Adenoviral VEGF-C overexpression induces blood vessel enlargement, tortuosity, and leakiness but no sprouting angiogenesis in the skin or mucous membranes

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ABSTRACT Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) are important regulators of blood and lymphatic vessel growth and vascular permeability. The VEGF-C/VEGFR-3 signaling pathway is crucial for lymphangiogenesis, and heterozygous inactivating missense mutations of the VEGFR-3 gene are associated with hereditary lymphedema. However, VEGF-C can have potent effects on blood vessels because its receptor VEGFR-3 is expressed in certain blood vessels and because the fully processed form of VEGF-C also binds to the VEGFR-2 of blood vessels. To characterize the in vivo effects of VEGF-C on blood and lymphatic vessels, we have overexpressed VEGF-C via adenovirus- and adeno-associated virus-mediated transfection in the skin and respiratory tract of athymic nude mice. This resulted in dose-dependent enlargement and tortuosity of veins, which, along with the collecting lymphatic vessels were found to express VEGFR-2. Expression of angiopoietin 1 blocked the increased leakiness of the blood vessels induced by VEGF-C whereas vessel enlargement and lymphangiogenesis were not affected. However, angiogenic sprouting of new blood vessels was not observed in response to AdVEGF-C or AAV-VEGF-C. These results show that virally produced VEGF-C induces blood vessel changes, including vascular leak, but its angiogenic potency is much reduced compared with VEGF in normal skin.—Saaristo, A., Veikkola, T., Enholm, B. Hytönen, M., Arola, J., Pajusola, K., Turunen, P., Jeltsch, M., Karkkainen, M. J., Kerjaschki, D., Bueler, H., Ylä-Herttuala, S., Alitalo, K. Adenoviral VEGF-C overexpression induces blood vessel enlargement, tortuosity, and leakiness but no sprouting angiogenesis in the skin or mucous membranes. *FASEB J.* 16, 1041–1049 (2002)

DRUG TARGETING of blood and/or lymphatic vessels is one approach for the treatment of many diseases, including cancer, ischemic diseases and tissue edema (1). The VEGF family currently includes five members, which are important regulators of angiogenesis and lymphangiogenesis: VEGF, placenta growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D (2, 3). VEGF is also known as vascular permeability factor, since it is more potent than histamine in increasing capillary permeability to plasma proteins (4, 5). Of the three known endothelial cell-specific receptor tyrosine kinases of the VEGF receptor family, VEGF binds selectively and with high affinity to VEGFR-1 and VEGFR-2 (5, 6). Angiopoietins (Angs) constitute another family of endothelial growth factors that are ligands for the endothelium-specific receptor tyrosine kinase, Tie-2 (Tek) (7, 8). Although Angs do not appear to induce new vessel growth, they may be involved in vessel stabilization. Vascular permeability induced by VEGF, for example, is known to be blocked by Ang-1 (9). Mice lacking Ang-2 also have defects in patterning and function of the lymphatic vessels (N. Gale, G. Thurston, and G. Yancopoulos, personal communication).

The VEGF-C receptor VEGFR-3 is expressed predominantly in the lymphatic endothelium of adult human tissues, and the VEGF-C/VEGFR-3 signaling pathway is critical for the growth and function of the lymphatic vessels (10). Recently, human hereditary lymphedema was shown to be associated with heterozygous inactivating missense mutations of the VEGFR-3 gene (11). Partially processed forms of VEGF-C are able to bind and activate VEGFR-3, whereas the fully processed short

form is a potent stimulator of VEGFR-2 expressed in blood and lymphatic vessel endothelia (12). Presumably through its interaction with VEGFR-2, VEGF-C can induce capillary endothelial cell migration and proliferation in culture (12, 13) and stimulate angiogenesis in the cornea and ischemic muscle (14, 15). The short form of VEGF-C has also been shown to increase capillary permeability in the Miles assay (12). Moreover, we have recently found that VEGFR-3 is expressed in some fenestrated blood vessel endothelia (16). Indeed, the VEGF-C expressed by respiratory epithelial cells may regulate the VEGFR-3-positive fenestrated blood vessels in the mucous membranes of the nasal cavity (17). In tumors, VEGF-C may stimulate lymphangiogenesis and lymphatic metastasis and act as an adjunctive stimulator of tumor angiogenesis (18, 19, 19a).

VEGF-C has been shown to induce hyperplasia of cutaneous lymphatic vessels in transgenic and adenoviral gene transduction models while causing little or no angiogenesis (10, 20, 21). Here we have studied the effects of adenovirus (Ad) and adeno-associated virus (AAV) -mediated VEGF-C overexpression in the skin and respiratory tract compared with gene transfer of control viruses encoding VEGF, enhanced green fluorescent protein (EGFP), or β-galactosidase.

MATERIALS AND METHODS

Generation and in vitro analysis of recombinant adenoviruses and adeno-associated viruses

For the adenovirus construct, the full-length human VEGF-C cDNA (Genbank accession number X94216) was cloned into the pAD *Bgl*II vector as described previously (21). Replication-deficient E1-E3 deleted adenoviruses were produced in 293 cells and concentrated by ultracentrifugation (22). Adenoviral preparations were analyzed to be free from helper viruses, lipopolysaccharide, and bacteriological contaminants (23). The full-length Ang-1 cDNA fused to a sequence encoding carboxyl-terminal Myc epitope was cloned under the CMV promoter in pShuttle vector containing the bovine growth hormone polyadenylation signal (Clontech, Palo Alto, CA). The AdAng-1 adenoviruses were produced using the Adeno-X Expression System kit according to the manufacturer's instructions (Clontech). The adenoviruses encoding VEGF$_{165}$ and nuclear targeted *LacZ* were constructed as described (22, 24, 25). In the AAV construct, the full-length human VEGF-C was cloned as a blunt-end fragment into the Mlu I site of psub-CMV-WPRE plasmid and the rAAV was produced as described (26). AAV encoding EGFP was used as a control (27).

