Lymphangiogenic Gene Therapy With Minimal Blood Vascular Side Effects

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Abstract

Recent work from many laboratories has demonstrated that the vascular endothelial growth factor-C/VEGF-D/VEGFR-3 signaling pathway is crucial for lymphangiogenesis, and that mutations of the *Vegfr3* gene are associated with hereditary lymphedema. Furthermore, VEGF-C gene transfer to the skin of mice with lymphedema induced a regeneration of the cutaneous lymphatic vessel network. However, as is the case with VEGF, high levels of VEGF-C cause blood vessel growth and leakiness, resulting in tissue edema. To avoid these blood vascular side effects of VEGF-C, we constructed a viral vector for a VEGFR-3–specific mutant form of VEGF-C (VEGF-C156S) for lymphedema gene therapy. We demonstrate that VEGF-C156S potently induces lymphangiogenesis in transgenic mouse embryos, and when applied via viral gene transfer, in normal and lymphedema mice. Importantly, adenoviral VEGF-C156S lacked the blood vascular side effects of VEGF and VEGF-C adenoviruses. In particular, in the lymphedema mice functional cutaneous lymphatic vessels of normal caliber and morphology were detected after long-term expression of VEGF-C156S via an adeno associated virus. These results have important implications for the development of gene therapy for human lymphedema.

Key words: lymphedema • lymphatic endothelium • VEGF-C • VEGFR-2 • VEGFR-3

Introduction

Proangiogenic gene therapy, developed first in the pioneering work of Dr. Jeffrey Isner, has shown great promise in the treatment of cardiovascular ischemic diseases (1–3). In such studies, angiogenesis has been stimulated for example by overexpression of vascular endothelial growth factor (VEGF)* or various fibroblast growth factors (FGFs). More recent developments also include the use of modified forms of the hypoxia-induced transcription factor (HIF)-1α, which may orchestrate the induction of several angiogenic mechanisms (4, 5). However, although VEGF is a potent inducer of angiogenesis, the vessels it helps to create are immature, tortuous, and leaky, often lacking perivascular support structures (6–8). Only a fraction of the blood vessels induced in response to VEGF in the dermis and in subcutaneous fat tissue were stabilized and functional after adenoviral treatment of the skin of nude mice (9, 10), while intramuscular vessels developed into an angioma-like proliferation or regressed with a resulting scar tissue (9, 11). Furthermore, edema induced by VEGF overexpression complicates VEGF-mediated neovascularization, although recent evidence suggests that it can be avoided by providing angiopoietin-1 for vessel stabilization (12, 13).

Lymphatic vessels play an important physiological role in homeostasis, regulation of tissue fluid balance, and in the immune responses to pathogens, yet the molecular mechanisms that control their development and function are only beginning to be elucidated. So far, only two peptide growth factors have been found capable of inducing the growth of new lymphatic vessels in vivo. These factors, VEGF-C and VEGF-D (14–16), belong to the larger VEGF family of growth factors which also includes VEGF, placenta growth factor (PlGF), and VEGF-B. VEGF-C and

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Abbreviations used in this paper: AAV, adeno-associated virus; PFA, paraformaldehyde; VEGF, vascular endothelial growth factor.

VEGF-D are ligands for the endothelial cell–specific tyrosine kinase receptors VEGFR-2 and VEGFR-3 (17, 18). In adult human as well as mouse tissues VEGFR-3 is expressed predominantly in the lymphatic endothelial cells which line the inner surface of lymphatic vessels (19, 20). Whereas VEGFR-2 is thought to be the main mediator of angiogenesis, VEGFR-3 signaling is crucial for the development and maintenance of the lymphatic vessels (21). Inhibition of VEGFR-3 signaling using soluble VEGFR-3 which competes for ligand binding with the endogenous receptors led to lymphatic vessel regression in a transgenic mouse model (22). Other molecules that have been reported to be necessary for normal lymphatic development include the transcription factor Prox-1 (23), the integrin $\alpha 9$ (24), and angiopoietin-2 (25).

Impairment of lymphatic function, which results in inadequate transport of fluid, macromolecules, or cells from the interstitium, is associated with a variety of diseases and leads to tissue edema, impaired immunity and fibrosis (26). Development of strategies for local and controlled induction of lymphangiogenesis would thus be of major importance for the treatment of such diseases. Adenoviral gene transfer of VEGF-C in the skin has been shown to result in a strong lymphangiogenic response (27, 28), but high levels of VEGF-C also lead to blood vascular effects such as increased vessel leakiness, presumably through the interaction of VEGF-C with the VEGFR-2 expressed on blood vascular endothelium (28). To develop a lymphatic-specific gene therapy approach without the unwanted blood vascular side effects, we have studied the potential of a VEGFR-3–specific mutant form of VEGF-C (VEGF-C156S) as a therapeutic agent in lymphedema. We demonstrate that stimulation of VEGFR-3 alone by VEGF-C156S potently induces lymphangiogenesis both in transgenic embryos and after virus-mediated gene transfer. In a lymphedema mouse model functional cutaneous lymphatic vessels formed after intradermal infection with adeno-associated virus (AAV) encoding VEGF-C156S. Most importantly, VEGF-C156S essentially lacked the blood vascular effects of native VEGF-C.

Materials and Methods

Generation and In Vitro Analysis of Recombinant Adenoviruses and AAVs. For the adenovirus construct, the full-length human VEGF-C156S cDNA (29) was cloned as a BamHI/NotI fragment into the corresponding sites of the pAD BglII vector. Replication-deficient E1-E3 deleted adenoviruses were produced in 293 cells and concentrated by ultracentrifugation (30). Adenoviral preparations were analyzed to be free of helper viruses, lipopolysaccharide, and bacteriological contaminants (31). The adenoviruses encoding human VEGF-C and nuclear targeted *LacZ* were constructed as described (27, 30). For the AAV construct, the full-length human VEGF-C156S was cloned as a blunt-end fragment into the MluI site of psub-CMV-WPRE plasmid and the rAAV type 2 was produced as described previously (32). AAVs encoding human VEGF-C and EGFP were used as controls (32, 33).

For the analysis of protein expression, 293EBNA cells were infected with recombinant adenoviruses for 2 h in serum-free medium or by AAVs 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–specific antibodies or to a binding assay using soluble VEGFR-2-Ig (R&D Systems) and VEGFR-3-Ig (18) fusion proteins. AdLacZ and AAV-EGFP infected cells were used as negative controls. The bound proteins were precipitated with protein G Sepharose, separated in 15% SDS-PAGE, and analyzed by autoradiography. To compare the protein production levels of AdVEGF-C156S and AdVEGF-C viruses, 20-μl aliquots of the media from AdVEGF-C156S, AdVEGF-C, and AdLacZ infected cell cultures were separated in 15% SDS-PAGE gel and subjected to Western blotting using polyclonal anti–VEGF-C antibodies (R&D Systems).

In Vivo Use and Analysis of the Viral Vectors. All the studies were approved by the Committee for Animal Experiments of the University of Helsinki. 5×10^8 pfu of the recombinant adenoviruses or 5×10^9-1×10^{11} rAAV particles were injected intradermally into the ears of NMRI nu/nu mice (Harlan) or Chy lymphedema mice (32). The infected nude mice were killed 3, 5, 7, 10, 14, 21, 42, or 56 d after adenoviral infection and 3, 6, or 8 wk after AAV infection. The AAV-infected Chy mice were killed 1, 2, 4, 6, or 8 mo after infection. Total RNA was extracted from the ears (RNAeasy Kit; QIAGEN) 1 to 8 wk after adenoviral infection and 10 wk after AAV-infection. 10 μg of RNA was subjected to Northern blotting and hybridization with a mixture of $[\alpha^{32}P]$dCTP (Amersham Biotech) labeled cDNAs specific for VEGF-C. The glyceraldehyde-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 (34) of the AdLacZ-infected ears 1 to 7 wk after gene transfer. The AAV-EGFP-infected ears were studied under the fluorescence microscope at 3 wk to 8 mo after infection.

