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Background—New revascularization therapies are urgently needed for patients with severe coronary heart disease who lack conventional treatment options.

Methods and Results—We describe a new proangiogenic approach for these no-option patients using adenoviral (Ad) intramyocardial vascular endothelial growth factor (VEGF)-B186 gene transfer, which induces myocardium-specific angiogenesis and arteriogenesis in pigs and rabbits. After acute infarction, AdVEGF-B186 increased blood vessel area, perfusion, ejection fraction, and collateral artery formation and induced changes toward an ischemia-resistant myocardial phenotype. Soluble VEGF receptor-1 and soluble neuropilin receptor-1 reduced the effects of AdVEGF-B186, whereas neither soluble VEGF receptor-2 nor inhibition of nitric oxide production had this result. The effects of AdVEGF-B186 involved activation of neuropilin receptor-1, which is highly expressed in the myocardium, via recruitment of G-protein-α interacting protein, terminus C (GIPC) and upregulation of G-protein-α interacting protein. AdVEGF-B186 also induced an antiapoptotic gene expression profile in cardiomyocytes and had metabolic effects by inducing expression of fatty acid transport protein-4 and lipid and glycogen accumulation in the myocardium.

Conclusions—VEGF-B186 displayed strikingly distinct effects compared with other VEGFs. These effects may be mediated at least in part via a G-protein signaling pathway. Tissue-specificity, high efficiency in ischemic myocardium, and induction of arteriogenesis and antiapoptotic and metabolic effects make AdVEGF-B186 a promising candidate for the treatment of myocardial ischemia. (Circulation. 2009;119:845-856.)

Key Words: angiogenesis ■ gene therapy ■ metabolism ■ myocardial infarction

Severe coronary heart disease is still a leading cause of death in developed countries in spite of improved management of risk factors and more effective treatments. It is estimated that approximately 5 million people in the United States and the European Union have ischemic heart disease; however, a steadily increasing number of patients fall into a category in which currently available revascularization techniques cannot be applied. This is especially true of elderly patients who have had multiple bypass and stenting operations.1 It is estimated that these patients represent up to 3% to 5% of all patients in specialty cardiology clinics. Thus, there is a clear need to develop efficient, minimally invasive procedures for the treatment of these no-option patients.

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Therapeutic vascular growth (ie, angiogenesis and arteriogenesis) with genes or proteins has been suggested as an alternative approach for the treatment of these patients.2 Vascular endothelial growth factors (VEGFs) are potent inducers of vascular growth via binding to 3 tyrosine kinase receptors (VEGFRs). VEGFR-2 is the main regulator of angiogenesis, exerting its function via nitric oxide production, whereas the role of VEGFR-1 is far less defined.3 VEGF-B4 and placental growth factor (PIGF)5 share structural and functional characteristics and bind to VEGFR-1, whereas VEGF-A6 binds to both VEGFR-1 and VEGFR-2.
Figure 1. AdVEGF-B_{186} induces myocardium-specific angiogenic response. A, AdVEGF-B_{186} induced angiogenesis in normoxic pig myocardium (a1–a5) and vessel growth in the infarction edge of ischemic myocardium (a6–a10). Platelet endothelial cell adhesion molecule-1 (PECAM-1) staining for endothelial cells (brown) 6 days after gene transfer, magnification 200×. AdLacZ gene transfer did not have any effect on capillary size or morphology (a1), whereas AdVEGF-A induced a strong angiogenic response in the myocardium (a2), (Continued)
VEGF-B has 2 isoforms, VEGF-B_{167} and VEGF-B_{186}. Both isoforms of VEGF-B, PlGF, and VEGF-A_{165} have been shown to bind neuropilin receptor-1 (Nrp-1), but their binding is not identical. VEGF-A_{165} binds to 2 different domains of Nrp-1, whereas PlGF only binds to one. Although both isoforms of VEGF-B bind to Nrp-1, the binding of VEGF-B_{167} is mediated via a heparin-binding domain, whereas the binding of VEGF-B_{186} requires proteolytic processing. Nrp-1 interactions with VEGFRs may alter their signaling properties, and recently, Nrp-1 has also been shown to convey intracellular signaling. In addition, diffusion properties of VEGFs regulate their biological effects. VEGF-A_{165} and VEGF-B_{186} bind to heparin sulfates, whereas VEGF-B_{167} and PlGF are more freely soluble.

In addition to enhancing blood flow, the altering of myocardial metabolism and inhibition of apoptosis may also help protect hibernating myocardium after myocardial infarction. Metabolism is altered in energy-depleted myocardium, and, for example, glycogen accumulation has been shown to protect ischemic myocardium and limit structural damage. Although several angiogenic and metabolic factors have been identified, none has shown relative specificity for the heart. Here, we describe a new cardiac-specific proangiogenic gene therapy approach that could be applicable for the treatment of patients with severe ischemic heart disease.