For analysis of protein expression, 293EBNA cells were infected by recombinant adenoviruses for 2 h in serum-free medium or by adeno-associated viruses for 8 h in 2% FCS medium. After 24–72 h, the cells were metabolically labeled for 8 h and subjected to immunoprecipitation with VEGF-C or Myc-specific antibodies or to binding assay with soluble VEGFR-3-Ig and Tek-Ig fusion proteins. AdLacZ and AAV-EGFP-infected cells were used as negative controls. The bound proteins were precipitated with protein G Sepharose, separated in 10% or 15% SDS-PAGE, and analyzed by autoradiography. To compare the protein production levels of AdVEGF-C and AdVEGF viruses, polypeptides of the AdVEGF-C-, AdVEGF-, or AdLacZ-infected cell media (20 μl) were separated in 15% SDS-PAGE gel and direct Western blotting was performed using polyclonal α-VEGF-C (R&D, Abingdon, UK) and α-VEGF (R&D) antibodies.

In vivo use and analysis of the viral vectors

All studies were approved by the Experimental Animal Committee of the University of Helsinki. 1–9 × 10^8 pfu of the recombinant adenoviruses or 5 × 10^9-1 × 10^{11} rAAV particles was injected into the ears or the nasal cavities (only adenoviruses) of NMRI nu/nu mice (Harlan, Horst, Netherlands) and the mice were killed 1 to 3 wk after adenovirus or 5 to 6 wk after AAV gene transfer. At least six mice were used in each study group for each analytical technique (AdVEGF-C, AdVEGF$_{165}$, AdLacZ, AdVEGF-C+AdAng-1, AAV-VEGF-C, AAV-EGFP). The ears, nasal conchae, endoturbinates, and lungs of the mice were dissected and either fixed in 4% paraformaldehyde or frozen in liquid nitrogen before the analysis.

Total RNA was extracted from the ears, nasal mucosa, and lungs (RNAeasy Kit; Qiagen Gmbh, Hilden, Germany) 1 to 3 wk after adenoviral infection. 6 μg of RNA was subjected to Northern blotting and hybridization with a mixture of [α^{32}P]dCTP (Amersham, Arlington Heights, IL) labeled cDNAs specific for VEGF-C, VEGF, or Ang-1. RNA extracted from HT1080 cells or from cells transfected with Ang-1 was used as a positive control. The glyseraldehyde-3-phosphate dehydrogenase cDNA probe was used as an internal control for equal loading. The adenoviral protein expression was confirmed by whole mount β-galactosidase staining (28) of the AdLacZ-infected ears or nasal cavities 1–3 wk after gene transfer. The AAV-EGFP-infected ears were studied under the fluorescent microscope at 5 and 6 wk after infection.

Lectin and β-galactosidase staining of vessels

In some of the infected mice, *L. esculentum* lectin staining was used to visualize the blood vessels in whole mount (29). Either fluorescent or biotinylated lectin (1 mg/ml; Vector Laboratories, Burlingame, CA) was injected into the femoral veins of the mice under anesthesia and after 2 min the mice were killed and perfusion fixed with 1% paraformaldehyde (PFA)/0,5% glutaraldehyde in PBS. The tissues were dissected and the biotinylated lectin was visualized by the ABC-DAP peroxidase method (Vectastain, Vector Laboratories; and Sigma, St. Louis, MO). Finally, the tissues were dehydrated and mounted on slides.

To study the expression of VEGFR-2 and VEGFR-3 genes in the different types of vessels, biotinylated lectin and whole mount β-galactosidase stainings were performed in the VEGFR-2 + /LacZ or VEGFR-3/LacZ mice, in which one VEGFR-2 or VEGFR-3 allele is replaced by the LacZ marker gene by a knock-in strategy (30, 31). To better visualize the blood vessels of the skin, some of the VEGFR-2 + /LacZ mice were mated with K14-VEGFR-3-Ig mice to eliminate the lymphatic vessels of the skin (32). The biotinylated lectin and β-galactosidase stainings were then carried out on these double transgenic mice.

Immunohistochemistry of whole mount tissues and sections

For blocking of endogenous peroxidase activity, the PFA fixed tissues were incubated in 5% H$_2$O$_2$ in methanol for 1 h. After this, tissues were blocked in 3% milk 0.3% Triton-X in PBS overnight and antibodies against the vascular endothelial marker PECAM-1 (BD PharMingen, Hamburg, Germany) or VEGFR-3 (R&D) were applied overnight at +4°C. Visualization was done with either the ABC-DAB peroxidase method (Sigma) or with ABC-alkaline phosphatase using substrate kit

II (Vector Laboratories). Finally, the tissues were flattened and mounted on slides.

