Adenoviral Catheter-Mediated Intramyocardial Gene Transfer Using the Mature Form of Vascular Endothelial Growth Factor-D Induces Transmural Angiogenesis in Porcine Heart

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Background—It is unclear what is the most efficient vector and growth factor for induction of therapeutic vascular growth in the heart. Furthermore, the histological nature of angiogenesis and potential side effects caused by different vascular endothelial growth factors (VEGFs) in myocardium have not been documented.

Methods and Results—Adenoviruses (Ad) at 2 doses (2×10^11 and 2×10^12 viral particles) or naked plasmids (1 mg) encoding LacZ control, VEGF-A165, or the mature, soluble form of VEGF-D (VEGF-D^NAC) were injected intramycardially with the NOGA catheter system into domestic pigs. AdVEGF-D^NAC gene transfer (GT) induced a dose-dependent myocardial protein production, as measured by ELISA, resulting in an efficient angiogenic effect 6 days after the injections. Also, AdVEGF-A165 produced high gene transfer efficacy, as demonstrated with immunohistochemistry, leading to prominent angiogenesis effects. Despite the catheter-mediated approach, angiogenesis induced by both AdVEGFs was transmural, with maximal effects in the epicardium. Histologically, strongly enlarged α-smooth muscle actin–positive microvessels involving abundant cell proliferation were found in the transduced regions, whereas microvessel density did not change. Myocardial contrast echocardiography and microspheres showed marked increases in perfusion in the transduced areas. VEGF-D^NAC but not matrix-bound VEGF-A165 was detected in plasma after adenoviral GT. A modified Miles assay demonstrated myocardial edema resulting in pericardial effusion with the higher AdVEGF doses. All effects returned to baseline by 3 weeks. Naked plasmid–mediated GT did not induce detectable protein production or vascular effects.

Conclusions—Like AdVEGF-A165, AdVEGF-D^NAC GT using the NOGA system produces efficient transmural angiogenesis and increases myocardial perfusion. AdVEGF-D^NAC could be useful for the induction of therapeutic vascular growth in the heart. (Circulation. 2004;109:1029-1035.)

Key Words: angiogenesis ■ echocardiography ■ gene therapy ■ perfusion ■ regional blood flow

Therapeutic angiogenesis induced by vascular growth factors may be a novel treatment for ischemic heart disease. Recombinant proteins, naked plasmids, and adenoviruses (Ads) have been used with intracoronary and intramyocardial injections in preclinical and clinical studies. However, the most efficient vector, growth factor, and route of delivery of angiogenic genes into the myocardium remain unclear. The feasibility of the intramyocardial injection strategy has been substantially improved by the introduction of the NOGA electromechanical mapping and injection system, which offers a percutaneous route to myocardial transduction as efficient as injections via thoracotomy.

Our objective was to study the nature of angiogenesis and potential side effects induced by 2 different vascular endothelial growth factors (VEGFs), the mature form of VEGF-D (VEGF-D^NAC) and VEGF-A165, using Ads and NOGA system–guided injections in pig heart. VEGF-A165 is a well-known angiogenesis factor with extracellular matrix–bind-
Miles assay. After the animals were euthanized, myocardial contrast echocardiography (MCE), microspheres, and modified perfusion and vascular permeability were studied with myocardial animals were euthanized 6 days or 3 weeks after gene transfer (GT), 

\[ \text{D consists of only the central VEGF homology domain.} \]

Before the N- and C-terminal propeptides of the full-length VEGF-D and 

\[ \text{the Experimental Animal Committee of Kuopio University.} \]

performed in a blinded manner. The study protocol was approved by 

samples were collected for histological analyses. All analyses were 

\[ \text{Both vector types encoded nuclear-targeted} \]

