VEGF-D Is the Strongest Angiogenic and Lymphangiogenic Effector Among VEGFs Delivered Into Skeletal Muscle via Adenoviruses


Abstract—Optimal angiogenic and lymphangiogenic gene therapy requires knowledge of the best growth factors for each purpose. We studied the therapeutic potential of human vascular endothelial growth factor (VEGF) family members VEGF-A, VEGF-B, VEGF-C, and VEGF-D as well as a VEGFR-3–specific mutant (VEGF-C156S) using adenoviral gene transfer in rabbit hindlimb skeletal muscle. The significance of proteolytic processing of VEGF-D was explored using adenoviruses encoding either full-length or mature (∆NAC) VEGF-D. Adenoviruses expressing potent VEGFR-2 ligands, VEGF-A and VEGF-D156S, induced the strongest angiogenesis and vascular permeability effects as assessed by capillary vessel and perfusion measurements, modified Miles assay, and MRI. The most significant feature of angiogenesis induced by both VEGF-A and VEGF-D156S was a remarkable enlargement of microvessels with efficient recruitment of pericytes suggesting formation of arterioles or venules. VEGF-A also moderately increased capillary density and created glomeruloid bodies, clusters of tortuous vessels, whereas VEGF-D156S–induced angiogenesis was more diffuse. Vascular smooth muscle cell proliferation occurred in regions with increased plasma protein extravasation, indicating that arteriogenesis may be promoted by VEGF-A and VEGF-D156S. Full-length VEGF-C and VEGF-D induced predominantly and the selective VEGFR-3 ligand VEGF-C156S exclusively lymphangiogenesis. Unlike angiogenesis, lymphangiogenesis was not dependent on nitric oxide. The VEGF-R1 ligand VEGF-B did not promote either angiogenesis or lymphangiogenesis. Finally, we found a positive correlation between capillary size and vascular permeability. This study compares, for the first time, angiogenesis and lymphangiogenesis induced by gene transfer of different human VEGFs, and shows that VEGF-D is the most potent member when delivered via an adenoviral vector into skeletal muscle. (Circ Res. 2003;92:1098-1106.)

Keywords: vascular permeability • magnetic resonance imaging • edema • pericyte • arteriogenesis

Angiogenesis (capillary growth), lymphangiogenesis (lymphatic vessel growth), arteriogenesis (enlargement of arteries), and vasculogenesis (in situ formation of blood vessels from vascular stem cells) are crucial for normal embryonic development, growth, and tissue repair. Further understanding of these processes may also contribute to the treatment of many disorders, such as cancer, tissue ischemia, and lymphedema. Vascular endothelial growth factors (VEGFs) are involved in all types of vascular growth.1-6 Currently, the human VEGF family consists of 5 members: VEGF-A, -B, -C, -D, and placenta growth factor (PIGF).7-13 which differ in their ability to bind to VEGF receptors that are primarily expressed in endothelial cells (ECs): VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), VEGFR-3 (Flt-4), and neuropilin-1. VEGF-A binds to VEGFR-1 and VEGFR-2 as well as to neuropilin-1, whereas PIGF and VEGF-B bind only to VEGFR-1 and neuropilin-1.1,14,15 VEGF-C and VEGF-D are synthesized and secreted as large precursor forms that are proteolytically processed into mature forms comprising the central VEGF homology domain.16,17 Unprocessed forms preferentially signal through VEGFR-3, whereas only the mature forms efficiently trigger VEGFR-2 signaling.16,17 Both VEGF-C and -D have been suggested to be mainly lymphangiogenesis factors; their angiogenic potential has been reported to be considerably weaker than that of VEGF-A.6,13,16 In addition to the naturally occurring forms, Joukov et al18 have generated a mutant factor (VEGF-C156S) that binds to VEGFR-3 but not to VEGFR-2.
VEGF receptors have distinct biological roles. VEGFR-2 is considered to mediate most of the angiogenic, survival, and vascular permeability effects of VEGFs and effects on endothelial progenitor cells (EPCs), whereas the role of VEGFR-1 is controversial. Most studies indicate that VEGFR-1 is mainly a decoy receptor existing also as a soluble form, and that it may downregulate VEGFR-2–mediated mitogenesis. However, others have reported that the VEGFR-1 ligand PIGF mobilizes EPCs, hematopoietic, and inflammatory cells, and that it may amplify VEGFR-2–mediated effects. There is extensive evidence that VEGFR-3–mediated signaling alone is sufficient for the growth and maintenance of lymphatic vasculature.