Deparaffinized sections (6 μm) of the tissues were subjected to heat-induced epitope retrieval (Target Retrieval Solution; Dako, Carpinteria, CA) for 20 min at 95[eg]C or to an alternative enzyme treatment. The endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 20 min. Monoclonal antibodies against VEGFR-3 (33), PECAM-1, podoplanin (34), or polyclonal antibodies against LYVE-1 (35) were applied overnight at +4°C and detected using reagents of the tyramide signal amplification kit (NEN Life Sciences, Frankfurt, Main, Germany). Peroxidase activity was developed with 3-amino-9-ethyl carbazole (Sigma) for 15 min and the sections were counterstained with hematoxylin. Double staining for proliferating cell nuclear antigen (PCNA) and VEGFR-3 was carried out by first staining the sections for PCNA using the PCNA staining kit (Zymed, San Francisco, CA); subsequently, VEGFR-3 staining was performed as detailed above. Diaminobenzidine and 3-amino-9-ethyl carbazole were used as chromogens for PCNA and VEGFR-3 antibodies, respectively.

Permeability assay

The right ear of each mouse was infected with AdVEGF-C, AdVEGF, AdVEGF-C+AdAng-1, or AdLacZ virus. The virus concentrations used are indicated in Fig. 6A. The left ear received either AdLacZ or PBS. Two weeks after the infection, a modified Miles permeability assay was performed as described (36). 1 μl/g of 3% Evans Blue was injected to the femoral vein of the mice and after 2 min the mice were perfusion fixed with 0.05M citrate buffer in 1% PFA pH 3.5. The ears were dissected, washed, weighed, and extracted in formamide at 55°C overnight. The Evans Blue absorbance of the formamide was calculated in a spectrophotometer set at 610 nm and leakage (ng/mg) to the right and left ear was compared in the same mouse.

RESULTS

Expression of the virally transduced genes in vitro and in vivo

The production of VEGF-C and Ang-1 proteins into the adeno or AAV-infected, metabolically labeled 293 EBNA cell culture media was confirmed by immunoprecipitation and by binding to soluble VEGFR-3-Ig and Tek-Ig fusion proteins. Gel electrophoresis showed that AdVEGF-C- and AAV-VEGF-C-infected cells produced the 30 kDa partially processed form of VEGF-C, whereas the 21 kDa mature form was also produced in AAV-VEGF-C-infected cultures (**Fig. 1A**). When we cultured the AdVEGF-C-infected cells 36 h instead of 24 h after infection, the 21 kDa band was found to accumulate (data not shown). Ang-1 polypeptides of 70 kDa were immunoprecipitated from the medium of AdAng-1-infected cells; these polypeptides were also precipitated by the Tek-Ig fusion protein (Fig. 1A). Protein production by the AdVEGF and AdLacZ viruses has been reported (21, 22, 24). Direct Western blotting analysis of the AdVEGF-C- and AdVEGF-infected cell media confirmed that the same viral titers of these two viruses produced comparable levels of protein in vitro (data not shown).
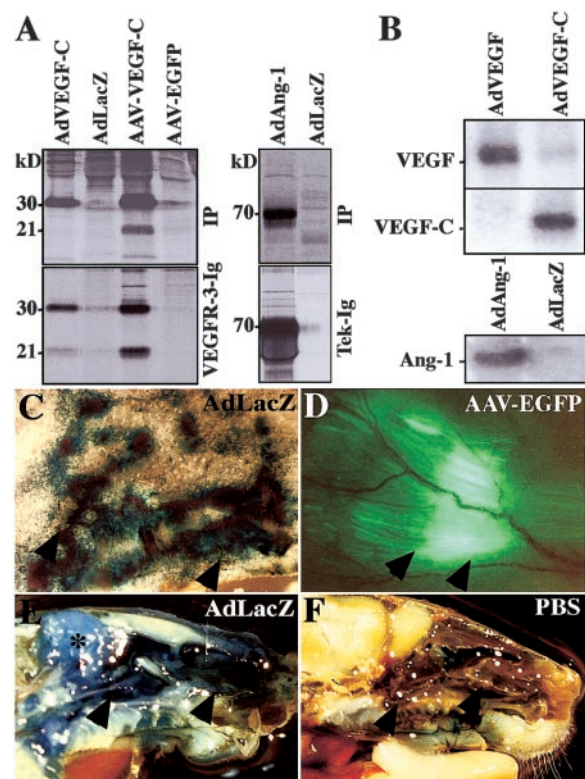

Figure 1. Adenoviral and AAV expression in vitro and in vivo. *A)* Ad-, AAV-VEGF-C-, and AdAng-1-infected cells were metabolically labeled and culture medium was subjected to precipitation with antibodies against VEGF-C or Myc (IP) or with soluble VEGFR-3-Ig or Tek-Ig fusion proteins. AdLacZ- and AAV-EGFP-infected media were used as negative controls. *B)* Northern blot analysis of total RNA from the mouse ears (*n*=8) was performed 7 days after adenoviral infection. The infecting adenoviruses and the probes used for hybridization are indicated. *C)* β-Galactosidase staining in stromal cells of the ear 7 days after infection with AdLacZ (*n*=6). *D)* GFP expression in fluorescence microscopy 6 wk after AAV-EGFP infection of the ear (*n*=10). *E, F)* LacZ expression in the epithelium of the nasal conchae and endoturbinates (arrowheads) 7 days after infection with AdLacZ (*E*) vs. the control (*F*) (*n*=10). Note that because of a very strong color development (*E*), surrounding tissues outside of the nasal cavity are also weakly stained (asterisk).

To analyze the expression of the adenovirally transduced genes in vivo, RNA samples from the infected ears, nasal mucosa, and lungs were analyzed by Northern blotting. High levels of the human VEGF-C, VEGF, and Ang-1 mRNAs were detected in the infected tissues (Fig. 1*B* and data not shown). When the ears or the nasal mucosa of the mice infected with AdLacZ were stained for β-galactosidase, a strong blue signal was observed in the infected tissues (Fig. 1*C, E, F*). Similarly, expression of green fluorescent protein was detected in the muscle fibers of the ear 3–6 wk after AAV-EGFP infection (Fig. 1*D*).

