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Abstract—The growth of blood and lymphatic vasculature is mediated in part by secreted polypeptides of the vascular endothelial growth factor (VEGF) family. The prototype VEGF binds VEGF receptor (VEGFR)-1 and VEGFR-2 and is angiogenic, whereas VEGF-C, which binds to VEGFR-2 and VEGFR-3, is either angiogenic or lymphangiogenic in different assays. We used an adenoviral gene transfer approach to compare the effects of these growth factors in adult mice. Recombinant adenoviruses encoding human VEGF-C or VEGF were injected subcutaneously into C57Bl6 mice or into the ears of nude mice. Immunohistochemical analysis showed that VEGF-C upregulated VEGFR-2 and VEGFR-3 expression and VEGF upregulated VEGFR-2 expression at 4 days after injection. After 2 weeks, histochemical and immunohistochemical analysis, including staining for the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), the vascular endothelial marker platelet–endothelial cell adhesion molecule-1 (PECAM-1), and the proliferating cell nuclear antigen (PCNA) revealed that VEGF-C induced mainly lymphangiogenesis in contrast to VEGF, which induced only angiogenesis. These results have significant implications in the planning of gene therapy using these growth factors. **(*Circ Res*. 2001;88:623-629.)**

Key Words: angiogenesis ■ immunohistochemistry ■ viruses ■ vessels ■ revascularization

Control of the vascular system by modulation of growth factor signaling is essential in attempts to treat diseases such as ischemic cardiovascular disease and cancer.[1–3] Perhaps the most important family of growth factors involved in the regulation of angiogenesis is the vascular endothelial growth factor (VEGF) family, which includes VEGF, VEGF-B, VEGF-C, VEGF-D, Orf virus–encoded VEGF-E, and the placenta growth factor.[4,5] These ligands bind to VEGF receptors (VEGFR)-1, VEGFR-2, and VEGFR-3 with partially overlapping receptor specificities. Both VEGF-C and VEGF-D bind VEGFR-2 and VEGFR-3 but are differentially regulated in cells and in tissues.[6–9] The affinity of VEGF-C and VEGF-D toward their receptors is regulated by proteolytic processing; the affinity of the mature, proteolytically processed forms toward VEGFR-3 is ≈40 times higher than the affinity toward VEGFR-2.[6,10] The importance of VEGFR-3 signals in the vascular system is indicated by targeted mutagenesis of the VEGFR-3 gene, which results in embryonic lethality despite the presence of an intact VEGFR-2.[11] The VEGFR-3 gene knockout leads to a disruption of the remodeling of primitive embryonic vasculature into a hierarchy of large and small vessels and results in cardiovascular failure of the embryos. However, in normal adult tissues, VEGFR-3 is largely absent from blood vessel endothelia and remains predominantly expressed in the lymphatic endothelium.[9,12,13]

Although a variety of angiogenic responses have been shown to be induced by adenoviral expression of VEGF in different mouse tissues,[14] the biological functions of VEGF-C in normal adult tissues are thus far less clear. Overexpression of VEGF-C or VEGF in the skin under the keratin 14 promoter induced hyperplasia of lymphatic vessels or angiogenesis, respectively.[15–17] In addition, recombinant VEGF-C was angiogenic in the early chick chorioallantoic membrane, but it induced exclusively lymphangiogenesis in the differentiated chorioallantoic membrane.[18,19] Furthermore, both VEGF and VEGF-C were angiogenic when expressed from a transfected plasmid vector in a rabbit model of hindlimb ischemia.[20] VEGF-C expression may thus in general lead to lymphangiogenesis, whereas in early embryonic stages[11] or when overexpressed in ischemic tissues, it may stimulate angiogenesis.

The results showing that VEGF-C can induce both angiogenic and lymphangiogenic responses in various settings of gene delivery have raised important questions about the specificity of VEGF-C–induced vascular effects in normal

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adult tissues. Although in at least some conditions, the goal of proangiogenic gene therapy may be to regenerate all components of the vascular system, in other conditions, such as in secondary lymphedema, only lymphangiogenesis may be desired. In fact, the development of specific lymphangiogenic gene therapy would be an important development for example for the tens of thousands of patients who suffer from lymphedema secondary to axillary evacuation of the lymph nodes or for the millions of patients who develop the disease after filariasis. To resolve questions about the angiogenic versus lymphangiogenic specificity of VEGF-C, we have investigated in the present study the effects of VEGF-C gene transfer on the skin vasculature of adult mice compared with gene transfer of VEGF and β-galactosidase in the same setting.