Methods

Animal Models

Acute myocardial infarction in pigs was induced in the anterolateral wall of the left ventricle by occluding the distal part of the left anterior descending coronary artery with a VortX-18 occlusion coil (Boston Scientific, Natick, Mass). After the appearance of ischemic ECG changes and detection of a wall-motion defect by intracardiac ultrasound (Acucard, Acuson Sequoia, Siemens, Erlangen, Germany), total occlusion of the coronary artery was confirmed by angiography. Hindlimb ischemia was induced in New Zealand White rabbits as described previously. Gene Transfer

Domestic pigs (n = 121) and New Zealand White rabbits (n = 68) received an intramuscular or intramyocardial gene transfer of adeno-viruses (Ad) encoding human VEGF-A_{165}, PlGF-2, VEGF-B_{167}, VEGF-B_{186}, or a marker gene, LacZ. Experiments were performed in 2 large-animal models to reduce the possibility of species-specific effects. In addition, a subgroup of animals received intramuscular or intramyocardial injections of adenosviruses encoding soluble (s) VEGFR-1, sVEGFR-2, or sNrp-1. In the myocardium, 10 injections with 10^11 viral particles (vp)/mL were given with a custom-made injection catheter in rabbits (injection volume 50 μL) and an 8F NOGA catheter system (Biosense Webster, Diamond Bar, Calif), with an injection volume of 200 μL in pigs, as described previously. In ischemic pigs, gene transfer was performed 30 minutes after acute myocardial infarction. A total of ten 200-μL injections were given around the infarct area, the border zone of the movement defect, and the normally contracting myocardium proximal to the infarction area where collateral vessel formation was expected (referred to as the maximal transduction area). In the hindlimbs, 5 injections with virus concentration of 10^11 vp/mL were given into the semimembranosus muscles (25-gauge needle, volume 0.1 mL in rabbits; 20-gauge needle, injection volume 1 mL in pigs). In ischemic hindlimbs, rectus femoris and tibialis anterior muscles were also transduced. Human clinical GMP-grade (first-generation, adenovirus type 5) replication-deficient adenosviruses analyzed to be free from contaminants were used. The nitric oxide synthase inhibitor L-NAME (N^ω-nitro-l-arginine methyl ester; 50 mg · kg^{-1} · d^{-1}; Sigma-Aldrich, St. Louis, Mo) was given to a subgroup of animals twice a day after the gene transfer. Propofol and fentanyl were used.
Figure 2. AdVEGF-B_{186} induces arteriogenesis in ischemic pig myocardium. A, The intracoronary occlusion coil causing the infarct is indicated by arrows. Increased collateral vessel formation was observed 6 days after gene transfer in the infarction border zone in AdVEGF-A– and AdVEGF-B_{186}–transduced hearts (area of collateral formation indicated by red circles). In AdLacZ and AdVEGF-B_{167}, vascularity in the periinfarct zone was low. B, AdVEGF-B_{186} and AdVEGF-A increased ejection fraction. Ejection fraction was comparable (Continued)
for pig anesthesia and medetomidin and ketamine for rabbit anesthesia. All animal experiments were approved by the Experimental Animal Committee, University of Kuopio.

**Echocardiography and Contrast-Enhanced Ultrasound Imaging of Perfusion**

Echocardiography was performed at baseline, after acute myocardial infarction, and before animals were euthanized on day 6 after gene transfer with an Acunav catheter inserted into the right atrium. Ejection fraction was quantified with ultrasound. Contrast-enhanced ultrasound perfusion measurement was done in skeletal muscles as described previously13 after a bolus injection of a contrast agent (SonoVue, Bracco, Milan, Italy) via the ear vein.

**Microsphere Perfusion and Angiography**

Myocardial perfusion was measured with red fluorescent microsphere particles (Molecular Probes/Invitrogen, Carlsbad, Calif; 15 μm, 5 × 10^6 particles) as described previously.12 Angiography of the left anterior descending coronary artery was used to visualize collateral vessels and the left images before venous filling are shown.

**Blood Vessel Measurements**

Mean blood vessel area (μm^2) was measured from CD31-immunostained sections of rabbit muscles13 and platelet and endothelial cell adhesion molecule-1–stained pig muscle sections at 200× magnification. Vessels from the infarction edge were analyzed at 100× magnification. All measurements were performed with AnalySIS software (Soft Imaging System) in a blinded manner from 5 to 10 different randomly selected fields from each section. Means of the measurements are reported.

**Immunoprecipitation and Western Blot**

Snap-frozen tissue samples from normoxic and ischemic transduced myocardium and skeletal muscles were homogenized in protein extraction buffer (T-Per, Pierce, Rockford, Ill). Immunoprecipitations were done with Nrp-1 antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, Calif) and protein A Sepharose (Amersham Biosciences, Uppsala, Sweden). Immunoblotting from native samples was performed with antibodies for Nrp-1 antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, Calif) and protein A Sepharose (Amersham Biosciences, Uppsala, Sweden). Immunoblotting from native samples was performed with antibodies for Nrp-1 antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, Calif) and protein A Sepharose (Amersham Biosciences, Uppsala, Sweden).

