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Abstract*—**Vascular endothelial growth factor (VEGF)-C and VEGF-D are composed of the receptor-binding VEGF homology domain and a carboxy-terminal silk homology domain that requires proteolytic cleavage for growth factor activation. Here, we explored whether the C-terminal heparin-binding domain of the $VEGF_{165}$ or $VEGF_{189}$ isoform also containing neuropilin-binding sequences could substitute for the silk homology domain of VEGF-C. Such VEGF-C/VEGF–heparin-binding domain chimeras were produced and shown to activate VEGF-C receptors, and, when expressed in tissues via adenovirus or adeno-associated virus vectors, stimulated lymphangiogenesis in vivo. However, both chimeras induced a distinctly different pattern of lymphatic vessels when compared with VEGF-C. Whereas VEGF-C–induced vessels were initially a dense network of small diameter vessels, the lymphatic vessels induced by the chimeric growth factors tended to form directly along tissue borders, along basement membranes that are rich in heparan sulfate. For example, in skeletal muscle, the chimeras induced formation of lumenized lymphatic vessels more efficiently than wild-type VEGF-C. We conclude that the matrix-binding domain of VEGF can target VEGF-C activity to heparin-rich basement membrane structures. These properties may prove useful for tissue engineering and attempts to regenerate lymphatic vessels in lymphedema patients. (Circ Res*. 2007;100:1468-1475.)**

Key Words: VEGF-C ■ VEGF-A ■ heparin-binding ■ lymphangiogenesis

The 5 mammalian vascular endothelial growth factor (VEGF) family members identified to date, VEGF, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor, are key effectors of physiological and pathological regulation of vasculogenesis, hematopoiesis, angiogenesis, lymphangiogenesis, and vascular permeability.[1–3] VEGF is a key growth factor for blood vessel formation and plays an essential role in this process via VEGF receptor (VEGFR)-1 and VEGFR-2.[1] VEGF-C and VEGF-D activate primarily VEGFR-3[4–8] and induce lymphangiogenesis in transgenic mice and in other in vivo models.[8–11]

VEGF is expressed as multiple forms, including the major forms $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, which result from alternative RNA splicing.[1] An important biological property that distinguishes these VEGF isoforms from each other is their different binding affinity to heparin and other heparan sulfates. Except for $VEGF_{121}$, all of the other forms described above contain a heparin-binding domain (HBD) encoded by exon 6 and/or exon 7. The 24-aa residues encoded by exon 6 contain the HBD and also elements that enable its binding to the extracellular matrix.[12] VEGF molecules containing the cationic polypeptide sequence encoded by exon 7 (44 aa) are also heparin-binding and remain bound to the cell surface and the extracellular matrix.[13] VEGF exon 7–encoded domain also enables its binding to neuropilin-1 (NP-1).[14] Other members of the VEGF family that contain a HBD include $VEGF\text{-}B_{167}$[15] and placenta growth factor-2.[16,17] There is increasing evidence pointing to the importance of the HBD for the biological activity of VEGF.[18,19]

VEGF-C and VEGF-D have a C-terminal domain homologous to certain silk proteins, plus an amino terminal propeptide. A proteolytic cleavage between the growth factor domain and the silk domain activates VEGF-C binding to VEGFR-3, and the N-terminally cleaved mature form (VEGF-CΔNΔC) can also activate VEGFR-2 in blood vessel endothelium, resulting in angiogenic activity.[20–24] However, in transgenic models in which both wild-type and mutant

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forms of VEGF-C induced lymphangiogenesis in the skin, no angiogenic effect was observed, suggesting that during embryonic development, VEGF-C may not be fully processed to a form that activates the VEGFR-2 in blood vessels.[7,9]

In this study, we investigated the contribution of a matrix-binding domain to the in vivo activity of VEGF-C. The exon 6 to 8 or exon 7 to 8 encoded domains from VEGF were fused to the C terminus of the fully processed VEGF-CΔNΔC lacking the N- and C-terminal propeptides. With these constructs, we wanted to investigate whether the heparin- and neuropilin-binding property can alter the lymphangiogenic or angiogenic effects of VEGF-CΔNΔC.

Materials and Methods

Cell Culture

293T cells from American Type Culture Collection were maintained in DMEM (HaartBio, Helsinki, Finland) supplemented with 2 mmol/L L-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% FBS (Autogen Bioclear). Hela cells were maintained in DMEM, and Ba/F3 cells[25] were grown in DMEM as above, with the addition of Zeocin (200 μg/mL) and recombinant human VEGF-CΔNΔC (corresponding to the fully processed mature short form, 100 ng/mL).