Lac 

\[ \text{p indicates naked plasmid.} \]

ing properties, whereas the effects of VEGF-D^{\text{Nac}}, a soluble member of the VEGF family, have not been documented previously in the myocardium. Furthermore, we compared the efficiency of naked plasmid with that of adenoviral vector, both used in clinical trials for therapeutic angiogenesis.

\[ \text{Methods} \]

NOGA Gene Transfer

Electromechanical mapping of the left ventricle of domestic pigs (National Experimental Animal Center, Kuopio, Finland) weighing 25 to 30 kg (n=45, Table) was performed with the NOGA system and an 8F NOGA injection catheter (Biosense-Webster, Johnson & Johnson). After \( \geq 70 \) points had been mapped, 10 intramyocardial injections (5 to 6 mm deep, 0.2 mL each, total volume 2.0 mL, \( \geq 5 \) mm apart from each other) were performed to the anterolateral wall. Human GMP-grade first-generation Ads1 at 2 doses (2\( \times 10^{11} \) or \( 2\times 10^{12} \) viral particles (vp) or naked plasmids (1 mg) were used. Both vector types encoded nuclear-targeted LacZ marker gene, human VEGF-A (AdVEGF-D^{\text{Nac}}), or the mature form of human VEGF-D (VEGF-D^{\text{Nac}}) driven by a CMV promoter. cDNA for VEGF-D^{\text{Nac}} lacks the N- and C-terminal propeptides of the full-length VEGF-D and consists of only the central VEGF homology domain. Before the animals were euthanized 6 days or 3 weeks after gene transfer (GT), perfusion and vascular permeability were studied with myocardial contrast echocardiography (MCE), microspheres, and modified Miles assay. After the animals were euthanized, myocardial samples were collected for histological analyses. All analyses were performed in a blinded manner. The study protocol was approved by the Experimental Animal Committee of Kuopio University.

Myocardial Contrast Echocardiography

Modified long-axis images to detect pericardial effusion were acquired with an Acuson Sequoia 512 and 3V2c transducer (Siemens). MCE for assessment of blood perfusion in the injected region was performed at 3.5 MHz and receiving second harmonics. Real-time reperfusion images (22 Hz, mechanical index=0.16) were obtained at the short-axis midpapillary level after destruction of intravenous bolus-administered contrast agent (1.0 mL, SonoVue, Bracco) with a high-energy Doppler wave. The end-systolic images representing maximal refilling of the treatment area compared with untreated segments of the left ventricle are presented.

Modified Miles Assay for Quantitative Measurement of Vascular Permeability

Plasma protein extravasation in the transduced myocardium was assessed with the modified Miles assay with Evans blue dye (30 mg/kg, Sigma). After perfusion fixation in diastolic arrest, the heart was photographed and samples were collected for calculation of the vascular permeability ratio between the target anterolateral and intact apical control regions.

Regional Myocardial Perfusion

The perfusion ratio between the transduced anterolateral and intact apical control regions was calculated at rest and during dobutamine stress (10 to 80 \( \mu \)g \( \cdot \) kg\(^{-1} \) \cdot \) min\(^{-1} \)) until a heart rate 2 times that of rest was achieved (Dobutrex, Lilly) by use of red and yellow-green fluorescent microspheres (n=6\( \times 10^{10} \), 15 \( \mu \)m in diameter, Molecular Probes) injected near the mitral valve.

Histology and Microvessel Measurements

The avidin-biotin–horseradish peroxidase system with DAB was used for single immunostainings on 4% PFA-fixed paraffin-embedded sections and, for double immunostainings, together with the alkaline phosphatase system and Vector blue color substrate (Vector Laboratories). Myocardial samples were immunostained with a mouse monoclonal antibody against \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA, clone 1A4, Sigma, 1:250), proliferating cell nuclear antigen (PCNA, clone PC10, NeoMarkers, 1:500), and VEGF-A (Santa Cruz Biotechnology [sc-7269], 1:500). Because of the lack of good markers for pig endothelium, myocardial microvessel density (microvessels/mm\(^2\)), microvessel mean area (\( \mu \)m\(^2\)), and total microvesSEL lumen area of myocardial area (%) in 2 transduced and 1 intact apical sections from each pig were measured from \( \alpha \)-SMA immunostained sections at \( \times 200 \) magnification from 5 fields of each section.

