Vascular Endothelial Growth Factor-B Acts as a Coronary Growth Factor in Transgenic Rats Without Inducing Angiogenesis, Vascular Leak, or Inflammation

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Background—Vascular endothelial growth factor-B (VEGF-B) binds to VEGF receptor-1 and neuropilin-1 and is abundantly expressed in the heart, skeletal muscle, and brown fat. The biological function of VEGF-B is incompletely understood.

Methods and Results—Unlike placenta growth factor, which binds to the same receptors, adeno-associated viral delivery of VEGF-B to mouse skeletal or heart muscle induced very little angiogenesis, vascular permeability, or inflammation. As previously reported for the VEGF-B167 isoform, transgenic mice and rats expressing both isoforms of VEGF-B in the myocardium developed cardiac hypertrophy yet maintained systolic function. Deletion of the VEGF receptor-1 tyrosine kinase domain or the arterial endothelial Bmx tyrosine kinase inhibited hypertrophy, whereas loss of VEGF-B interaction with neuropilin-1 had no effect. Surprisingly, in rats, the heart-specific VEGF-B transgene induced impressive growth of the epicardial coronary vessels and their branches, with large arteries also seen deep inside the subendocardial myocardium. However, VEGF-B, unlike other VEGF family members, did not induce significant capillary angiogenesis, increased permeability, or inflammatory cell recruitment.

Conclusions—VEGF-B appears to be a coronary growth factor in rats but not in mice. The signals for the VEGF-B–induced cardiac hypertrophy are mediated at least in part via the endothelium. Because cardiomyocyte damage in myocardial ischemia begins in the subendocardial myocardium, the VEGF-B–induced increased arterial supply to this area could have therapeutic potential in ischemic heart disease. (Circulation. 2010;122:1725-1733.)

Key Words: angiogenesis ■ coronary disease ■ hypertrophy

Coronary artery disease leads to compromised myocardial blood supply and the typical symptoms of stress-induced angina. Although pharmaceutical therapy and revascularization of stenotic epicardial coronary arteries are the standard therapy for coronary artery disease, many patients with advanced disease or small-vessel disease respond poorly to these treatments. Novel therapeutic strategies for promoting collateral artery formation, or arteriogenesis, are in high demand. Although vascular endothelial growth factor (VEGF) is the most potent angiogenic factor for possible therapy of myocardial ischemia, it also promotes vascular leakage, inflammation, and the formation of angioma-like vascular structures, which has hampered its utility in therapeutic angiogenesis. Among the VEGF family members, placenta growth factor (PlGF) has shown the most promise for promoting therapeutic arteriogenesis in preclinical studies.

Clinical Perspective on p 1733

VEGF-B, isolated in 1995, has been an exceptional member of the VEGF family in that efforts to discover a blood vascular function for VEGF-B have had largely nega-
tive results. VEGF-B knockout (KO) mice are viable and display at most mild cardiac phenotypes such as a slightly smaller heart size in 1 genetic background or a mild delay in atrioventricular coupling in another strain. Transgenic (TG) or adenoviral overexpression of VEGF-B in the skin or skeletal muscle increased blood vessel density only minimally. Interestingly, however, strong overexpression of VEGF-B by adenoviral transduction induced heart-specific capillary dilation and increased collateral blood vessel growth after myocardial infarction in pigs.

VEGF-B is expressed as 2 RNA splice isoforms. Recently, we demonstrated that when overexpressed in the mouse heart, the heparin-binding VEGF-B isoform induced cardiac hypertrophy without overt angiogenesis, although it caused mild enlargement of myocardial blood capillaries. The other isoform, VEGF-B, is O-glycosylated, proteolytically processed, and freely diffusible. Both isoforms bind to and activate VEGF receptor-1 (VEGFR-1) and neuropilin-1. Studies have indicated that the VEGF-B isoforms are expressed simultaneously in various tissues. PIGF binds to the same receptors as VEGF-B and has been shown to promote angiogenesis and arteriogenesis in pathological conditions.

The aim of this study was to investigate the mechanisms of VEGF-B action in the heart in more depth. A critical question about the effects of VEGF-B in the myocardium is how they differ from those of PIGF and whether VEGF-B could provide a means to improve myocardial function in the failing heart. Because adenoviral transduction in an immunocompetent host is short-lived and results in nonspecific inflammation, we have instead used adenoviral and TG delivery of VEGF-B to the mouse and rat heart.

**Methods**

A detailed description of the methods used is provided in the online-only Data Supplement.

**Construction and Preparation of the Recombinant Adeno-Associated Virus Vectors**

Mouse VEGF, PIGF, VEGF-B, and human serum albumin complementary DNAs were cloned into the psbCMV-WPRE recombinant adeno-associated virus (AAV) expression vector. AAV particles were injected into tibialis anterior muscles or the left ventricle.

**Generation of αMHC–VEGF-B TG Mice and Rats**

A fragment of the human VEGF-B gene and the mouse VEGF-B promoter expression vector (a kind gift from Dr Jeffrey Robbins). TG animals were generated by microinjection of fertilized oocytes from FVB/N mice or HsdBr:WH Wistar rats. All animal experiments were approved by the Provincial State Office of Southern Finland and carried out in accordance with institutional guidelines.

**Immunohistochemistry, Microscopy, and Image Analysis**

The antibodies and methods used are detailed in the online-only Data Supplement.

**Blood Pressure Measurements and Echocardiography**

Blood pressure was measured with the CODA Non-Invasive Blood Pressure System for Mice and Rats (Kent Scientific Corp, Torrington, Conn). Transthoracic echocardiography was performed with an Acuson Sequoia 512 Ultrasound System and an Acuson Linear 15L8 transducer (Siemens Medical Solutions, Mountain View, Calif).