β-Galactosidase and Lectin Staining of Vessels. For visualization of the superficial lymphatic vessels in the K14-VEGF-C156S and K14-VEGF-C embryos (15, 16), staged VEGFR-3+/LacZ embryos were dissected, fixed in 0.2% glutaraldehyde, and stained with X-gal (Sigma-Aldrich) for β-galactosidase activity at +37°C. For the analysis of the adult cutaneous lymphatic phenotype, β-galactosidase staining was performed for dissected adult mouse ear skin.

In some of the adenovirus-infected mice, *Lycopersicon esculentum* lectin staining was used to visualize the blood vessels in whole mount (12). Biotinylated lectin (1 mg/ml; Vector Laboratories) 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-DAB peroxidase method (Vectastain and Sigma-Aldrich). Finally the tissues were dehydrated and mounted on slides.

For the gene expression studies of different types of lymphatic vessels, a combination of biotinylated lectin and whole mount β-galactosidase staining was performed for VEGFR-2+/LacZ (35) and VEGFR-3+/LacZ (36) adult mouse tissues.

Analyses of the Lymphatic and Blood Vessels. For immunohistochemical analysis the mouse ears were dissected and fixed in 4% PFA. Those ears that were analyzed in whole mount were incubated in 5% H_2O_2 in methanol for 1 h to block endogenous peroxidase activity. The tissues were then blocked in 3% milk 0.3% Triton-X in PBS overnight, and antibodies against the vascular

endothelial marker PECAM-1 (BD Biosciences) or VEGFR-3 (R&D Systems) were applied overnight at +4°C. The visualization was achieved with either the ABC-DAB peroxidase method or with ABC-alkaline phosphatase using the alkaline phosphatase substrate kit II (Vector Laboratories). Finally the tissues were flattened and mounted on slides.

5-μm deparaffinized tissue sections were subjected to heat induced epitope retrieval treatment or to an alternative enzyme treatment. The endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 20 min. Antibodies against VEGFR-3 (19), PECAM-1, podoplanin (a gift from Dr. Miguel Quintanilla, Alberto Sols Biomedical Research Institute, Madrid, Spain), or LYVE-1 (a gift from Dr. Erkki Ruoslahti, Burnham Institute, La Jolla, CA) were applied overnight at +4°C and staining was performed using the tyramide signal amplification kit (NEN Life Science Products) and 3-amino-9-ethyl carbazole (Sigma-Aldrich). Hematoxylin was used for counterstaining.

To study the function of the cutaneous lymphatic vessels in the Chy lymphedema mice, a small volume of FITC-labeled dextran (MW 464 000; Sigma-Aldrich) was injected intradermally to the periphery of mouse ear. Drainage of the dye via the lymphatic vessels was followed under a fluorescence microscope.

Quantification of the Lymphangiogenic Response. To quantify the number of lymphatic vessels and branch points at 1 wk after adenoviral infection, six histological sections from ear midline with the highest vessel density were chosen from each study group (AdVEGF-C156S, AdVEGF-C, AdLacZ). The number of LYVE-1–positive vessels and the number of branches in these vessels were counted under a high power microscope. The area analyzed in each sample was 4 mm².

Permeability Assay. The right ear of each mouse was infected with AdVEGF-C156S, AdVEGF-C, or AdLacZ virus (5 × 10⁸ pfu). The left ear received either AdLacZ (5 × 10⁸ pfu) or PBS (in the AdLacZ group). 2 wk after the infection a modified Miles permeability assay was performed as described previously (12). 1 μl per gram of mouse weight of 3% Evans Blue was injected into the femoral vein and after 2 min the mice were perfusion fixed

with 0.05 M citrate buffer (pH 3.5) in 1% PFA. The ears were dissected, washed, weighed and extracted in formamide at +55°C overnight. The Evans Blue absorbance of the formamide was then measured with a spectrophotometer set at 610 nm, and the leakage (ng/mg) was compared between the right and left ear of the same mouse.

Results

Expression of the Virally Transduced Genes In Vitro and In Vivo. The production of active VEGF-C156S and VEGF-C proteins into the cell culture media of adenovirus (Ad)– or AAV–infected, metabolically labeled 293EBNA cells was confirmed by immunoprecipitation and by binding to soluble VEGFR–2-Ig and VEGFR-3-Ig fusion proteins. Both the partially processed 30 kD and the fully processed 21-kD forms of VEGF-C156S and VEGF-C bound to VEGFR-3-Ig, but only the 21-kD form of VEGF-C was capable of binding to VEGFR–2-Ig (Fig. 1 A, and unpublished data). Furthermore, Western blotting analysis of media from the infected cultures confirmed that the same viral titers of AdVEGF-C156S and AdVEGF-C gave rise to comparable levels of the corresponding proteins in vitro (Fig. 1 A). To analyze the expression of adenovirus and AAV transduced genes in vivo, RNA samples from infected mouse ear skin were analyzed by Northern blotting. High levels of human VEGF-C156S and VEGF-C mRNAs were detected in the AdVEGF-C156S and AdVEGF-C infected tissues 1 wk after infection (Fig. 1 B). 3 wk after infection transgene expression in the control AdLacZ infected ears was still strong (Fig. 1 C). Thereafter the transgene expression was gradually down-regulated, and by 8 wk expression was no longer detected in the adenovirus-infected ear. Somewhat weaker, but more sustained
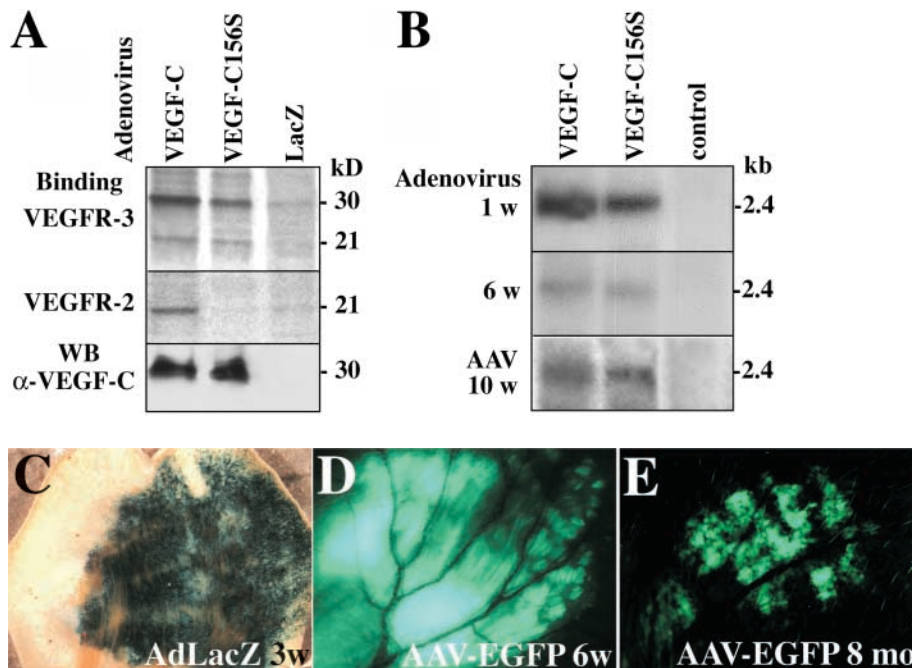

