the Cellular Types

Five-micrometer sections were hybridized for 45 minutes with antibody against monoclonal α smooth muscle actin to identify smooth muscle cells (clone 1A4, DAKO, dilution 1/50, M0851), as previously described. Adjacent sections were also hybridized for 45 minutes with antibody against CD31 to identify endothelial cells (Santa Cruz, dilution 1/20). The bound biotin-labeled antibodies were then detected by the addition of horseradish peroxidase–streptavidin conjugate and AEC substrate.

Mice Ischemic Hindlimb Model

Male C57Bl/d mice (Iffa Credo, Lyon, France) underwent surgery to induce unilateral hindlimb ischemia, as previously described. Animals were anesthetized by isoflurane inhalation. The ligature was performed on the proximal origin of the right deep femoral artery, just below the origin of the circumflex femoris lateralis.

Intramuscular Electrotransfer of Expression Plasmid Containing Growth Factor cDNA

Mice were injected at day 1 after femoral artery occlusion with 25 μg of expression plasmid coding for the human form of growth factor, as follows: pCOR-VEGF-B<sub>145</sub> (pXL3579, phVEGF-B<sub>145</sub>), pCOR-VEGF-B<sub>167</sub> (pXL3601, phVEGF-B<sub>167</sub>), and pCOR-VEGF-A<sub>165</sub> (pXL 3212, phVEGF-A<sub>165</sub>) and with the control empty plasmid, pCOR (pXL 3296, Gentell SA, France), into tibial cranial muscles of ischemic and nonischemic legs of the mouse (6 animals per group). Specific role of NO in VEGF-B–induced neovascularization was assessed by treatment with phVEGF-B<sub>167</sub> + NO synthase inhibitor (L-NAME, 10 mg/kg per day in the drinking water, Sigma). In an additional set of experiments, the specific role of VEGF-R1 was assessed by treatment with phVEGF-B<sub>165</sub> + Anti-R1 (neutralizing antibody against mouse VEGF-R1, 50 μg twice a week, IP, R&D Systems, n=4).

Quantification of Neovascularization

Microangiography

After 28 days of ischemia, vessels density was evaluated by high-definition microangiography, as previously described. Briefly, animals were anesthetized (isoflurane inhalation) and a contrast medium (barium sulfate, 1 g/mL) was injected through a catheter introduced into the abdominal aorta. The catheter was connected to a syringe placed on a pump (Harvard apparatus, PHD 2000), allowing constant perfusion pressure of 100 mm Hg. Images acquired by a digital radiograph transducer were assembled to obtain a complete view of the hindlimbs (see Figure 6). The angiographic score was expressed as a percentage of pixels per image occupied by vessels in the quantification area.

Arteriole and Capillary Densities

Microangiographic analysis was completed by assessment of capillary and arteriole densities. Ischemic and nonischemic gastrocnemius muscles were dissected and progressively frozen in isopentane solution cooled in liquid nitrogen. Sections (7 μm) were first incubated for 30 minutes in PBS containing 5% BSA at room temperature and then for 1 hour with either mouse monoclonal antibody directed against human smooth muscle actin α (dilution 1:50) to identify arterioles or with rabbit polyclonal antibody directed against total fibronectin (dilution 1:50) to identify capillaries. Arteriole immunohistochemistry was achieved by treating section with H<sub>2</sub>O<sub>2</sub> 3% and with a biotinylated secondary antibody with streptavidin-conjugate and AEC substrate. Capillaries were then identified by the addition of horseradish peroxidase–streptavidin conjugate and AEC substrate.
were calculated in five randomly chosen fields of a definite area for each animals using Optilab/Pro software.

Laser Doppler Perfusion Imaging
Ischemia-induced changes in vascularization were also assessed by Laser Doppler Perfusion Imaging, as previously described. Protein content was determined by the method of Bradford. The same amount of each proteic sample was then loaded on denaturing SDS/9% polyacrylamide gels. Membranes were stripped and stained with Ponceau Red for 10 minutes, as a second protein-loading control.