**Micro–Computed Tomography Imaging of the Cardiac Vessels**

Coronary angiographies were performed with the Inveon micro–computed tomography scanner (Siemens, Knoxville, Tenn). The ascending aorta was cannulated and clamped, and iodinated intravascular contrast agent eXIATM160XL (Binitio Biomedical Inc, Ottawa, Ontario, Canada) was carefully injected manually to fill the cardiac blood vessels, avoiding very high pressure.

**Assessment of Myocardial Perfusion, Oxygen Consumption, and Efficiency of Work**

Eight rats were given a slow bolus of 30±24 MBq of [11C]acetate and imaged with the Inveon micro–positron emission tomography scanner (Siemens, Knoxville, Tenn).

**Statistical Analysis**

Values are presented as mean±SD unless otherwise indicated. Statistical analysis was performed with 1-way ANOVA (post hoc with Tukey test) or with the 2-tailed unpaired Student t test unless otherwise specified in the Results. Differences were considered statistically significant at P<0.05.

**Results**

Unlike PIGF, VEGF-B Fails to Induce Capillary Angiogenesis or Arteriogenesis in Mouse Skeletal or Cardiac Muscle

To compare the effects of VEGF-B and PIGF, which bind to VEGFR-1 and neuropilin-1, we injected recombinant AAVs encoding VEGF-B, VEGF-B, and PIGF, as well as VEGF and human serum albumin as positive and negative controls, respectively, into mouse tibialis anterior muscles. Immunofluorescence staining of the muscles for platelet endothelial cell adhesion molecule (PECAM)-1 and α-smooth muscle actin (SMA) 4 weeks after injection indicated strong capillary angiogenesis with primarily smooth muscle cell–coated vessels in PIGF-injected muscles, whereas no evidence of angiogenesis was seen in muscles overexpressing either VEGF-B isoform (Figure 1). In the VEGF-injected muscles, endothelial cell proliferation resembled angioma formation (Figure 1). As expected on the basis of prior work with adenoviral vectors, PIGF and VEGF increased blood perfusion in the muscles, whereas even a combination of VEGF-B and VEGF-B had no effect on perfusion (Figure IA in the online-only Data Supplement). Interestingly, Evans Blue dye injection experiments indicated that although PIGF and VEGF increased vascular permeability, VEGF-B and VEGF-B did not (Figure IB in the online-only Data Supplement). Similar effects were obtained when the same AAV vectors were expressed in the myocardium (Figure II in the online-only Data Supplement). Importantly, mice injected with PIGF or VEGF vectors had to be...
euthanized within 2 weeks because of edema, whereas the VEGF-B–expressing mice showed no signs of distress (data not shown).

VEGF-B, in Contrast to PlGF, Does Not Induce Inflammation in Skeletal or Cardiac Muscle

Immunofluorescence staining of sections from mouse skeletal muscles transduced with the AAVs encoding PlGF or VEGF revealed marked infiltration of CD45-positive leukocytes and especially F4/80-positive macrophages. In contrast, overexpression of either VEGF-B isoform could at best only mildly increase macrophage infiltration (Figure IIIA and IIIB in the online-only Data Supplement). A similar difference was observed in parallel experiments in which the myocardium was transfected (Figure IIIC in the online-only Data Supplement).

Both Mice and Rats Overexpressing the Human VEGF-B Gene in the Myocardium Exhibit Cardiac Hypertrophy

To test the therapeutic potential of VEGF-B both in mice and in a larger animal model, we adopted a gain-of-function approach. Because several studies have indicated that both VEGF-B isoforms are simultaneously expressed,14 we overexpressed the full-length human VEGF-B gene under the αMHC promoter in mice and rats. The schematic structure of the transgene is shown in Figure IVA in the online-only Data Supplement. Analysis of the TG mice and rats at 3 to 6 months of age indicated robust transgene expression in Western blot analysis of the heart lysates (Figure IVC in the online-only Data Supplement and data not shown). As can be seen from that figure, all 5 TG rat founder hearts contained both full-length and processed VEGF-B167 polypeptides (32 and 14 kDa, respectively), as well as VEGF-B186 polypeptides (22 kDa). Immunofluorescence staining for VEGF-B in the rat hearts demonstrated a mosaic pattern of expression in the cardiomyocytes (TG2), except for the highest expressing founder (TG3), which showed staining in all cardiomyocytes (Figure IVE in the online-only Data Supplement). Three of these founder lines (TG2 through TG4) were used in subsequent experiments with essentially similar results.

Both the TG mice and rats developed cardiac hypertrophy, with an increased ratio of heart to body weight and cardiomyocyte size as determined by staining of the cardiomyocyte plasma membrane with anti-dystrophin antibodies at 4 to 5 months of age (Figure 2A through 2C). Echocardiographic analysis of the TG rat hearts revealed a significantly increased left ventricular wall thickness at 5 months of age (Figure 2D and the Table), which was associated with a decrease in end-systolic volume (the Table). Interestingly, however, the TG rats maintained heart function, as shown by analysis of the ejection fraction and fractional shortening (the Table). In addition, the TG rats tended to have lower blood pressure and heart rate than wild-type (WT) controls (Figure 2E and the Table). No degenerative changes comparable to those seen previously in αMHC–VEGF-B167 mouse hearts12 were seen in the rat cardiomyocytes at 4 months of age in electron microscopy or in older rats, whereas cardiomyocyte damage and fibrosis were evident in light microscopy of 1-year-old αMHC–VEGF-B mice (Figure V in the online-only Data Supplement and data not shown).