Comparison of the effects of adenoviral VEGF-C and VEGF in the skin

To study the in vivo effects of VEGF-C, 1–9 $\times 10^8$ pfu of adenoviruses encoding VEGF-C, VEGF, or LacZ were

injected intradermally to the ears of the nu/nu mice. The injection wound healed in less than 1 wk. When the study groups were observed by in situ images 1–2 wk after injection, tortuous and enlarged blood vessels were detected in the ears of AdVEGF-C and AdVEGF-infected mice compared with the AdLacZ or noninfected ears (data not shown). These effects were dose and time dependent; for example, only a few curly and enlarged blood vessels were seen with the lowest viral titers of AdVEGF-C (1×10^8 pfu). Some petechiae were also observed with the highest viral titers of AdVEGF, whereas petechiae were present in only one AdVEGF-C-infected mouse (data not shown). The morphology of the vessels in the whole mount PECAM-1 staining indicated that the enlarged vessels were veins and venules (**Fig. 2***A*, arrowheads). In the AdVEGF-infected ears, a dense network of new capillaries was stained for PECAM-1; in the VEGF-C or LacZ-infected ears, no such network was observed (Fig. 2*A*–*C*).

The enlargement of the blood vessels in response to AdVEGF-C was noted in the skin sections stained for PECAM-1 (Fig. 2*D*, arrowheads). There were large numbers of new capillary sprouts in the VEGF overexpressing ears 7 days after infection, but such capillaries were not observed in AdVEGF-C- or AdLacZ-infected samples (arrows in Fig. 2*D*–*F*). Double staining for the PCNA and VEGFR-3 revealed that a majority of the endothelial cells (ECs) in the lymphatic vessels and several ECs in the enlarged blood vessels were proliferating 7 days after the AdVEGF-C infection (Fig. 2*G*). This was most obvious in the samples infected with the highest viral titers of AdVEGF-C, and the transient response was down-regulated at 2 wk when the veins had already started to regress to their normal size. AdVEGF-infected ears showed increased proliferation of the blood vascular ECs but much less stimulation of the lymphatic ECs was detected (Fig. 2*H*). In the ears infected with AdLacZ, only a few of the EC nuclei stained positive for PCNA (Fig. 2*I*). Staining for lymphatic markers podoplanin and LYVE-1 confirmed the formation of numerous large sprouting lymphatic vessels in the AdVEGF-C-infected ears (Fig. 2*J*–*L* and data not shown) and whole mount VEGFR-3 staining demonstrated new lymphatic vessels at 1 and 2 wk after AdVEGF-C infection (Fig. 1*M*). The lymphangiogenic response was also obvious with the low viral doses of AdVEGF-C. Although the VEGFR-3-positive lymphatic vessels of the AdVEGF-infected ears appeared dilated compared with the AdLacZ control, the lymphatic vessel density was not increased (Fig. 2*N*, *O*).

Effects of AAV-derived VEGF-C in the skin

To confirm the results obtained with AdVEGF-C, AAVs encoding VEGF-C or EGFP were injected intradermally into the ears of the nu/nu mice. Indeed, enlargement and tortuosity of blood vessels was observed in the AAV-VEGF-C-infected ears but not in the AAV-EGFP-infected controls (**Fig. 3***A*, *B*). However, the response was weaker than with the adenoviral VEGF-C. VEGFR-3
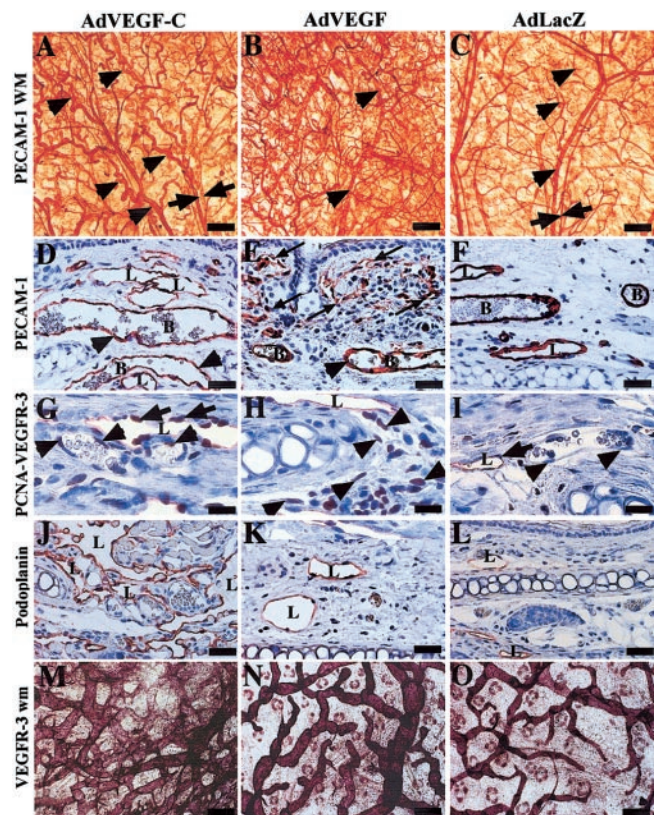