Figure 1. Viral gene expression in vitro and in vivo. (A) Comparison of Ad-VEGF-C156S and AdVEGF-C recombinant protein production in vitro. Top panels: the infected cells were metabolically labeled and cell culture media were subjected to precipitation with VEGFR-3-Ig or VEGFR-2-Ig fusion proteins. Medium from AdLacZ-infected cells was used as a negative control. Bottom panel: culture media of Ad-VEGF-C, AdVEGF-C156S, and AdLacZ infected cells were separated in 15% PAGE gel followed by Western blotting using antibodies against VEGF-C. (B) Viral transgene expression in vivo. Northern blot analysis of total RNA from mouse ears infected with recombinant adenoviruses or AAVs. The infecting virus and the duration of infection are indicated. (C) β-galactosidase staining of the ear three weeks after infection with AdLacZ. (D and E) EGFP expression in the ear 6 wk and 8 mo after AAV-EGFP infection.

mRNA and protein expression was obtained with the AAV vectors (Fig. 1, B and D). Furthermore, at 8 mo after infection, the latest time point studied, EGFP fluorescence was still detected in the ear skin of the Chy mice infected with the AAV–EGFP control virus (Fig. 1 E).

Comparison of the Lymphangiogenic Effects of VEGF-C156S and VEGF-C in Transgenic Embryos. To analyze the effects of VEGF-C156S and VEGF-C gene transfer in vivo, the K14–VEGF-C156S and K14–VEGF-C transgenic mice, which overexpress the factors in the basal cells of the epidermis and have a severe hyperplasia of the dermal lymphatic vessels (15, 16), were crossed with the VEGFR–3+/LacZ mice. In the latter mouse strain one allele of VEGFR–3 has been replaced by the LacZ marker gene by a knock-in strategy, allowing the visualization of the VEGFR–3 expressing cells by β–galactosidase staining. We collected a series of staged K14–VEGF-C156S × VEGFR–3+/LacZ and K14–VEGF-C × VEGFR–3+/LacZ embryos and analyzed the superficial lymphatic vessel phenotype after whole mount β-galactosidase staining. Both types of transgenic embryos developed normally up to embryonic day 12.5 (E12.5). The first signs of lymphatic hyperplasia were detected in the transgenic embryos at E13.5 (unpublished data), consistent with the low level of activation of the K14-promoter at about E13 (37). At E14.5, the cutaneous lymphatic vessels of both the K14-VEGF-C156S × VEGFR–3+/LacZ and K14–VEGF-C × VEGFR–3+/LacZ embryos were clearly hyperplastic (Fig. 2, A–C). Interestingly, whereas VEGF-C156S mainly induced enlargement of the preexisting lymphatic capillaries, VEGF-C caused extensive lymphatic vessel sprouting (Fig. 2, D–F). This effect was not due to a shift of VEGF-C signaling from VEGFR–3 to VEGFR–2 in the VEGFR–3+/LacZ heterozygous knock out embryos, as a similar phenotype was observed with VEGFR–3 immunostaining in histological sections of K14–VEGF-C156S and K14–VEGF-C transgenic embryos (Fig. 2, G–I). Furthermore, in two independent K14–VEGF-C156S founder lines studied, the higher VEGF-C156S dose resulted in an even more severe hyperplasia but no increase in lymphatic sprouting.

To determine whether the differences in VEGF-C156S and VEGF-C induced phenotypes were retained in adult mice, whole mount β-galactosidase staining of the adult skin was performed. Also, in the adult transgenic mice a clear in-
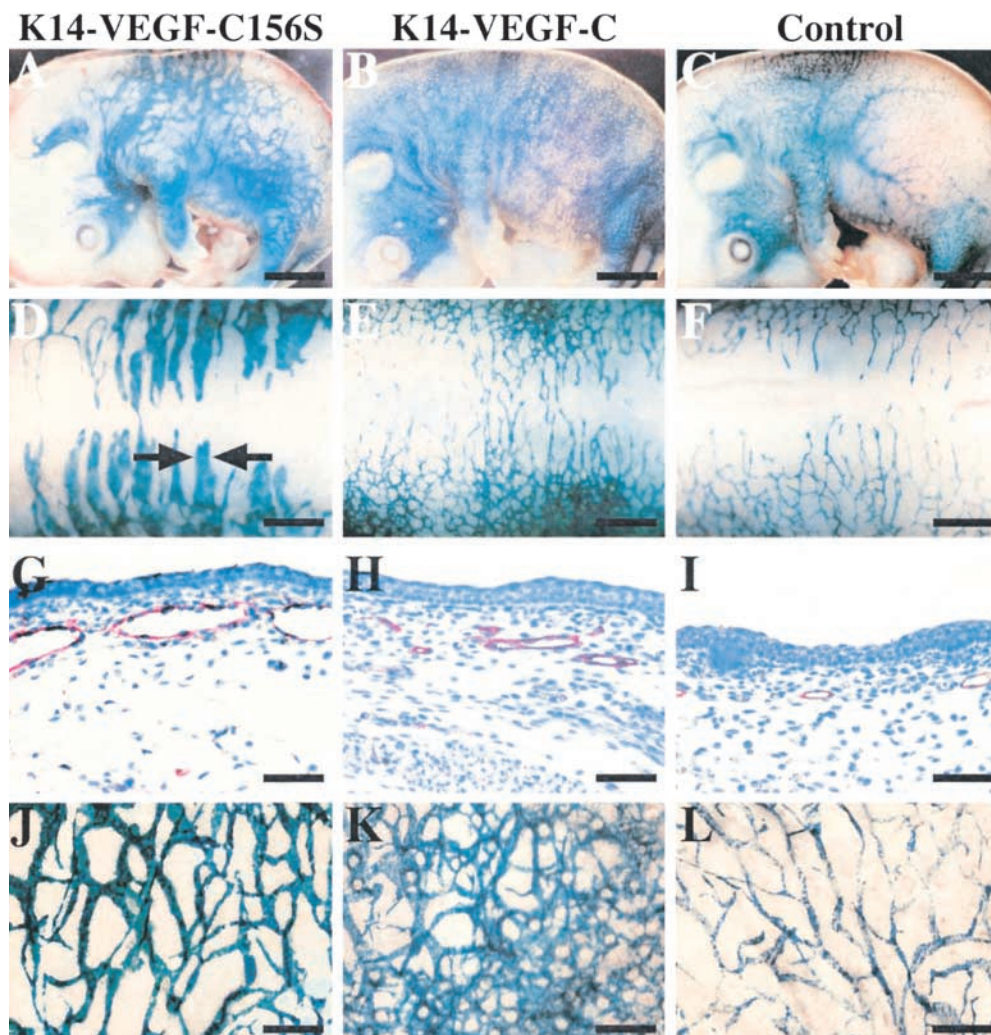

K14-VEGF-C156S   **K14-VEGF-C**   **Control**

Figure 2. Lymphangiogenesis in the skin of K14-VEGF-C156S and K14-VEGF-C transgenic embryos. (A–C) Superficial lymphatic vessels of VEGFR–3+/LacZ heterozygous E14.5 embryos visualized by β–galactosidase staining. Note the increase in the number of lymphatic capillaries in B and lymphatic capillary hyperplasia in A (arrows). (D–F) Dorsal views of the same embryos. (G–I) Cross sections of the superficial lymphatic vessels of E14.5 K14-VEGF-C156S and K14-VEGF-C transgenic embryos stained with antibodies against VEGFR–3. (J–L) Cutaneous lymphatic vessels of adult (age 8 wk) VEGFR–3+/LacZ mice. The lymphatic phenotype established during embryonic development is retained. Scale bars: A–C, 650 μm; D–F, 250 μm; G–I, 35 μm; J–L, 150 μm.

crease in the lymphatic vascularity of the skin was observed, but again VEGF-C156S induced mainly lymphatic vessel hyperplasia, whereas VEGF-C induced a strong increase in the number of lymphatic vessels (Fig. 2, J–L).