Materials and Methods

Generation of Recombinant Adenoviruses Encoding the VEGFs

For the construction of an adenovirus vector encoding VEGF-C, the full-length human VEGF-C cDNA (GenBank accession No. X94216) was cloned under the cytomegalovirus promoter in the pcDNA3 vector (Invitrogen). The SV40-derived polyadenylation signal of the vector was then exchanged for that of the human growth hormone gene, and the transcription unit was inserted into the pAd *Bgl*II vector[21] as a *Bam*HI fragment. Replication-deficient recombinant E1-E3– deleted adenoviruses were produced in human embryonic kidney 293 cells and concentrated by ultracentrifugation as previously described.[22] Recombinant adenoviruses encoding $VEGF_{165}$ and β-galactosidase were constructed as previously described.[22–26] Adenoviral preparations were confirmed to be free from helper viruses, lipopolysaccharide, and bacteriological contaminants.[26]

Construction, Expression, and Purification of VEGFR Ig Fusion Proteins

The expression plasmids encoding human VEGFR-1-Ig and VEGFR-3-Ig were constructed by polymerase chain reaction-amplifying the first three Ig homology domains of the extracellular portions of VEGFR-1 and VEGFR-3 with the primer pairs 5′-TCTCGGATCCTCTAGTTCAGGTTCAAAATT-3′ (*Bam*HI site underlined)/5′-GATGAGATCTTTATCATATATATGCACTGA-3′ (*Bgl*II site underlined) and 5′-CCTGGGATCCCTGGTGAGTGGCTACTCCATGAC-3′/5′-GATGAAGAGATCTTCATGCACAATGACCTCGG-3′, respectively. The products were cloned into the *Bgl*II site of the pMT/BiP · V5 · HisC vector (Invitrogen), and the cDNA coding for the Fc-tail of human IgG1 was cloned in frame with the VEGFR Ig homology domains into the same vector. The expression plasmids were cotransfected with the pCO · Hygro selection plasmid (Invitrogen) into *Drosophila* S2 cells, and stable cell pools were selected in 150 μg/mL hygromycin B (Calbiochem). The expression of the Ig fusion proteins was induced with 500 μmol/L $CuSO_4$ in serum-free DES medium (Invitrogen) and after 4 days, they were purified from the conditioned medium by protein A affinity chromatography (Amersham Pharmacia). VEGFR-2-Ig was obtained from R&D Systems (catalogue No. 357-KD).

Expression of Recombinant Adenoviral VEGF-C, VEGF, and β-Galactosidase

Cells (293EBNA) grown in 10% FCS were transfected with pREP7 (Invitrogen) expression vectors encoding $VEGF_{165}$ or VEGF-C[6], using the calcium phosphate precipitation method or infected by incubation with 2×10^7 pfu/10^6 cells (multiplicity of infection=20) of the respective adenoviruses in serum-free medium for 1 hour. The medium was then changed to medium containing 10% FCS, the cells were incubated overnight, and metabolically labeled with ^{35}S-methionine and cysteine (Promix, Amersham) for 6 hours. The media were collected, and labeled VEGF proteins were precipitated using soluble VEGFR-Ig domain fusion proteins. Before VEGF-C precipitation using VEGFR-2-Ig, endogenous VEGF was removed from the supernatants by preadsorption using anti-VEGF monoclonal antibodies (R&D catalogue No. MAB293). The bound proteins were precipitated with protein G Sepharose, washed three times in PBS, dissolved in Laemmli sample buffer, and analyzed by 12.5% or 15% SDS-PAGE. Gels were then dried and analyzed by phosphorimaging and autoradiography.

Analysis of the Adenovirus-Encoded Transcripts In Vivo

Adenovirus (2×10^8 pfu) encoding VEGF, VEGF-C, or β-galactosidase was injected into the tail veins of two C56/Bl6 mice. The mice were sacrificed 4 days later and RNA was extracted from the livers (RNAeasy Kit, Qiagen). Total RNA (15 μg) was subjected to Northern blotting and hybridization with a mixture of ^{32}P-labeled cDNAs specific for VEGF (nucleotides 57 to 639, GenBank accession No. NM003376), VEGF-C (nucleotides 495 to 1661, GenBank accession No. X94216), or LacZ (nucleotides 529 to 977 pBluscript SK+, Stratagene).

All experimental procedures involving laboratory animals were approved by the Helsinki University Ethical Committee and by the Provincial State Office of Southern Finland (permit No. HY 312).