The VEGF-B Transgene Induces Strong Coronary Arteriogenesis in Rats

A striking observation made in the analysis of histochemically stained sections of TG rat, but not mouse, hearts was the presence of numerous large arteries of nearly the caliber of epicardial coronary arteries (Figure 3A and 3D, arrowheads). They were located deep in the myocardium and often close to the endocardium, whereas similarly sized arteries were seen only on the epicardial side in WT hearts (Figure 3D, arrow). The total number of arteries as analyzed by Masson trichrome staining for the adventitial layer was significantly increased in TG heart sections at 4 months of age, especially in the subendocardial myocardium (Figure 3A through 3D). The number of arteries in TG rat hearts was significantly increased also at 6 to 8 weeks of age (83±14 total arteries per heart section in αMHC–VEGF-B rat hearts versus 60±2 arteries per heart section in WT hearts; n=6 in both groups; P<0.005).
The average arterial vessel area was significantly increased in the TG heart sections (Figure 3C), and the arterial phenotype was also associated with dilation of the epicardial veins (Figure 3A, arrows). Consistent with the arteriogenesis, VEGF-B induced a number of cytokines that have been associated with angiogenesis and tissue remodeling. Notably, VEGF-B increased the expression of the delta-like 4 (Dll4) notch ligand, which has been associated with arteriogenesis (Figure VI in the online-only Data Supplement). We also saw the induction of plasminogen activator inhibitor (PAI)-1, which is involved in matrix remodeling and, for example, myoendothelial junction formation. Immunostaining revealed that PAI-1 was expressed mainly in the arterial walls (Figure 4C). Resorcin Fuchsin staining for elastin indicated that most of the smooth muscle cell proliferation had occurred in the media of the arteries of 1-month-old VEGF-B TG hearts, whereas some fragmentation of the internal elastic lamina was seen in many of the arteries from 4-month-old TG rats (Figure 3E, white arrowheads). In line with the results from the AAV-transfected mice, immunostaining with antibodies against the macrophage marker ED-1 did not indicate significant inflammatory cell infiltration (39 ± 4 cells in WT heart sections; P > 0.05, n = 5 in both groups). No increase in fibrosis could be seen in Masson trichrome staining of the hearts at 1 or 4 months (Figure 3D and data not shown).

Immunofluorescent SMA staining confirmed the presence of a thick periendothelial smooth muscle cell layer in the TG arteries (Figure 4A and B). In contrast, staining for the rat endothelial cell antigen RECA-1 did not reveal increased blood capillary density (immunostained area, 11.7 ± 2.9 cells in TG versus 10.2 ± 1.3 cells in WT heart sections; P > 0.05, n = 5 in both groups). However, similar to previous observations in TG mice,12 we could observe some increase in mean capillary vessel luminal areas (data not shown).

To analyze whether the myocardial arteries in the TG hearts were functional and connected to the coronary arterial tree, we performed contrast-enhanced micro–computed tomography angiography of the rat hearts. The coronary arteries and veins and the epicardial vessels were found in anatomically normal locations, but remarkably, the number and size of coronary vessel branches were strikingly increased in TG hearts compared with controls (Figure 5, arrows), with large vessels also seen deep inside the myocardium (Figure 5, arrowhead, and data not shown). In vivo positron emission tomography showed homogeneous uptake of $^{18}$F-labeled and $^{11}$C-labeled microspheres throughout the left ventricular myocardium of the rats. The average myocardial perfusion was comparable in the TG and

### Table. Echocardiography of Cardiac Dimensions and Blood Pressure Analysis of Transgenic Rats and Wild-Type Controls

<table>
<thead>
<tr>
<th></th>
<th>TG</th>
<th>WT</th>
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<tbody>
<tr>
<td>LVPWTD, mm</td>
<td>2.4 ± 0.3†</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>IVSTD, mm</td>
<td>1.7 ± 0.2</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>LV EF, %</td>
<td>83 ± 8†</td>
<td>76 ± 7</td>
</tr>
<tr>
<td>LV FS, %</td>
<td>48 ± 12</td>
<td>41 ± 9</td>
</tr>
<tr>
<td>ESV, mL</td>
<td>0.12 ± 0.09†</td>
<td>0.19 ± 0.07</td>
</tr>
<tr>
<td>SV, mL</td>
<td>0.55 ± 0.14</td>
<td>0.59 ± 0.08</td>
</tr>
<tr>
<td>EDV, mL</td>
<td>0.68 ± 0.21</td>
<td>0.78 ± 0.12</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>345 ± 65</td>
<td>377 ± 27</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>98 ± 10</td>
<td>104 ± 14</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>138 ± 12</td>
<td>144 ± 16</td>
</tr>
</tbody>
</table>

LVPWTD indicates diastolic left ventricular posterior wall thickness; IVSTD, diastolic interventricular septal thickness; LV, left ventricle; EF, ejection fraction; FS, fractional shortening; ESV, end-systolic volume; SV, stroke volume; EDV, end-diastolic volume; and BP, blood pressure.

*Under anesthesia.
†P < 0.05; ‡P < 0.005; ††P < 0.0005; n = 12 in each group.
WT rats (K1, 5.1±0.4 versus 5.0±0.7 per minute; n=4 in each group; P=0.86). TG and WT rats also demonstrated comparable myocardial oxygen consumption (Kmono, 0.50±0.11 versus 0.58±0.06 per minute; P=0.24) and efficiency of work (154±71 versus 138±42 mm Hg · L−1 · g−1; P=0.69).