**Statistical Analyses**

Results are expressed as mean ± SEM. Statistical significance was evaluated with linear mixed models (SPSS version 14.0, SPSS, Inc, Chicago, Ill), which are based on generalized estimating equations, or by ANOVA followed by Student’s t test. P < 0.05 was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**AdVEGF-B186 Induces Myocardium-Specific Angiogenesis**

It has remained unknown which growth factor(s) might be best suited for the treatment of myocardial ischemia.1,2,12,13 In the present study, we tested several VEGF-R-1 ligands by side in large-animal models using a new pig infarction model based on a percutaneous coil-mediated occlusion of the left anterior descending coronary artery. The infarction area was confirmed with a wall-motion defect and ECG changes. In contrast to previous infarction models based on thoracotomy and surgical coronary occlusion, this model preserves an intact pericardium, avoids excess fibrous scar formation in the thorax and around the heart, and allows precise imaging of the myocardium with ultrasound. Gene delivery was done percutaneously into the infarction border zone and adjacent myocardium with a NOGA mapping-injection catheter.12

Of the VEGFR-1 ligands (VEGF-B167, VEGF-B186, PI GF, and VEGF-A165), both VEGF-B isoforms showed angiogenic effects only in the myocardium (Figure 1A through 1E). Both normal and in ischemic skeletal muscles, the 2 AdVEGF-B isoforms were ineffective (Figure 1D, d4–5, d9–10, and d14–15), whereas AdVEGF-A and AdPIGF had significant angiogenic effects that increased tissue perfusion (Figure 1D, d2, d7, and d12 and d3, d8, and d13, respectively; Data Supplement Figure I). AdVEGF-B186 was particularly efficient in myocardium after acute myocardial infarction (Figure 1A, a6 through a10). Mean vessel size in the infarction border zone was increased almost 3-fold compared with the AdLacZ controls (Figure 1C). AdPIGF also increased the mean vessel number in the infarction edge (Figure 1A, a8 and data not shown), but the increase in mean vessel area was not significant (Figure 1C). The angiogenic effects of AdVEGF-B186 and AdVEGF-A persisted in the maximal transduction area in the peri-infarct zone 3 weeks after gene therapy (Data Supplement Figure II). Transient production of angiogenic factors by adenaloviral overexpression is therefore sufficient to induce angiogenic neovessels that are further regulated by blood flow and the metabolic needs of the surrounding tissue.

The angiogenic phenotypes induced by the different VEGFs were clearly distinct. AdVEGF-B186 predominantly stimulated proliferation of abundant α-smooth muscle actin–positive cells (Figure 1F and 1G, g4 and 1g10), whereas AdVEGF-A and AdVEGF-A mainly induced proliferation of endothelial cells (Figure 1F and 1G, g5 and 1g3) in normal myocardium. AdVEGF-B167–induced vessels had low pericyte coverage, and only a few proliferating endothelial cells were seen (Figure 1F and 1G, g2 and g9). Thus, the effects of AdVEGF-B186 in the myocardium were clearly different from the other VEGFs.

AdVEGF-B186–induced angiogenesis also improved the functional outcome in the myocardial ischemia model. AdVEGF-B186 induced the formation of angiographically visible collateral arteries around the infarction area (Figure 2A). Ejection fraction improved by 18% and 21% after AdVEGF-B186 and AdVEGF-A treatments, respectively (Figure 2B), as well as blood flow in the myocardium as measured by microsphere trapping (Figure 2C). Thus, AdVEGF-B186 treatment is a promising new candidate for acute
Figure 3. Angiogenic effect of VEGF-B$_{186}$ is dependent on VEGFR-1 and Nrp-1, whereas AdPIGF requires VEGFR-2 activation. A through D, Three-fold excess of sVEGFR-2 or sVEGFR-1 completely blocked the angiogenic effects of AdVEGF-A in rabbit skeletal muscle

Continued
AdVEGF-B Induces Angiogenesis Through VEGFR-1 and Nrp-1 but Not VEGFR-2

Even though both VEGF-B_{186} and PlGF bind to VEGFR-1, the angiogenic effects induced by these 2 ligands were clearly distinct. The effect of AdVEGF-B_{186} was dependent on VEGFR-1, but unlike AdPlGF, it was not dependent on VEGFR-2 or upregulation of endogenous VEGF-A, as determined by simultaneous transductions with AdsVEGFR-1 or AdsVEGFR-2 (Figure 3A through 3D). Moreover, angiogenesis induced by AdVEGF-B_{186} was not blocked by inhibition of nitric oxide production by L-NAME, which completely inhibits the angiogenic effects of AdPIGF and AdVEGF-A (Data Supplement Figure III).