Cloning

cDNAs encoding the fusion proteins comprising the VEGF-C signal sequence (amino acids 1 to 31), the VEGF-CΔNΔC domain (amino acids 103 to 225) and the C terminus of VEGF (exon 6 to 8 encoded polypeptide fragment, named CA89; or exon 6 to 7 encoded fragment, named CA65) were constructed by PCR amplification using the following primers: VEGF-CΔNΔC, 5′-ACATTGGTGTGCACCTCCAAGC-3′ and 5′-AATAATGGAATGAACTTGTCTGTAAAC-3′; VEGF C-terminal regions, 5′-AAATCAGTTCGAGGAAAGGGAAAG-3′ or 5′-CCCTGTGGGCCTTGCTCAGAG-3′ and 5′-ACCATGCTCGAGAGT-CTTTCCTGGTGAGAGATCTGG-3′. The PCR products were digested with *Hind*III (5′-HindIII/3′-blunt) or *Xho*I (5′-blunt-3′-*Xho*I) and cloned into the pEBS7 expression vector that had been digested with the same enzymes, resulting in constructs pEBS7/CA89 and pEBS7/CA65.

Transfection and Immunoprecipitation

293T cells were transfected with pEBS7/CA89, pEBS7/CA65, pEBS7/VEGF-CΔNΔC, or pEBS7 vector using liposomes (FuGENE 6, Roche). Transfected cells were cultured for 24 hours and were then metabolically labeled in methionine-free and cysteine-free modified Eagle's medium supplemented with [^{35}S]methionine/[^{35}S]cysteine (Promix, GE Healthcare) at 100 μCi/mL for 8 hours. Cells transfected with pEBS7/CA89 were cultured with or without heparin (20 U/mL). Conditioned medium was harvested, cleared of particulate material by centrifugation, depleted of VEGF by immunoprecipitation with anti-VEGF antibody (R&D Systems), and incubated with soluble VEGFR1-Ig, VEGFR2-Ig, VEGFR3-Ig, or polyclonal antibodies against VEGF-C.[20] The formed growth factor–antibody or –receptor complexes were bound to protein A–Sepharose or protein G–Sepharose (GE Healthcare), which were washed twice with 0.5% BSA/0.02% Tween-20 in PBS and once with PBS and analyzed in SDS-PAGE under reducing conditions.

For the analysis of neuropilin-binding, conditioned medium from transfected cells was used. Briefly, both NP-1–Ig and NP-2(a22)–Ig fusion proteins[26] were transiently transfected into 293T cells. The transfected cells were cultured in serum-free medium for 48 hours, the conditioned medium was collected, cleared by centrifugation, and used for binding analysis as described above.

Production and In Vivo Delivery of CA89 and CA65 by Viral Vectors

The adeno-associated virus (AAV) vector psub-CAG-WPRE has been derived by substituting the cytomegalovirus (CMV) promoter fragment of psub-CMV-WPRE with the CMV–chicken β-actin insert. The cDNAs encoding CA89 and CA65 were cloned as blunt-end fragments into the psub-CAG-WPRE plasmid, and the recombinant AAVs (AAV-CA89 and AAV-CA65, AAV serotype 2) were produced as previously described.[26] The cDNAs encoding CA89 and CA65 were also cloned into the pAdBglII vector (AdCA89 and AdCA65), and recombinant adenoviruses were produced as described previously.[27] HeLa cells used for expression analysis were transduced with AAVs (2000 multiplicities of infection) or adenoviruses (50 multiplicities of infection). Expression of the recombinant proteins was examined by metabolic labeling and immunoprecipitation, followed by SDS-PAGE analysis as described above.

All animal experiments were approved by the Committee for Animal Experiments of the District of Southern Finland. Approximately 3×10^8 plaque forming units of AdCA89, AdCA65, AdVEGF-C, AdCΔNΔC,- or β-galactosidase-encoding AdLacZ control virus or AAVs (AAV-CA89, AAV-CA65, AAV-VEGF-C, AAV-CΔNΔC, AAV-VEGF-B_{167}, or AAV-EGFP; approximately 1×10^{10} viral particles) were injected subcutaneously into mouse ears. Tissues were collected for histological analysis 2 weeks after adenoviral, or 6 weeks or 2 years after AAV transduction. Semimembranosus muscles of rabbit hindlimbs were transduced with adenoviral vectors, as previously.[28]