Comparison of the Lymphangiogenic Potential of Recombinant VEGF-C156S and VEGF-C Viruses. We then wanted to compare the lymphangiogenesis induced by viral delivery of VEGF-C156S and VEGF-C. For this purpose, adenoviruses encoding VEGF-C156S, VEGF-C, or LacZ were injected intradermally into the ears of nu/nu mice. The first sprouting lymphatic vessels were detected in the infected ear skin by VEGFR–3 whole mount staining 3 to 4 d after the adenoviral infection. The lymphatic sprouting was more abundant in the AdVEGF-C–infected skin than in the AdVEGF-C156S–infected skin at all time points studied. In the AdVEGF-C156S–infected skin the lymphatic vessels enlarged progressively, and 1 wk after the infection several lymphatic vessels appeared to be splitting to form new daughter vessels (Fig. 3 A). At this time point nearly all lymphatic vessels in the AdVEGF-C–infected skin exhibited sprouting and bridging; some enlarged vessels were also detected, whereas no signs of lymphangiogenesis were seen in the AdLacZ-infected control skin (Fig. 3, B and C). 2 wk after AdVEGF-C156S infection the lymphatic vessels network was vastly enlarged and most of the large vessels had formed new sprouts or bridges (Fig. 3 D). At the same time point the AdVEGF-C–infected skin was filled with very actively sprouting, relatively small lymphatic vessels, while only a few enlarged lymphatic vessels were detected (Fig. 3 E). A few new lymphatic sprouts were detected in only one AdLacZ-infected control mouse around the injection wound (*n* = 15; unpublished data), otherwise the

lymphatic vasculature in the control skin remained unaltered (Fig. 3 F). The staining of histological sections for VEGFR–3, LYVE-1, and podoplanin confirmed that the sprouting of the lymphatic vessels was more efficient in the AdVEGF-C than in the AdVEGF-C156S infected samples (Fig. 3, G–I, and unpublished data). The newly formed lymphatic vessels regressed gradually with time as expression of the viral genes was downregulated, and by 8 t to 10 wk after the infection the lymphatic vessel network had returned to its normal architecture (unpublished data).

To quantify the lymphangiogenic response in the skin after adenoviral infection, we counted in histological sections the number of LYVE-1–positive vessels and the number of branch points in these vessels. Both AdVEGF-C and AdVEGF-C156S induced a significant increase in the number of lymphatic vessels in comparison to the AdLacZ controls (Fig. 4 A). Furthermore, the AdVEGF-C–infected samples contained more LYVE-1–positive vessels than the AdVEGF-C156S infected samples ($P = 0.02$), consistent with the results obtained from the transgenic experiments. The LYVE-1–positive vessels in the AdVEGF-C–infected skin also had more branch points than the vessels in the AdVEGF-C156S infected samples ($P = 0.003$; Fig. 4 B).

AAV vectors may be better suited for gene therapy than adenoviral vectors due to the apparent lack of cell-mediated immune response against AAV. We therefore tested the ability of AAV-encoded VEGF-C156S to induce lymphangiogenesis in the mouse ear skin model. In VEGFR–3 whole mount staining of AAV-VEGF-C156S and AAV-VEGF-C infected ear skin, sack-like enlargements in the lymphatic vessels were detected 3 to 8 wk after the viral infection (Fig. 5, A–F). In both cases most of these enlarged
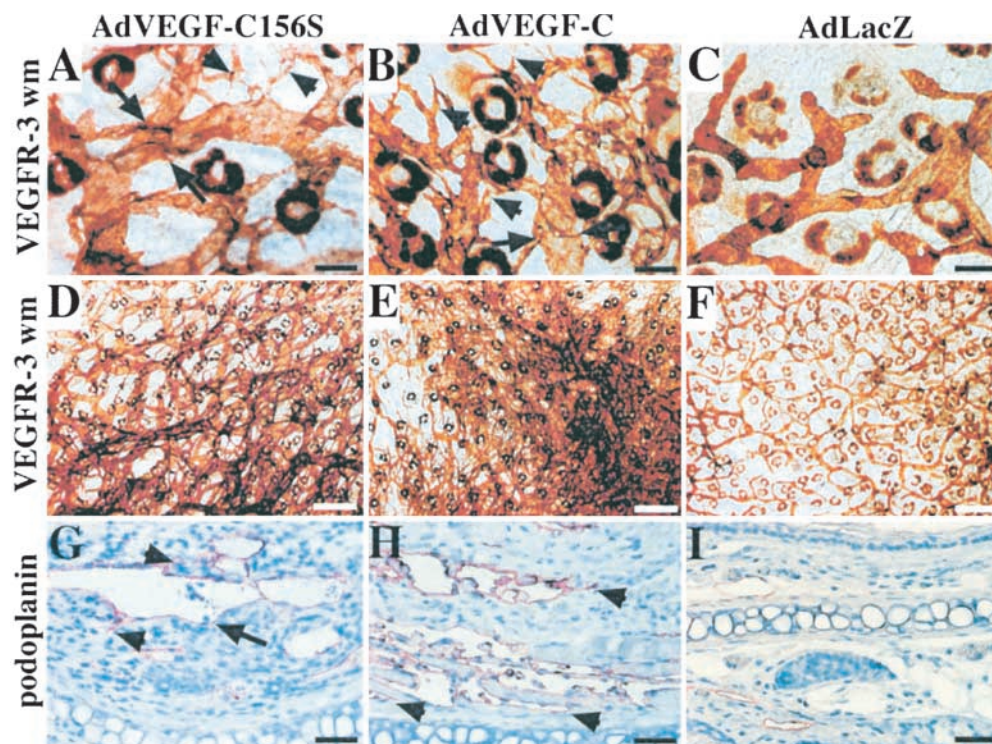