The angiogenic effects of AdVEGF-B_{186} in normal myocardium were instead dependent on membrane-bound Nrp-1, because AdsNrp-1 significantly reduced AdVEGF-B_{186}-induced capillary growth (Figure 3E, e4, and 3F). Interestingly, AdsNrp-1 enhanced the angiogenic effects of AdVEGF-A (Figure 3Em, e3, and 3F), which may be due to enhanced VEGFR-2 activity, further suggesting different angiogenic mechanisms for VEGFR-1 and VEGFR-2.

Mechanistic studies were also performed in ischemic myocardium. AdsNrp-1 increased mean vessel area by 2.3-fold compared with AdLacZ (Figure 3G, g3, and 3H), which was likely due to an enhancement of the vessel growth induced by endogenous VEGF-A produced in the ischemic tissue (data not shown). In contrast, AdsVEGFR-1 referred angiogenesis was reduced both by AdsNrp-1 (by 69%; Figure 3G, g4) and by AdsVEGFR-1 (by 47%; Figure 3G, g6).

In line with the cardiac-specific effects of VEGF-B_{186}, Nrp-1 was abundantly present in both normal (Figure 4A and 4B) and ischemic myocardium (data not shown) but not in skeletal muscle (Figure 4A and 4B). In AdLacZ-, AdVEGF-A-, and AdPIGF-transduced hearts, Nrp-1 was observed mainly in the capillary endothelium in the angiogenic area and in the endothelial layer of arteries and small veins (Figure 4A, a5 through a7), whereas in AdVEGF-B_{186}-transduced myocardium, expression was also seen in cardiomyocytes (Figure 4A, a8 and a12). Western blot analysis confirmed strong expression of Nrp-1 in both AdLacZ- and AdVEGF-B_{186}-transduced myocardium but not in skeletal muscle (Figure 4B).

The role of Nrp-1 in VEGF-B_{186}-induced cardiac angiogenesis warranted analysis of the intracellular signaling events downstream of the VEGF-B_{186} binding to Nrp-1. Immunostainings, Western blotting, and pull-down assays of GIPC, an intracellular Nrp-1 adaptor protein, and GAIP, a GIPC interacting protein and a regulator of G-protein signaling (also known as RGS19), were performed. GIPC expression was mainly localized to endothelial cells in AdLacZ-, AdVEGF-A-, and AdPIGF-transduced myocardium (Figure 4C, c5 through c7), whereas no expression was detected in skeletal muscle (Figure 4C, c1 through c4); however, in AdVEGF-B_{186}-transduced hearts, polarized GIPC expression was also found in the cardiomyocytes (Figure 4C, c12).

VEGF-B_{186} bound to Nrp-1 in AdVEGF-B_{186}-transduced myocardial samples, as shown by pull-down assays of tissue lysates with Nrp-1 antibodies followed by Western blotting with antibodies to VEGF-B (Figure 4D). Pull-down assays with Nrp-1 antibodies also showed GIPC binding to Nrp-1 in AdVEGF-B_{186}-transduced myocardium but not in skeletal muscle (Figure 4E). Small amounts of Nrp-1-associated GIPC were seen in AdLacZ-transduced myocardium (Figure 4E), which suggests a VEGF-B-dependent recruitment of GIPC to Nrp-1. Similar activation of Nrp-1 in ischemic myocardium was also confirmed by Western blot (data not shown).

GIPC was expressed in endothelial cells in AdLacZ-, AdVEGF-A-, AdPIGF-, and AdVEGF-B_{186}-transduced myocardium (Figure 4F, f5 through f8). Cardiomyocytes in AdLacZ-, AdVEGF-A-, and AdPIGF-transduced hearts were positive for GAIP (Figure 4F, f9 through f11); however, in AdVEGF-B_{186}-transduced myocardium, a strongly polarized, granular GAIP staining was observed in cardiomyocytes in an overlapping staining pattern with Nrp-1 and GIPC (Figure 4F, f12). Upregulation of GAIP was confirmed by Western blot analyses that showed strong GAIP expression in AdVEGF-B_{186}-transduced myocardium but not in AdLacZ-transduced myocardium or skeletal muscle (Figure 4G). These findings suggest that Nrp-1 signaling via G proteins at least partially mediates the cardiac effects of VEGF-B_{186} and that this effect is specific for VEGF-B compared with the other VEGFs.

AdVEGF-B_{186} Induces Secondary Changes in Metabolism and Apoptotic Signaling in Cardiomyocytes

Because proliferation was largely seen in nonendothelial, α-smooth muscle actin–positive cells in vivo (Figure 1F and 1G, g5, and g10), possible effects of VEGF-B on cardiomyo-
Figure 4. VEGF-B binds to and activates Nrp-1. A and B, Nrp-1 was strongly expressed in the myocardium but not in skeletal muscles. Nrp-1 immunostaining (brown, magnification ×400) of pig tissues showed expression in endothelial cells in normoxic myocardium (a5–a8) (Continued)
cyte growth and metabolism were also addressed. Recently, both VEGF-B and Nrp-1 have been implicated in antiapoptotic signaling.\(^{15,16}\) We therefore studied apoptosis in the infarction edge of ischemic myocardium using terminal dUTP nick end-labeling staining. The number of apoptotic cells was significantly decreased after AdVEGF-B\(_{186}\) gene transfer compared with AdLacZ-, AdPigF-, and AdVEGF-A–transduced hearts (Data Supplement Figure IV).