Nitric oxide (NO) is crucial in VEGF–mediated angiogenesis and vascular permeability, but it is not known whether NO is necessary for lymphangiogenesis.

For optimal gene therapy, it is essential to determine which VEGF family member is most suitable for angiogenesis/lymphangiogenesis in a given tissue. To address this issue, we compared adenoviruses encoding human VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-C156S in rabbit hindlimb skeletal muscle. The objective was to study the direct effects of these VEGFs on local blood and lymphatic vessel growth, blood perfusion, and vascular permeability in the injected muscles and thus establish a basis for the selection of most suitable growth factors for each purpose. In addition, the significance of proteolytic processing of VEGF-D in vivo was studied by using adenoviruses expressing both its full-length and mature (∆NΔC) forms. We further explored the effects of these growth factors on vascular smooth muscle cells (SMCs) and pericytes, and evaluated the role of NO in vascular permeability, angiogenesis, and lymphangiogenesis induced by VEGF-D in vivo.

The superficial femoral artery of New Zealand White rabbits (n=66) was excised and two reentry arteries for collaterals near the knee joint were ligated in order to induce collateral artery growth in the hindlimb as described. The nonischemic thigh region lacking connective tissue between muscle bundles and in muscle connective tissue was excised and two reentry arteries for collaterals near the knee joint were ligated in order to induce collateral artery growth in the hindlimb as described. Animals received intramuscular (IM) injections of human clinical grade adenoviruses (total dose 10¹¹ viral particles vp) encoding human VEGF-A35, VEGF-B35, full-length VEGF-C, full-length or mature (∆NΔC) VEGF-D, or VEGF-C156S18 (n=3 to 8 in each group). Nω-Nitro-L-arginine methyl ester (LNAME, NO synthase inhibitor) was used to study whether angiogenesis, lymphangiogenesis, and vascular permeability promoted either by the full-length or mature form of VEGF-D are dependent on NO production. Transduced hindlimbs were monitored for vascular permeability and subsequent edema with gadodiamide (GdDTPA-BMA)-contrast agent-enhanced T₂*-weighted MRI (MRI) 5 days after gene transfer (GT). Muscle perfusion and vascular permeability were assessed quantitatively with fluorescent microspheres and a modified Miles assay, respectively, 6 days after GT. Thereafter, muscle samples were collected for histological analyses. All animal experiments were approved by the Experimental Animal Committee, University of Kuopio.

For further details, see the expanded Materials and Methods in the online data supplement available at http://www.circresaha.org.

**Results**

**VEGF-2 Ligands VEGF-A and VEGF-DΔNAC Induce the Strongest Angiogenesis Effects in Skeletal Muscle**

Effects of VEGFs were evaluated in nonischemic muscles in areas outside the needle track in order to exclude the confounding effects of endogenous growth factors induced by injection trauma or ischemia. Control virus (10¹¹ vp/mL) encoding the LacZ marker gene did not induce angiogenesis or significant inflammation in rabbit skeletal muscle (Figures 1a and 1c), whereas an angiogenesis response characterized by a remarkable microvessel enlargement was induced by both AdVEGF-A and AdVEGF-DΔNAC (Figures 1b, 1d, and 1i). In AdVEGF-DΔNAC transduced muscles, the size of enlarged vessels sometimes exceeded that of surrounding skeletal myocytes. Adenoviruses encoding full-length VEGF-C and VEGF-D also enlarged the microvessels, although less than AdVEGF-A and AdVEGF-DΔNAC (Figures 1f and 1h), whereas AdVEGF-B and AdVEGF-C156S had no effects on blood vessels (Figures 1e and 1g). VEGF-2 and αβ3 integrin were not detectable with immunostaining in capillaries of intact and AdLacZ transduced muscles (data not shown). In contrast, both VEGF-A (data not shown) and VEGF-DΔNAC upregulated VEGFR-2 and αβ3 integrin expression in the ECs (Figures 1j and 1k).

