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-B_{167} 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.[1,2] Although vascular endothelial growth factor (VEGF) is the most potent angiogenic factor for possible therapy of myocardial ischemia,[3] it also promotes vascular leakage, inflammation, and the formation of angioma-like vascular structures,[4–7] 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.[7]

Clinical Perspective on p 1733

VEGF-B, isolated in 1995,[8] 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-

Received March 29, 2010; accepted August 18, 2010.

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The online-only Data Supplement is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.110.957332/DC1.

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Circulation **is available at http://circ.ahajournals.org** **DOI: 10.1161/CIRCULATIONAHA.110.957332**

tive results.[6] 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.[9,10] Transgenic (TG) or adenoviral overexpression of VEGF-B in the skin or skeletal muscle increased blood vessel density only minimally.[11,12] 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.[13]

VEGF-B is expressed as 2 RNA splice isoforms.[14] Recently, we demonstrated that when overexpressed in the mouse heart, the heparin-binding VEGF-B_{167} isoform induced cardiac hypertrophy without overt angiogenesis, although it caused mild enlargement of myocardial blood capillaries.[12] The other isoform, VEGF-B_{186}, is O-glycosylated, proteolytically processed, and freely diffusible. Both isoforms bind to and activate VEGF receptor-1 (VEGFR-1) and neuropilin-1.[15,16] Several studies have indicated that the VEGF-B isoforms are expressed simultaneously in various tissues.[14] PlGF binds to the same receptors as VEGF-B and has been shown to promote angiogenesis and arteriogenesis in pathological conditions.[17]

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 PlGF 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 adeno-associated viral 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 $VEGF_{120}$, PlGF, VEGF-B_{167}, VEGF-B_{186}, and human serum albumin complementary DNAs were cloned into the psubCMV-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-$B_{Ex1–5}$ fragment were isolated and cloned into the α-myosin heavy chain (αMHC) promoter expression vector (a kind gift from Dr Jeffrey Robbins). TG animals were generated by microinjection of fertilized oocytes from FVB/N mice or HsdBrl: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 [^{11}C]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 (posthoc 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 PlGF, VEGF-B Fails to Induce Capillary Angiogenesis or Arteriogenesis in Mouse Skeletal or Cardiac Muscle

To compare the effects of VEGF-B and PlGF, which bind to VEGFR-1 and neuropilin-1, we injected recombinant AAVs encoding VEGF-B_{167}, VEGF-B_{186}, and PlGF, 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 PlGF-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, PlGF and VEGF increased blood perfusion in the muscles, whereas even a combination of VEGF-B_{167} and VEGF-B_{186} had no effect on perfusion (Figure IA in the online-only Data Supplement). Interestingly, Evans Blue dye injection experiments indicated that although PlGF and VEGF increased vascular permeability, VEGF-B_{167} and VEGF-B_{186} 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 PlGF or VEGF vectors had to be