Immunohistochemistry and Morphometry

Recombinant adenovirus or buffer (2×10^8 pfu) was injected subcutaneously into the backs of C56/Bl6 mice or into the ears of NMRI nude (nu/nu) mice (Harlan). The mice were sacrificed at various time points after injection. Skin from the site of injection was fixed in 4% paraformaldehyde and embedded in paraffin, and 6-μm sections were stained using monoclonal antibodies against VEGFR-2,[27] VEGFR-3,[28] or polyclonal antibodies against the lymphatic marker LYVE-1, a receptor for hyaluronan and a homologue to the CD44 glycoprotein,[29] or mouse platelet–endothelial cell adhesion molecule-1 (PECAM-1) (BD Pharmingen, catalogue No. 01951D), the mouse homologue of the human vascular endothelial antigen CD31. Sections were also stained using polyclonal antibodies against laminin.[30] The tyramide signal amplification (TSA) kit (NEN Life Sciences) was used to enhance staining. Negative controls were done by omitting the primary antibodies. Double staining of sections was carried out by first staining sections for proliferating cell nuclear antigen (PCNA) (ZYMED, catalogue No. 93-1143) and subsequently for LYVE-1 and PECAM-1 as detailed above. The results were viewed with an Olympus AX80 microscope and photographed. For quantification, the vessels in the sections were counted using square grids (area=0.16 mm^2, ×200 magnification), and the mean and probability value were calculated using the Student's *t* test. Eight visual fields were quantified in sites of active angiogenesis or lymphangiogenesis in five different ears injected with AdVEGF-C or AdVEGF. For controls, 15 to 20 visual fields in five different ears injected with AdLacZ were quantified. For morphometric quantification of vessel volume, quantitative densitometry of 70 to 80 vessels in 8 to 10 visual fields was performed according to Weibel's principles using a CAS200 (Becton-Dickinson) automated image analyzer and the proprietary software. Blood vessels were visualized and photographed in situ using a Leica MZ APO microscope.

Results

Expression of VEGF-C and VEGF by Recombinant Adenoviruses In Vitro

To confirm that adenoviral gene transfer of VEGF-C results in secretion of polypeptides that bind to their receptors, 293EBNA cells were infected with the respective adenoviruses. Cells infected with the VEGF-C adenovirus (AdVEGF-C) produced major polypeptides of ≈29/31 kDa that bound to the VEGFR-3-Ig fusion protein (Figure 1A,

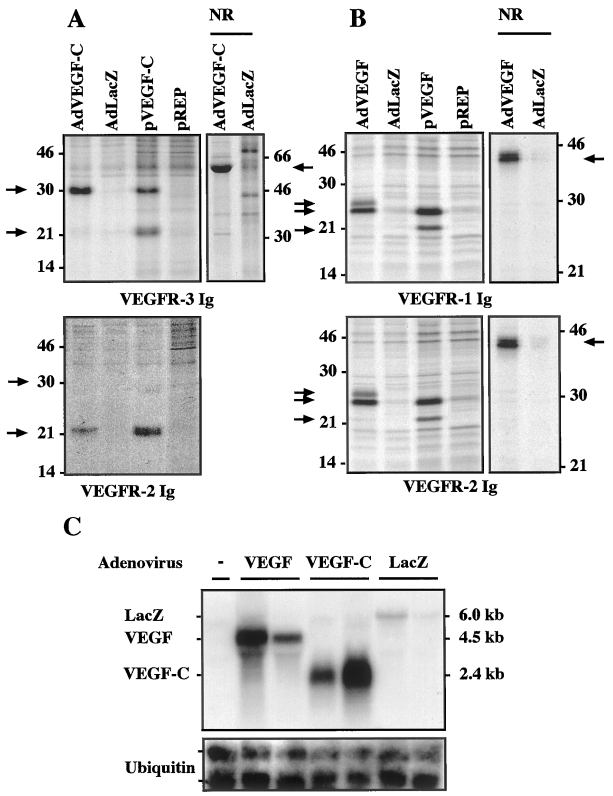

Figure 1. Expression of VEGF-C and $VEGF_{165}$ by the recombinant adenoviruses in vitro and in vivo. A and B, Receptor binding by adenovirally produced VEGF-C and VEGF in vitro. VEGFR Ig fusion proteins were used to precipitate polypeptides from the media of metabolically labeled 293EBNA cells infected with AdVEGF-C, AdVEGF, or AdLacZ, as indicated. Plasmid controls are indicated as pREP for empty vector backbone, pVEGF-C for the vector encoding VEGF-C, and pVEGF for the vector encoding VEGF. Analysis by SDS-PAGE is shown under reducing and nonreducing (NR) conditions (the exposure in the lower panel in A was about twice as long as the exposure in other panels). Molecular size markers in the figure are indicated in kilodaltons, and arrows indicate bands representing the precipitated VEGF polypeptides. C, Analysis of adenovirus-encoded RNAs in the livers of infected mice. About 15 μg of total RNA extracted from livers of two mice injected with the indicated recombinant adenoviruses was subjected to Northern blotting and hybridization with a mixture of the corresponding cDNA probes. The first lane contains RNA extracted from the liver of an untreated mouse. The blot was stripped and reprobed for ubiquitin mRNA to confirm equal loading. The sizes of the different RNAs are indicated in kilobases on the right.

top). Under nonreducing conditions, these polypeptides migrated as an ≈60-kDa band (Figure 1A, lanes NR). In comparison to cell cultures transfected with a VEGF-C plasmid expression vector, very small amounts of the mature, proteolytically processed 21/23 kDa-form of VEGF-C were observed in the culture media of Ad VEGF-C–infected cells, suggesting incomplete proteolytic processing. However, the mature 21/23-kDa species was the predominant VEGF-C form that bound to VEGFR-2-Ig (Figure 1A, bottom), as previously reported.[6]