Interestingly, there were differences in the angiogenesis patterns promoted by AdVEGF-A and AdVEGF-DΔNAC. The strongest effects after AdVEGF-A treatment occurred in connective tissue between muscle bundles and in muscle fascias, whereas angiogenesis stimulated by AdVEGF-DΔNAC was more diffuse (Figures 2b, 2c, 2f, and 2g). Furthermore, AdVEGF-A generated more glomeruloid bodies (clusters of tortuous vessels) than AdVEGF-DΔNAC (Figure 2b).
VEGFR-3 Ligands VEGF-C, VEGF-C\(^{156S}\), and VEGF-D Promote Lymphangiogenesis

Histochemical staining of lymphatic endothelial cells for 5'-nucleosidase activity\(^{29}\) and an intraarterial injection of Ricinus Communis lectin (data not shown) revealed that the CD31-positive but \(\alpha\)SMA-negative vessels in the interstitial connective tissue between the muscle bundles were lymphatics, not blood vessels (Figures 2d through 2j).

Adenovirus encoding the full-length VEGF-D induced the strongest lymphatic vessel growth as almost all the interstitial connective tissue in transduced muscles was filled with lymphatic vasculature 6 days after GT (Figures 2d and 2h). AdVEGF-D\(^{ANAC}\), AdVEGF-C, and AdVEGF-C\(^{156S}\) were also potent lymphangiogenesis inducers (Figures 2g, 2i, and 2j). Remarkably, the adenovirus encoding the VEGFR-3 selective mutant VEGF-C\(^{156S}\) stimulated exclusively lymphangiogenesis, whereas AdVEGF-A (Figures 2f and 2j) and AdVEGF-B (data not shown) had no effects on lymphatic vessels.

**VEGF-A and VEGF-D\(^{ANAC}\) Induce Arteriogenesis**

In AdLacZ muscles, only a portion of the capillaries had \(\alpha\)SMA-positive pericytes and there were few proliferating SMCs (Figures 2k and 2o). In contrast, both in AdVEGF-A- and AdVEGF-D\(^{ANAC}\)-transduced muscles, nearly all enlarged capillaries had a complete or almost complete \(\alpha\)SMA-positive pericyte coverage, indicating that \(\alpha\)SMA expression was upregulated (Figures 2b, 2c, and 2l). Furthermore, BrdU labeling showed a high proliferation rate of ECs and pericytes after AdVEGF-A (data not shown) and AdVEGF-D\(^{ANAC}\).
In these muscles, collateral arteries showed active remodeling with abundant numbers of proliferating SMCs (Figure 2p), especially in regions of plasma protein extravasation (see following sections).

**AdVEGF-A and AdVEGF-D^\text{ANAC} Increase Vascular Permeability and Cause Edema**

Extensive vascular permeability and subsequent edema were observed in AdVEGF-A and AdVEGF-D^\text{ANAC} transduced muscles 5 days after GT by contrast agent–enhanced MRI (Figures 3b and 3g). Extravasated contrast agent was detected under the skin, in the semimembranosus muscle and in its fascias, and in the fat tissue between the medial and lateral muscle compartments. In contrast, AdLacZ or other AdVEGFs did not induce significant vascular permeability effects (Figures 3a, 3c through 3f, 4a, 4c, and 4e). The modified Miles assay demonstrated strong and quite diffuse vascular permeability in AdVEGF-D^\text{ANAC} transduced muscles (Figure 4b). Microscopically, extravasated plasma proteins were retained, especially in connective tissue between muscle bundles, around large blood vessels, as well as within muscle fibers surrounded by enlarged, leaky microvessels (Figures 4d and 4f).

**Angiogenesis and Lymphangiogenesis Profiles of Adenovirally Delivered VEGFs**

A quantitative comparison of capillary density and mean area, total capillary area, plasma protein extravasation, regional muscle perfusion, and total lymphatic vessel area in the transduced muscles 6 days after GT gave an angiogenesis or lymphangiogenesis profile for each VEGF. Surprisingly, only AdVEGF-A GT significantly increased the capillary density (Figure 5a). In contrast, AdVEGF-D^\text{ANAC} enlarged the mean capillary area as much as 14-fold as compared with AdLacZ control without inducing any increase in the capillary number (Figures 5a and 5b). The corresponding increase in the mean capillary size was 6.4-fold with AdVEGF-A. Effects on microvessel enlargement reached a statistical significance also with AdVEGF-C and AdVEGF-D with 2.5- and 2.2-fold increases, respectively (Figure 5b). AdVEGF-D^\text{ANAC} and AdVEGF-A GT resulted in dramatic 24- and 13-fold increases, respectively, in plasma protein extravasation as measured by the modified Miles assay (Figure 5b). Although AdVEGF-C and AdVEGF-D had weak effects on capillary enlargement, they did not significantly enhance plasma protein extravasation.