Figure 2. Blood vessel enlargement and lymphangiogenesis in response to adenoviral VEGF-C in the skin. *A)* Whole mount PECAM-1 staining shows enlargement of the veins and venules (arrowheads) in response to adenoviral VEGF-C (5×10^8 pfu) 2 wk after infection (n=8); double arrows mark arteries. *B)* A dense blood capillary network is seen in the AdVEGF-infected ear (n=6) but not in AdVEGF-C-infected ear (*A*). *C)* AdLacZ control (n=6). *D*–*F)* PECAM-1 staining of histological sections. Note enlargement of the blood vessels (arrowheads) in response to VEGF-C (*D*) (n=10). Many new sprouting capillaries are seen in the AdVEGF-infected ear (*E*, arrows) compared with the AdVEGF-C (*D*) and AdLacZ (*F*) (n=6) infected ears. *G, H)* Staining for VEGFR-3 and proliferating cell nuclear antigen (PCNA) 1 wk after infection (n=6 in each group). Note that many lymphatic (arrowheads) and some blood (arrows) EC nuclei are proliferating in AdVEGF-C-infected tissue. *H)* VEGFR-3 negative vascular sprouts formed in response to AdVEGF contain PCNA-positive nuclei (arrows). *I)* AdLacZ control. *J*–*L)* Podoplanin staining 2 wk after adenoviral infection (n=6 in each group). Note formation of the hyperplastic lymphatic network in response to adenoviral VEGF-C (*J*) compared with AdVEGF (*K*) and AdLacZ (*L*). VEGFR-3 whole mount staining shows formation of new lymphatic vessels in AdVEGF-C-infected ear (n=10) 1 wk after infection (*M*). Note enlargement of the lymphatic vessels in response to AdVEGF (*N*) (n=5) compared with AdLacZ (n=8) sample (*O*). Scale bars: *A*–*C)* 150 μm; *D*–*F)* 40 μm; *G*–*I)* 20 μm; *J*–*L)* 40 μm; *M*–*O)* 150 μm.

whole mount staining demonstrated a lymphangiogenic response in the AAV-VEGF-C-infected ears 6 wk after infection (Fig. 3*C*, *D*), but the effect was weaker than the lymphangiogenesis obtained with the adenoviral VEGF-C 1–2 wk after infection. In histological sections, numerous VEGFR-3-positive lymphatic vessels
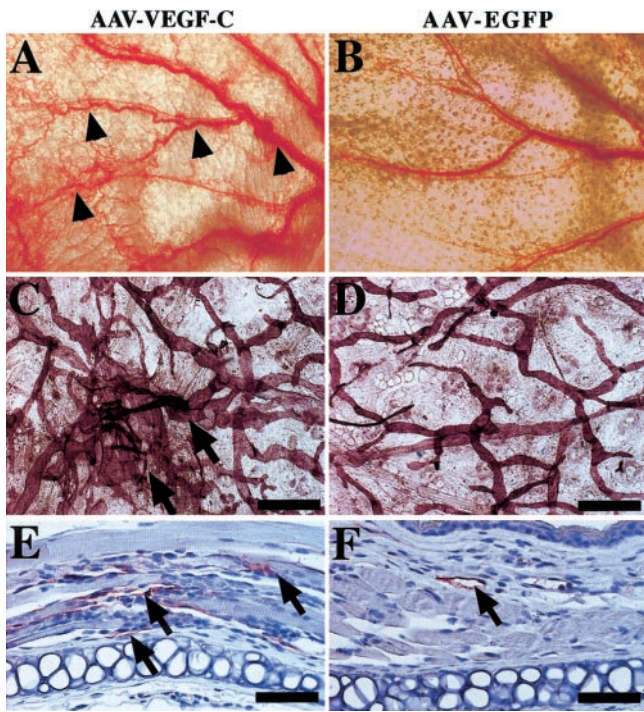
Figure 3. Blood and lymphatic vessel responses to AAV-mediated VEGF-C overexpression. *A)* Enlargement and tortuosity of the blood vessels in AAV-VEGF-C-infected skin (*n*=10) 6 wk after infection compared with the AAV-EGFP control (*n*=10) (*B*). VEGFR-3 whole mount staining (*C, D*) shows formation of new lymphatic vessels after AAV-VEGF-C infection (*n*=6). VEGFR-3 staining of the histological sections shows a dense lymphatic network inside the muscle layer in the AAV-VEGF-C-infected ear (*E*) but not in the AAV-EGFP-infected ear (*F*) (*n*=6 in both groups). Scale bars: *C–D)* 150 µm; *E–F)* 40 µm.

were seen in between muscle fibers of the AAV-VEGF-C-infected ears (Fig. 3*E, F*) whereas AdVEGF-C induced growth of lymphatic vessels mostly in the connective tissue layer of the ear. In the AAV-VEGF-C-infected ears, blood vessel enlargement was observed around the muscle layer (data not shown). These results are consistent with the finding that AAV-mediated gene expression occurs in the muscle cells (Fig. 1*D*).

VEGF-C receptor expression in the skin vessels

The proteolytically processed mature form of VEGF-C is known to bind to VEGFR-2 in addition to its lymphatic endothelial receptor VEGFR-3 (12). To characterize VEGFR-2 expression in adult mouse skin, we performed lectin perfusion and β-galactosidase double staining of skin from heterozygous VEGFR-2 + /LacZ mice, which are phenotypically normal. For a better visualization of the blood vessels in the absence of the lymphatic vessels, we also used VEGFR-2+/LacZ x K14-VEGFR-3-Ig double transgenic mice, which lack the lymphatic vessels in the skin (31). The results showed β-galactosidase expression in the veins, capillaries, and deep collecting lymphatic vessels of the ears whereas the arteries were negative for the VEGFR-2 marker (**Fig.**

4*A–C*). For comparison, β-galactosidase staining of the VEGFR-3+/LacZ mice showed strong expression in the superficial lymphatic vessels of the ear (Fig. 4*D*, arrows), whereas VEGFR-2 marker was only weakly expressed in these lymphatic capillaries (Fig. 4*B*, arrows).