Neuropilin-1 Does Not Transduce VEGF-B Signals Essential for the Hypertrophic Response

To establish the VEGF-B signal transduction pathway involved in the hypertrophy response, we first concentrated on the 2 receptors of VEGF-B in mice. A C-terminally truncated form of VEGF-B containing the first 5 exons and thus lacking the isoform-specific sequences and therefore also the neuropilin-1– and heparin-binding domain15 was expressed in mouse heart (αMHC–VEGF-B167; Figure IVB and IV in the online-only Data Supplement). Analysis of the TG hearts indicated that they undergo a degree of hypertrophy similar to that of hearts overexpressing the full-length gene or the indicated that they undergo a degree of hypertrophy similar to the online-only Data Supplement). Analysis of the TG hearts quantified with the aid of Masson trichrome (TRI) staining of the vessel adventitia. *P<0.05; **P<0.005. C, The average arterial vessel area in VEGF-B–overexpressing rats (n=11) and controls (n=10). D, Higher-magnification heart sections stained with Herovici and Masson trichrome. Arrowheads indicate arteries in TG rats compared with a similarly sized epicardial artery in a WT rat (arrow). Scale bar=400 μm. Asterisks indicate the left ventricle. E, Representative images of Resorcin Fuchsin–stained elastin in heart sections at 1 and 4 months of age. White arrowheads indicate fragmentation of the internal elastic lamina of subendocardial arteries in the 4-month-old TG heart. The asterisk indicates the left ventricle. Scale bar=200 μm.

Deletion of the VEGFR-1 Tyrosine Kinase Domain Attenuates the Hypertrophic Effect of VEGF-B

To analyze the role of VEGF-B signal transduction in the VEGF-B–induced cardiac hypertrophy, we mated the αMHC–VEGF-B167 TG mice with mice having a deletion of the VEGFR-1 tyrosine kinase domain (VEGFR-1 TK−/−) but no obvious phenotype.20 Analysis of offspring 3 to 4 months after birth revealed that cardiac overexpression of VEGF-B had no effect on ratios of heart to body weight in VEGFR-1 TK−/− mice (heart to body weight, 0.49±0.04% in αMHC–VEGF-B167;VEGFR-1 TK−/− [n=8] mice versus 0.48±0.04 in VEGFR-1 TK−/− mice [n=4]; P>0.5), indicating that VEGF-B does not induce hypertrophy in mice lacking the VEGFR-1 tyrosine kinase domain. However, as previously observed, the ratios of heart to body weight of VEGF-B–overexpressing VEGFR-1–WT mice were increased very significantly compared with non-TG VEGFR-1–WT mice (heart to body weight, 0.53±0.04% in αMHC–VEGF-B167;Bmx −/0, WT, and Bmx −/0 (KO) mice. At the age of 3 to 3.5 months, when the hypertrophic phenotype of αMHC–VEGF-B167 mice is apparent,12 the mice were euthanized and the heart tissues analyzed. Loss of Bmx attenuated the cardiac hypertrophy induced by VEGF-B (Figure 6A and 6C). In addition, the average cardiomyocyte area was significantly reduced in the αMHC–VEGF-B167;Bmx KO hearts compared with the αMHC–VEGF-B167 hearts (Figure 6B and 6D). These results indicate that Bmx mediates at least some of the signals for the VEGF-B–induced hypertrophy via the arterial endothelium.

Loss of Bmx Reduces VEGF-B–Induced Cardiac Hypertrophy

The cytoplasmic bone marrow kinase in chromosome X (Bmx) is expressed in the arterial endothelium and myeloid cell lineage; its deletion from mice does not lead to any apparent phenotype,21 but interestingly protects mice from cardiac hypertrophy induced by aortic constriction.22 To evaluate whether Bmx deficiency inhibits the VEGF-B–induced hypertrophy, we crossed Bmx gene–targeted mice with the αMHC–VEGF-B167 mice. We then compared mice of 4 genetic backgrounds: αMHC–VEGF-B167, αMHC–VEGF-B167;Bmx −/0, WT, and Bmx −/0 (KO) mice. At the age of 3 to 3.5 months, when the hypertrophic phenotype of αMHC–VEGF-B167 mice is apparent,12 the mice were euthanized and the heart tissues analyzed. Loss of Bmx attenuated the cardiac hypertrophy induced by VEGF-B (Figure 6A and 6C). In addition, the average cardiomyocyte area was significantly reduced in the αMHC–VEGF-B167;Bmx KO hearts compared with the αMHC–VEGF-B167 hearts (Figure 6B and 6D). These results indicate that Bmx mediates at least some of the signals for the VEGF-B–induced hypertrophy via the arterial endothelium.
Discussion

In the present study, we show that VEGF-B induces a striking growth of the coronary vascular tree in the rat, but not mouse, heart and promotes myocardial hypertrophy in both species. Importantly, VEGF-B, unlike PlGF or VEGF, did not significantly increase vascular permeability or inflammatory cell recruitment into cardiac or skeletal muscle. The VEGF-B–induced hypertrophy did not compromise myocardial contractile function at least in rats up to 5 months of age, and the impressive arteriogenesis was not associated with intimal thickening, although the internal elastic lamina of the arteries had undergone some pathological changes in older rats.