To study whether VEGF-B had a direct antiapoptotic effect on cardiomyocytes or whether it mediated its effects through VEGFR1- and Nrp-1–expressing endothelial cells, bEnd3 endothelial cells and HL-1 cardiomyocytes were studied in vitro. Whereas bEnd3 cells express VEGFR-1, VEGFR-2, and Nrp-1, HL-1 cardiomyocytes express Nrp-1 but do not express VEGFR-1 (data not shown), similar to the situation in vivo. Treatment of HL-1 cardiomyocytes with VEGF-B\(_{186}\) did not alter levels of the fatty acid transporters Fatp1 and Fatp4 or of the apoptotic factors Bik1, Bmf1, or Bad1 (Figure 5A), which suggests that VEGFR-1 indeed is essential for VEGF-B–mediated signaling. In contrast, VEGF-B\(_{186}\) treatment of bEnd3 endothelial cells increased Fatp4 expression by 1.7-fold, whereas it had no direct effect on the apoptotic genes (Figure 5B) (C. Rosenlew and U. Eriksson, unpublished data, 2007). Most importantly, we found that although VEGF-B\(_{186}\) (Figure 5B) (C. Rosenlew and U. Eriksson, unpublished data, 2007). Higher accumulation of lipids (4-fold compared with AdLacZ) was also observed in cardiomyocytes after AdVEGF-A– and AdVEGF-B\(_{186}\) gene transfer (Figure 5E, e1 through 5e4, and 5F), compared with AdLacZ–transduced hearts in the vicinity of the needle track (a12), whereas no such finding was observed after AdVEGF-A (a10) or AdPlGF-transduced (a11) gene transfers. Needle tracks are shown on the left in a9–12. Accordingly, Western blot analysis showed strong expression of Nrp-1 both in intact and in AdVEGF-B\(_{186}\)–transduced normoxic myocardium but not in skeletal muscles (B). L indicates AdLacZ–transduced tissue; B, AdVEGF-B\(_{186}\)–transduced tissue. C, VEGF-B induces GIPC expression in cardiomyocytes. In AdVEGF-B\(_{186}\)–transduced myocardium, we propose a model for VEGF-B function in the myocardium; B, AdVEGF-B\(_{186}\)–transduced tissue; and P, recombinant human VEGF-B\(_{186}\)–positive control. Immunoblotting with GIPC antibodies (E) showed weak association with Nrp-1 in AdLacZ- and strong binding of GIPC to Nrp-1 in VEGF-B\(_{186}\)–transduced myocardium (F). GIPC staining was localized to the capillary endothelium in AdLacZ–, AdVEGF-A–, AdPlGF–, and AdVEGF-B\(_{186}\)–transduced hearts (f5–f8). In addition, a polarized, granular GIPC staining was detected in AdVEGF-B\(_{186}\)–transduced cardiomyocytes (f12), GAIP staining in AdLacZ– (f9), AdVEGF-A– (f10), and AdPigF–transduced (f11) cardiomyocytes was diffuse. No GAIP expression was found in skeletal muscles (f1–f4). Accordingly, Western blot analysis showed strong expression of GAIP in AdVEGF-B\(_{186}\)–transduced hearts, whereas expression in AdLacZ–transduced hearts was very low, and no GAIP was detected in skeletal muscle samples (G). L indicates AdLacZ–transduced tissue; B, AdVEGF-B\(_{186}\)–transduced tissue.