Comparison of the effects of VEGF-C and VEGF in the nasal mucosa

The nasal mucosa allows efficient adenoviral infection of the respiratory epithelium without associated tissue trauma due to needle injection. Whole mount fluorescent lectin staining of the blood vessels showed formation of enlarged and tortuous blood vessels in AdVEGF-C- and AdVEGF-infected nasal conchae and endoturbinates 1 wk after infection (**Fig. 5*A*, *B**, compare with Fig. 5*C*). Small blood vessel sprouts indicative of ongoing angiogenesis were seen in AdVEGF-infected mucosa (Fig. 5*B*), but such vessels were not present in AdVEGF-C- or AdLacZ-infected tissues (Fig. 5*A*, *C*). Staining for the panendothelial cell marker PECAM-1 showed enlargement of blood vessels located right beneath the respiratory epithelium in AdVEGF-C- and AdVEGF-infected nasal tissues when compared with the AdLacZ-infected tissues (Fig. 5*D–F*). Apparently, only some of the dilated vessels seen in whole mount preparations were preserved in histological sections because of the collapse of the vessels during the tissue dissection and fixation procedures. Staining of the mucosal sections for LYVE-1 revealed hyperplastic lymphatic vessels
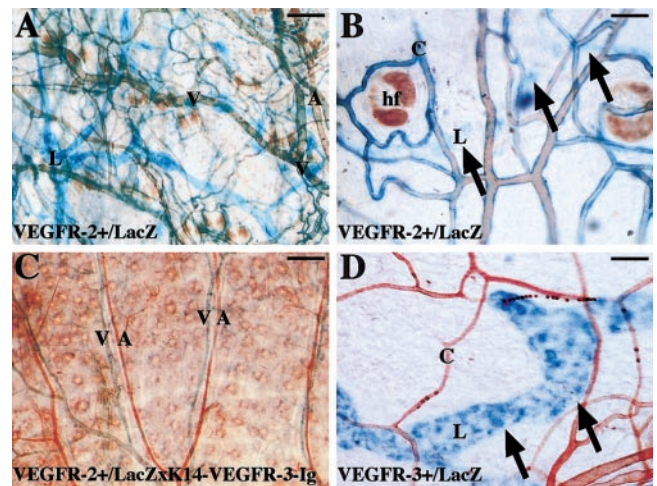

Figure 4. VEGFR-2 expression in the veins, capillaries, and deep collecting lymphatic vessels in the mouse skin (*n*=6 in each group). *A, B)* β-galactosidase and lectin staining of skin from heterozygous VEGFR-2+/LacZ mice and *C)* VEGFR-2+/LacZ x K14-VEGFR-3-Ig double transgenic mice as well as from *D)* heterozygous VEGFR-3+/LacZ mice. Note blue staining for β-galactosidase as a marker for VEGFR-2 expression in the collecting lymphatics (marked L), veins (V), and capillaries (C), whereas arteries (A) are only stained brown for biotinylated lectin. Small superficial lymphatic vessels are stained weakly for VEGFR-2 marker (arrows, *B*) but strongly for VEGFR-3 marker (arrows, *D*). The hair follicles (hf, *B*) give unspecific brown staining. Scale bars: *A)* 150 µm; *B)* 60 µm; *C)* 170 µm; *D)* 60 µm.
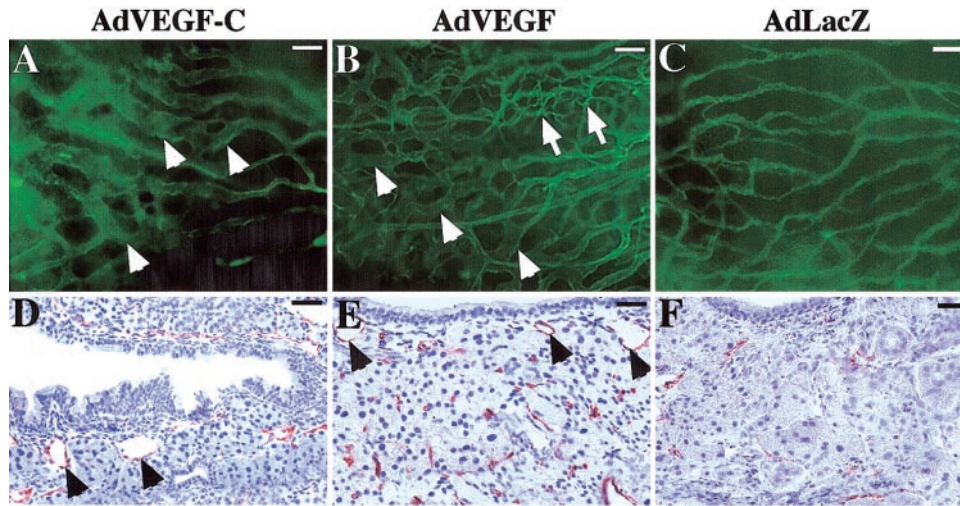
Figure 5. Enlargement of blood vessels in the nasal mucosa in response to VEGF-C 1 wk after adenoviral infection. *A–C)* Fluorescent lectin staining of the nasal conchae shows enlargement of the blood vessels (arrowheads) in mice infected with AdVEGF-C (*A*) (*n*=10) and AdVEGF (*B*) (*n*=6) compared with the AdLacZ control (*C*) (*n*=6). Note the new vascular sprouts in AdVEGF-infected mucosa (*B*, arrows). Staining of corresponding section for the panendothelial marker PECAM-1 is shown in panels *D–F* (*n*=6 in each group). Note enlargement of venules in AdVEGF-C- and AdVEGF-infected tissues (arrowheads, *D, E*), especially in the vessels closest to the respiratory epithelium. Scale bars: *A-C)* 150 μm; *D–F)* 60 μm.