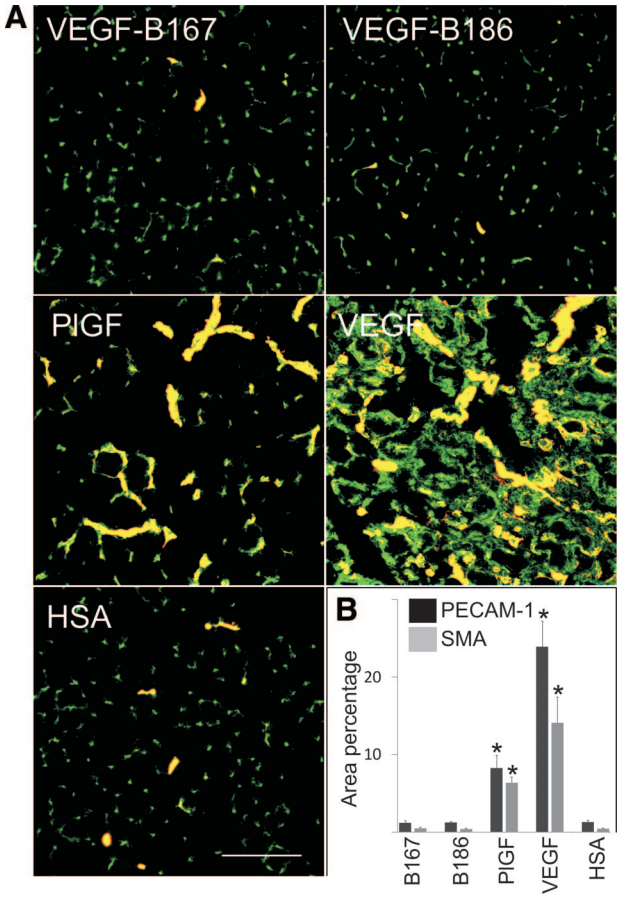

Figure 1. Comparison of the effects of VEGF-B and PlGF expressed in skeletal muscle via AAV vector delivery. A, Representative PECAM-1– (green) and SMA- (orange) stained sections of tibialis anterior muscles injected with AAVs encoding VEGF-B_{167}, VEGF-B_{186}, PlGF, VEGF, or human serum albumin (HSA). B, Quantification of the total PECAM-1– and SMA-positive surface areas in muscle sections. Scale bar=100 μm. *$P<0.05$.

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-B_{186} polypeptides (32 and 14 kDa, respectively), as well as VEGF-B_{167} 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-B_{167} mouse hearts[12] 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; $P<0.005$).