It has previously been shown that myocardial hypertrophy in the absence of other stimuli can be induced by angiogenesis in mice. Heart-specific overexpression of both VEGF-B isoforms in mice and rats reproduced the hypertrophic phenotype previously seen in αMHC–VEGF-B167 mice. Both the TG mice and rats had significantly increased ratios of heart to body weight and hypertrophy of the cardiomyocytes. Even though increases in capillary density were not observed in the TG animals, it is conceivable that endothelium-derived functions activated by VEGF-B mediate the cardiac hypertrophy. Echocardiography confirmed the increased left ventricular mass in the TG rats and, similar to our previous analysis of mice, showed that despite circumferential hypertrophy, VEGF-B overexpression did not compromise systolic function. Indeed, VEGFR-1 activation by VEGF-B has been found to elicit a gene expression profile typical of the compensatory, hypertrophic response both in cultured cardiomyocytes and in infarcted hearts. In mice, the hypertrophy ultimately resulted in exhaustion of triglycerides and accumulation of toxic lipid species, resulting in mitochondrial autophagy/lysis and cardiomyopathy (Reference 12 and our unpublished observations). The cardiomyocytes in αMHC–VEGF-B rats, however, have not shown signs of lipotoxic damage, perhaps because of a preserved perfusion of the hypertrophic myocardium induced by the strong coronary arteriogenesis in this species. Importantly, functioning arteries are essential for the sufficient perfusion of tissues.

In contrast to VEGF-B, PlGF strongly increased vascular permeability. In addition, a novel snake venom VEGF has been shown to induce vascular permeability through preferential signaling via VEGFR-1. Another major difference observed between VEGF-B and PlGF was the lack of inflammatory cell recruitment into skeletal or cardiac muscle by VEGF-B. In previous work, the recruitment of bone marrow–derived cells, mainly monocytes/macrophages, correlated with arteriogenesis, and this required the neuropilin-1–binding domain of VEGF. The hypertrophic effects of VEGF-B did not require activation of neuropilin-1; overexpression of a truncated form of VEGF-B capable of binding to VEGFR-1 but lacking the neuropilin-1–binding domain induced significant cardiac hypertrophy. On the other hand, deletion of the VEGFR-1

Figure 4. Immunofluorescence staining of thick cardiac sections. A, Tile scan images of thick cardiac sections stained for SMA. Arrowheads indicate large subendocardial arteries in VEGF-B TG hearts. Scale bar=1 mm. LV indicates left ventricle; RV, right ventricle. B, Representative images of SMA- and RECA-1–stained cardiac sections. Scale bar=200 μm. C, Representative images of SMA- and PAI-1–stained cardiac sections. Arrows indicate colocalization of SMA and PAI-1. Scale bar=200 μm.
tyrosine kinase domain in the VEGF-B TG mice inhibited hypertrophy induced by VEGF-B, indicating that VEGFR-1 signaling provides the major pathway. This finding is of interest because the VEGFR-1 tyrosine kinase function seems dispensable for normal vascular development.27

Strikingly, in the TG rats but not in the mice, VEGF-B induced a remarkable growth of large arterial blood vessels that were found deep in the subendocardial myocardium, without causing capillary proliferation. These arteries were often larger than or equal in size to epicardial coronary arteries in control animals and were covered by a thick smooth muscle cell wall, with additional cell layers in the arterial media. Three-dimensional micro–computed tomography angiography suggested that the myocardial arteries formed part of an extended coronary arterial tree continuous with the epicardial coronary vessels, which in the TG hearts had larger and more numerous branches. The resting myocardial perfusion, oxygen consumption, and efficiency of work were preserved in the TG rats. Thus, VEGF-B induced a strong arteriogenic response in the coronary vessels, which was also associated with the upregulation of Dll4 in the TG rat hearts. Importantly, this occurred without capillary angiogenesis, vascular leakage, or inflammation. Increased PAI-1 protein was detected in the TG rat hearts, which likely resulted from the arterial growth because PAI-1 was expressed mainly in the arterial walls. A recent report has also indicated the importance of PAI-1 in myoendothelial junction formation.19 Further studies in animals with atherosclerosis or myocardial infarction are needed to demonstrate the potential beneficial effects of arteriogenesis on myocardial function.

We do not yet know why the human VEGF-B gene induces coronary arteriogenesis in rat but not mouse hearts. However, it is interesting that the results we obtained in the rats are almost a mirror image of those reported by Bellomo et al3 in the VEGF-B gene–targeted mice that developed slightly smaller hearts than wild-type mice during the first postnatal month. In vitro perfusion experiments of the VEGF-B gene–targeted mice indicated that their isolated hearts display vascular dysfunction after coronary occlusion and impaired recovery from experimentally induced myocardial ischemia.8 This finding and our present data warrant detailed analysis of the arteriogenic potential of VEGF-B in the rat heart in utero, during early postnatal coronary maturation, and in the adult heart. It is also interesting to note that blockage of VEGFR-1 signal transduction by use of a soluble form of the receptor that captures VEGF, VEGF-B, and PlGF leads to myocardial stunning or hibernation, which has been described in chronic myocardial ischemia.38 On the other hand, VEGF-B167 was reported to exert a powerful antiapoptotic effect on cardiomyocytes both in cell culture and in vivo after myocardial infarction.24 In addition, VEGF-B167 was also shown to preserve cardiac function in dogs developing tachypacing-induced dilated cardiomyopathy and to prevent oxidative...
stress and loss of mitochondrial membrane potential in neonatal rat cardiomyocytes exposed to angiotensin II. These results suggest important cardioprotective roles for VEGF-B in heart failure.

Recent reports indicate that pressure overload–induced cardiac hypertrophy generated by thoracic aortic constriction in mice is mediated at least in part through Bmx tyrosine kinase, which is expressed in the atrial endocardium and arterial endothelium. Furthermore, previous in vitro data from our laboratory show that Bmx phosphorylation can be stimulated by activated VEGFR-1, indicating a role in the downstream signaling of this receptor. Here, we show that Bmx deletion also reduces the cardiac hypertrophy induced by VEGF-B overexpression. These results provide the first evidence for the interaction between the VEGF-B and Bmx signaling pathways in vivo. Interestingly, the rescue of the phenotype in the Bmx KO background implicates the arterial endothelium as an important source for the hypertrophic signals. However, further studies need to be performed to improve our understanding of the mechanisms involved.