About 6% and 8% of the AdVEGF-A– and AdVEGF-D^\text{ANAC}–transduced muscles were covered by microvessel lumens, respectively (Figure 5c). These figures were much greater than in AdVEGF-C– (1.8%), AdVEGF-D– (1.7%), and AdLacZ-treated muscles (0.7%). Regional perfusion was increased accordingly in the transduced muscles. AdVEGF-A, AdVEGF-D^\text{ANAC}, and AdVEGF-C induced statistically significant 4.0-, 3.2-, and 2.0-fold increases in perfusion, respectively (Figure 5c). As shown in Figure 5d, lymphatic vessel area of muscles (%) was strongly increased with adenosinoreceptors expressing the VEGFR-3 ligands VEGF-C (1.5%), VEGF-C^156S (1.3%), VEGF-D (2.6%), and VEGF-D^\text{ANAC} (1.4%) as compared with AdLacZ (0.12%).

Finally, angiogenesis and lymphangiogenesis indices were calculated for each VEGF. These indices illustrate the balance between blood and lymphatic vessel growth (Figure 6). VEGF-A was the only to induce angiogenesis but not lymphangiogenesis, whereas full-length VEGF-C and VEGF-D were found to be mainly, and VEGF-C^156S exclusively, lymphangiogenic. The mature form VEGF-D^\text{ANAC} induced significant growth of both vessel types. VEGF-B was inefficient both for angiogenesis and lymphangiogenesis.
Angiogenesis but not Lymphangiogenesis Is Dependent on NO Production

NO synthase inhibitor L-NAME significantly blocked capillary enlargement and increases in vascular permeability after AdVEGF-D\textsuperscript{ΔN LC} treatment as shown in Figures 3h and 7a. In contrast, NO synthase inhibition did not affect lymphangiogenesis induced by full-length VEGF-D (Figure 7b).

Vascular Permeability Correlates With Microvessel Size

A positive correlation was found when the mean microvessel area calculated from each transduced muscle was plotted against the respective vascular permeability ratio (Figure 7c). A positive correlation also existed between the total capillary area and the regional muscle perfusion, indicating that the anatomical observation about capillary vessel growth is in line with a physiological measure of blood flow (Figure 7d). However, the correlation was best with physiological (small) capillary sizes because microspheres (diameter 15 μm) do not likely get stuck in strongly enlarged capillaries (diameter >15 μm), which may underestimate perfusion values obtained with this method.

Discussion

We analyzed the human VEGF family members VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-D\textsuperscript{ΔN LC}, and a VEGFR-3–specific mutant VEGF-C\textsuperscript{Δ156S} for their effects on angiogen-
esis, lymphangiogenesis, and vascular permeability in a rabbit hindlimb model using adenoviral GT. We found striking inductions in blood vessel growth and vascular permeability with adenoviruses encoding VEGF-A and VEGF-D^{ΔNAC}. Intriguingly, AdVEGF-A and AdVEGF-D^{ΔNAC} induced not only EC, but also pericyte and SMC proliferation, and 3- to 4-fold increases in muscle perfusion at rest. A positive correlation was observed between capillary size and vascular permeability. Adenoviruses expressing the full-length VEGF-C and VEGF-D induced mainly, and the VEGFR-3–specific mutant VEGF-C^{156S} exclusively, lymphangiogenesis. AdVEGF-D generated the most powerful lymphatic vessel growth, which, unlike angiogenesis, was not affected by NO synthase inhibition. Finally, we found that the VEGFR-1 ligand VEGF-B was ineffective both for angiogenesis and lymphangiogenesis.

We performed GT of different VEGFs in the nonischemic thigh muscles in this rabbit model of collateral artery growth because necrosis, inflammation, and expression of endogenous growth factors caused by ischemia may mask differences between transduced growth factors and make this kind of comparison impossible. Furthermore, therapies designed for augmentation of arteriogenesis should also be effective in nonischemic muscles upstream to ischemia, which is usually the site of collateral growth.