In an experiment similar to the one outlined above, we also confirmed that adenoviral gene transfer of VEGF results in the expression of VEGF polypeptides that form disulfide-bonded homodimers and bind to VEGFR-1 and VEGFR-2. Figure 1B shows SDS-PAGE analysis of the polypeptides precipitated from the conditioned medium of metabolically labeled AdVEGF-infected cells. A major VEGF polypeptide of ≈24 kDa and a minor one of ≈26 kDa are specifically precipitated using VEGFR-1-Ig or VEGFR-2-Ig. The former band comigrated with the major band in similar precipitates from the conditioned media of cells transfected using a plasmid expression vector for $hVEGF_{165}$. The minor band of 22 kDa in the media of transfected cultures and the 26 kDa-form in cultures infected with the adenovirus probably represent differentially glycosylated polypeptide species. The same bands were also precipitated by monoclonal antibodies against VEGF (data not shown). Under nonreducing conditions, the adenovirally expressed polypeptides migrated in the range of 43 to 45 kDa, indicating a disulfide-stabilized dimeric structure (Figure 1B, lanes NR).

Expression of Adenovirally Encoded VEGF-C and VEGF In Vivo

The expression of VEGF-C and VEGF adenoviruses in vivo was tested by injecting the viruses into the tail veins of C56/Bl6 mice. Because most of the gene expression after intravenous injection of recombinant adenovirus occurs in the liver,[31] we extracted RNA from the liver and analyzed it by Northern blotting and hybridization with a combination of probes specific for the adenoviral inserts. As can be seen in Figure 1C, the adenoviruses efficiently express mRNAs of 4.5 and 2.4 kb, encoding VEGF and VEGF-C, respectively, whereas somewhat lower amounts of mRNA of 6.0 kb encoding β-galactosidase were produced by the control virus. The liver of an uninfected mouse showed no signal.

AdVEGF-C and AdVEGF Stimulate VEGFR Expression

The effects of the adenoviruses in vivo were tested by subcutaneous injection into mouse skin and by analyzing skin sections 4 days later by immunohistochemistry for the VEGF-C receptors VEGFR-2 and VEGFR-3 and for the vascular marker PECAM-1. As can be seen from Figure 2A and from the enclosed insets at higher magnification, adenoviral expression of VEGF-C for 4 days induced the expression of VEGFR-2 and VEGFR-3 in endothelial cells of blood vessels (containing erythrocytes), whereas VEGF gene transfer induced the expression of VEGFR-2 but not VEGFR-3 (Figure 2D). In contrast, the blood vessels in mice injected with AdLacZ (Figure 2B) or PBS (Figure 2C) did not stain for VEGFR-2 or VEGFR-3; only the lymphatic vessels were positive for VEGFR-3 in these mice. Analysis after 2 weeks showed an inflammatory response in all adenovirus-injected samples from the C57/Bl6 mice, confounding immunohistochemical analysis (data not shown). For this reason, we continued our studies in the immunocompromised athymic mice.

Lymphangiogenic and Angiogenic Responses to the Adenoviruses

Five ears of three nu/nu mice were injected with each of the adenoviruses. Shown in Figure 3 are AdVEGF-C, AdVEGF, or AdLacZ injection sites of mouse ears photographed in situ

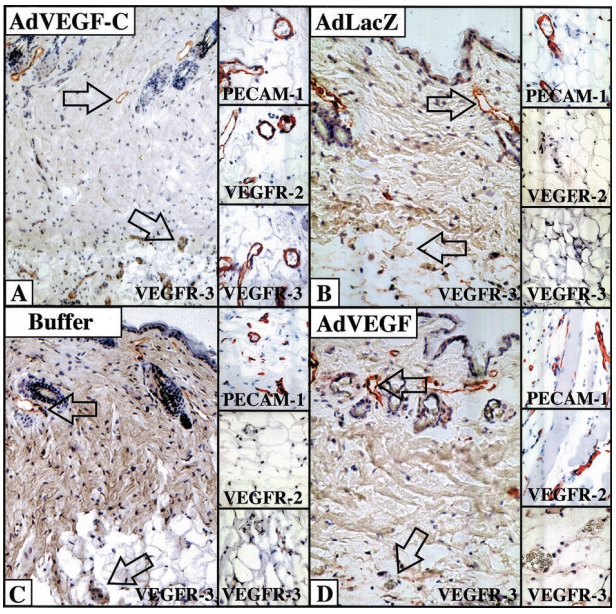

Figure 2. Induction of VEGFR expression in response to adenoviral gene transfer of VEGF-C and VEGF. Sections of skin from mice injected subcutaneously with AdVEGF-C (A), AdVEGF (D), AdLacZ (B), or buffer (C) were stained for VEGFR-3. To evaluate the expression of the different receptors in blood vessels, adjacent sections stained for VEGFR-3, VEGFR-2, and PECAM-1 were observed at ×400 magnification (insets). Open arrows indicate vessels staining for VEGFR-3; the upper arrows point to the superficial dermal lymphatic vessels and the lower ones to the blood vessels in the interface of dermis and subcutaneous fat tissue. In panel A, note VEGFR-3 expression in both blood vessels (containing erythrocytes) and lymphatic vessels, whereas in panels B, C, and D, VEGFR-3 expression is restricted to lymphatic vessels.