Figure 3. Lymphangiogenesis in AdVEGF-C156S and AdVEGF-C infected adult skin. Whole mount VEGFR–3 staining of the lymphatic vessels 1 wk (A–C) and 2 wk (D–F) after infection. Note enlarged lymphatic vessels (arrows) and sprouting or splitting lymphatic vessels (arrowheads) in AdVEGF-C156S (A) and AdVEGF-C (B) infected ears. 2 wk after infection, AdVEGF-C156S infected ear (D) contains large lymphatic vessels apparently still undergoing sprouting and splitting, whereas AdVEGF-C (E) infected ear is filled with small lymphatic sprouts. (G–I) Podoplanin stained tissue sections at 2 wk after adenoviral infection. Note the formation of a hyperplastic lymphatic network in response to adenoviral VEGF-C156S (G) and VEGF-C (H) compared with AdLacZ control (I). Sprouting and splitting of the vessels is especially clear in the AdVEGF-C infected ear (H). Scale bars: A–C, 60 μm; D–F, 220 μm; G–I, 70 μm.
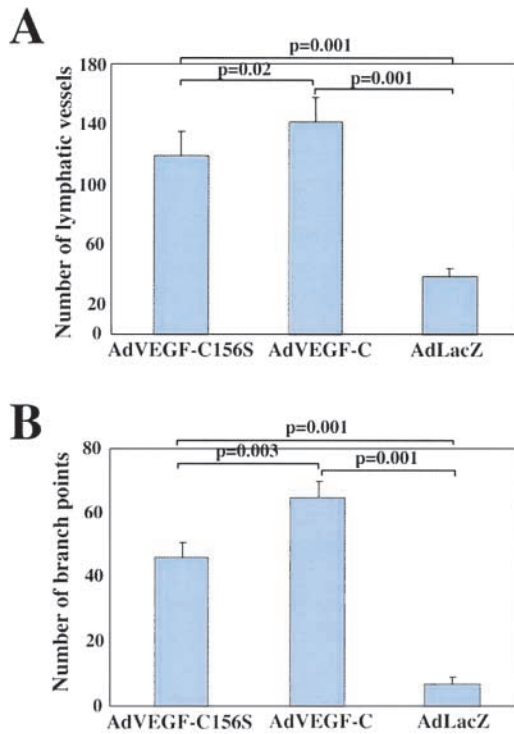
Figure 4. Quantification of the lymphangiogenic response in the adenovirus-infected samples. Number of LYVE-1 positive vessels in skin histological sections (A) and number of branch points in these vessels (B) were counted under a high power microscope.

vessels also exhibited new sprouts, but as in the adenoviral experiments, the sprouting effect was more pronounced in the AAV-VEGF-C–infected samples. This difference in phenotype was also apparent in podoplanin-stained histological sections (Fig. 5, G and H). Overall, the lymphangiogenic response to both AAV-derived VEGF-C156S and VEGF-C was weaker than that obtained with the adenoviral vectors.

Adenoviral VEGF-C156S Lacks the Blood Vascular Effects of VEGF-C. We have recently demonstrated that local adenoviral VEGF-C expression induces blood vessel enlargement, tortuosity, and leakiness (28). To see if a similar blood vascular response results from adenoviral expression of VEGF-C156S, we compared the dermal blood vessels in the ear skin 1 to 2 wk after infection with AdVEGF-C156S, AdVEGF-C, or AdLacZ. The vessels appeared normal in AdVEGF-C156S and AdLacZ infected ears, whereas in the AdVEGF-C–infected samples they were tortuous and enlarged (Fig. 6, A–C). Also, in whole mount PECAM-1 staining there were no obvious differences between the AdVEGF-C156S and AdLacZ infected samples in the caliber or morphology of veins, arteries, or capillaries (Fig. 6, D and F). However, the veins and venules of the AdVEGF-C–infected samples were clearly dilated and tortuous (Fig. 6 E).

To determine the effect of AdVEGF-C156S on blood vessel permeability, AdVEGF-C156S, AdVEGF-C, or AdLacZ viruses were injected into the right ears of nu/nu mice, while AdLacZ infected left ears served as controls. Extravasation of Evans Blue dye from ear blood vessels was measured 2 wk after infection. There was no statistically significant difference in the blood vessel permeability between the control ears and the AdVEGF-C156S–infected ears (Fig. 7 A), whereas the leakage of Evans Blue in the AdVEGF-C–treated ears was on an average 1.4
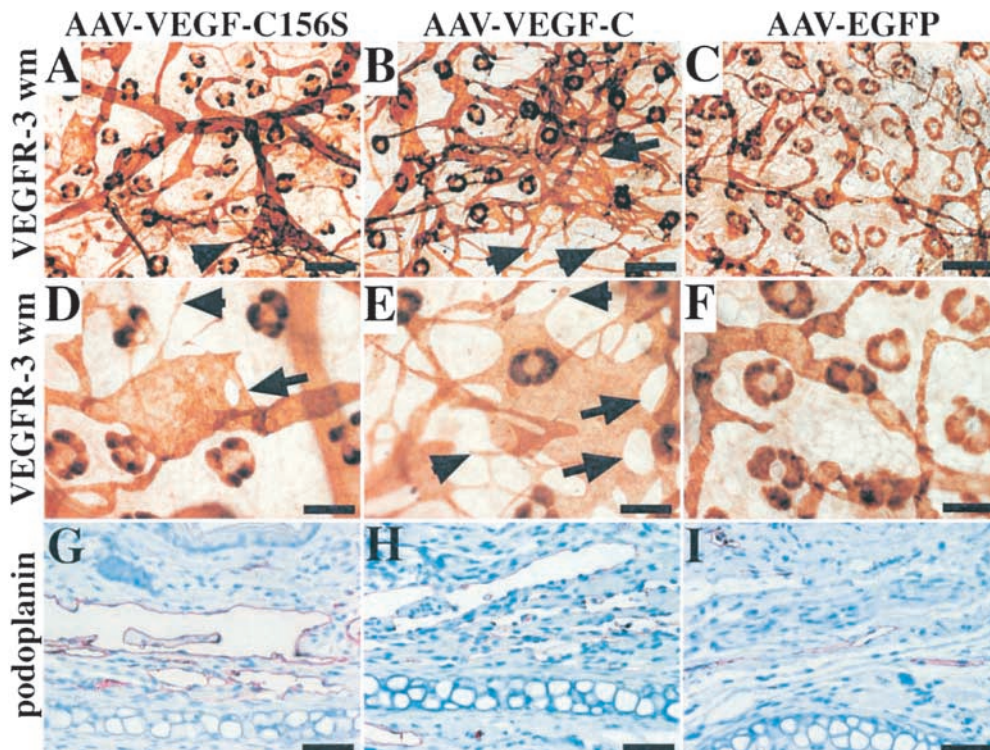