Bioassay for Growth Factor–Mediated Cell Survival

Ba/F3 cells expressing the VEGFR-3/EpoR chimeric receptor[25] were seeded in 96-well plates at 15 000 cells per well in triplicate, and supplied with conditioned medium from Hela cells transduced with AdLacZ, AdVEGF-C, AdVEGF-CΔNΔC, AdCA65, or AdCA89 (1:80 dilution). Cell viability was quantified by a colorimetric assay after 48 hours. Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma; 0.5 mg/mL) was added into each well and incubated for 4 hours at 37°C. The reaction was terminated by adding lysis buffer (10% SDS, 10 mmol/L HCl), and the resulting formazan products were solubilized overnight at 37°C in a humid atmosphere. The absorbance, at 540 nm, was measured with a Multiscan microtiter plate reader (Labsystems).

Generation of Antisera

We generated novel rabbit antisera to the core domain of human VEGF-C (VEGF-CΔNΔC), designated VEGF-CΔNΔC no. 3 and VEGF-CΔNΔC no. 4. Recombinant, mature hexahistidine-tagged VEGF-C (amino acids 103 to 215) was produced using the FastBac system (Invitrogen) and purified using Ni^{2+}–nitrilotriacetic acid affinity chromatography, followed by dialysis against PBS. Polyclonal rabbit antiserum was obtained using standard procedures.[29]

Immunohistochemistry

For whole-mount staining, mice were fixed by intracardiac perfusion with 1% paraformaldehyde, blocked with 3% milk in PBS, and incubated with polyclonal antibodies against the lymphatic endothelial–specific hyaluronan receptor LYVE-1,[30] full-length VEGF-C,[31] VEGF-CΔNΔC (#3 and #4) or VEGFR-3 (R&D Systems) or monoclonal antibodies against platelet endothelial cell adhesion molecule (PECAM)-1 (PharMingen) or phosphohistone H3 (Upstate) overnight at 4°C.[32] Appropriate Alexa 647–, Alexa594–, or Alexa488– conjugated secondary antibodies (Molecular Probes) were used for staining. All fluorescently labeled samples were mounted with Vectashield containing DAPI (4′,6-diamidino-2-phenylindole) (VectorLabs) and analyzed with a compound fluorescent microscope (Zeiss 2, Carl Zeiss, Göttingen, Germany; ×10 objective with 0.30 numerical aperture [NA]) or a confocal microscope (Zeiss LSM 510; 40× objective with 1.3 NA and ×63 objective with 1.4 NA) by using multichannel scanning in frame mode. Three-dimensional projections were digitally constructed from confocal z-stacks. For staining of tissue sections, tissues were fixed in 4% paraformaldehyde overnight at 4°C and paraffin sections