ELISA Analyses From Myocardial and Plasma Samples and Clinical Chemistry

VEGF-D^{\text{Nac}} and VEGF-A ELISAs were performed from homogenized snap-frozen myocardial samples and plasma (catalog No. DVED00 and DVE00, sensitivities 9 pg/mL and 11 pg/mL, respectively; Quantikine, R&D Systems). Plasma troponin T (TnT),

\[ \text{Figure 1. Adenoviral GT results in a dose-dependent VEGF-D^{\text{Nac}} production and presence in plasma as measured by ELISA. a, Human VEGF-D^{\text{Nac}} is not detectable in myocardium of controls or after naked plasmid-mediated GT but is abundantly present after AdVEGF-D^{\text{Nac}} GT. b, Intramyocardial AdVEGF-D^{\text{Nac}} GT produces substantial increases in plasma levels 6 days after transduction. P<0.05 and **P<0.01 vs AdLacZ (unless otherwise indicated). ND=not detectable.} \]
creatine kinase (CK), and CK-MBm (CK-MBm) were measured at Kuopio University Hospital Central Laboratory. Protein concentration was measured and electrophoresis was performed from pericardial effusate and plasma samples.

Statistical Analyses
Results are expressed as mean ± SEM. Statistical significance was evaluated by 1-way ANOVA followed by independent-samples t test or Kruskal-Wallis test followed by the Mann-Whitney U test when appropriate. For correlation analyses, Pearson’s test was performed. A value of *P* < 0.05 was considered statistically significant.

Results
Dose-Dependent Myocardial VEGF-D<sup>ANAC</sup> Production After Adenoviral GT
No deaths occurred during the injection procedure or the follow-up. AdVEGF-D<sup>ANAC</sup> but not pVEGF-D<sup>ANAC</sup> GT induced a dose-dependent protein production in myocardium and leakage to plasma as measured by ELISA 6 days after GT (Figure 1, a and b). Transduced human VEGF-A<sub>165</sub>, which is a strongly matrix-bound protein, was not detectable with ELISA from myocardial or plasma samples (data not shown, Figure 1b), but high GT efficacy could be demonstrated with immunohistochemistry after adenoviral GT (see below).

Transmural Effects of NOGA-Guided GT
AdLacZ did not change vascular permeability in the myocardium (Figure 2, b and c). In contrast, AdVEGF-D<sup>ANAC</sup> and AdVEGF-A increased vascular permeability in the target areas, as shown by the modified Miles assay 6 days after GT (Figure 2, c and d). Interestingly, the strongest response was located in the epicardium, despite the intraventricular route of injections (Figure 2, f and g). MCE revealed marked increases in perfusion in the corresponding regions (Figure 2, i and j). Despite strong myocardial edema, there were no increases in TnT, CK, or CK-MBm levels (data not shown).