Whereas this in vivo study supports the role for VEGFR-2 signaling as the major regulator of angiogenesis and vascular permeability, our data suggest that VEGFR-1 signaling alone may not be capable of initiating angiogenesis because its ligand VEGF-B was ineffective. Nevertheless, distinct histological characteristics were associated with AdVEGF-A–induced angiogenesis in comparison with that obtained with AdVEGF-D^{ΔNAC}. AdVEGF-A increased capillary density and induced the formation of glomeruloid bodies more frequently than AdVEGF-D^{ΔNAC}, which exerted more diffuse effects and strongly enlarged the preexisting microvessels. These findings may relate to the fact that VEGF-A binds to VEGFR-1 and VEGFR-2, whereas VEGF-D^{ΔNAC} binds to VEGFR-2 and VEGFR-3. However, it is more likely that the different affinities of VEGF-A and VEGF-D^{ΔNAC} for the extracellular matrix are responsible for these biological differences. In contrast to VEGF-A, there is no heparan-binding domain in the sequence of VEGF-D^{ΔNAC}. Further-
modify the biological outcomes, cannot be excluded because human VEGFs were tested in the rabbit. However, according to our previous studies and this work, human and mouse VEGF-A and human VEGF-C have all shown expected potency in rabbits. Further studies are needed to clarify the role of VEGFR-1 in angiogenesis because PIGF, another VEGFR-1 ligand, is angiogenic at least in some circumstances.

As expected from in vitro receptor binding profiles and recent in vivo work, we found that VEGFs activating VEGFR-3 induced lymphangiogenesis in rabbit skeletal muscle. The counting of lymphatic vessels was done from CD31 and α-SMA double-immunostained sections because of the possibility that molecular markers used for the detection of lymphatic endothelium, such as VEGFR-3 and LYVE-1, may also be expressed on activated ECs of blood vessels or may not be expressed in all lymphatic ECs. VEGF-C stimulated predominantly, and the VEGFR-3-specific mutant VEGF-C156S exclusively, lymphatic vessel growth. However, lymphangiogenesis induced by the full-length VEGF-D was strongest as nearly all the space between muscle bundles was filled with lymphatics in AdVEGF-D–treated muscles. The ability of the processed form VEGF-DΔNAC to induce substantially more angiogenesis and less lymphangiogenesis than the full-length form can be explained by the fact that proteolytic processing increases its affinity toward VEGFR-2 more (290-fold) than toward VEGFR-3 (40-fold). Our data also suggest that, at least in nonischemic skeletal muscle, VEGF-D is not efficiently cleaved by proteases. Taken together, our results implicate that VEGF-C, VEGF-D, and VEGF-C156S could be applicable in the treatment of lymphatic disorders such as primary and secondary lymphedema.

Blood vessels formed in response to VEGF-A have been suggested to lack pericytes, and it has been proposed that because of this defect they are prone to regression when VEGF-A levels are decreased. However, our data demonstrate that the expanded vessels induced both by AdVEGF-A and AdVEGF-DΔNAC are accompanied by an α-SMA–positive pericyte coverage, which, together with the large diameter (up to 50 μm), suggests a shift from the midcapillary phenotype toward arterioles, venules, or possibly arteriovenous shunts. Nevertheless, in spite of the efficient pericyte recruitment, our recent study shows that at least in nonischemic skeletal muscle, where excess blood perfusion is not necessary, the enlarged capillaries induced by AdVEGF-A or AdFGF-4 regress after the termination of gene expression.

To the best of our knowledge, it has not been shown that VEGFs could induce SMC or pericyte proliferation in vivo and thus these actions are likely indirect. Arterial SMCs and pericyte proliferation occurred in areas of increased plasma protein extravasation, which suggests that protein efflux from the vasculature to the extravascular space may contribute to these processes. For example, extravasation of plasma proteins triggers the clotting cascade, leading to deposition of fibrin gel in the interstitial space. In addition to causing edema, the hydrated fibrin gel is proangiogenic because it provides matrix for cell migration and growth. Additional growth factors that are mitogenic for SMCs, such as PDGFs, may also be upregulated. Furthermore, increased shear stress in the enlarged microvessels and enhanced integrin signaling may further contribute to the transformation of capillaries toward bigger vessels.

The NO synthase inhibitor L-NAME significantly blocked AdVEGF-DΔNAC–induced vascular effects including capillary enlargement and extravasation of plasma proteins, indicating that, like in the case of VEGF-A, NO is crucial for blood vascular effects stimulated by VEGF-DΔNAC. Furthermore, VEGFR-2 and α,β2 integrin were upregulated on ECs by VEGF-DΔNAC, suggesting that its angiogenic signaling mechanisms are similar to those of VEGF-A. Furthermore, efficient angiogenesis and VEGFR-2 upregulation by its ligands in nonischemic skeletal muscle shows that ischemia is not requisite for blood vessel growth. In contrast to angiogenesis, inhibition of NO did not affect lymphangiogenesis. To the best of our knowledge, this is the first demonstration that NO synthases are not crucial for lymphangiogenesis. Perivascular cells may be important for such a specific requirement of NO in angiogenesis as blood vascular, but not small lymphatic vessels, have a pericyte coverage.