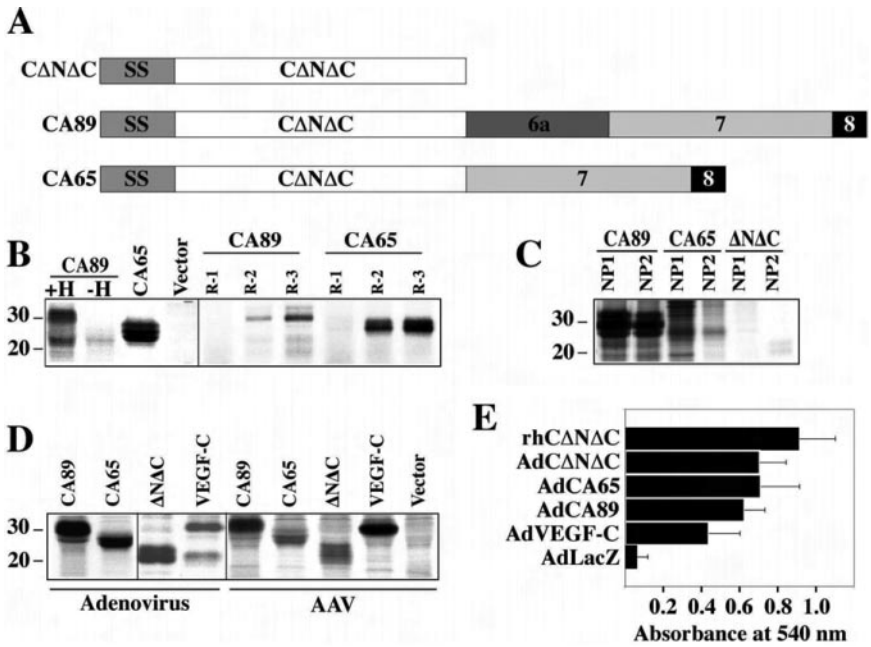

Figure 1. Biochemical analysis of the chimeric proteins. A, Schematic illustration of the VEGF-C/VEGF chimeric molecules comprised of the signal sequence (SS) of VEGF-C, VEGF-CΔNΔC, and VEGF exon 6 to 8 or exon 7 to 8 encoded sequences (CA89 and CA65, respectively). The depictions are not drawn to scale. B through D, Immunoprecipitation and polyacrylamide gel electrophoresis of metabolically labeled proteins from the conditioned medium of 293T cells transfected with pEBS7/CA89 (CA89) (B), pEBS7/CA65 (CA65) (B), pEBS7/VEGF-CΔNΔC (ΔNΔC) (D), or the pEBS7 vector with anti–VEGF-C serum (B), VEGFR-1–Ig (R-1) (B), VEGFR-2–Ig (R-2) (B), and VEGFR-3–Ig (R-3) (B) or NP-1–Ig (NP1) (C) and NP-2–Ig (NP2) (C). +H and −H indicate medium with or without heparin (20 U/mL), respectively. D, Recombinant AAV and adenoviral expression of CA89, CA65, VEGF-CΔNΔC, and VEGF-C were analyzed by immunoprecipitation of metabolically labeled proteins with anti–VEGF-C serum as above. E, The biological activity of the VEGF-C chimeric proteins assessed in a bioassay using Ba/F3 cells expressing a chimeric VEGFR-3/erythropoietin receptor (EpoR) that mediates Ba/F3/VEGFR-3 cell survival as detailed in Materials and Methods. Data represent the mean values from 3 triplicate assays.

(6 μm) were immunostained with anti–LYVE-1 as described previously[26] and were analyzed with a Leica DMLB microscope.

Statistical Analysis

The number of LYVE-1–positive lumenized vessels was counted from ×400 micrographs containing the highest vessel density (3 micrographs/section). Proliferating blood vascular endothelial cells were counted as phosphohistone H3 and PECAM-1 double positive cells from ×400 confocal micrographs of whole mount preparations of the AAV-tranduced mouse ears. Colocalization was assessed from single confocal z-sections, as previously.[32] The cross-sectional surface area of capillaries in rabbit semimembranosus muscle was counted from PECAM-1–stained sections. All quantifications were counted from three ×400 microscopic fields in each ear section and at least 3 sections from each ear or muscle, and the analytical technique and time point were analyzed. At least 3 animals were used for each time point and analytical technique, and each experiment was repeated at least 3 times. Statistical analysis was performed using Student's unpaired *t* test. A probability value of less than 0.05 was considered to be statistically significant.

Results

Fusion of the HBD of VEGF to the C Terminus of Mature VEGF-C

To investigate whether the HBD of VEGF, which is known to play an important role in regulating VEGF activity,[33,34] would alter the lymphangiogenic or angiogenic effects of VEGF-CΔNΔC, we constructed plasmids encoding chimeric proteins comprising the VEGF-C signal sequence, followed by the VEGF-CΔNΔC domain and VEGF exon 6 to 8 or exon 7 to 8 encoded sequences. These constructs, named CA89 and CA65, are schematically shown in Figure 1A. We also made constructs where both N- and C-terminal propeptides were swapped (ACA), but these were very poorly expressed, suggesting protein folding problems (data not shown).

CA65 and CA89 Proteins Bind to VEGFR-2, VEGFR-3, and Neuropilins

Production of the chimeric VEGF-C proteins into the media of transfected cells was tested by immunoprecipitation using polyclonal antibodies against VEGF-C. The results showed that CA65 is secreted into the medium, whereas CA89 was not released from the cells unless heparin was included in the culture medium (Figure 1B, compare lanes +H and −H), indicating that this form was strongly bound to cell surface heparan sulfate similar to what has been described for $VEGF_{189}$. Analysis of the receptor binding profiles of the chimeric molecules indicated that similar to VEGF-CΔNΔC, both CA89 and CA65 bound to VEGFR-2 and VEGFR-3, but not to VEGFR-1 (Figure 1B). In agreement with the binding of VEGF exon 7 containing sequences to neuropilin,[14,35] both CA89 and CA65 also bound strongly to NP-1 and more weakly to NP-2, whereas VEGF-CΔNΔC showed barely detectable binding to NP-2 but not to NP-1 (Figure 1C).