Figure 5. Lymphangiogenesis in AAV-VEGF-C156S and AAV-VEGF-C infected adult skin. (A–F) Lymphatic vessels visualized with VEGFR-3 whole mount staining 6 wk after infection with the recombinant AAVs. Note the enlargement, sprouting (arrowheads), and splitting (arrows) of the lymphatic vessels in response to VEGF-C156S and VEGF-C. Lymphatic sprouting is more obvious in the AAV-VEGF-C infected ear (B and E) than in the AAV-VEGF-C156S infected ear (A and D). (G–I) Podoplanin staining of histological sections at the same time point. Note the hyperplastic lymphatic vessel network within the subcutis and muscle cell layers of the skin in the AAV-VEGF-C156S (G) and AAV-VEGF-C (H) infected samples. Scale bars: A–C, 150 μm; D–F, 60 μm; G–I, 70 μm.
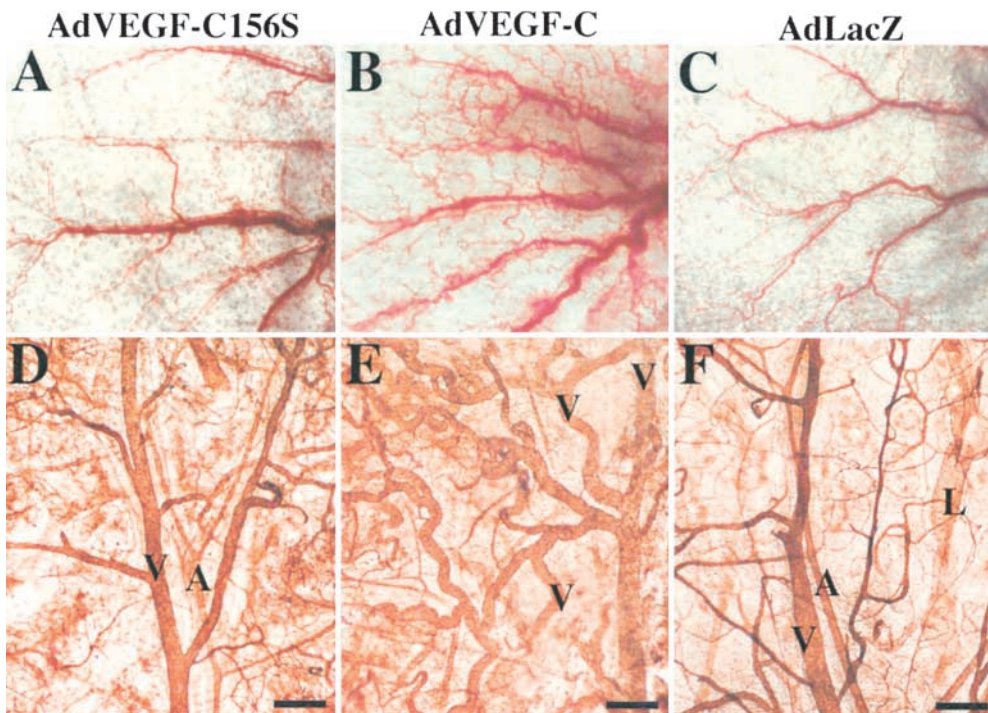
Figure 6. Adenoviral VEGF–C156S does not affect blood vessel morphology. (A–C) Ears photographed 1 wk after adenoviral infection. Note the dilation and tortuosity of blood vessels in the AdVEGF-C infected skin (B) compared with the AdVEGF-C156S (A) and AdLacZ (C) infected skin. (D–F) Whole mount PECAM-1 staining of the ear skin blood vessels at the same time point. Compared with the AdLacZ control (F), the veins (V) and arteries (A) in the AdVEGF-C156S infected skin (D) appear morphologically normal, whereas in the AdVEGF-C infected ear (E) the veins are large and tortuous. Note weak staining of lymphatic vessels (L) in F. Scale bars: D–F, 150 μm.

fold greater than in the AdLacZ–infected control ears (Fig. 7 A).

The systemic effects of AdVEGF-C156S, AdVEGF-C, and AdLacZ were studied by injecting 5×10^8 or 1×10^9 pfu of the viruses into the tail veins of C57/Bl6 mice. All mice that had received intravenous AdVEGF-C156S or AdLacZ appeared healthy during the 2-wk follow-up period, whereas all mice that had received different doses of
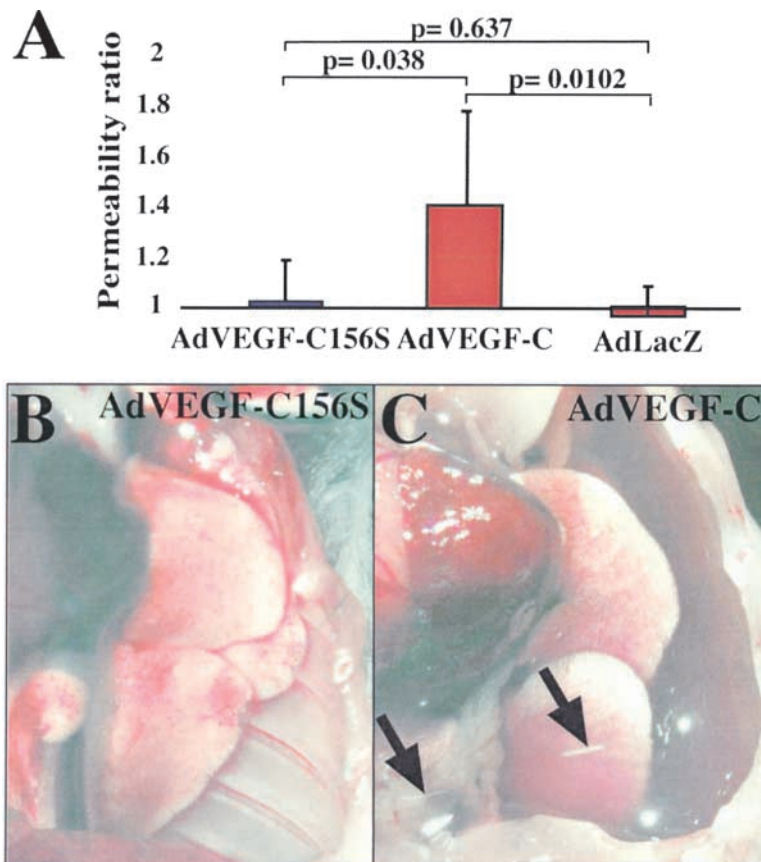

Figure 7. Adenoviral VEGF–C156S has a minimal effect on vascular permeability. (A) The difference in permeability between the treated versus the control ear measured by Evans Blue concentration ratio (ng/mg). (B and C) Mouse thoracic cavities photographed after systemic administration of AdVEGF-C156S or AdVEGF-C (1×10^9 pfu). Note the accumulation of pleural fluid in the AdVEGF-C infected mouse (C) but not in the AdVEGF-C156S infected mouse (B).

AdVEGF-C became ill on day 3 after infection. Three of the five mice that received the higher dose of AdVEGF-C died during the first week. All mice infected with Ad-VEGF-C had dose-dependent accumulation of fluid in the thoracic cavity, but intrathoracic fluid was not observed in any of the AdVEGF-C156S or AdLacZ infected mice (Fig. 7, B and C). Two of the five mice that received the higher dose of AdVEGF-C also had fluid in the peritoneal cavity.

AAV-mediated VEGF-C156S Gene Transfer Results in Formation of Functional Lymphatic Vessels in the Chy Lymphedema Mice. Similar to certain patients with Milroy's disease, the Chy lymphedema mice have a germline inactivating mutation of VEGFR-3, which results in hypoplasia of cutaneous lymphatic vessels (32). We have previously reported that adenoviral or AAV-encoded VEGF-C is capable of restoring a functional cutaneous lymphatic vessel network in the Chy mice (32). To assess the lymphangiogenic activity of viral VEGF-C156S in the Chy mice, their ears were injected with AAVs encoding VEGF-C156S, VEGF-C, or EGFP. Fluorescent microlymphangiography using FITC-dextran revealed that functional, normal-looking, but slightly dilated lymphatic vessels had formed in the AAV-VEGF-C156S infected mice during the 2-mo follow-up period (Fig. 8, A–D); we also observed that in the AAV-VEGF-C–infected Chy mice such vessels persisted at least for 8 mo. As a rule, in Chy mice infected with AAV-EGFP, FITC-dextran remained at the injection site, except that a single draining lymphatic vessel was seen in 2/12 of the mice (Fig. 8 C, and unpublished data). This finding may reflect the occasional finding of isolated lymphatic vessel segments in untreated Chy mice.