We found an interesting correlation between mean capillary size and plasma protein extravasation. VEGFR-2 ligands induce plasma protein extravasation probably by multiple mechanisms. For example, they increase EC surface area, capillary blood flow and pressure, and also have direct effects on EC ultrastructure and vesicle transport.

As shown in this study, cell proliferation is involved in capillary enlargement. Thus, in addition to hemodynamic changes in enlarged microvessels, an increased number of intercellular clefts between dividing ECs, and possibly decreased integrity of the basement membrane may be of importance in vessel leakiness occurring 5 to 6 days after AdVEGF GT. In contrast to this kind of vascular permeability related to angiogenesis, ultrastructural changes and vesicle transport in ECs may be more important in acute plasma protein leakage induced by VEGFs. Ang-1 has been reported to improve the EC barrier function to plasma proteins. However, our data suggest that edema after VEGF GT could be reduced by hindering the excess enlargement of developing vessels. On the other hand, this may not be possible without the restriction of EC and SMC proliferation, which would likely compromise the success of therapeutic angiogenesis. Furthermore, as discussed above, increased plasma protein extravasation may be essential for efficient angiogenesis and arteriogenesis.

Our findings have several important implications. Firstly, they show that at least in nonischemic skeletal muscle the angiogenic effects of VEGF family members comprise microvessel enlargement, pericyte recruitment, and increases in vascular permeability, and not necessarily increases in capillary density. This relationship between angiogenesis and vascular permeability could be used for the judgment of successful gene transfer and for the evaluation of edema after clinical VEGF gene therapy with noninvasive imaging methods such as MRI or ultrasound. Secondly, unlike angiogenesis, lymphangiogenesis is not dependent on VEGFR-2 and NO. Most importantly, this unique comparison of the biological effects generated by different human VEGF family members in vivo provides a classification of human VEGFs as blood or lymphatic vessel growth factors depending on
their receptor specificity. The VEGFR-1 ligand VEGF-B is not able to trigger either angiogenesis or lymphangiogenesis in rabbit skeletal muscle, whereas VEGFR-2 and VEGFR-3 ligands are strongly angiogenic and lymphangiogenic, respectively. In conclusion, VEGF-A, VEGF-C, VEGF-C\textsuperscript{165}, VEGF-D, and VEGF-D\textsuperscript{-SMA} have potential as vascular therapeutics and the most suitable VEGF for each treatment can be chosen based on the need for angiogenesis and/or lymphangiogenesis.

Acknowledgments

This study was supported by grants from the Ludwig Institute for Cancer Research, the Sigrid Juselius Foundation, Academy of Finland, Novo Nordisk Foundation, and Finnish Medical Foundation. Marc Achen and Steven Stacker are supported by the National Health and Medical Research Council of Australia and the Anti-Cancer Council of Victoria. The α-SMA antibody was a generous gift from Dr. Giulio Gabbiani (Department of Pathology, Centre Medical Universitaire, Geneva, Switzerland). Anne Martikainen, Mervi Nieminen, Janne Kokkonen, Marja Poikolainen, and Dr Martin Kavec are acknowledged for their expert technical help.

References

20. Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG. Iso-


VEGF-D Is the Strongest Angiogenic and Lymphangiogenic Effector Among VEGFs Delivered Into Skeletal Muscle via Adenoviruses

Circ Res. 2003;92:1098-1106; originally published online April 24, 2003;
doi: 10.1161/01.RES.0000073584.46059.E3
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/92/10/1098

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2003/05/27/92.10.1098.DC1

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

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

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses

ONLINE DATA SUPPLEMENT –
EXPANDED MATERIALS AND METHODS

Tuomas T. Riissanen*1, Johanna E. Markkanen*1, Marcin Gruchala1, Tommi Heikura1, Antti Puranen1, Mikko I. Kettunen2, Ivana Kholová1, Risto A. Kauppinen2, Marc G. Achen3, Steven A. Stacker3, Kari Alitalo4 and Seppo Ylä-Herttuala1