The recent findings that VEGFR-1 activation by VEGF-B increases cardiac mass and promotes maintenance of cardiac contractility over time have obvious therapeutic implications. Prolonged cardiac hypertrophy leads to diastolic insufficiency, whereas concurrent cardiac angiogenesis in such conditions has been shown to be important for preservation of cardiac function. VEGF-B may provide this essential function, as we demonstrate that overexpression of both isoforms of VEGF-B in the rat heart induces concurrent myocardial growth and growth of arteries that penetrate into the myocardium. Importantly, VEGF-B does not promote vascular leakage or tissue inflammation, both of which have in part compromised previous attempts to use growth factors of the VEGF family in therapeutic angiogenesis. Thus, VEGF-B seems to be a more promising therapeutic candidate than PlGF or VEGF for patients with myocardial ischemia.

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Disclosures
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References


CLINICAL PERSPECTIVE

Coronary heart disease is the leading cause of death in the Western world. Although pharmaceutical therapy and revascularization of stenotic coronary arteries are the standard therapy, many patients with advanced disease or small-vessel disease respond poorly to these treatments. Novel therapeutic strategies for promoting collateral artery formation, or arteriogenesis, are in high demand. Although vascular endothelial growth factor (VEGF) is the most potent angiogenic factor for possible therapy of myocardial ischemia, VEGF also promotes vascular leakage and inflammation, hampering its therapeutic utility. Our present findings show that VEGF-B, a hitherto poorly understood member of the VEGF family, acts as a coronary growth factor in the rat heart, stimulating the growth of epicardial vessels and large arteries deep inside the myocardium. Because cardiomyocyte damage in myocardial ischemia begins in the subendocardial myocardium, the VEGF-B–induced arterial supply to this area could have great therapeutic potential in ischemic heart disease. VEGF-B also does not cause increased vascular permeability or inflammatory cell recruitment, making it a promising candidate for therapeutic translation.
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SUPPLEMENTAL MATERIAL

Supplementary Methods

Construction and preparation of the recombinant adeno-associated virus (AAV) vectors. Mouse VEGF\textsubscript{120}, PlGF, VEGF-B\textsubscript{167}, VEGF-B\textsubscript{186} and human serum albumin (HSA) cDNAs were cloned into blunted MluI and NheI restriction sites of the psubCMV-WPRE recombinant AAV expression vector\textsuperscript{1}. The AAVs (serotype 9) were produced as described previously\textsuperscript{2}. Six to seven-week-old female FVB/NJ, ICR and C57Bl/6J mice were anesthetized with xylazine (Rompun, Bayer)-ketamine (Ketalar, Pfizer), and $5 \times 10^{10}$ AAV particles (in 30 µl volume) were injected into \textit{tibialis anterior} muscles. In parallel experiments, $2 \times 10^{11}$ AAV particles (in 120 µl volume) were injected into the left ventricle.

\textit{In vivo} ultrasound measurement of perfusion in transduced muscles. One \textit{tibialis anterior} muscle per mouse was injected as above and expression continued for four weeks. Values are presented as ratios between pools of treated muscles and a pool of untreated muscles (VEGF-B, \textit{n}=6; PlGF, \textit{n}=6; VEGF, \textit{n}=6; HSA, \textit{n}=3). The mice were anesthetized, and perfusion in \textit{tibialis anterior} muscles was measured with an Acuson Sequoia 512 ultrasound apparatus (Siemens). Intensities of the ultrasound signals were analyzed with the Datapro 2.13 (Noesis) program.

Evans Blue permeability assay. Mouse \textit{tibialis anterior} muscles were injected with AAVs encoding mouse VEGF-B (both isoforms, \textit{n}=3), PlGF (\textit{n}=5), VEGF (\textit{n}=4), HSA (\textit{n}=7), or were left untreated (\textit{n}=7). Eight weeks later, mice were anesthetized with xylazine-ketamine, and 300 µl of 1% Evans Blue-PBS was injected into the left ventricle. The dye was allowed
to circulate for 30 min, after which the mice were euthanized and both tibialis anterior muscles dissected. The extravasated dye was released by incubating the muscles in formamide overnight at 55°C and quantitated by spectrophotometry at 620 nm.

**Generation of αMHC-VEGF-B transgenic mice and rats.** A fragment of the human VEGF-B gene (corresponding to nucleotides 745-5059 of Genbank accession number AF468110) was isolated from the K14-VEGF-B construct and cloned into the alpha myosin heavy chain (αMHC) promoter expression vector (a kind gift from Dr. Jeffrey Robbins). Transgenic animals were generated by microinjection of fertilized oocytes from FVB/N mice or outbred HsdBrL:WH Wistar rats. Positive founder animals were identified using PCR analysis of ear biopsies taken at the time of weaning. The primer pair 5’-TCAGAGAGGTGGTGAAGCCT-3’ and 5’-CTCCTCACTGGTTTTCCTGC-3’ was used for genotyping. All animal experiments were approved by the Provincial State Office of Southern Finland and carried out in accordance with institutional guidelines.

**Generation of αMHC-mVEGF-Bex1-5 mice.** The mVEGF-Bex1-5 fragment (last four coding amino acids: VKPD) was isolated from a mVEGF-Bex1-5-pSubCMV-WPRE vector with MluI and blunted. The fragment was cloned into the αMHC promoter expression vector, digested with SalI and blunted with Klenow. The plasmid was further digested with BamHI, and the fragment was purified and injected into fertilized mouse oocytes of the FVB/N background. The primers 5’-CCAGAAATGACAGACAGATCC-3’ and 5’-GCTTCTAGTTAGTCAGTCGACG-3’ were used for genotyping.