Determination of Protein Expression
Western Blot
Phospho-Akt, Akt, and eNOS protein expression were determined by Western blot in treated and untreated Matrigels and in ischemic and nonischemic legs, as previously described. Protein content was determined by the method of Bradford. The same amount of each proteic sample was then loaded on denaturing SDS/9% polyacrylamide gels. Membranes were stripped and stained with Ponceau Red for 10 minutes, as a second protein-loading control.

Determination of VEGF-R1 Phosphorylation
Aliquots of tissue lysates (containing 100-μg proteins) were pre-cleared for 1.5 hours at 4°C with 20 μL of protein A/G-agarose beads (Santa Cruz Biotechnology). Supernatants were incubated overnight with goat anti-mouse VEGF-R1 (8 μg/mL, AF471, R&D Systems). Protein A/G-agarose beads were then added for an additional 1.5-hour incubation at 4°C. Bound immune complexes were washed three times with lysate buffer. Immunoprecipitated proteins were eluted by boiling for 5 minutes in Laemmli sample buffer and loaded on a SDS-polyacrylamide gel for Western blot analysis. Phosphorylation of VEGF-R1 was then assessed by using antibody against the phospho-VEGF receptor-1 (Ab-2) (PC459, Bio-technological) or mouse anti-p-Tyr (PV99, sc-7020, Santa Cruz Biotechnology).

Immunohistochemistry
Frozen tissue sections (7 μm) were incubated with rabbit polyclonal antibody directed against VEGF-A (Santa-Cruz, dilution 1:25) and goat polyclonal antibodies directed against mouse VEGF-R1 (R&D Systems, dilution 1:30), mouse VEGF-R (R&D Systems, dilution 1:30), or mouse VEGF-B (R&D Systems, dilution 1:50). Immunostaining was visualized by using avidin-biotin horseradish peroxidase visualization systems (Vectastain ABC kit elite, Vector Laboratories) for VEGF-A and VEGF-B staining or by incubation with a fluorescent CY3 anti-goat antibody (Sigma, dilution 1:30) for VEGF-R1 and VEGF-R staining. Immunostains were then analyzed in randomly chosen fields of a definite area using Histolab software.

Zymography
MMP activity was analyzed by zymography, as previously described. Densitometric analysis was performed using NIH Image software.

Statistical Analysis
Results are expressed as mean±SEM. One-way ANOVA was used to compare each parameter. Post hoc Bonferroni’s t test comparisons were then performed to identify which group differences account for the significant overall ANOVA. χ² test was also performed to analyze the difference observed in the score established for semi-quantitative evaluation of angiogenesis (see Figure 2).

Results
VEGF-B–Induced Cell Ingrowth in the Matrigel Model
Quantification of Angiogenesis
Histological Score
The histological analysis showed that rhVEGF-B167 treatment increased cellular infiltration and proliferation in the plug (score 2 and 3) compared with control (score 1, P<0.01). Similarly, addition of rhVEGF-A165 enhanced the histological score compared with control (score 2 and 3, P<0.01). Furthermore, in rhVEGF-B167–treated Matrigel, the cells within the Matrigel formed numerous tube-like structures and the presence of erythrocytes was evidenced in the lumen, demonstrating the existence of a functional vascular structure (Figures 1 and 2).

Cellular Density
Histological data were confirmed by cellular density measurement. Cellular number was significantly higher by 2.0- and 2.5-fold in rhVEGF-B167–treated and rhVEGF-A165–treated Matrigel, respectively, compared with that of control (P<0.01) (Figure 2C).

Cell Type Determination in Matrigel Plug
To determine the type of cells involved in rhVEGF-B–induced cell migration and proliferation into the Matrigel, specific staining was performed in the different treated groups. Analysis of serial section stained with CD31 revealed that most cells were endothelial cells in the VEGF-B–treated Matrigel. The number of CD31-positive cells was increased by 80% and 90% in rhVEGF-B167–treated and rhVEGF-A165–treated Matrigel, respectively, compared with that of control (P<0.01) (Figures 1B and 2B). We also observed α actin smooth muscle cell staining; indicating presence of few smooth muscle cells and the development of a mature defined vascular structure (Figure 1B).