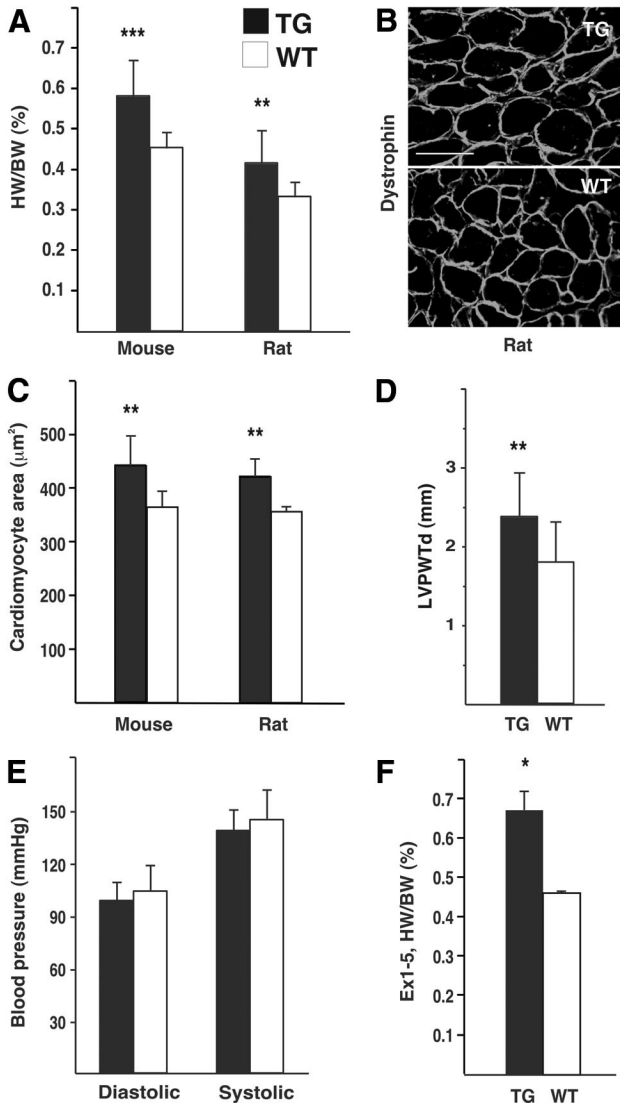

Figure 2. Cardiac hypertrophy in αMHC–VEGF-B mice and rats. A, Quantification of ratios of heart to body weight (HW/BW) in the αMHC–VEGF-B TG and WT rats (n=13 in both groups) and mice (TG, n=10; WT, n=12). B, Representative dystrophin-stained sections of αMHC–VEGF-B rat and control rat hearts. Scale bar=100 μm. C, Quantification of cardiomyocyte areas (n=5 in all groups). D, Echocardiographic analysis of the rat left ventricular posterior wall thickness in diastole (LVPWTd; n=12 in each group). E, Blood pressure analysis of the αMHC–VEGF-B and control rats (n=12 in each group). F, Ratios of heart to body weight of αMHC–VEGF-$B_{Ex1–5}$ (n=3) mice and WT littermates (n=4). *P<0.05; **P<0.005; ***P<0.0005.

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[18] (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.[19] 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±14 cells in αMHC–VEGF-B versus 36±9 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).

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

	TG	WT
LVPWTd, mm	2.4±0.5‡	1.8±0.5
IVSTd, mm	1.7±0.2	1.7±0.1
LV EF, %	83±8†	76±7
LV FS, %	48±12	41±9
ESV, mL	0.12±0.09†	0.19±0.07
SV, mL	0.55±0.14	0.59±0.08
EDV, mL	0.68±0.21	0.78±0.12
Heart rate, bpm	345±65	377±27
Heart rate, bpm*	237±38†	289±56
Diastolic BP, mm Hg	98±10	104±14
Systolic BP, mm Hg	138±12	144±16

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; n=12 in each group.

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±2.2% in TG [n=4] versus 11.8±4.9% in WT hearts [n=5]; P>0.05). 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 [^{11}C]acetate throughout the left ventricular myocardium of the rats. The average myocardial perfusion was comparable in the TG and

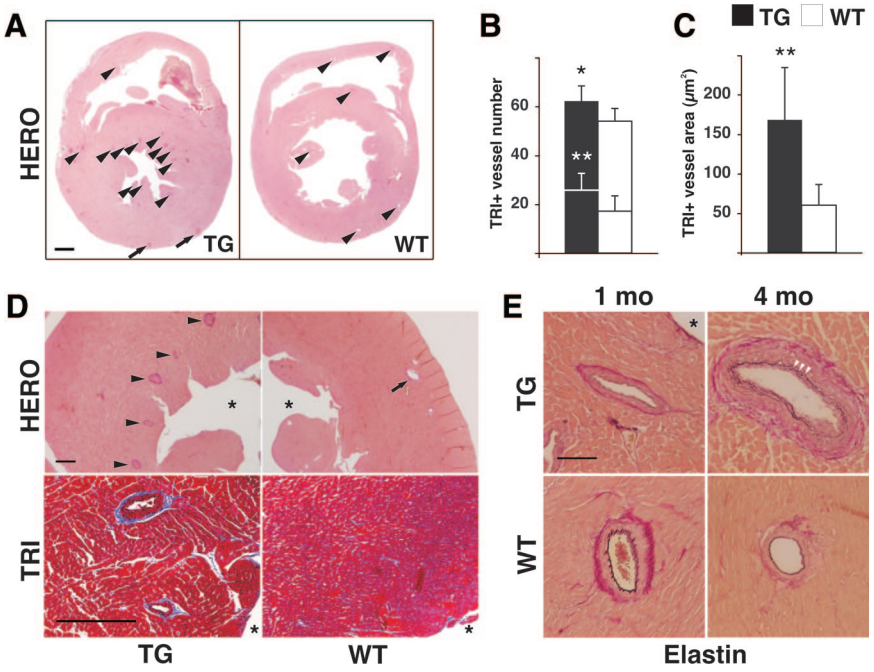

Figure 3. The VEGF-B transgene induces strong arteriogenesis in rat myocardium. A, Representative Herovici-stained (HERO) sections of 4-month-old rat hearts. Arrowheads indicate arteries; arrows indicate enlarged epicardial veins. B, Quantification of the total (top bars) and subendocardial (bottom bars) arterial vessel number in VEGF-B–overexpressing rats (n=11) and controls (n=10) 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.