beneath the respiratory epithelium 1 and 2 wk after AdVEGF-C infection compared with AdVEGF- and AdLacZ-infected samples, but the lymphatic vessel response was not as obvious as that seen in the skin (data not shown).

VEGF-C-induced blood vessel leakiness is reversed by Ang-1

To study the effect of full-length VEGF-C on the permeability of the blood vessels, AdVEGF-C or AdVEGF virus was injected to the right ear; the left ear served as a control. The extravasation of the Evans Blue dye in the ears was investigated 2 wk after infection.

The blood vessel permeability was measured as an accumulation of the Evans Blue dye to the tissues after circulation for 2 min. This period is probably too short for lymphatic vessels to contribute to its drainage. About 1.2- and 1.6-fold greater leakage occurred in the AdVEGF-C- and AdVEGF-infected ears, respectively, at a dose of 3×10^8 pfu when compared with AdLacZ- or PBS-infected control ears (**Fig. 6A**). At 9×10^8 pfu of AdVEGF-C, this ratio was increased to 1.7 and in AdVEGF-C+AdAng-1-infected mice the ratio was reduced to 0.95, which is similar to the ratio obtained in the AdLacZ-infected mice (0.97). As shown in Fig. 6*A*, these differences were statistically significant between the AdVEGF-C/AdLacZ, AdVEGF/AdLacZ, and
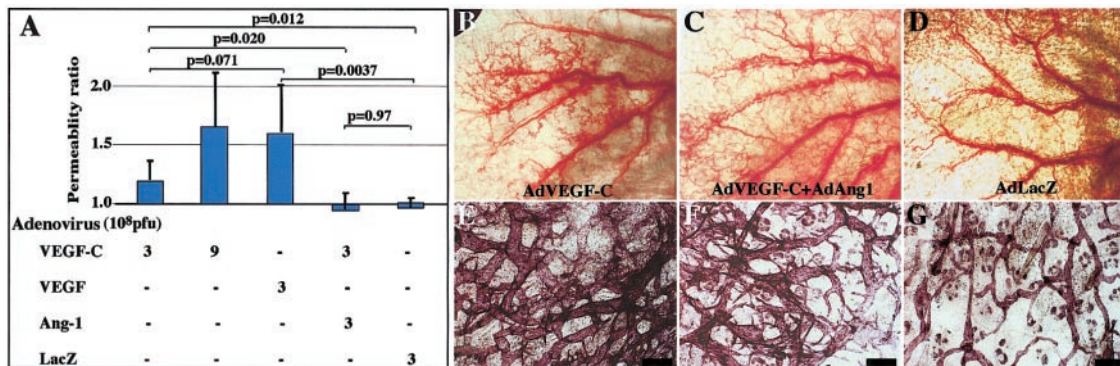

Figure 6. Ang-1 blocks VEGF-C mediated vascular leak but not lymphangiogenesis or blood vessel enlargement (*n*=10 in each group). *A)* Permeability ratio measured as the Evans Blue concentration ratio (ng/mg) in the right treated ear vs. the left control ear. The adenoviral doses used are indicated. Note that AdVEGF is threefold more potent in increasing the blood vessel permeability than AdVEGF-C and that the AdVEGF-C effect is blocked by coadministration of AdAng-1. *B–D)* The ears of the nu/nu mice were photographed 2 wk after injection of VEGF-C, VEGF-C+Ang1, or AdLacZ. Note enlargement of the blood vessels in response to VEGF-C (*B*) and VEGF-C+Ang-1 (*C*) compared with the AdLacZ control (*D*). *E–G)* VEGFR-3 whole mount staining shows strong lymphangiogenic response in AdVEGF-C (*E*) and AdVEGF-C+Ang-1 (*F*) -infected ears compared with AdLacZ control (*G*). Scale bars: *E–G)* 150 μm.

AdVEGF-C/AdVEGF-C+AdAng-1 groups, but there was no significant difference between the AdVEGF-C/Ad-VEGF and AdVEGF-C+AdAng-1/AdLacZ groups. The morphology of the blood vessels was similar in the AdVEGF-C- and AdAng-1+AdVEGF-C-infected ears. Both contained enlarged, tortuous blood vessels compared with the AdLacZ control ears (Fig. 6B–D). In VEGFR-3 whole mount staining, the AdVEGF-C- and AdVEGF-C+AdAng-1-infected ears showed a strong lymphangiogenic response when compared with the AdLacZ control (Fig. 6E–G).

DISCUSSION

Our present study shows that overexpression of the full-length VEGF-C coding region via adenovirus or adeno-associated virus results in the formation of enlarged and tortuous veins. VEGF-C also increased blood vessel permeability in a dose-dependent manner, although VEGF was threefold more potent as a permeability factor. The permeability effect of AdVEGF-C was blocked by coadministration of AdAng-1, whereas AdVEGF-C-induced lymphangiogenesis or the effects on blood vessel morphology were not affected. Similar results have been previously reported for the effect of Ang-1 on VEGF-induced vascular leak (9).