**Western blotting of cardiac extracts.** Corresponding pieces of the heart were sliced into small pieces, mixed with 1 mL RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% DOC,
0.1% SDS, 50 mM Tris pH 8.0, 20 μg/mL leupeptin, 3.4 μg/mL aprotinin, 1 mM Na$_3$VO$_4$, 1 mM PMSF) in Lysing Matrix tubes (MP Biomedicals) and homogenized. The total protein concentrations were measured using the BCA Protein Assay Kit (Thermo Scientific). Lysates were boiled in Laemmli sample buffer (LSB) and equal amounts of total protein samples (25 μg) were subsequently separated in SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with anti-VEGF-B (AF751, R&D Systems) antibodies, followed by an HRP-conjugated secondary antibody. Antibody complexes were visualized on X-ray film using chemiluminescent substrate (Thermo Scientific).

**Angiogenesis antibody array analysis.** Snap-frozen left ventricular samples of four-month-old rat hearts were lysed and analyzed with the R&D Proteome Profiler™ Mouse Angiogenesis Array Kit (#ARY015) according to the manufacturer’s instructions. Lysates from three different transgenic and wildtype hearts, respectively, were pooled before analysis. Intensities were quantified with the ImageJ software (NIH).

**Histochemistry.** Formalin-fixed mid-ventricular paraffin sections were stained with Resorcin Fuchsin for the internal elastic lamina, Herovici’s stain for collagen, and Masson’s Trichrome. The sections were viewed and imaged with a Leica DM LB research microscope with Olympus DP50 color camera.

**Immunohistochemistry, microscopy and image analysis.** 6-8 μm frozen transverse sections were fixed with cold acetone, washed, and blocked in TNB (PerkinElmer) or 5% donkey serum and 0.2% BSA. Thick 200 μm sections of hearts were fixed with 4% paraformaldehyde, washed and blocked with TNB. The primary antibodies used for immunostaining were: rat anti-mouse PECAM-1 (BD Pharmingen), mouse anti-human
dystrophin (Dys2, Novocastra), goat anti-human VEGF-B (AF751, R&D Systems), mouse anti-SMA (Cy3-conjugated, clone 1A4, C6189, Sigma), mouse anti-rat RECA-1 (MCA970, Serotec), rabbit anti-mouse PAI-1 (a kind gift from Peter Andreasen), mouse anti-rat ED-1 (22451D, BD Pharmingen), rat anti-mouse CD45 (BD Pharmingen), and rat anti-mouse F4/80 (AbD Serotec). Alexa Fluor 488, 594 and 647-conjugated secondary antibodies (Molecular Probes) were used for detection. Sections were post-fixed with 4% paraformaldehyde, washed, and mounted with Vectashield with DAPI (Vector Laboratories). Immunofluorescence stainings were imaged using an Axioplan2 fluorescence microscope (Zeiss) or a confocal LSM 510 Meta or LSM 5 Duo microscope (Zeiss). Alternatively, the peroxidase ABC method (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA) was used and the reaction revealed by 3-amino-9-ethylcarbazole (AEC; Vector Laboratories) for light microscopy.

Image analysis was carried out using the ImageJ software (NIH) from several randomly chosen photographic fields from each section. The number of arteries in rat hearts was quantified visually from whole transverse heart sections with the aid of Masson’s Trichrome staining for the arterial adventitia. PECAM-1 and SMA-positive surface areas as well as CD45 and F4/80 immunostainings were quantified from transverse muscle sections. Two to three photographic fields (10x magnification) were analyzed from each section from left and right tibialis anterior muscles of three to four mice per group. Cardiomyocyte areas were quantified from dystrophin-stained sections with ImageJ. Four photographic fields (20x magnification) from five mice per group were analyzed.

**Electron microscopy.** Tissue samples from the left ventricle were fixed with 2.5% glutaraldehyde, postosmicated and embedded in epon. Semithin sections were stained with
toluidine blue, and on the basis of initial analysis in light microscopy, regions of interest were selected for thin (100 nm) sectioning and analysis using a JEOL 1400 EX Transmission Electron Microscope equipped with Morada CCD Camera (Olympus SIS).

**Blood pressure measurements and echocardiography.** The blood pressure of the rats was measured with the CODA Non-Invasive Blood Pressure System for Mice and Rats (Kent Scientific Corporation, Torrington, Connecticut, USA) on non-anesthetized animals restrained in a rodent holder. Measurements were performed in three sets of six cycles with five-second breaks between cycles and thirty-second breaks between sets. Transthoracic echocardiography was performed with an Acuson Sequoia 512 Ultrasound System and an Acuson Linear 15L8 14 MHz transducer (Siemens Medical Solutions, Mountain View, CA, USA). Rats were anesthetized with xylazine 10 mg/kg i.m. (Rompun 20 mg/mL Bayer) and ketamine 40 mg/kg i.m. (Ketalar 50 mg/mL Pfizer). Normal body temperature was maintained.