3 days after the injection. As can be seen from this figure, VEGF induced the formation of enlarged, tortuous vessels (Figure 3B, arrows) in contrast to VEGF-C (Figure 3A) or β-galactosidase (Figure 3C), which did not seem to affect at least the larger blood vessels.

The adenovirus-injected ears were processed for immunohistochemistry and stained for PECAM-1 and the lymphatic-specific antigen LYVE-1. As can be seen from the LYVE-1 staining shown in Figures 4A and 4B, AdVEGF-C transfer induced the formation of LYVE-1–positive hyperplastic lymphatic vessels (arrows), which did not stain for laminin, a component of the basal laminae of blood vessels (data not shown), whereas AdVEGF (Figure 4D) or AdLacZ (Figure 4C) did not have any effects on the lymphatic vessels. In contrast, AdVEGF induced the formation of blood vessels (Figures 4E and 4F, arrows) whereas the AdLacZ (Figure 4G) did not have any effects on the blood vasculature. The effects of AdVEGF-C on blood vessels were more difficult to evaluate because of the strong lymphangiogenic response. However, there was a small increase of PECAM-1–positive vessels in the AdVEGF-C–injected ears (see Figures 4H and 5B). Some of these may represent newly formed, very weakly PECAM-1–positive lymphatic vessels (Figure 4H, asterisk).

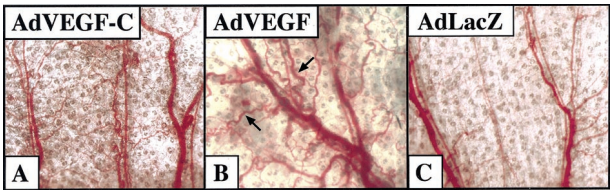

Figure 3. Comparison of vascular changes in response to VEGF, VEGF-C, and ß-galactosidase in situ. Mouse ears were photographed 3 days after injection with AdVEGF-C (A), AdVEGF (B), AdLacZ (C). Note the prominent enlarged and tortuous blood vessels (arrows) in response to AdVEGF compared with the vasculature in ears injected with AdVEGF-C or AdLacZ.