WT rats (K_1, 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 (K_{mono}, 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 domain[15] was expressed in mouse heart (αMHC–VEGF-$B_{Ex1–5}$; 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 VEGF-B_{167} form ($P<0.05$, Mann-Whitney U test; Figure 2F). This indicated that, despite its expression at least in fetal myocardium,[15] neuropilin-1 binding does not mediate the hypertrophic response.

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

To analyze the role of VEGFR-1 signal transduction in the VEGF-B–induced cardiac hypertrophy, we mated the αMHC–VEGF-B_{167} 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-B_{167};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-B_{167} (n=10) versus 0.42±0.03% in WT (n=7) mice; $P<0.0005$). These results indicate that the VEGFR-1 pathway is essential for transduction of the hypertrophic signals of VEGF-B.

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-B_{167} mice. We then compared mice of 4 genetic backgrounds: αMHC–VEGF-B_{167}, αMHC–VEGF-B_{167};Bmx-/0, WT, and Bmx-/0 (KO) mice. At the age of 3 to 3.5 months, when the hypertrophic phenotype of αMHC–VEGF-B_{167} 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-B_{167};Bmx KO hearts compared with the αMHC–VEGF-B_{167} 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.

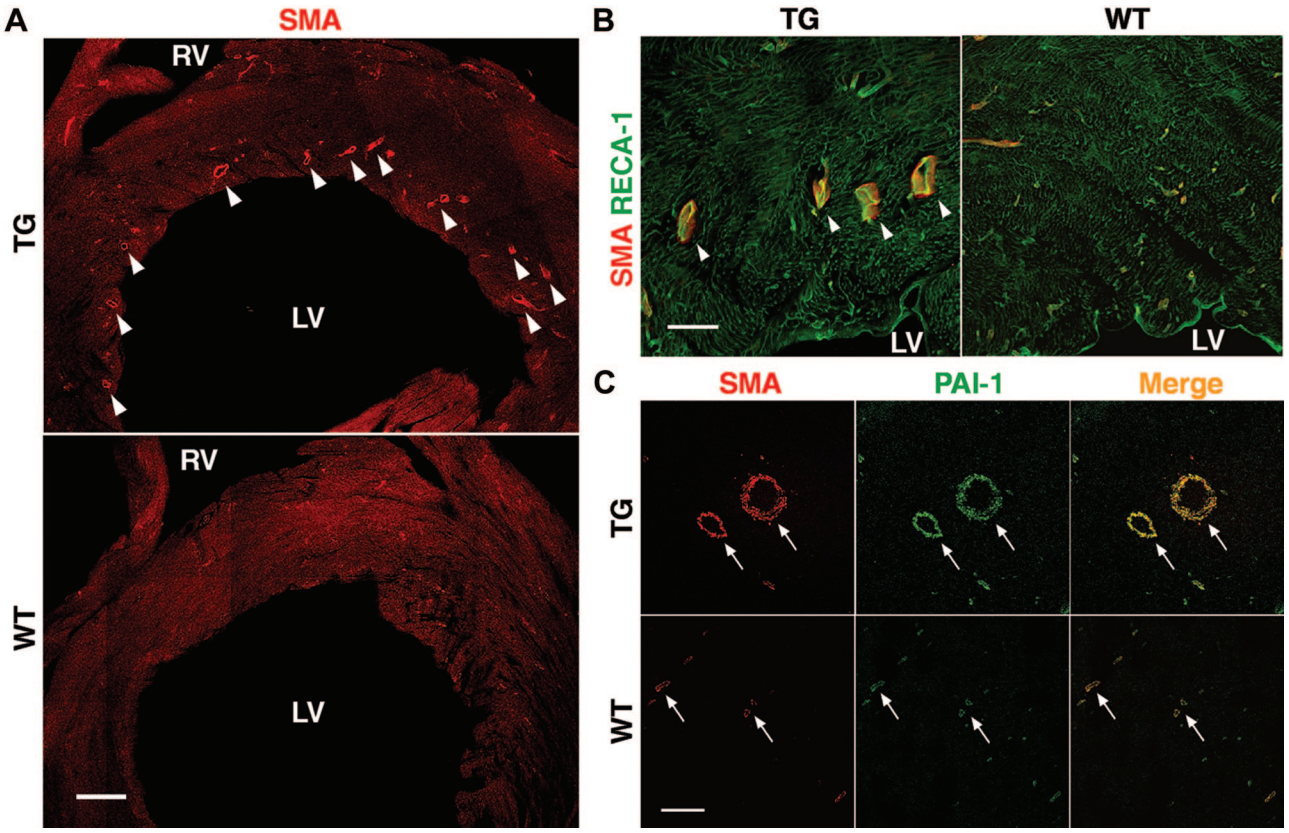