VEGF-B Angiogenic Effect Was Associated With Phospho-Akt/eNOS Pathway Activation
Protein level variations might result from the increase in cell number and subsequently might not mediate VEGF-B effect. To cope with changes in cell density, protein content variations are then expressed according to the number of cells within the Matrigel.

Phospho-Akt/Akt
In animals injected with Matrigel+rhVEGF-B167, phospho-Akt/Akt protein level was increased by 1.8-fold when compared with untreated plug (P<0.01). rhVEGF-A165 treatment also enhanced by 1.8-fold phospho-Akt/Akt protein content when compared with control (P<0.01) (Figure 3).

eNOS
rhVEGF-B167 angiogenic effect was also associated with variation in eNOS protein level. In animals injected with Matrigel+rhVEGF-B167, eNOS content was higher by 80% compared with that of untreated Matrigel (P<0.05). Similarly, rhVEGF-A165 treatment rose by 60% eNOS protein level in reference to that of untreated control (P<0.05) (Figure 3). Interestingly, treatment with NOS inhibitor hampered VEGF-B–induced cell ingrowth, indicating that eNOS mediated, in part, VEGF-B proangiogenic effect (Figures 1 and 2).

MMP Activity
The gelatin zymographic analysis revealed a lytic band at 62 kDa corresponding with the active form of MMP-2 and a lytic band of 72 kDa consistent with the proform of MMP-2. Gelatin zymographic analysis also revealed 92- and 82-kDa gelatinolytic activities consistent with the proform and active form of MMP-9, respectively. However, no significant changes in MMP-2 and MMP-9 activity were observed in either group (Figure 4).

VEGF-B Angiogenic Effect Was Mediated by VEGF-R1 Signaling
VEGF-R1-positive cells were present in the Matrigel plug, demonstrating that infiltrated cells expressed VEGF-R1 (Figure 1A). In addition, rhVEGF-B167 increased by 3.8-fold VEGF-R1 phosphorylation, supporting the hypothesis that VEGF-R1 mediates VEGF-B biological function (Figure 1C). In this view, treatment with VEGF-R1 neutralizing antibody completely hampered the rhVEGF-B167–induced VEGF-R1
phosphorylation and cell ingrowth (Figures 1C and 2, \( P<0.05 \) versus rhVEGF-B_{167}–treated animals). Treatment with VEGF-R neutralizing antibody did not affect rhVEGF-B_{167} proangiogenic effect. In addition, rhVEGF-B_{167}–induced increase in phospho-Akt and eNOS levels was blocked by VEGF-R1 neutralizing antibody (Figure 3, \( P<0.05 \) versus rhVEGF-B_{167}–treated animals) but not by VEGF-R1 neutralizing antibody. Treatments with VEGF-B and anti-R1 or anti-R did not affect the activity of MMP (Figure 4).
VEGF-B–Induced Neovascularization in Ischemic Hindlimb

**In Vivo Expression of VEGFs and Their Receptors**

After 7 days of ischemia, we showed that endogenous VEGF-B186 protein content was 1.6-fold increased in ischemic leg in reference to nonischemic leg of control animals (158±17% versus 100±12%, respectively; \( P < 0.05 \), \( n = 5 \)) (Figure 5A). We could not detect significant changes in VEGFB186 protein level after 28 days of ischemia (data not shown). In mice electrotransferred with phVEGF-B167, we showed that VEGFB167 protein content was raised by 4-fold in ischemic leg of treated animals as soon as 2 days after treatment, and VEGFB167 level was detectable up to 21 days after the treatment (Figure 5A). Finally, we also evidenced that after plasmid electrotransfer, VEGFB186 was mainly expressed in myocyte of ischemic tissue (Figure 5B).