Figure 4. (Continued) but not in skeletal muscle (a1–a4). Nrp-1 expression was most prominent in arteries, arterioles, and veins, but strong expression was also detected in capillary endothelium in the gene transfer areas of AdVEGF-A– (a6), AdPigF– (a7), and AdVEGF-B\(_{186}\)–transduced (a8) myocardium. In addition, polarized, granular Nrp-1 staining was seen in cardiomyocytes in AdVEGF-B\(_{186}\)–transduced hearts in the vicinity of the needle track (a12), whereas no such finding was observed after AdVEGF-A (a10) or AdPigF– (a11) gene transfers. Needle tracks are shown on the left in a9–12. Accordingly, Western blot analysis showed strong expression of Nrp-1 both in intact and in AdVEGF-B\(_{186}\)–transduced normoxic myocardium but not in skeletal muscles (B). L indicates AdLacZ–transduced tissue; B, AdVEGF-B\(_{186}\)–transduced tissue. C, VEGF-B induces GIPC expression in cardiomyocytes. In AdVEGF-B\(_{186}\)–transduced myocardium, a polarized, granular GIPC staining was found in cardiomyocytes in the vicinity of the needle tract in an overlapping pattern with Nrp-1 staining (c12), but this was not found in the skeletal muscle (c1–c4) or in AdLacZ– (c9), AdVEGF-A– (c10), or AdPigF–transduced (c11) myocardium. GIPC staining was strongly positive for GIPC. D and E, VEGF-B binds to and activates Nrp-1. Tissue extracts were immunoprecipitated with an Nrp-1 antibody and resolved on an SDS-PAGE gel. Western blotting with a VEGF-B antibody (D) showed that VEGF-B bound to Nrp-1 in transduced myocardium, whereas no Nrp-1–bound VEGF-B was detected in skeletal muscle. L indicates AdLacZ–transduced tissue; B, AdVEGF-B\(_{186}\)–transduced tissue; and P, recombinant human VEGF-B\(_{186}\)–positive control. Immunoblotting with GIPC antibodies (E) showed weak association with Nrp-1 in AdLacZ–transduced myocardium and strong binding of GIPC to Nrp-1 in AdVEGF-B\(_{186}\)–transduced hearts. No binding was observed in skeletal muscles. L indicates AdLacZ–transduced tissue; f, AdVEGF-B\(_{186}\)–transduced tissue; and g, AdVEGF-B\(_{186}\) upregulates GAIP in the endothelium and induces GAIP expression in cardiomyocytes. GAIP staining was localized to the capillary endothelium in AdLacZ–, AdVEGF-A–, AdPigF–, and AdVEGF-B\(_{186}\)–transduced hearts (f5–f8). In addition, a polarized, granular GAIP staining was detected in AdVEGF-B\(_{186}\)–transduced cardiomyocytes (f12), GAIP staining in AdLacZ– (f9), AdVEGF-A– (f10), and AdPigF–transduced (f11) cardiomyocytes was diffuse. No GAIP expression was found in skeletal muscles (f1–f4). Accordingly, Western blot analysis showed strong expression of GAIP in AdVEGF-B\(_{186}\)–transduced hearts, whereas expression in AdLacZ–transduced hearts was very low, and no GAIP was detected in skeletal muscle samples (G). L indicates AdLacZ–transduced tissue; B, AdVEGF-B\(_{186}\)–transduced tissue.

Discussion

VEGF-B\(_{186}\) induced a myocardium-specific angiogenic effect that was qualitatively and mechanistically different from that induced by other VEGF-1 ligands. In previous studies, we have shown that AdVEGF-B\(_{167}\) and AdVEGF-B\(_{186}\) do not have any angiogenic effects in periventricular tissue around carotid arteries.\(^{16}\) In contrast to our results, VEGF-B has been shown to induce angiogenesis in mouse hindlimb ischemia.\(^{14}\) This discrepancy may be explained by the use of a small-animal model and differences in capillary growth mechanisms between small- and large-animal species. Interestingly, a recent publication by Li et al\(^{19}\) also suggests a restricted function for VEGF-B in mouse myocardium. It is interesting to note that VEGF-B–deficient mice appear healthy and fertile and display only minor heart phenotypes.\(^{20,21}\) PIGF-deficient mice are also healthy and fertile.\(^{22}\) Although neither growth factor is regulated by hypoxia, both VEGF-B and PIGF knockout mice are reported to have an impaired angiogenic response in ischemic tissues.\(^{20,22}\) However, the specificity of VEGF-B in the myocardium has not been reported previously.
Figure 5. VEGF-B induces antiapoptotic signaling and a changed metabolic pattern indirectly in cardiomyocytes. A and B, VEGF-B treatment in vitro induces direct gene expression changes in bEnd3 endothelial cells but not in HL-1 cardiomyocytes. mRNA levels, determined by real-time reverse-transcription polymerase chain reaction, of fatty acid transport proteins (FATPs) and BH3-only genes Bik1, Bmf1, and Bad1 in cultured HL-1 cardiomyocytes (A) and mouse endothelial bEnd3 cells (B) treated with VEGF-B186 or sVEGFR-1. VEGF-B186 induced a 1.7-fold upregulation of Fatp4 mRNA in bEnd3 cells but no expressional changes of any studied genes in HL-1.

(Continued)
In addition to angiogenesis, AdVEGF-B186 also induced collateral artery growth in the ischemic heart (Figure 2). Collateral artery growth may have been induced by either direct effects on smooth muscle cells (Figure 1G) or increased shear stress in collateral arteries induced by increased blood flow in the infarction edge. Nrp-1 expression was strongly positive in the capillary endothelium in angiogenic areas and in the endothelium of arterioles and venules in the myocardium, but it was below the detection limit in the skeletal muscles. It is possible that Nrp-1 localizes the downstream effects of VEGF-B186 to the myocardium because of the differences in Nrp-1 expression in the myocardium and skeletal muscle (Figure 4A). Nrp-1 has been shown to be involved in embryonic angiogenesis. In contrast to its functions in the developing nervous system, intracellular signaling events have recently been shown to play a role in angiogenic functions of Nrp-1. Interestingly, AdVEGF-B186 also induced Nrp-1 expression in cardiomyocytes in the gene transfer area (Figure 4A).