Biological Activity of CA65 and CA89 Expressed via Adenovirus Vector

To further characterize the biological functions of the chimeric proteins in vivo, the cDNAs encoding CA89 and CA65 were used to generate recombinant adenoviruses (AdCA89 and AdCA65). Recombinant AAVs (AAV-CA89 and AAV-CA65) were produced to study the effect of long-term expression of the chimeric proteins in skeletal muscle. Shown in Figure 1D is the analysis of polypeptides produced by the recombinant adenoviruses and AAVs. The biological activity

of the chimeric proteins was demonstrated in a bioassay using Ba/F3 cells expressing a chimeric VEGFR-3/erythropoietin receptor (Ba/F3-VEGFR-3/EpoR).[25] Conditioned medium from adenovirus-transduced cells containing CA89 or CA65 was shown to induce survival and proliferation of these cells (Figure 1E).

A Distinct Pattern of Lymphatic Vessels Is Induced by the CA65 and CA89 Chimeric Proteins

For comparison of their vascular effects, adenoviruses encoding CA89, CA65, VEGF-CΔNΔC, and VEGF-C were injected subcutaneously into the ears of nude mice. Two weeks after virus transduction, tissues were collected for whole-mount immunostaining of lymphatic vessels using antibodies against the lymphatic endothelial–specific hyaluronan receptor LYVE-1. Both AdCA89 and AdCA65 were shown to induce robust lymphangiogenesis (Figure 2A and 2B) in comparison with the AdLacZ control virus (Figure 2E). However, the lymphangiogenic responses to both CA65 and CA89 appeared different from those induced by full-length VEGF-C or mature VEGF-C (CΔNΔC) (Figure 2A through 2D). The CA65- and CA89-generated vessels were thicker and formed a more sparse network, whereas a robust lymphangiogenic response consisting of a dense network of very-fine lymphatic capillaries was induced with the full-length VEGF-C (Figure 2C), whereas VEGF-CΔNΔC induced a weaker widespread lymphangiogenic effect characterized by lymphatic sprouting and hyperplasia (Figure 2D). The average number of LYVE-1–positive vessels determined from 3 microscopic fields of the highest vessel density is shown in Figure 2F. Assuming that roughly similar levels of growth factor proteins were expressed in each case from the adenovirus vector, it appeared that the ability of VEGF-CΔNΔC, CA65, and CA89 to induce formation of lymphatic vessels was significantly weaker than that of full-length VEGF-C ($P<0.001$).

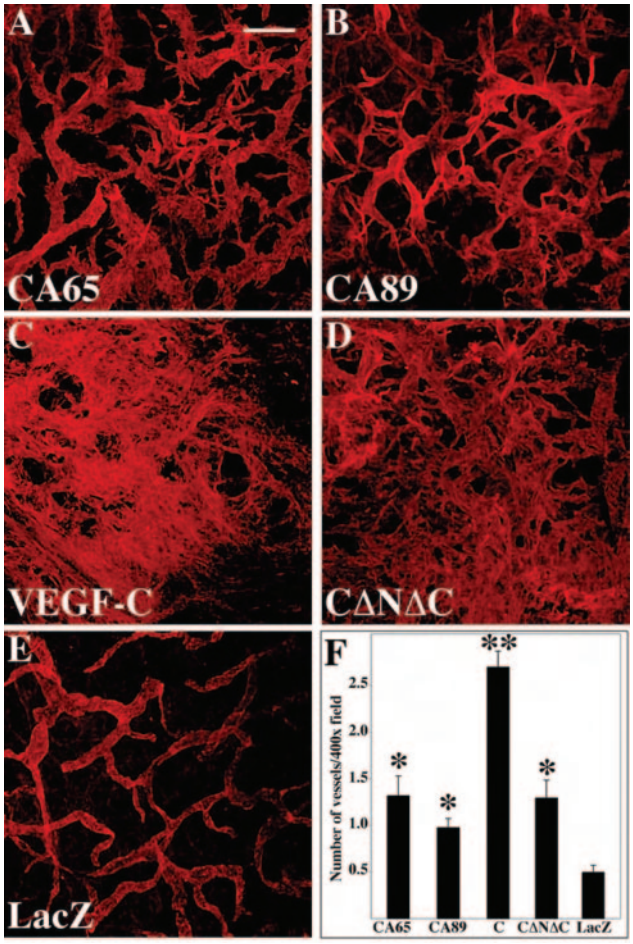

Figure 2. In vivo effects of the chimeric growth factors after adenoviral gene transduction. A through E, Whole-mount staining for LYVE-1 (red) of the mouse ears 2 weeks after transduction with AdCA65 (A), AdCA89 (B), AdVEGF-C (C), AdVEGF-CΔNΔC (D), or AdLacZ (E). F, Quantification of LYVE–positive lumenized vessels from sections of the ear skins transduced with adenoviruses. *$P<0.05$ compared with LacZ, **$P<0.05$ compared with all other groups. Scale bar=100 μm.