We also performed VEGF-A immunostaining and demonstrated a marked VEGF-A staining, mainly in myocyte of ischemic legs (Figure 5B). Conversely, VEGF-R1–positive and VEGF-R–positive stainings were mainly localized in capillaries. We also demonstrated the presence of VEGF-R1–positive cells in arterioles (Figure 5B).

**Quantification of Neovascularization**

**Microangiography**

The ischemic/nonischemic leg angiographic score ratio was increased by 1.4- and 1.5-fold in phVEGF-B167–treated and phVEGF-B186–treated mice, respectively, compared with control \( (P < 0.05) \). Conversely, administration of phVEGF-A165 did not significantly affect the angiographic score (Figures 4 and 5). No significant changes were observed in the nonischemic hindlimb (data not shown).

**Capillary Density**

The ischemic/nonischemic leg capillary number ratio was enhanced by 1.7- and 1.6-fold in phVEGF-B167–treated and phVEGF-B186–treated mice, respectively, compared with control \( (P < 0.01 \) and \( P < 0.05 \), respectively). phVEGF-A165 administration increased by 1.5-fold the ischemic/nonischemic leg capillary number ratio in reference to control \( (P < 0.05) \) (Figures 4 and 5). No significant changes were observed in the nonischemic hindlimb (data not shown). Similar results were obtained with CD31 immunostaining, a specific staining of endothelial cells (data not shown).
Arteriole Density
The ischemic/nonischemic leg arteriole number ratio was enhanced by 1.8- and 1.7-fold in phVEGF-B167–treated and phVEGF-B186–treated mice, respectively, compared with control (*P<0.05). phVEGF-A165 administration raised by 1.4-fold the ischemic/nonischemic leg arteriole density ratio in reference to control, but this did not reach statistical significance (Figures 6C and 7).

Laser Doppler Perfusion Imaging
Microangiographic and vessels density measurements were associated with changes in blood perfusion. At day 28 of treatment, the ischemic/nonischemic foot blood perfusion ratio was increased by 1.5-fold in phVEGF-B167–treated and phVEGF-B186–treated mice, respectively, compared with control (P<0.05). phVEGF-A165 administration raised by 1.4-fold the ischemic/nonischemic leg arteriole density ratio in reference to control, but this did not reach statistical significance (Figures 6C and 7).

Molecular Mechanisms Associated With VEGF-B–Related Effects

Phospho-Akt/Akt
In the nonischemic legs, the phospho-Akt/Akt protein level was unaffected in either group. In the ischemic hindlimbs, phVEGF-B167 and phVEGF-B186 administration enhanced phospho-Akt/Akt protein content by 1.7- and 1.6-fold, respectively, over that of control (P<0.05). phVEGF-A165 also raised phospho-Akt/Akt ratio by 1.7-fold compared with control mice (P<0.05) (Figure 6).

eNOS
In the nonischemic legs, eNOS protein level was unaffected in either group. In contrast, in the ischemic hindlimbs, eNOS content was raised by 84% and 57% in phVEGF-B167–treated and phVEGF-B186–treated mice, respectively compared with control (P<0.05). In addition, phVEGF-A165 treatment enhanced eNOS protein content by 59% over that of control (P<0.05) (Figure 6). Treatment with NOS inhibitor abro-
gated VEGF-B–induced vessel growth, confirming that eNOS mediated, in part, VEGF-B proangiogenic effect (Figures 5 and 8).

MMP Activity
In the nonischemic legs, MMP-2 activity was unaffected in either group. In the ischemic hindlimbs, no significant changes in pro-MMP-2 activity were observed in phVEGF-B167–treated, phVEGF-B186–treated, and phVEGF-A165–treated mice in reference to control group (97 ± 11%, 103 ± 21%, and 94 ± 20% versus 100 ± 12%, respectively). Similarly, MMP-2 activity was unchanged (94 ± 23%, 97 ± 18%, and 104 ± 12% versus 100 ± 17% in phVEGF-B167–treated, phVEGF-B186–treated, and phVEGF-A165–treated mice compared with control group, respectively). In addition, we did not evidence any MMP-9 activity in either group (data not shown).