In the present study, we showed an activation of the Nrp-1 signaling pathway after AdVEGF-B186 gene transfer in vivo. In contrast, the effects of AdPIGF were mediated primarily via upregulation of endogenous VEGF-A, which could be blocked by simultaneous transduction with AdsVEGFR-2 (Figure 3A). The angiogenic effects of AdVEGF-A and AdPIGF were also blocked by the nitric oxide synthase inhibitor L-NAME, whereas this treatment had no effect on the angiogenic effects of AdVEGF-B186. It is therefore likely that AdVEGF-B186 and AdPIGF induce different signaling events, and AdVEGF-B186-dependent recruitment of the Nrp-1 downstream target molecules GAIP and GIPC to myocardial cell membranes was detected only after AdVEGF-B186 administration (Figure 4a12, 4c12, and 4f12). On the basis of cell culture studies, the effects of AdVEGF-B186 were mediated via endothelial cells, and no direct effect on cultured cardiomyocytes could be detected. Interestingly, VEGF-B186 also has indirect metabolic effects on cardiomyocytes, increasing Fatp4 expression and lipid and glycogen accumulation in AdVEGF-B186 hearts, which may make cardiomyocytes more resistant to ischemia. Karpanen at al. also recently published a report on the metabolic effects of VEGF-B. However, we cannot exclude other possibilities, such as the presence of a new VEGF receptor, a coreceptor, or another type of structure capable of preferentially binding VEGF-B186 in cardiac cells, nor can we rule out the possibility of secretion of an unidentified molecule from endothelial cells that mediates the effects of VEGF-B186 on cardiomyocytes. In addition to Nrp-1 activation, different diffusion kinetics of the transduced growth factors contribute to the different angiogenic profiles, because VEGF-B186 is known to be freely soluble and capable of diffusing from the site of transduction, whereas VEGF-B167 and VEGF-A bind tightly to heparan sulfate proteoglycans.

The exact mechanisms for the myocardial specificity of VEGF-B186 and the collateral artery formation in the AdVEGF-B186-treated hearts need to be clarified further; however, the increased proliferation and appearance of apoptosis-resistant cardiomyocytes around the infarction border zone in the AdVEGF-B186-treated hearts, together with increases in Fatp4 expression and lipid and glycogen accumulation, clearly suggest that VEGF-B186 may have favorable cardioprotective properties in addition to its unique heart specificity. We conclude that AdVEGF-B186 is a promising new therapeutic candidate for the treatment of patients with severe coronary heart disease.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

Severe coronary heart disease is still a leading cause of death in developed countries in spite of improved management of risk factors and effective treatments. A steadily increasing number of patients fall into a category in which currently available revascularization techniques cannot be applied. Thus, there is a clear need to develop efficient, minimally invasive procedures for the treatment of these no-option patients. In this study, we report a heart-specific angiogenic gene therapy approach for the treatment of myocardial ischemia. An adenosine encoding vascular endothelial growth factor-B (VEGF-B) was injected directly into the ischemic area in the left ventricular wall via an injection catheter. In this pig model of acute myocardial infarction, VEGF-B induced growth of neovessels in the infarction edge, which rescued hibernating myocardium in the perinfarction zone and induced growth of angiographically visible collateral vessels to form a biological bypass to transport blood to the infarction edge. VEGF-B also increased blood flow and increased ejection fraction in the ischemic heart. Moreover, VEGF-B induced metabolic changes by increasing glycogen accumulation, fatty acid transport, and lipid content in cardiomyocytes. VEGF-B also inhibited apoptosis of cardiomyocytes, thus protecting the cells from ischemia. These effects were mediated at least in part by a novel mechanism via VEGF receptor-1 activation of neuropilin receptor-1 and consequent activation of G-protein–mediated signaling.


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VEGF-B induces myocardium-specific angiogenesis and arteriogenesis via VEGF receptor-1- and Neuropilin receptor-1-dependent mechanisms

SUPPLEMENTAL MATERIAL

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Supplemental methods

Histology

Tissues were perfusion fixed(12) and samples were collected from maximal gene transfer areas determined by the presence of a needle track. From ischemic tissues samples were also collected from both infarction scar area (macroscopically necrotic tissue) and from the infarction edge (50% of the cut surface necrotic, 50% viable). Immunostainings were done for CD31 (JC70A, Dako Cytomation, USA), PECAM-1 (M-20, Santa Cruz, USA), α-SMA (Clone 1A4, Sigma Aldrich, USA), Nrp-1 (C-19, Santa Cruz, USA), GIPC (N-19, Santa Cruz, USA), GAIP (N-17, Santa Cruz, USA) and PCNA (Promega, USA) as described(12). Glycogen was visualized with Periodic acid-Schiff (PAS) staining and lipid accumulation with Oil-red-O staining from snap-frozen samples. Photographs of histological sections were taken with an Olympus AX70 microscope (Olympus, Japan) and AnalySIS software (Soft Imaging System, USA), and were further processed with Adobe Photoshop 7.0 (Adobe, USA).