CA65 and CA89 Delivered via the AAV Vector Induce Lymphangiogenesis Along Muscle Fibers

Consistent with the fact that AAV mainly transduces muscle cells,[36] we detected expression of the growth factors only in the thin layer of skeletal muscle of the mouse ear by staining with antibodies that detect the VEGF homology domain of VEGF-C (VEGF-C/VHD) (Figure 3A through 3C). These antibodies did not detect skeletal muscle fibers transduced with AAV-VEGF-B_{167} used as a control (Figure 3D). At 6 weeks after transduction, the AAV-CA65–induced lymphatic capillaries were organized mostly in parallel along the muscle fibers (arrowheads, Figure 3A and 3E), whereas the AAV-VEGF-C and AAV-CΔNΔC–induced a very dense network of thin, rather unorganized vessels (Figure 3B, 3C, 3F, and 3G). Few lymphatic vessels forming along the muscle fibers were seen in the AAV-VEGF-C–transduced ears (arrowheads, Figure 3F). AAV-VEGF-B_{167} transduction did not stimulate lymphangiogenesis, and only a few quiescent lymphatic capillaries were observed in the muscle layer (Figure 1D and 1H). Even tighter association of developing lymphatic vessels with the muscle fibers was seen in samples treated with AAV-CA89 (Figure 3I), when compared with those induced in response to AAV-VEGF-C (Figure 3J).

The VEGF-C–induced lymphatic capillaries reorganized slowly along the muscle fibers, as seen when mice injected with the AAV vectors were analyzed at 6 weeks and at 2 years after transduction (Figure 3K and 3L). In striking contrast, the CA65-induced vessels were already well-oriented along the fibers at 6 weeks (Figure 3M). For comparison, shown are lymphatic capillaries in the overlying skin in Figure 3N. In histological sections, the AAV-CA65– and AAV-CA89–induced LYVE-1–positive vessels were observed in between the myofibers (Figure 3O), whereas only a few lymphatic vessels were found in corresponding sections from the control mice (Figure 3P). We found very few lymphatic vessels in the skeletal muscle layer of mouse ears of mice that received AAV-VEGF-B167, whereas abundant lumenized vessels were found in AAV-CA65– or AAV-CA89–transduced ears (Figure 3Q). Importantly, more vessels containing a lumen were found in the muscle layer of ears expressing the chimeric growth factors when compared with