Adenoviral VEGF-D<sup>ANAC</sup> and VEGF-A GTs Induce Efficient Myocardial Angiogenesis
In contrast to AdLacZ control GT, which caused only mild inflammation (Figure 3, a, d, and g), AdVEGF-D<sup>ANAC</sup> and AdVEGF-A stimulated remarkable transmural angiogenic effects in the injected regions, as shown by α-SMA immunostainings 6 days after the GT (Figure 3, b and c). Unlike AdVEGF-A–induced angiogenesis, which was oriented along the muscle bundles and involved clusters of α-SMA–positive vessels, the effect of AdVEGF-D<sup>ANAC</sup> was more diffuse (Figure 3, b, c, e, and f). Higher magnification demonstrates strong enlargement of α-SMA–positive microvessels with both AdVEGFs (Figure 3, h through k) involving proliferation of both endothelial cells and pericytes (Figure 3, o and p). Three weeks after the AdVEGF-D<sup>ANAC</sup> GT, angiogenic effects were no longer detected (Figure 3l). In contrast to Ad, which showed a high efficacy, only sporadic positive cells were observed in X-Gal staining 6 days after pLacZ GT (data not shown). In line with the low GT efficiency, pVEGF-A or pVEGF-D<sup>ANAC</sup> did not induce angiogenic effects in the heart (Figure 3n). The damage caused by injection resulted in sporadic endogenous VEGF-A production in myocytes surrounding the needle track in AdLacZ control animals (Figure 3q). After AdVEGF-A<sub>165</sub> GT, however, the VEGF-A was abundantly expressed by transduced myocytes (Figure 3r).

Myocardial Angiogenesis Is Primarily Microvessel Enlargement, Not Increases in Microvessel Number
As measured from α-SMA–immunostained sections 6 days after GT, the angiogenesis effects were composed of enlarge-
ment of preexisting microvessels (Figure 4a). Apex control and AdLacZ-transduced regions had similar microvessel mean areas showing that Ad itself did not cause any microvessel enlargement. The low and high doses of AdVEGF-A caused 1.7-fold \((P=0.017)\) and 2.3-fold \((P=0.004)\) increases, respectively, in the microvessel mean area compared with the AdLacZ control 6 days after GT. Both doses of AdVEGF-D\(^{\Delta NAC}\) increased the microvessel mean area 2.1-fold over AdLacZ \((P=0.003\) and \(P=0.02\), respectively). The microvessel enlargement, at its best, led to a 2.0-fold increase in the total area of the myocardium covered by microvessel lumens (from 6.7% in AdLacZ group to 13.5% in AdVEGF-A \(10^{12}\)-vp group, \(P=0.004\). pVEGF-A or pVEGF-D\(^{\Delta NAC}\) GT did not induce changes in microvessel morphology. No changes in \(\alpha\)-SMA–positive microvessel density were detected between the study groups (Figure 4b).

Increased Vascular Permeability and Myocardial Perfusion in Regions Injected With AdVEGFs

The modified Miles assay using Evans blue dye showed a marked increase in the myocardial vascular permeability with AdVEGFs (Figure 4c). Whereas the \(10^{11}\)-vp dose of AdVEGF-A did not induce permeability significantly, the same dose of AdVEGF-D\(^{\Delta NAC}\) caused a 4.8-fold increase compared with AdLacZ \((P=0.032)\). Higher doses of AdVEGF-A and AdVEGF-D\(^{\Delta NAC}\) caused 4.3- and 3.8-fold increases \((P=0.008\) and 0.003\), respectively, in vascular permeability. pVEGF-A or pVEGF-D\(^{\Delta NAC}\) GT did not increase vascular permeability over the pLacZ control. A positive correlation was found when the vascular permeability ratio of each heart was plotted against the respective microvessel mean area (Pearson correlation 0.687, \(P<0.01\)).
Compared with AdLacZ, the higher dose of AdVEGF-A and the lower dose of AdVEGF-D resulted in statistically significant 2.0- and 1.6-fold increases (P=0.006 and P=0.037), respectively, in the perfusion ratio between the transduced and apical control regions at rest (Figure 4d). Also, with the higher dose of AdVEGF-D, a trend toward an increase in perfusion (1.6-fold, P=0.064) was found. During dobutamine stress, the perfusion ratios between the transduced and apical control areas were at the same level in all groups (Figure 4d).

Excess Myocardial VEGF Causes Pericardial Effusion
Microvessel hyperpermeability with the higher dose of AdVEGFs resulted in substantial pericardial effusion detected with echocardiography (Figure 2, i and j, and Figure 5). In some cases, effusion even resulted in tamponade of the right atrium (Figure 5b).