Figure 4. Immunofluorescence staining of thick cardiac sections. A, Tile scan images of thick cardiac sections stained for SMA. Arrowheads indicate large subendocardial arteries in VEGF-B TG hearts. Scale bar=1 mm. LV indicates left ventricle; RV, right ventricle. B, Representative images of SMA- and 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.

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.[23] Heart-specific overexpression of both VEGF-B isoforms in mice and rats reproduced the hypertrophic phenotype previously seen in αMHC–VEGF-B_{167} mice.[12] 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.[24] 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.[2]

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.[25] 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,[2] and this required the neuropilin-1–binding domain of VEGF.[26]

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[15] induced significant cardiac hypertrophy. On the other hand, deletion of the VEGFR-1

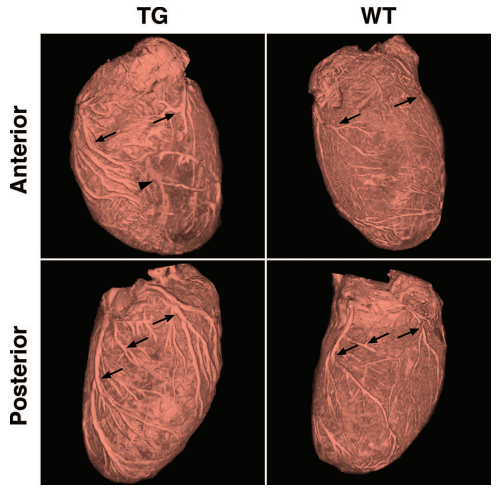

Figure 5. Representative 3-dimensional rendered micro–computed tomography images of vessels in VEGF-B TG rats and controls. Arrows indicate corresponding branching points in the TG and control hearts; arrowhead, large artery deep inside the myocardium of a TG rat.

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 al[9] 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–