Enlargement of blood vessels in response to AdVEGF-C and AdVEGF in the skin may result in part from receptor-mediated vasodilation as the veins and venules of the skin were shown to express VEGFR-2. VEGF-C has been shown to stimulate the release of endothelial nitric oxide, which is a potent mediator of vasodilation and could contribute to enhanced vascular permeability (15). However, proliferation of the endothelial cells (intercalated or circumferential growth) could also contribute significantly to the enlargement of the vessels. Consistent with this, some ECs of the blood vessels were stained for the cell proliferation marker PCNA 1 wk after infection with the highest concentrations of AdVEGF-C (9×10^8 pfu). However, despite some proliferation response in the ECs, new sprouting angiogenic blood vessels were not seen in AdVEGF-C-infected tissues, unlike the AdVEGF-infected tissues. AdVEGF-mediated sprouting angiogenesis has been reported to involve the detachment of VEGFR-1-expressing pericytes, which are considered to stabilize blood vessels (37) Failure of VEGF-C to activate VEGFR-1 thus might contribute to the lack of sprouting angiogenesis in VEGF-C-overexpressing tissues, although enlargement of blood vessels can be induced by the high concentrations of VEGF-C.

Smaller concentrations (1×10^8 pfu) or longer periods (14–21 days) of adenoviral VEGF-C expression resulted in a weaker blood vascular response although the lymphangiogenic response was maintained. This is consistent with previous studies in which VEGF-C was reported to be mainly lymphangiogenic in the skin when expressed either as a transgene or via an adenovirus (10, 20, 21). Although VEGF was unable to induce the growth of new lymphatic vessels, it induced some enlargement of the lymphatic vessels. This might be related to the VEGF-induced increased permeability and tissue edema resulting in lymphatic vessel volume overload. However, VEGFR-2 was found to be expressed in the deep collecting lymphatic vessels and weakly in the lymphatic capillaries. Therefore, VEGF-induced enlargement of the lymphatic vessels might be related to intercalated growth of these vessels.

Adenoviruses infect typically epithelial cells of the respiratory tract (38). Therefore, adenoviral vectors are efficient for gene expression in the respiratory mucosa. Thus, topical infection of airway epithelial cells provided a convenient way of confirming and extending the studies of VEGF-C overexpression without the associated tissue trauma due to needle injection. In the mucosa, essentially similar blood vessel enlargement was obtained with AdVEGF-C and AdVEGF as in the skin whereas new blood vessel sprouts occurred only in AdVEGF-infected mucosa. However, the lymphatic vessel response to AdVEGF-C was weaker in the respiratory epithelium than in the skin, suggesting tissue-specific differences in the lymphangiogenic responses.

In the nasal cavity, small amounts of VEGF-C are expressed by the respiratory epithelial cells and VEGFR-3 is expressed in the fenestrated mucosal blood vascular ECs (17). Blood vessels of the nasal respiratory mucosa play an important role in the normal physiology of the respiratory tract in thermal regulation and humidification of the inhaled air, as well as in the control of the lumen of the nasal cavity. As VEGF-C has been shown to be up-regulated by proinflammatory cytokines (39); as it causes vascular dilation and leak (the present findings), it could be one of the inflammatory effectors contributing to respiratory allergy and infections. Thus, the blocking of the VEGF-C or VEGF signaling might have implications in the treatment of respiratory diseases associated with inflammatory cytokines and edema where they could act as inducers of increased permeability and tissue edema.

Lymphatic and blood vessel responses to VEGF-C were stronger with adenoviral than with AAV vectors. Adenovirus vectors are known to induce a strong inflammatory response whereas AAV vectors are much less immunogenic (40). The strong inflammatory response to adenoviral vectors may contribute to the effects of VEGF-C, which, like VEGF, is capable of recruiting macrophages (41); these could contribute to the additional inflammation. On the other hand, the VEGF-C expression levels are also likely to be higher in tissues infected with AdVEGF-C than in those infected with AAV-VEGF-C vectors. Another difference that modulates the biological response is the target tissue in which the vector is optimally expressed. AAV has a high tropism for muscle cells (40) and Witzenbichler et al. have reported that VEGF-C is angiogenic in ischemic muscle (15). In the ear model we used in this study, the muscle cell layer is very thin and lymphatic vessel network of the skin is located near the muscle cells. However, the effects of AAV-derived VEGF-C might be

more angiogenic in large skeletal or myocardial muscles in which very few lymphatic vessels occur. The differences in proteolytic processing of VEGF-C may also regulate the receptor binding and biological effects of VEGF-C in different tissues.

For therapeutic approaches, the permeability effect of VEGF-C acting via VEGFR-2 presents a concern of possible complications due to tissue edema. The biological role of the VEGF-C-induced enlargement and increased permeability of the blood vessels is unknown. One can speculate that fibrin and other factors needed for efficient lymphanogenesis are delivered in this way from the circulation to the tissue. However, in this study we have shown that Ang-1 blocks the blood vascular permeability effect of VEGF-C but that Ang-1 does not inhibit lymphangiogenesis. For VEGF-C gene therapy approaches in patients with lymphatic dysfunctions and edema, the VEGFR-3-specific mutant form of VEGF-C, called VEGF-C156S, would seem like a more attractive molecule. As VEGFs have both beneficial and harmful effects (42), these effects need to be carefully analyzed before the clinical use of these growth factors. **FJ**

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