The total protein and fibrinogen concentration of the effusate was smaller than in plasma of the same animals (total protein concentration, 38±3 versus 49±4 g/L, P=0.022; electrophoresis data not shown). Specific ELISAs showed high amounts of VEGF-D\textsuperscript{2NAC} (6320±687 pg/mL) and VEGF-A (796±108 pg/mL) in the effusate after adenoviral GT with the respective Ads. There was no effusion either after AdLacZ with the higher dose or after AdVEGFs with the lower dose (Figure 2h and Figure 5, a and c).

All Angiogenic Effects and Side Effects Return to Baseline by 3 Weeks After GT
VEGF-D\textsuperscript{2NAC} production in the myocardium was no longer detectable 3 weeks after the adenoviral GT (data not shown). Consequently, the increases in microvessel mean area, vascular permeability, and regional perfusion had returned to baseline by 3 weeks (Figure 3l, data not shown).

Discussion
This study demonstrates for the first time that intramyocardial injections of Ad encoding a novel VEGF, VEGF-D\textsuperscript{2NAC}, with the NOGA system promote efficient transmural angiogenesis and a marked increase in myocardial perfusion. However, the strong microvessel enlargement resulted in substantial myocardial edema and, with the higher AdVEGF doses, pericardial fluid accumulation. Interestingly, VEGF-D\textsuperscript{2NAC} promoted more diffuse angiogenesis than VEGF-A\textsuperscript{165}. Naked plasmid encoding either VEGF-D\textsuperscript{2NAC} or VEGF-A did not induce detectable protein production, angiogenesis, or vascular permeability.

We used nonischemic pig heart because comparison of the angiogenic effects and quantitative measurements of the transduced growth factors could be significantly interfered with endogenous VEGF-A\textsuperscript{13} and other angiogenic factors induced by ischemia. Moreover, the amiodar constrictor model of myocardial ischemia produces unpredictable ischemia\textsuperscript{14} and requires thoracotomy and pericardial incision, making it impossible to analyze pericardial effusion reliably. In contrast to adenoviral GT, we did not find detectable protein production or biological effects with naked plasmid–mediated VEGF-D\textsuperscript{2NAC} or VEGF-A GT in nonischemic myocardium. However, in preclinical and clinical trials, naked plasmid has been reported to stimulate therapeutic angiogenesis in ischemic heart and skeletal muscle.\textsuperscript{3,15,16} Even though naked plasmid–mediated GT may be enhanced by tissue damage and ischemia,\textsuperscript{17} collateral artery growth, which should be one of the main goals of proangiogenic gene therapy, occurs in nonischemic areas of the heart.\textsuperscript{18} Furthermore, patients with stable angina pectoris have myocardial...
ischemia only during exercise. Thus, the GT vector used for therapeutic vascular growth should be efficient also in nonischemic tissue.

One of the most important findings in our study was that widespread transmural and epicardially prominent angiogenesis was achieved with the catheter-mediated intraventricular approach. This is probably because of an intramyocardial pressure gradient, the systolic subendocardial pressure being greater than the subepicardial pressure, leading to the movement of the GT solution and secreted growth factors toward the epicardial surface. Our findings imply that the epicardial surface can also be reached for therapeutic purposes via the percutaneous route without the need for thoracotomy.

In agreement with previous findings in nonischemic skeletal muscle, the predominant response of microvessels to adenovirally administered VEGFs is the enlargement of preexisting vessels rather than an increase in the number of microvessels. In addition to microvessel dilatation, VEGFs promoted efficient proliferation of pericytes and endothelial cells. Although the role of these enlarged vessels in the transfer of oxygen and nutrients to myocardium remains unclear, perfusion was enhanced up to 2-fold in the treated area compared with the apical control region at rest but not during dobutamine stress. The lack of perfusion difference between these regions during stress can be at least partly explained by the relaxation of arterioles in the nontransduced control region as well.