**MicroCT imaging of the cardiac vessels.** Coronary angiographies were performed using the Inveon micro-computed tomography (CT) scanner (Siemens, Knoxville, TN, USA). The rats were anesthetized with isoflurane, heparinized (0.1 mL, 60 IU) via a tail vein, and euthanized with carbon dioxide. The ascending aorta was cannulated, clamped and 0.3 mL of iodinated intravascular contrast agent eXIATM160XL (working dilution 1:5, Binitio Biomedical Inc., Ottawa Ontario, Canada) was carefully injected manually to fill the cardiac blood vessels, avoiding very high pressure. Then, both the vena cava and pulmonary artery were clamped, and the heart was excised and placed in a cylindrical container. The CT acquisition consisted of 721 projections acquired with an exposure time of 1000 ms, X-ray voltage of 80 kVp, and current of 500 µA for a full 360° rotation and a total scan time of 20 min. Images were
reconstructed using a standard filtered backprojection algorithm. The resulting matrix was 768 x 768 pixels with 512 transverse slices (pixel size 0.04×0.04×0.04 mm). The coronary arterial and venous trees were segmented using the ADW 4.4 Workstation (General Electric, Milwaukee, Wis., USA) and visualized as 3D volume rendered images.

**Assessment of myocardial perfusion, oxygen consumption and efficiency of work.** Eight rats were given a slow bolus of 30±24 MBq of \[^{11}\text{C}\]\text{acetate} (0.4–1.0 ml) and imaged for ten minutes using the Inveon microPET/CT scanner (Siemens, Knoxville, TN, USA). Myocardial blood flow was estimated using the single compartment model and expressed as rate constant K\(_1\) (1/min). Myocardial oxygen consumption was assessed by applying monoexponential fitting to calculate the \[^{11}\text{C}\]\text{acetate} clearance rate (Kmono). Myocardial efficiency of forward work was estimated as forward LV work power per gram/LV Kmono\(^4\). LV mass was calculated from LV dimensions in the long axis M-mode image and cardiac output from LV outflow tract pulsed Doppler measurements using the Visualsonics Vevo 770 ultrasound apparatus.

**Statistical Analysis.** Values are indicated as mean±SD unless otherwise indicated. Statistical analysis was performed with one-way ANOVA (post-hoc with Tukey’s test), or with the two-tailed unpaired Student’s \(t\)-test unless otherwise specified in the Results. Differences were considered statistically significant at \(P<0.05\).
Supplementary References


Supplementary Figure Legends

Supplementary Figure 1. Perfusion and permeability of muscles after transduction of AAVs encoding VEGF-B (both isoforms), PlGF, VEGF or HSA. A. Blood perfusion in tibialis anterior muscles as quantified by ultrasound. B. Evans Blue dye was injected into the left ventricle of mice and dye leakage from muscles was quantified as the ratio between the absorbance at 620 nm and muscle weight. Significance values were determined between the test groups and the HSA control group. **, P<0.005; ***, P<0.0005.

Supplementary Figure 2. Comparison of the vascular effects of VEGF-B and PlGF overexpressed for two weeks in cardiac muscle via AAV vector delivery. Representative PECAM-1 and SMA-stained sections of hearts transduced with AAVs encoding VEGF-B_{167}, PlGF or HSA. Scale bar = 100 µm.

Supplementary Figure 3. Inflammatory cell infiltration in mouse skeletal and cardiac muscles transduced with AAVs. A. CD45 and F4/80-staining of skeletal muscles injected with AAVs encoding VEGF-B_{167}, VEGF-B_{186}, PlGF, VEGF or HSA. B. Quantification of A. C. CD45-staining of hearts injected with AAVs encoding VEGF-B (1:1 mixture of both isoforms), PlGF or HSA. Scale bars = 100 µm. **, P<0.01; *, P<0.05.

Supplementary Figure 4. Expression and structure of the αMHC-VEGF-B transgenes. A. Shown are exons and introns with the alternative splice acceptor (SA) sites that produce the VEGF-B_{167} and VEGF-B_{186} isoforms. Arrowhead indicates the site of proteolytic processing of VEGF-B_{186}. hGH pA, human growth hormone polyadenylation signal. Red, sequence encoding the VEGF homology domain. B. Schematic structure of the αMHC-VEGF-B_{Ex1-5}
cDNA transgene. **C.** Western blot analysis of VEGF-B in heart lysates from five transgenic rat founder lines (TG1-5) and a wildtype control (WT). Founder lines 2-4 were used for subsequent analyses. The polypeptides generated from the 167 and 186 transcripts are indicated. **D.** Western blot analysis of VEGF-B in heart lysates from αMHC-VEGF-B_{Ex1-5} (TG) and wildtype mice. **E.** Immunofluorescence staining of heart sections with antibodies against VEGF-B. Blue, DAPI staining of the nuclei. Scale bar = 50 µm.

**Supplementary Figure 5. Degenerative changes in αMHC-VEGF-B mouse but not rat hearts.**

**A.** Representative hematoxylin-eosin stained heart sections from one-year-old αMHC-VEGF-B mice and rats. Note the vacuolar lesions indicated by arrows in the mouse hearts. Scale bar = 50 µm. **B.** Transmission electron micrographs from four-month-old αMHC-VEGF-B rat hearts. Scale bar = 800 nm.

**Supplementary Figure 6. Angiogenesis antibody array analysis of VEGF-B transgenic and wildtype rat heart lysates.** In the TG rat, the expression of Cyr61, Dll4, Osteopontin and PAI-1 was upregulated more than 2.5-fold when compared to WT rat. VEGF-B expression was 6-fold higher in TG rat.
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genomic hVEGF-B fragment (~3.6 kb)

αMHC promoter (~5.5 kb)

VEGF-B cDNA
Ex1-5

32 - 22 - 14 - kD

BamHI

1 2 3 4 5 6A 6B 7

SA: 186 167

VEGF-B

A

B

C

TG1 TG2 TG3 TG4 TG5 WT

D

Ex1-5 TG WT

14 - kD

E

VEGF-B DAPI

TG2 TG3 WT

Scale bar