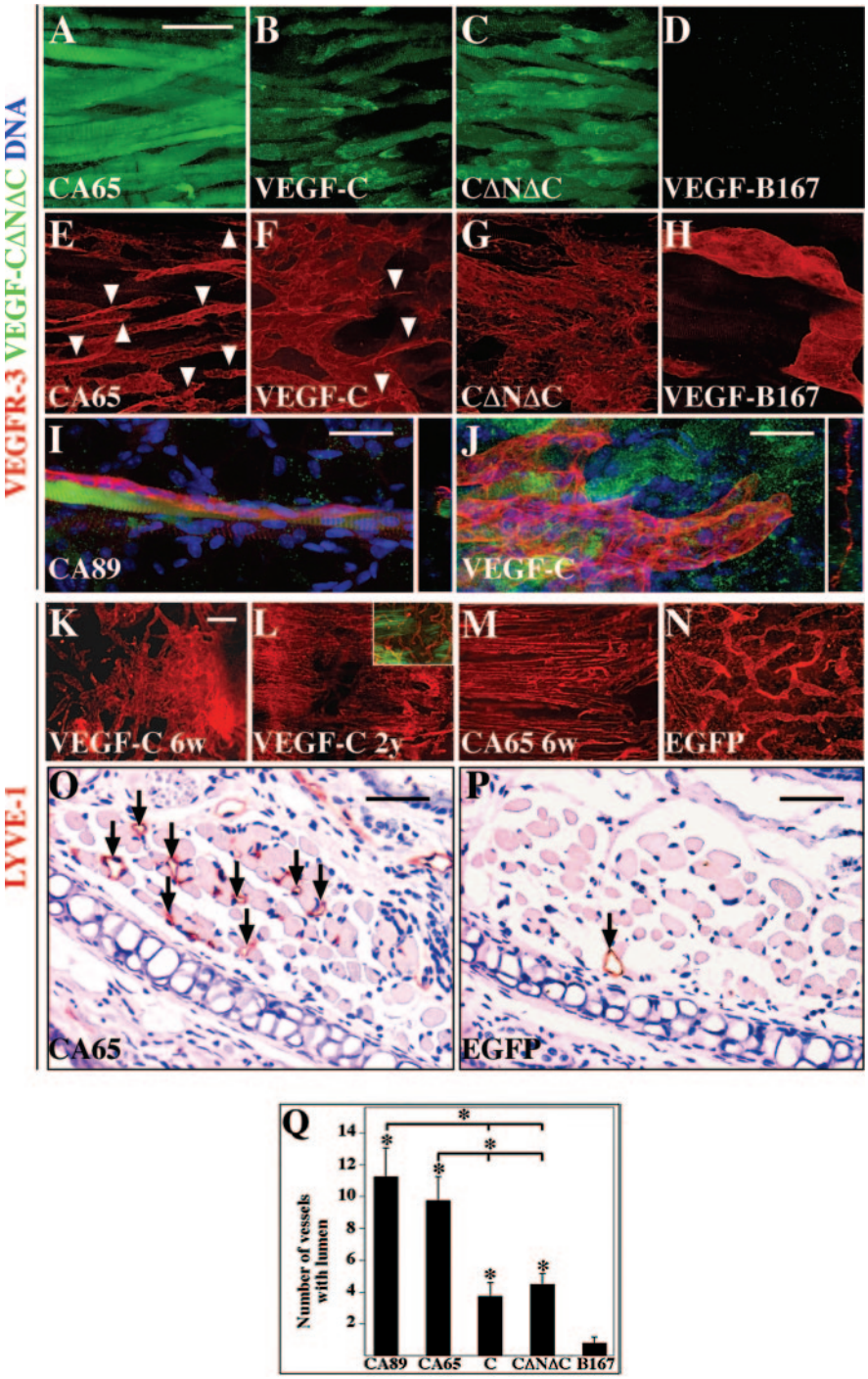

Figure 3. Induction of lymphatic vessel growth along muscle fibers by AAV-CA65 and AAV-CA89. A through H, Whole-mount immunostaining of VEGFR-3 (red) and VEGF-C ΔNΔC (green), included in all chimeric and wild-type VEGF-C constructs used in the study, in the ears of mice transduced 6 weeks before analysis with AAV-CA65 (A), AAV-VEGF-C (B), AAV-CΔNΔC (C), or AAV-VEGF-B167 (D). Arrowheads indicate lymphatic vessels forming along muscle fibers transduced with the vector. I and J, Very-high-resolution confocal analysis of ears transduced with AAV-CA89 (I) or AAV-VEGF-C (J). The smaller images at right in I and J are cross-sections of confocal z-stacks. VEGFR-3 staining is in red, VEGF-CΔNΔC in green, and DNA in blue. K through N, Lymphatic vessels visualized by whole-mount LYVE-1 immunostaining (red) in the ears 6 weeks (K and M) or 2 years (L and N) after transduction with AAV-VEGF-C. Normal LYVE-1–positive lymphatic vessels (red) and AAV-EGFP expression (green) 2 years after transduction are shown in the inset in L. O and P, LYVE-1 immunostaining (red) and hematoxylin counterstaining (blue) of paraffin sections from ears transduced with AAV-CA65 or AAV-EGFP. LYVE-1–positive lymphatic vessels with a lumen are indicated with arrows. Scale bars: 100 μm (A through H and K through P); 10 μm (I and J). Q, Quantification of the number of LYVE-1–positive vessels containing a lumen in mouse ears 6 weeks after transduction with AAV vectors. *$P<0.05$.

AAV-VEGF-C– or AAV-CΔNΔC–transduced ears (Figure 3Q).

CA65 and CA89 Induce Mild Changes in the Blood Vessels

A weak angiogenic response characterized by increased arterial tortuosity was observed in PECAM-1–stained skin injected with AdCA65, AdCA89, AdVEGF-C, or AdVEGF-CΔNΔC (arrowheads in Figure 4A through 4E; data not shown). We did not detect statistically significant changes in vessel area density in any of the growth factor–transduced ears when compared with control (data not shown), although all 4 factors also stimulated proliferation of blood vascular endothelial cells when expressed in the ear via AAV vectors, with CA89 displaying a trend toward the strongest response ($P=0.119$; Figure 4F). The CA65 and CA89 adenoviruses induced a barely detectable widening of muscle capillaries in rabbit hindlimb (Figure 4G through 4I). CA65 increased the cross-sectional capillary surface area 1.9-fold ($P=0.011$) and CA89 1.6-fold ($P=0.0072$) when compared with AdLacZ. We also observed a slight trend toward an increase in capillary permeability, as measured by the Miles permeability assay,[28] but the increase was not statistically significant compared with the control (data not shown).