VEGF-B–Induced Neovascularization Was Mediated by VEGF-R1 Signaling
VEGF-R1 blockade totally hampered phVEGF-B167–induced raise in angiographic score, capillary and arteriole density, and limb perfusion (Figure 7), suggesting that VEGF-R1 mediates VEGF-B signaling in ischemia-induced revascularization reaction.

Discussion
The main results of this study are that VEGF-B promotes cell ingrowth in the Matrigel plug and neovascularization in ischemic hindlimb. The proangiogenic effect of VEGF-B is likely mediated by VEGF-R1 signaling and is associated with an activation of phospho-Akt and eNOS-related pathways.

We evidenced that VEGF-B induced cell ingrowth within the Matrigel plug. Because the Matrigel plug is initially avascular, vascular-like structures located within the Matrigel plug must be newly formed, reflecting the angiogenic process.19 This effect was associated with an increase in phospho-Akt and eNOS protein levels. In addition, blockade of VEGF-B–induced cell ingrowth by administration of NOS inhibitor suggests that eNOS mediates in part VEGF-B signaling. We also assessed VEGF-B angiogenic effect in another model of in vivo angiogenesis, the mice ischemic hindlimb model. We demonstrated that intramuscular administration of VEGF-B promoted neovascularization in the ischemic hindlimb. VEGF-B–related effect was associated with an activation of the proangiogenic Akt and eNOS-related pathways. We could not detect any significant differences between the proangiogenic effect of the two protein isoforms VEGF-B167 and VEGF-B186. Surprisingly, in the ischemic hindlimb model, phVEGF-A165 administration raised capillary density and blood flow but did not change angiographic score and arteriole number in the ischemic leg. The reason for this discrepancy is unclear. Nevertheless, previous studies indicate that delivery of VEGF-A in ischemic tissues rescues blood perfusion but induces formation of abnormal vascular structure and did not affect the number of precapillary arterioles and collateral arteries.22–25 In contrast, similar doses of the VEGFR-1–selective ligand placenta growth factor (PIGF) have been shown to raise angiographic

Figure 4. A, Representative gelatin zymographic analysis of protein extract from control and treated Matrigel. For each group, 50 μg of total proteins was used. B, Densitometric analysis of zymographic gels. Values are mean ± SEM, n = 7 per group. Abbreviations as in Figure 2.
score, number of collateral arteries, and limb perfusion in ischemic legs of treated animals. It is therefore likely that the VEGFR-1–selective ligands PlGF or VEGF-B are superior to VEGF-A to activate the neovascularization process and that higher doses of VEGF-A are required to affect the revascularization reaction to a similar extent. Alternatively, based on the fact that VEGFR-1 is the only VEGF receptor expressed on inflammatory cells, one putative explanation for this discrepancy would be that postischemic neovascularization of not only endothelial cells but also inflammatory cells is essentially required.

We also focused on the role of the activation of MMP as a putative molecular target in VEGF-B–induced angiogenesis. Nevertheless, we could not observe any significant changes in the activity of MMP after VEGF-B treatment, indicating that, in our experimental conditions, VEGF-B angiogenic effect is not related to changes in MMP-related pathway. Taken together, these results underscore that VEGF-B is a potential candidate in the regulation of the angiogenic process. The key role of Akt and eNOS signaling in VEGF-B proangiogenic effect is also highlighted. A strong correlation between angiogenesis, VEGF-A, Akt activation, and eNOS expression has already been extensively described; we can then hypothesize a putative common pathway involving VEGF family members and Akt/NO-related pathways in angiogenesis in vivo.