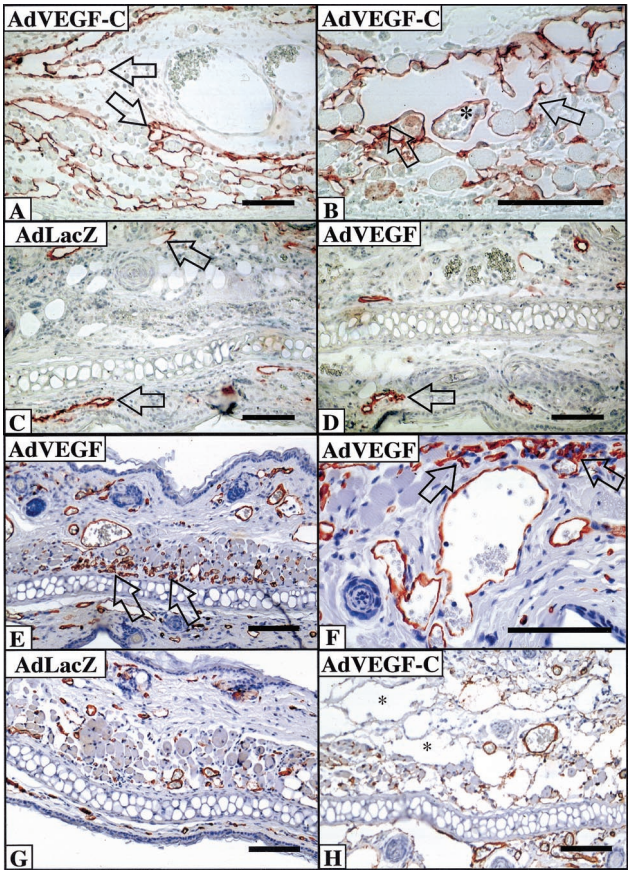

Figure 4. AdVEGF-C induces lymphangiogenesis and AdVEGF induces angiogenesis in vivo. The ears of nude mice were injected with AdVEGF-C (A and B), AdLacZ (C), or AdVEGF (D) and analyzed immunohistochemically 2 weeks after injection for the lymphatic endothelial-specific antigen LYVE-1. Note the abundant formation of enlarged, hyperplastic lymphatic vessels (arrows) in panel A. In panel B, ×400 magnification of the lymphatic vessels shows extensive growth of these vessels around the muscle fibers. Both panels A and B contain blood vessels that are negative for LYVE-1, ie, the two large blood vessels (note erythrocytes) in panel A and the arteriole (asterisk) encroached by a LYVE-1–positive lymphatic vessel in panel B. In contrast, AdVEGF (D) or AdLacZ (C) did not affect the lymphatic vessels (arrows). In AdVEGF-injected mice, PECAM-1 staining revealed angiogenesis as represented by new capillary formation (E; arrows) and at ×400 magnification (F). AdLacZ did not have effects on the vasculature (G) whereas AdVEGF-C mainly induced the formation of enlarged, very weakly PECAM-1–positive vessels (marked by asterisks in panel H), which were demonstrated to be lymphatic vessels by LYVE-1 staining (data not shown). Bar=500 μm.

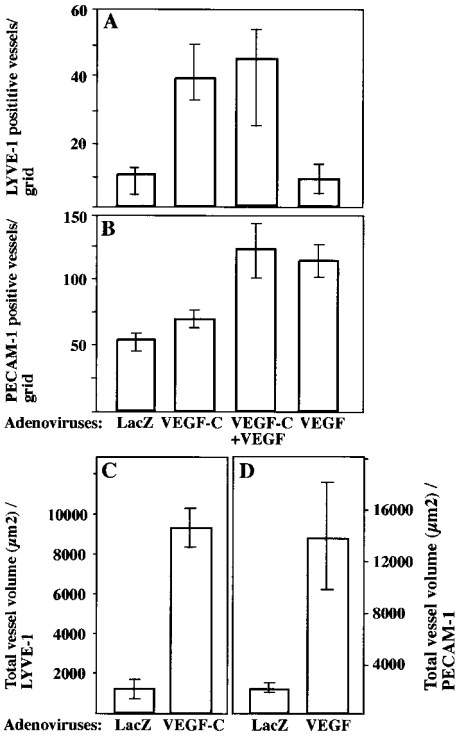

Figure 5. Quantification of adenovirus-induced vascular responses. The LYVE-1–positive (A) and PECAM-1–positive (B) vessels were counted at sites of adenoviral injection as detailed in Materials and Methods. VEGF-C and VEGF induced distinct lymphangiogenic and angiogenic responses, respectively, whereas a combination of VEGF and VEGF-C did not markedly potentiate either lymphangiogenic or angiogenic responses. C and D, Quantification of the volume of vessels staining for LYVE-1 and of strongly PECAM-1–staining vessels 2 weeks after injection. Error bars are ±2 SD.

Quantitatative Analysis of the Adenovirus-Induced Lymphatic and Blood Vessels

As can be seen from the results of counting the LYVE-1–positive and strongly PECAM-1–staining vessels with lumens in Figure 5A, AdVEGF-C induced an ≈4-fold increase ($P<0.01$) of lymphatic vessel density (Figure 5A) whereas VEGF induced a 2-fold increase ($P<0.01$) of blood vessel density (Figure 5B). The combination of AdVEGF and AdVEGF-C did not significantly ($P>0.5$) potentiate either of these responses. VEGF-C increased the total volume of the LYVE-1–positive vessels by 7.5-fold ($P<0.01$) (Figure 5C), whereas VEGF increased the volume of the blood vessels by 5.7-fold ($P<0.01$) (Figure 5D).

Endothelial Cell Proliferation in Lymphangiogenesis Induced by VEGF-C

As can be seen in Figure 6A and at higher magnification in Figures 6B and 6C, sequential staining for both LYVE-1 and PCNA revealed that the lymphatic vessels in AdVEGF-C–injected ears contained proliferating lymphatic endothelial cells. For example, the lymphatic endothelial cells surrounding a small arteriole in Figure 6C stain for PCNA (closed arrowhead), whereas the blood vascular endothelial cells do not (open arrowhead). Figure 6D shows PCNA-positive nuclei in the wall of a blood vessel in an ear injected with