VEGFR-3 whole mount staining revealed only a few large sack-like vessel structures in the AAV-EGFP–treated Chy mice (Fig. 8 G). 5 wk after infection with AAV-VEGF-C156S or AAV-VEGF-C, new VEGFR-3–positive vessels were seen sprouting from these lymphatic remnants (inset in Fig. 8 E). From 2 mo after VEGF-C156S or VEGF-C gene transfer, branched lymphatic vessel network was detected in the skin (Fig. 8, E and F). However, in most cases the lymphatic vessels that formed in the skin of the Chy mice in response to the lymphangiogenic gene therapy appeared slightly hyperplastic in comparison with those of wild-type mouse skin (Fig. 8, E–H).

VEGF-C Receptors Are Differentially Distributed in Lymphatic Vessels. The results with the VEGFR-3–specific form of VEGF-C suggest that VEGFR-2 signaling is not obligatory for lymphangiogenesis, although it has been reported to be expressed in lymphatic endothelial cells in vivo (28). To better understand these results, we compared the lymphatic endothelial expression patterns of VEGFR-2 and VEGFR-3 in the respective heterozygous β-galactosidase knock-in mice. In the skin of VEGFR-3+/LacZ mice, the superficial lymphatic capillaries were strongly stained, whereas in VEGFR-2+/LacZ mice, the lymphatic staining was strongest in the collecting lymphatic vessels (Fig. 9, A and B). To determine whether such distribution patterns are specific to the skin, we analyzed other organs. In the diaphragm of the VEGFR-3+/LacZ mice, β-galactosidase staining was also found in the initial lymphatic capillaries, whereas in the VEGFR-2+/LacZ mice the staining was mostly seen in the collecting lymphatic vessels (Fig. 9, C–F). However, in the mesenteric lymphatic vessels of the small intestine the receptors were expressed at equally high
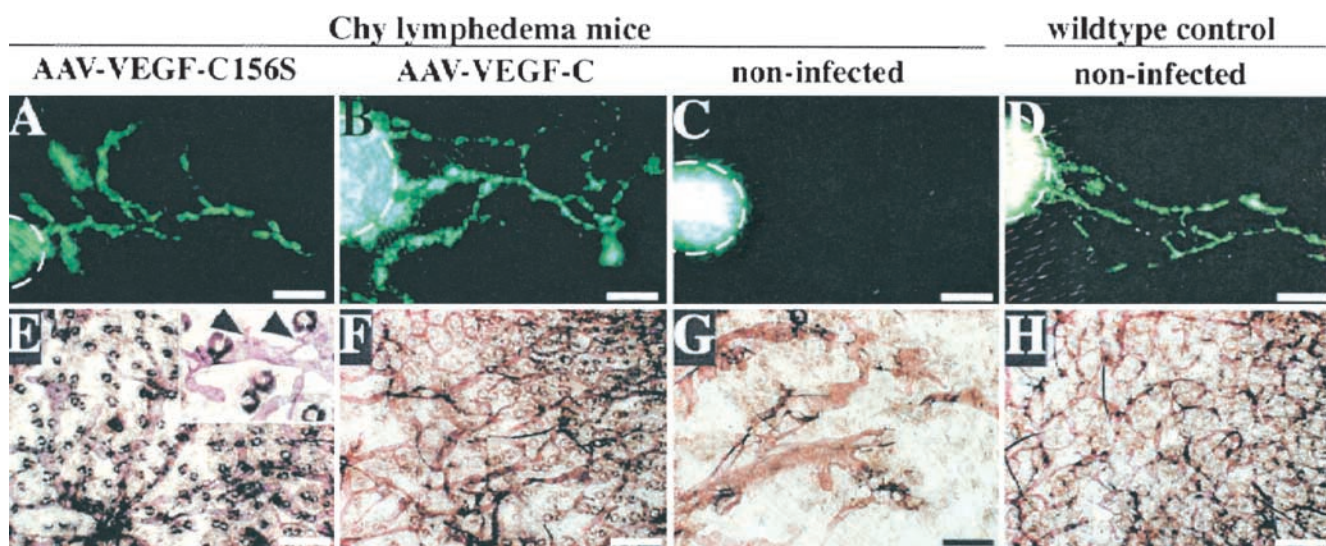

Figure 8. VEGF-C156S gene therapy in the Chy lymphedema mice. (A and B) AAV-mediated overexpression of VEGF-C156S (2 mo) and VEGF-C (8 mo) induces the formation and maintenance of a functional lymphatic vessel network, as analyzed by fluorescent microlymphangiography. C and D show comparison with noninfected Chy mouse ear and wild-type control ear, respectively. (E–H) VEGFR-3 whole mount staining of the ears 2 mo after AAV-infection. Note the formation of a tree-like network of lymphatic vessels in the skin of Chy mice in response to VEGF-C156S (E) and VEGF-C (F). Inset in E shows lymphatic sprouting and splitting (arrowheads) in the AAV-VEGF-C156S infected Chy mouse skin 5 wk after infection. The control Chy mouse skin (G) contains few abnormal VEGFR-3–positive vessel structures. (H) VEGFR-3 staining of wild-type mouse skin. Scale bars: A–D, 250 μm; E–H, 200 μm.

VEGFR-3+/LacZ   VEGFR-2+/LacZ

Skin — A, B

Diaphragm — C, D

Diaphragm — E, F
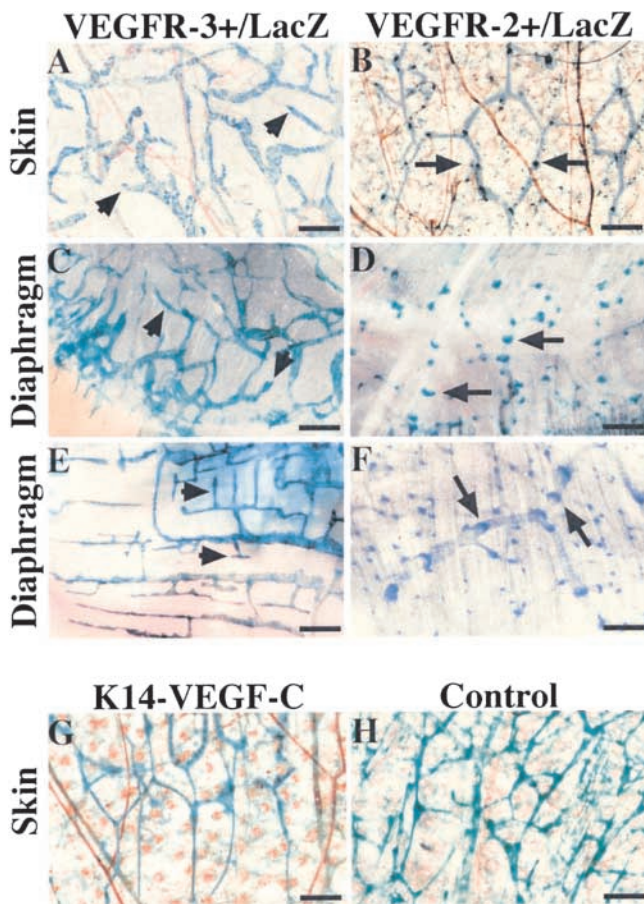
K14-VEGF-C   Control

Skin — G, H

Figure 9. Molecular markers of adult lymphatic vessels. (A and B) Visualization of cutaneous lymphatic vessels in VEGFR-3+/LacZ and VEGFR-2+/LacZ mice. Blood vessels are stained brown for biotinylated lectin while the blue staining marks the expression of the β galactosidase gene. Note that whereas VEGFR-2 is found in the collecting lymphatic vessels with valves (arrows), VEGFR-3 expression is strong in the initial lymphatic capillaries (arrowheads). (C and D) Lymphatic vessels on the membranous part and (E and F) on the muscular part of the diaphragm. (G and H) The collecting lymphatic vessels of a K14-VEGF-C × VEGFR-2+/LacZ mouse and a VEGFR-2+/LacZ littermate control. Scale bars: A and B, 320 μm; C and D, 250 μm; E and F, 120 μm; G and H, 180 μm.

levels (unpublished data). We have previously reported the in the adult K14-VEGF-C mice the superficial cutaneous lymphatic capillaries are hyperplastic (15), but the phenotype of the skin collecting lymphatic in these mice has not been characterized. To study the collecting lymphatic vessel phenotype in the K14-VEGF-C mice, we crossed them with VEGFR-2+/LacZ mice. However, the collecting lymphatic vessels were not affected by the overexpression of VEGF-C in the basal keratinocytes (Fig. 8, G and H).