Cell culture experiments

bEnd3 cells (ATCC) were cultured in high glucose DMEM (Gibco) supplemented with 10% FCS. Murine HL-1 cardiomyocyte cell line was kindly provided by Dr. W.C. Claycomb (Louisiana State University Medical Center)(4) and were cultured in Claycomb medium (JRH Biosciences Ltd.) supplemented with 10% FCS according to the instructions of Dr. Claycomb. mVEGF-B_{186}, mVEGF-B_{167},
mPlGF2 and sVEGFR1 were purchased from R&D systems (Minneapolis, MN). For the treatment of HL-1 and bEnd3 cells with VEGF-B or PlGF2, cells were starved in Claycomb medium containing 0.5% FCS for 4 h followed by addition of 100 ng/ml of the growth factors, or 1 µg/ml of sVEGFR1, and further incubated for 20-25 h. For experiments with conditioned media, the media from growth factor treated bEnd3 cells was transferred to pre-starved HL-1 cells, and the HL-1 cells were further incubated for 8h. Total RNA fractions were isolated, reverse transcribed and analyzed by real-time RT-PCR. All experiments were performed in triplicates, and were repeated three times. Primers for the analyzed genes are shown below (Table 1).

<table>
<thead>
<tr>
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<th>Fwd primer</th>
<th>Rev primer</th>
</tr>
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<tbody>
<tr>
<td>mL19</td>
<td>GGTGACCTGGATGAGAAGGA</td>
<td>TTCAGCTTGTTGGATGCTC</td>
</tr>
<tr>
<td>mFatp1</td>
<td>TCAATGTACCAGGAATTACAGAAGG</td>
<td>GAGTGAGAAGTGGCGAC</td>
</tr>
<tr>
<td>mFatp4</td>
<td>GCAAGTCCCATCAGCAACTG</td>
<td>GGGGAAATCACAGGCTTCTC</td>
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<tr>
<td>ssFatp4</td>
<td>TGGTCCGTGTCAAGCGAGG</td>
<td>GGTGGACACGTTCTCGCC</td>
</tr>
<tr>
<td>ssBeta-Actin</td>
<td>ATGGATACTTGCGGCCATC</td>
<td>CTTGCTGATCCGACATCTGC</td>
</tr>
</tbody>
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**Supplemental table S1.** Primer sequences. All sequences are written 5’→ 3’

Expression levels in cultured cells were normalized to the expression of mL19, whereas beta-actin was used as normalization gene for pig samples. For detection of Mouse BH3-only genes (Bik1, Bmf1 and Bad1), Qiagen QuantiTect specific primers were used (Qiagen, Germany). m, Mouse; ss, Sus scrofa.
Supplemental Figures

Supplementary figure 1. AdVEGF-B$_{186}$ does not increase perfusion in skeletal muscle: Perfusion measured by contrast-enhanced ultrasound imaging six days after gene therapy showed significant increases in perfusion in AdVEGF-A and AdPIGF transduced animals (29- and 44-fold as compared to AdLacZ, respectively). Both AdVEGF-B$_{167}$ and AdVEGF-B$_{186}$ were ineffective.

Supplementary figure 2. Angiogenic effects of AdVEGF-B$_{186}$ persist three weeks after gene transfer. Pecam-1 staining for endothelial cells (brown), magnification 200X. 21 days after gene transfer mean capillary size was normal in AdLacZ transduced animals. In the AdVEGF-A group, the mean capillary size was still significantly larger compared to the control. In the AdPIGF group a small increase in mean capillary size persisted on d21 while in AdVEGF-B$_{167}$ group mean vessel size had returned to baseline. In AdVEGF-B$_{186}$ transduced myocardium thick-walled enlarged vessels were still present on d21.
Supplementary figure 3. AdVEGF-B\textsubscript{186} induced angiogenesis in the pig myocardium is not dependent on nitric oxide: The angiogenic effects of AdVEGF-A and AdPIGF were significantly reduced by the nitric oxide synthase inhibitor, L-NAME. The mean capillary sizes were reduced 54% and 50%, respectively. Administration of L-NAME did not reduce the mean capillary sizes in AdVEGF-B\textsubscript{167}, and AdVEGF-B\textsubscript{186} transduced hearts.

Supplementary figure 4. AdVEGF-B\textsubscript{186} inhibits apoptosis in the infarction edge. Tunel apoptosis stainings were performed from transduced myocardial sections. The number of tunnel positive cells was decreased in the border zone of the infarction scar after AdVEGF-B\textsubscript{186}, AdVEGF-A and PIGF gene transfers.
Representative images from the infarction border zone are shown (magnification 100X).