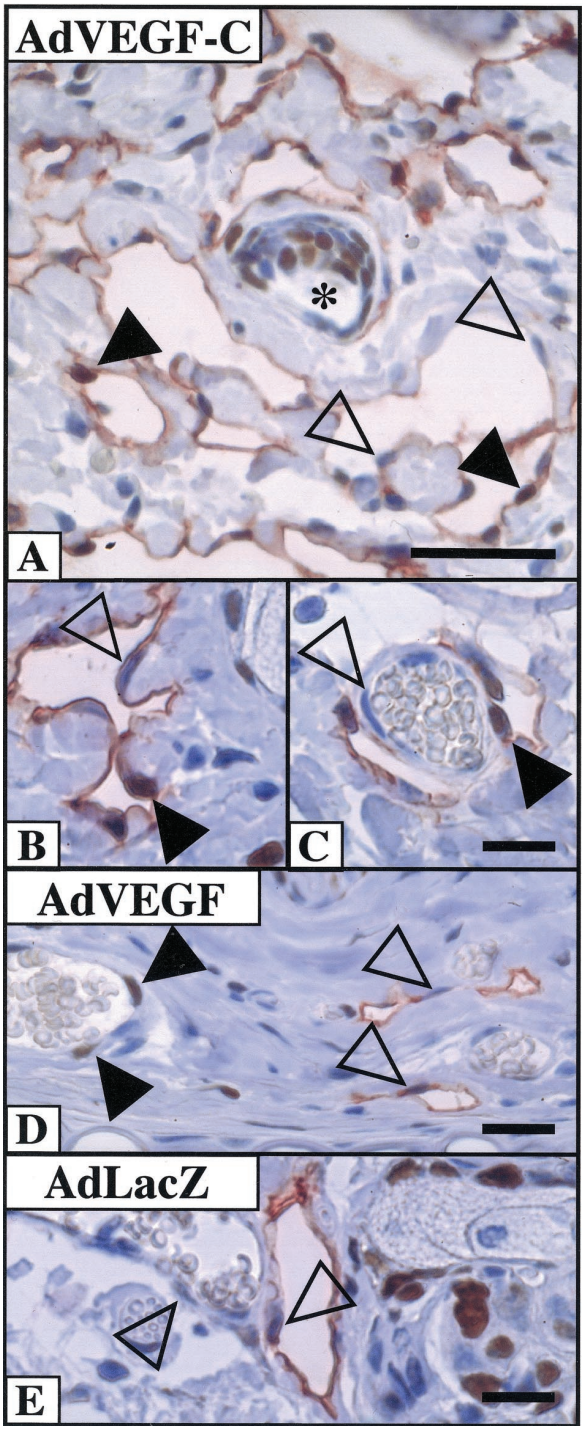

Figure 6. VEGF-C induces endothelial cell proliferation in the lymphatic vessels. Shown are sections sequentially stained for both PCNA and LYVE-1. Arrowheads indicate PCNA-positive and open arrowheads PCNA-negative nuclei. In panel A and at higher magnification in panels B and C, the lymphatic vessels formed in response to VEGF-C contain several PCNA-positive nuclei. Note the PCNA-negative nuclei in the arteriole surrounded by two lymphatic vessels containing PCNA-positive nuclei in panel C. Gene transfer of VEGF had no effect on the lymphatic vessels as can be seen in panel D. However, blood vessels in these sections contained PCNA-positive nuclei, in contrast to the ß-galactosidase control shown in panel E, in which the blood and lymphatic vessels were negative. Panels A and E also contain numerous PCNA-positive nuclei in hair follicles as indicated by an asterisk in panel A. Bar=500 μm in panel A and 100 μm in panels B through E.

AdVEGF. In contrast, the lymphatic vessels in the ears injected with AdVEGF or AdLacZ did not stain for PCNA. Approximately 30% (n=50) of the nuclei in the lymphatic vessels formed in response to VEGF-C stained positive for LYVE-1, whereas the proportion of PCNA-positive nuclei in blood vessels in ears injected with AdVEGF was only 6% (n=50). This low figure may reflect the fact that the peak in endothelial cell proliferation in the blood vessels occurs earlier during angiogenesis induced by VEGF.[14]

Discussion

The present study shows that VEGF-C expressed subcutaneously by adenoviral gene transfer induces proliferation and enlargement of lymphatic vessels in a process that we refer to herein as lymphangiogenesis. VEGF-C also strongly upregulates VEGFR-2 and VEGFR-3 expression in blood vessels. In contrast, adenoviral gene transfer of VEGF induced VEGFR-2 upregulation in the endothelial cells of blood vessels and angiogenesis, as described earlier.[14,24]

The vessel density in foci of lymphatic vessel formation in the ears infected with AdVEGF-C increased ≈4-fold in comparison to ears injected with AdVEGF, AdLacZ, or buffer control as measured by quantification of LYVE-1–positive vessels. The lack of smooth muscle cells around the vessels and erythrocytes within the vessels generated in 2 weeks was in accordance with the lymphatic vessel morphology. Furthermore, these vessels did not stain for laminin, a component of the basal laminae (data not shown). The density of strongly PECAM-1–positive vessels in the ears infected with AdVEGF increased ≈2-fold compared with ears infected with AdVEGF-C, AdLacZ, or buffer. It may also be noted that LYVE-1 expression was not upregulated in blood vessels in AdVEGF-induced angiogenesis (eg, see Figure 3A). Thus, the response to AdVEGF-C was primarily lymphangiogenic, whereas very little angiogenesis was seen, unlike in the experiments in which plasmid expression vectors were used in ischemic rabbit muscle.[20]

In cell culture, the majority of the adenovirally produced VEGF-C consisted of the partially processed 29/31-kDa form, which binds VEGFR-3 but only very weakly to VEGFR-2.[6] In our in vivo assay in normal dermis, this could be the predominant form, whereas in ischemic tissue the 21-kDa form of VEGF-C, which has a higher binding affinity toward VEGFR-2, may predominate because of increased expression of VEGF-C processing enzymes in the latter. A major difference between our assay conditions and those used in experiments using ischemic hindlimb as a target for plasmid delivery is the presence of abundant amounts of endogenous VEGF induced by hypoxia in the latter. However, at least in our initial experiments, simulation of such conditions by coinjection of AdVEGF and AdVEGF-C did not result in a substantial potentiation of the angiogenic response.