As in skeletal muscle, the largest vessels are also the leakiest to plasma proteins, because a positive correlation exists between microvessel size and vascular permeability. The large size (diameter up to 50 μm) and pericycle proliferation suggest that VEGF-DΔNε and VEGF-A induce transformation of capillaries toward vessels resembling arterioles, venules, or arteriovenous shunts. Despite cell proliferation and the quite complete pericycle coverage, enlarged vessels returned to normal size after withdrawal of the growth factors. Actually, no signs of transduced proteins, microvessel enlargement, edema, or increased perfusion were detectable 3 weeks after GT, suggesting that demonstrable biological effects occur only during the time course of adenoviral expression (<2 weeks). This finding clearly alleviates the safety concerns related to therapeutic angiogenesis. Conversely, a longer VEGF overexpression may be required for more persistent therapeutic effects, although Mack et al reported that in ischemic pig heart, the effects of AdVEGF-A, are still present 4 weeks after GT.

Interestingly, there were differences in the biological responses to VEGF-A165 and VEGF-DΔNε. VEGF-A165 created clusters of α-SMA-positive vessels, previously described as glomeruloid bodies, that grew along myocardial muscle bundles. Conversely, VEGF-DΔNε promoted a more uniform effect throughout the bundles. Furthermore, transduced VEGF-DΔNε but not VEGF-A165 was detected in plasma. Unlike AdVEGF-A, both doses of AdVEGF-DΔNε were equally angiogenic despite the dose-dependent VEGF-DΔNε production. These findings may relate to the efficient matrix-binding properties of VEGF-A165, whereas there is no heparan-binding domain in the sequence of VEGF-DΔNε, making it soluble. Also, the distinct receptor (R)-binding profiles may contribute to these differences. VEGF-A is a ligand for VEGFR-1 and VEGFR-2, whereas VEGF-DΔNε activates primarily VEGFR-2 and to some extent VEGFR-3, VEGFR-1 can serve as a decoy receptor preventing

Figure 5. High doses (10^{12} vp) but not low doses (10^{11} vp) of AdVEGFs cause pericardial effusion. a through d, Modified long-axis echocardiography 6 days after GT. LV indicates left ventricle; RV, right ventricle; and RA, right atrium. a, High dose of AdLacZ control virus does not cause effusion. b, Significant effusion (asterisks) after transduction with high dose of AdVEGF-A. Note compressed right atrium, suggesting tamponade (arrowhead). c, No pericardial effusion (arrowhead) after low-dose AdVEGF-D^{ΔNε} treatment. d, Marked effusion (asterisks) after high dose of AdVEGF-D^{ΔNε} GT.
VEGF-A but not VEGF-D<sub>ANSC</sub> from binding to VEGFR-2, which is the principal mediator of the angiogenic effects.<sup>10,11</sup>

Intramyocardially administered adenovector itself caused only mild inflammation but no effusion, and only the higher doses of AdVEGFs caused extravasation of plasma to the pericardium. Our data indicate that the correct dose of adenovirus is very important for safe myocardial angiogenesis, especially in the heart, where a long-term VEGF-A expression has been shown to result in significant problems.<sup>23</sup> However, myocardial edema induced by AdVEGFs did not increase the plasma levels of TnT, CK, and CK-MB. It has been shown previously that NOGA-system-mediated injections themselves do not cause effusion or increases in plasma CK-MB levels.<sup>7</sup> Although the pig hearts tolerated the transient pericardial effusion, the situation may be different in diseased human hearts. On the basis of our results, the maximal dose of AdVEGF-D<sub>ANSC</sub> and AdVEGF-A in clinical trials targeting myocardium should be <10<sup>-2</sup> vp.

We conclude that adenoviral GT of VEGF-D<sub>ANSC</sub> delivered via the NOGA system is an effective novel approach for the induction of widespread transmural angiogenesis that results in markedly increased perfusion in the myocardium.

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