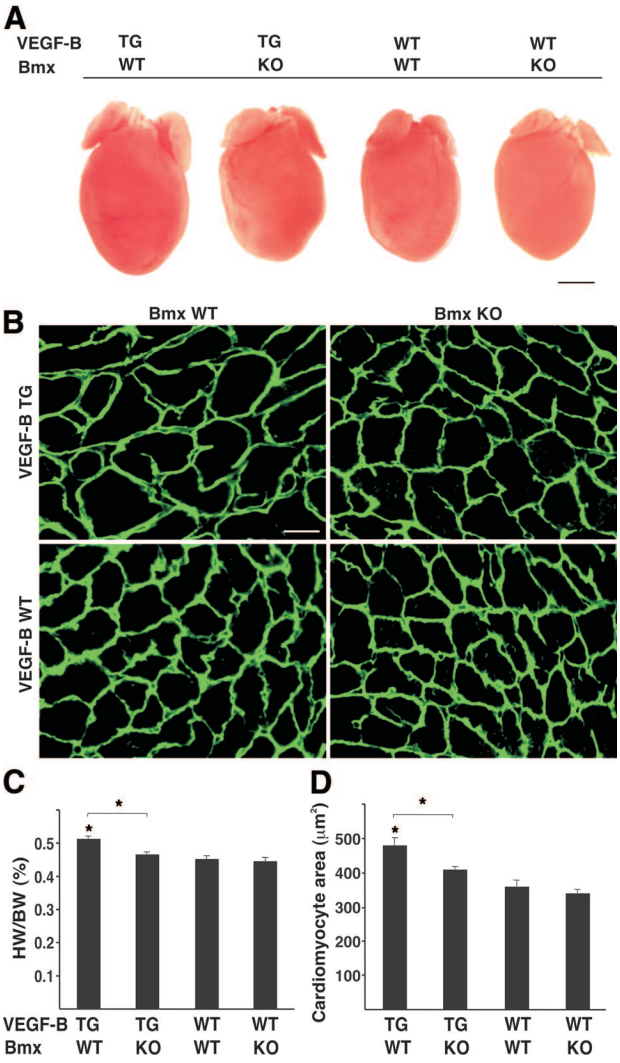

Figure 6. Loss of Bmx tyrosine kinase reduces VEGF-B–induced cardiac hypertrophy. A, Representative ex vivo images of hearts from αMHC–VEGF-B_{167} (VEGF-B TG/Bmx WT) (n=6), VEGF-B TG/Bmx KO (n=21), WT (n=11), and Bmx KO (n=9) mice. B, Representative images of dystrophin-stained cardiac sections. C, Quantification of ratios of heart to body weight. Values are presented as mean±SEM. D, Quantification of cardiomyocyte areas. *$P<0.05$. Scale bar=2 mm (A) and 20 μm (B).

targeted mice indicated that their isolated hearts display vascular dysfunction after coronary occlusion and impaired recovery from experimentally induced myocardial ischemia.[9] 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.[28] On the other hand, VEGF-B_{167} was reported to exert a powerful antiapoptotic effect on cardiomyocytes both in cell culture and in vivo after myocardial infarction.[24] In addition, VEGF-B_{167} 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.[29] 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.[21,22] 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.[21] 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.[12,24,29] 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.[30,31] 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.

Acknowledgments

We thank Denis Tvorogov and Antti Nykänen for professional advice; Peter Andreasen for the PAI-1 antibody; and Tapio Tainola, Katja Salo, Karita Viita-aho, Ulla Kiiski, Eeva Rouvinen, Riitta Sinervirta, Marita Heikkinen, Tuula Reponen, Marko Tirri, and Päivi Leinikka for technical assistance. We also thank the Molecular Imaging Unit at Biomedicum Helsinki and Fang Zhao for microscope support and Nicolas Durant-Schaefers (GE Healthcare) for help with image processing.

Sources of Funding

This study was supported financially by the Academy of Finland, Sigrid Juselius Foundation, Helsinki University Hospital Funds, Turku University Hospital Funds, Finnish Foundation for Cardiovascular Research, and Centre of Excellence for Molecular Imaging in Cardiovascular and Metabolic Research. M. Bry has personally been supported by grants from Nylands Nation, Finska Läkaresällskapet, the Aarne Koskelo Foundation, Paulo Foundation, Emil Aaltonen Foundation, and Finnish Foundation for Cardiovascular Research. Dr Kivelä has personally been supported by the Finnish Cultural Foundation and the Academy of Finland. Dr Holopainen has been supported by the Maud Kuistila Memorial Foundation, Finnish Foundation for Cardiovascular Research, Aarne Koskelo Foundation, and Paulo Foundation.

Disclosures

Dr Alitalo is the principal investigator of research grants relevant to the topic of this study from the Academy of Finland, Sigrid Juselius Foundation, and Helsinki University Hospital Funds. The other authors report no conflicts.

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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 increased 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.