The mechanisms of lymphangiogenesis in adult tissues have not been elucidated. The generation of lymphatic vessels could in principle require endothelial cell sprouting from or splitting of preexisting lymphatic vessels or blood vessels, in situ differentiation of endothelial cells, or recruitment and lymphatic differentiation of endothelial precursor cells, as has been described in other models.[32–34] In embryos, lymphatic vessels are mainly formed by the process of sprouting from certain venous structures, although in the avian species, mesenchymal precursor cells called lymphangioblasts also exist.[35,36] We do not yet know the mechanisms of lymphangiogenesis in the adult, but the present results are compatible with the process of sprouting lymphatic vessels from preexisting ones and perhaps splitting of such enlarged lymphatic vessels that we observed in the AdVEGF-C–treated ears. The upregulation of VEGFR-2 and VEGFR-3 in blood vessels in response to VEGF-C raises the interesting possibility that endothelial cells in blood vessels could also participate in lymphangiogenesis by the process of migration and transdifferentiation. Such upregulation of both VEGF-C receptors in the blood vascular endothelium should also be considered when using gene therapy in the setting of tissue ischemia.

It has been shown that the angiogenic response induced by AdVEGF is a highly dynamic process involving the initial formation of mother vessels and endothelial glomeruloid bodies.[14] Thus, our analysis at the 2-week time point does not reveal the kinetics of possible transient blood vessel responses. The responses to VEGF-C in blood vessel endothelia, which upregulate both receptors for VEGF-C, remain to be characterized. Therapeutic angiogenesis ultimately requires the induction of entire vascular structures consisting of arteries, veins, and lymphatics. Thus, proangiogenic therapy could consist of different growth factors that cover the entire genetic program for the induction of new vessels.[37] Our studies in transgenic mouse embryos and newborn mice have revealed that the developing lymphatic vasculature is dependent on VEGF-C for survival signals and when the embryonic tissues are deprived of such signals by blocking both VEGF-C and VEGF-D, the forming lymphatic vessels regress by specific lymphatic endothelial apoptosis (T. Makinen et al, unpublished observations, 2000). Therefore, further studies are needed to determine the long-term effects of the transient viral expression of VEGF-C, whether this results in permanent and functional lymphatic vasculature and whether stable changes of the blood vasculature can also be observed.

Acknowledgments

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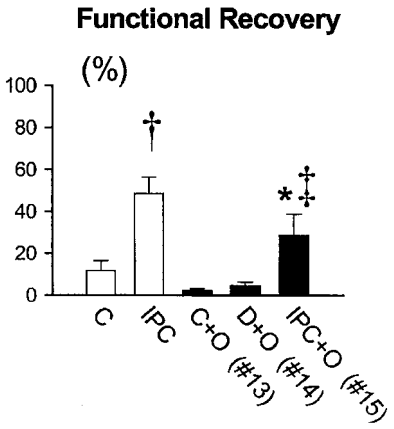
Corrections

In the article by Wen et al, "Overexpression of 12-Lipoxygenase Causes Cardiac Fibroblast Cell Growth" (*Circ Res*. 2001;88:70–76), the numbers on the ordinate scale of the left panel of Figure 8 (page 74) are not correct. The correct numbers of the ordinate scale of the left panel are 5, 10, 15, and 20 $\mu g/10^6$ cells. The numbers on the ordinate scale of the right panel of Figure 8 are incorrect as well. The correct numbers of the ordinate scale of the right panel are 1, 2, 3, and 4 $\mu g/10^6$ cells.

In the article by Enholm et al, "Adenoviral Expression of Vascular Endothelial Growth Factor-C Induces Lymphangiogenesis in the Skin" (*Circ Res*. 2001;88:623–629), the designation of lanes "VEGF" and "VEGF-C" in Figure 1C (page 625) should be exchanged. The molecular weights on the right should be 3.6 kb, 2.4 kb, and 0.6 kb (instead of 6.0 kb, 4.5 kb, and 2.4 kb), respectively.

In the article by Imahashi et al, "Role of Intracellular Na^+ Kinetics in Preconditioned Rat Heart" (*Circ Res*. 2001;88:1176–1182), the statistical symbols in Figure 5C (page 1179) are mislabeled. The correct version of Figure 5C is shown below.



(*Circ Res.* 2001;89:e15.)

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