1 Department of Biotechnology and Molecular Medicine, A.I. Virtanen Institute, Kuopio University, Finland
2 National NMR facility, A.I. Virtanen Institute, Kuopio University, Finland
3 Ludwig Institute for Cancer Research, Royal Melbourne Hospital, Victoria, Australia
4 Molecular/Cancer Biology Laboratory, Haartman Institute, University of Helsinki, Finland

* These authors contributed equally to this work

Address for correspondence and reprint requests:
Seppo Ylä-Herttuala, M.D., Ph.D
Department of Molecular Medicine
A. I. Virtanen Institute
University of Kuopio
P.O. Box 1627
FIN-70211 Kuopio
Finland
Phone: +358-17-162075
Fax: +358-17-163751
E-mail: Seppo.Ylaherttuala@uku.fi
Materials and methods

Gene transfer

The superficial femoral artery of New Zealand White rabbits (n=66) was excised and two re-entry arteries for collaterals near the knee joint were ligated in order to induce collateral artery growth in the hindlimb as described.\(^1\) The non-ischemic thigh region lacking confounding endogenous upregulation of VEGF-A and VEGFR-2\(^2\) was used for analysis of the gene transfer (GT).

Seven days after surgery, animals received intramuscular (i.m.) injections of adenoviruses [total dose 10\(^{11}\) viral particles (vp)] encoding human VEGF-A\(_{165}\)\(^3\), VEGF-B\(_{167}\)\(^4\), full-length VEGF-C\(^5\), full-length or mature VEGF-D\(^6\), or VEGF-C\(_{156}\)\(^7\) under control of the cytomegalovirus (CMV) promoter (n=3-8 in each group). Adenovirus expressing the full-length VEGF-D contains the sequence for the unprocessed prepropeptide VEGF-D including both N- and C-terminal propeptides whereas the adenovirus encoding the fully processed mature form (VEGF-D\(^{N\Delta C}\)) lacks these domains and consists only of the central VEGF homology domain.\(^6\);\(^8\) Human clinical grade (first generation, serotype 5) replication-deficient adenoviruses produced under GMP conditions and analyzed to be free from contaminants were used.\(^9\) Before the in vivo experiments, Western blotting was used\(^10\) to determine that rabbit aortic smooth muscle cells (RaSMCs) transduced with the different adenoviruses secreted proteins with the correct molecular weights (data not shown). I.m. GT was performed in the semimembranosus muscle on the medial thigh and in the abductor cruris cranialis muscle on the lateral thigh using a 1 ml syringe and a 25-gauge needle (10\(^{11}\) vp/ml divided into 10 separate injections, each 0.1 ml, 5 injections per individual muscle).

No-Nitro-L-arginine methyl ester (L-NAME, an NO synthase inhibitor)\(^11\) was used to study whether the angiogenic and lymphangiogenic signaling pathways of the full-length and
mature forms of VEGF-D are dependent on NO production. L-NAME (Sigma, 100mg/kg/day) was administered twice daily per os. Animals were monitored for edema with magnetic resonance imaging (MRI) five days after GT. Six days after GT, muscle perfusion and vascular permeability measurements were performed, and samples were collected for histological analyses. All animal experiments were approved by the Experimental Animal Committee, University of Kuopio.

*Magnetic resonance imaging (MRI)*

MRI for edema and vascular permeability effects of AdVEGFs was performed with a Varian INOVA (Varian Inc, Palo Alto, CA) imaging console interfaced to a 4.7T horizontal magnet (Magnex Scientific Ltd., Abingdon, UK) with actively shielded gradients (Magnex Scientific Ltd.) five days after GT. MRI data were acquired 3 min after gadodiamide-contrast agent (GdDTPA-BMA, Omniscan, Nycomed, the molecular weight 574 Da) injection (0.25 mmol/kg i.v.) with a custom built surface coil (50mm in diameter) placed between the thighs and using a 3D flow compensated T₂* weighted gradient-echo sequence (FOV 6x8x6 cm³, matrix 256x128x64, tr=25ms, te=80ms).¹ ²

*Modified Miles assay for vascular permeability*

Six days after GT, Evans Blue dye (30 mg/kg) was injected i.v. 30 min before sacrifice. After sacrifice the animals were perfusion-fixed with 1.5 L of 1% paraformaldehyde (PFA) in 0.05M citrate buffer (pH 3.5) via the left ventricle. Extravasated Evans Blue dye bound to plasma proteins (mostly albumin) was extracted from transduced and contralateral intact semimembranosus muscle samples by incubation in formamide overnight at 60°C. The amount of Evans Blue dye was
determined on the basis of absorbance at 610 nm and the ratio between transduced and intact muscle samples was calculated.