Figure 5. A, Representative Western blot of VEGFB186 protein level in control and phVEGFB186–treated mice. Mb indicates membrane stained with Ponceau Red as protein-loading control. B, Representative photomicrographs of ischemic muscle sections from control (VEGF-A, VEGF-R1, and VEGF-R stainings) or phVEGFB186–treated animals (VEGFB186 staining) stained with antibody directed against VEGFB186 (brown), VEGF-A (brown), VEGF-R1 (red), and VEGF-R (red).
VEGF-B binds specifically to VEGFR-1. We evidenced that VEGF-B–induced cell ingrowth and activation of the postischemic neovascularization reaction was blocked by VEGFR-1 neutralizing antibody. Similarly, PlGF and its receptor VEGF-R1 stimulate formation of mature vessels in the ischemic heart and enlarge collateral arterioles in the ischemic limb. Anti–VEGF-R1 therapy has also been shown to suppress neovascularization in tumor and ischemic retina. Taken together, these results suggest that VEGF-R1–mediated signaling may have a significant role in the angiogenic process.

VEGF-B and its receptor may thus constitute an alternative pathway for postnatal angiogenesis and may activate new vessel growth via several complementary mechanisms. VEGF-R1 has been shown to mediate monocyte migration and recruitment. Macrophages and T lymphocytes promote angiogenesis through the release of proinflammatory cytokines that heighten the production of MMPs, leading to matrix degradation, and through the expression of angiogenic factors, including VEGF and fibroblast growth factor-2. Hence, VEGF-B might regulate the angiogenic process by modulating the inflammatory reaction. Genetic truncation of the VEGF-R1 tyrosine kinase domains or antisense-mediated downregulation of VEGF-R1 suppresses tumor angiogenesis and VEGF-driven tumor angiogenesis. Similarly, VEGF-B–induced changes in phospho-Akt and eNOS protein levels was prevented by anti–VEGF-R1, suggesting that VEGF-R1 could also transmit its own intracellular angiogenic signals.

Homologous binding of VEGF-B to neuropilin 1, a receptor for collapsins/semaphorins, which functions as an isoform-specific receptor for VEGF-A165. Neuropilin-1 has been reported to enhance the mitogenic effect of VEGF-R on VEGF-A165 stimulation. Binding of VEGF-B to neuropilin 1 might also mediate biological responses of VEGF-B.

In conclusion, the present study demonstrates for the first time that VEGF-B and its receptor VEGF-R1 promote angiogenesis in association with an activation of Akt and eNOS-related pathways. Additional studies will be necessary to fully characterize the molecular events associated with such an effect. These findings also provide a rationale for evaluating VEGF-B as potential candidate for promoting therapeutic neovascularization of ischemic tissues.

Acknowledgments

This study was supported by grants from INSERM, Gencell SA, and Université Paris 7. R.T. was supported by a grant from Fondation pour la Recherche Médicale. The authors also thank AMRAD (Victoria, Australia) for providing rhVEGF-B167.

References


Figure 8. A, Top, Representative Western blot of phospho-Akt and Akt protein contents in ischemic leg 28 days after femoral artery occlusion. Bottom, Quantitative evaluation of phospho-Akt/Akt protein levels expressed as a percentage of non-ischemic control. Values are mean ± SEM, n = 6 per group. *P < 0.05 vs control rats. Abbreviations as in Figure 5. Mb indicates membrane stained with Ponceau Red as protein-loading control.

B, Top, Representative Western blot of eNOS protein content in ischemic leg 28 days after femoral artery occlusion. Bottom, Quantitative evaluation of eNOS protein levels expressed as a percentage of non-ischemic control. Values are mean ± SEM, n = 6 per group. *P < 0.05 vs control rats.


Vascular Endothelial Growth Factor-B Promotes In Vivo Angiogenesis
Jean-Sébastien Silvestre, Radia Tamarat, Teni G. Ebrahimian, Aude Le-Roux, Michel Clergue, Florence Emmanuel, Micheline Duriez, Bertrand Schwartz, Didier Branellec and Bernard I. Lévy

Circ Res. published online June 12, 2003;

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2003/06/12/01.RES.0000081594.21764.44.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/