Discussion

Our study shows that overexpression of VEGF-C156S, a VEGFR-3–specific mutant form of VEGF-C, potently induces lymphangiogenesis both during embryonic develop-

ment and in adult skin. Recombinant VEGF-C156S viruses were capable of inducing growth of lymphatic vessels and the formation of new lymphatic sprouts, although the latter effect was seen only in adults and was less pronounced than with VEGF-C. In addition, we show that VEGF-C156S does not have obvious effects on blood vessels in contrast to VEGF-C, which also binds VEGFR-2 expressed on the blood vascular endothelium. In the Chy lymphedema mice AAV-mediated gene transfer of VEGF-C156S induced the formation of a functional lymphatic vessel network into the skin.

The whole mount images of ongoing embryonic and adult lymphangiogenesis that we present here suggest that the mechanisms of lymphangiogenesis may be very similar to those of angiogenesis (38) in that lymphangiogenesis also appears to proceed via vessel enlargement, sprouting and splitting. Also, like the angiogenic blood vessels, the newly formed lymphatic vessels seem to require a continuous growth factor stimulation in order to be maintained. Although VEGF-C156S and VEGF-C both induced an increase of lymphatic vascularity, their lymphangiogenic mechanisms seemed to differ. The difference between the capacities of VEGF-C156S and VEGF-C to induce new lymphatic sprouts was most obvious in the transgenic skin model. This suggests that activation of VEGFR-2 is required for the induction of lymphatic sprouting during embryonic development. Indeed, we have evidence that VEGFR-2 is expressed in the embryonic lymph sac endothelium (unpublished data). Although VEGFR-3 activation via viral VEGF-C156S expression seems sufficient for the induction of lymphatic sprouting in adult tissues, a more effective sprouting response may require VEGFR-2 signaling also in adult tissues. An interesting possibility is that signaling via VEGFR-2 and VEGFR-3 receptor heterodimers is needed for efficient formation of vessel sprouts. Gene expression studies in cultured lymphatic endothelial cells after VEGF-C156S and VEGF-C stimulation should reveal at least some of the mechanisms behind the differences in such lymphangiogenic phenotypes observed in vivo. On the other hand, it is also possible that VEGF-C can recruit other effector cells and factors needed for sprouting in adult tissues better than VEGF-C156S.

The biological significance of VEGF-C–induced blood vessel dilation and increased permeability is unknown. These effects may at least in part result from receptor-mediated vasodilation as veins and venules in adult skin have been shown to express VEGFR-2 (28). Several angiogenic growth factors including VEGF and bFGF are known to be potent stimulators of nitric oxide production, and in vivo studies have documented endothelium-dependent hypotension in response to treatment with these growth factors (39, 40). VEGF-C has also been reported to stimulate the release of endothelial nitric oxide, which could contribute to enhanced vascular permeability (41). The results we obtained with systemic administration of AdVEGF-C resemble those reported after systemic administration of AdVEGF by Thurston and coworkers (13). Also, patients with tissue ischemia developed edema after VEGF gene

transfer (42). In contrast, we have thus far observed minimal blood vascular effects with VEGF-C156S.

The lymphangiogenesis in response to AAV-VEGF-C156S and AAV-VEGF-C was always weaker and slower than lymphangiogenesis obtained with the corresponding adenoviral vectors. This finding probably relates to differences in the biological properties of these two viral vectors. It has been demonstrated that after an intramuscular AAV infection, the level of transgene expression increased gradually over 5 to 10 wk after an initial lag phase of 1 to 2 wk (43). With adenovirus vectors, transgene expression has been detected already 24 h after infection, but after reaching its peak during the first week after infection, the expression is rapidly downregulated (10), probably due to direct cytopathic effects of the virus resulting in the elimination of the virally transduced cells. In the present study adenoviral gene expression was extinguished by eight weeks after infection of nude mice. The lower transgene expression levels and slower kinetics of AAV-VEGF-C156S and AAV-VEGF-C infection seemed to result in more controlled lymphangiogenesis than the acute high-level expression obtained by the use of adenoviral vectors. AAV could therefore be more suited for lymphangiogenic therapy in humans. Importantly, AAV-mediated transgene expression seems very stable as demonstrated by persistent fluorescence in the AAV–EGFP–infected samples. We have followed the AAV-VEGF-C–infected Chy mice for up to 8 mo after infection and found that functional skin lymphatic vessels are still present at this time point. This probably reflects continuous transgene expression from the integrated viral vector rather than actual stabilization of the newly formed lymphatic vessels. At present it is unclear whether a single infection with a recombinant AAV vector will produce life-long transgene expression or whether vector readministration would be required.

The first lymphangiogenic growth factors were discovered some time ago, but the molecular control of the formation of the patterned hierarchy of lymphatic vessels is still largely unknown. Here we report for the first time that the endothelial cells of lymphatic vessels of different calibers express different growth factor receptors on their surface. This probably reflects subtle differences in the function of the lymphatic endothelium of the various vessels and their abilities to respond to different types of stimuli. Interestingly, in the K14-VEGF-C mice only the superficial lymphatic capillaries were hyperplastic, whereas the collecting, VEGFR-2–positive lymphatic vessels appeared normal. Previous microlymphography studies have indicated that the function of the superficial lymphatic capillaries in the K14-VEGF-C mice may be partly compromised (15). As both capillaries and collecting vessels are needed for the lymphatic drainage function, more attention should be paid to the development of strategies to specifically target the different types of lymphatic vessels. This issue will be of major importance when designing molecular therapies for human lymphedema.

Angiogenic gene therapy has raised concerns regarding the potential stimulation of dormant tumor growth due to increased tumor angiogenesis in response to elevated systemic VEGF levels. In addition, high local levels of VEGF after viral gene transfer have been shown to result in the formation of hemangioma-like vascular tumors in murine skeletal muscle, heart, and skin (10, 11, 44). VEGF-C has also been shown to increase tumor angiogenesis and the recruitment of inflammatory cells (45, 46), but the increased angiogenesis was reversed by inhibition of VEGFR-2 (45). The safety of angiogenic gene therapy, in particular VEGF gene transfer, would be improved by a brief duration and low level of localized transgene expression. In a lymphatic vascular specific gene therapy without blood vascular side effects the safety margin would be wider. For patients with lymphatic hypoplasia, dysfunction, and edema, VEGF-C156S gene therapy would thus seem like an attractive choice. However, as tumor-induced lymphangiogenesis has been associated with enhanced metastasis to the lymph nodes (47–52) the risk of enhanced growth and spread of dormant metastases needs to be carefully evaluated.

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