*Regional perfusion*

Regional perfusion in transduced semimembranosus muscles was measured at rest with fluorescent microspheres (15μm in diameter, FluoSpheres, Molecular Probes) six days after GT.¹ 2x10⁶ red microspheres were injected into the left ventricle through a 4F catheter (Cordis, Johnson & Johnson). After sacrifice, microspheres were extracted from semimembranosus muscle samples with the sedimentation method according to the manufacturer’s instructions. The perfusion ratio between the transduced and contralateral intact muscle was then calculated on the basis of the amount of fluorescence in each sample and using yellow-green microspheres as an internal control for pipeting errors.

*Histology*

Avidin-biotin-HRP and alkaline-phosphatase systems (Vector Laboratories) with 3’-5’-diaminobenzidine (DAB, Zymed) and Vector Blue (Vector) color substrates were used, respectively, for immunohistochemistry on 6 μm thick 4% paraformaldehyde-fixed paraffin-embedded sections. The endothelium was immunostained using a mouse mAb against CD31 (DAKO, dilution 1:50) and pericytes with a mAb against α-smooth muscle actin (α-SMA, clone 1A4, a gift from Dr. Giuliano Gabbiani, 1:250). VEGFR-2 and αβ₁ integrin were immunostained with mAbs (VEGFR-2: Santa Cruz Biotechnology, clone sc-6251, 1:500 and αβ₁: Biogenesis, 1:200) on frozen sections using the tyramide signal amplification system (TSA-kit, NEN Lifesience). Rhodamine labeled Ricinus Communis lectin (Vector, 1 mg) was injected in the
hindlimb vasculature via a 4F right coronary catheter in a subset of animals before sacrifice. CD31 α-SMA double staining was performed on the lectin-labeled sections using FITC (Perkin Elmer Life Science) and ACNC (Vector) fluorescent dyes according to the manufacturer's instructions.

Proliferation marker BrdU (20mg/kg, Sigma) given 3h before sacrifice was stained with a mAb (Clone Bu20a, DAKO, 1:100) followed by CD31 staining on the same sections. Sections for histological detection of Evans blue dye were deparaffinized in xylene, mounted with Dapi (Vector) for nuclear counter staining, and viewed in fluorescence microscopy using a 610 nm excitation filter. Only Evans blue bound to proteins fluoresces red whilst unbound dye does not fluoresce. 5'-nucleosidase staining for lymphatic vessels was performed as previously described. Photographs of histological sections were taken with an Olympus AX70 microscope (Olympus Optical) and analySIS software (Soft Imaging System), and were further processed with Adobe Photoshop 7.0 (Adobe).

**Blood and lymphatic vessel measurements**

Although the enlarged vessels formed from capillaries in response to AdVEGFs (see the results) are quite different from normal capillaries of skeletal muscle, for sake of simplicity we call these vessels “capillaries”. Capillary density (capillaries/mm²) and mean capillary area (μm²) were measured from CD31 immunostained sections of semimembranosus muscles at 200X magnification from areas covered by skeletal muscle tissue. Total capillary area (%) and total lymphatic vessel (%) denote the percentage of muscle area covered by capillary or lymphatic vessel lumens, respectively, and demonstrate the total angiogenic and lymphangiogenic activity of each VEGF. Except for large collecting trunks, lymphatic vessels can be distinguished from blood vessels by the lack of pericytes and they were counted in the connective tissue between muscle bundles from α-SMA-CD31 double immunostainings at 100X magnification. All measurements were performed in
a blinded manner from 10 different randomly selected fields from each muscle section. Means of the measurements are reported.

Statistical analyses

Results are expressed as means ± SEM. Statistical significance was evaluated using one-way ANOVA followed by independent samples t-test or Kruskal-Wallis test followed by Mann-Whitney U-test where appropriate. Correlation analyses were performed by the Pearson test. $P<0.05$ was considered statistically significant.
References


6. Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stackar SA. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci USA.* 1998;95:548-553.


13. Casley-Smith JR. The fine structure and functioning of tissue channels and lymphatics. 