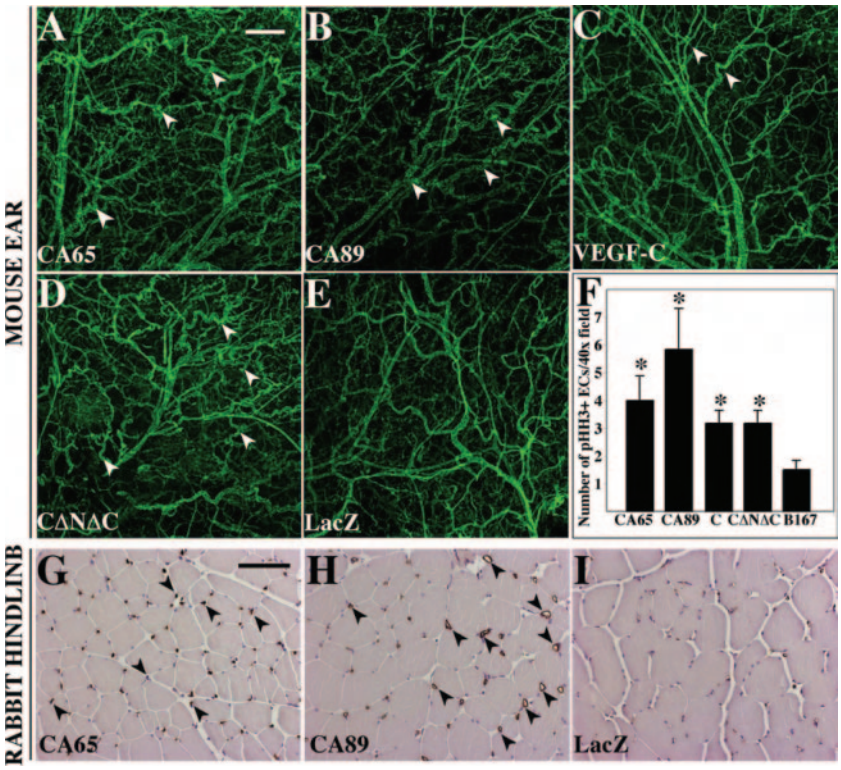

Figure 4. Blood vascular effects of the chimeric growth factors. A through E, Whole-mount analysis of blood vascular changes (PECAM-1, green) in the mouse ear 2 weeks after adenoviral gene transduction. Arrowheads indicate tortuous vessels. F, Quantitative analysis of proliferating blood vascular endothelial cells (phosphohistone H3/PECAM-1 double-positive cells) from ×400 confocal micrographs 6 weeks after AAV-mediated gene transfer. G through I, PECAM-1 immunostaining of the rabbit semimembranosus muscle 6 days after gene transfer. Arrowheads indicate enlarged capillaries. Scale bars=100 μm.

Discussion

Here, we have sought to determine the ability of a strong pericellular matrix-binding domain to influence the in vivo activity of VEGF-C. We demonstrated that the chimeric CA89 and CA65 proteins accommodating the growth factor domain of VEGF-C and the HBD of VEGF stimulated distinct patterning of the growth factor–induced lymphatic vessels.

The altered biological activity of CA89 and CA65 in comparison with VEGF-C may result from redistribution of the growth factors by binding of the HBD to heparin-rich pericellular matrix structures that typically are present in basal lamina and on the surface of certain cells. It is possible that by fusion with the HBD the 3D diffusion of VEGF-CΔNΔC is largely replaced by 2D mobility in the plane of the cell surface heparan sulfate matrix, which leads to more of the growth factor available for the high-affinity signal–transducing receptors. The importance of the exon 6 and 7 encoded sequences in $VEGF_{165}$ and $VEGF_{189}$ was emphasized when Carmeliet et al observed impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the corresponding mouse VEGF isoforms.[18] In fact, the lymphangiogenic response we obtained with VEGF-CΔNΔC is highly similar to the angiogenic response that has been observed with $VEGF_{121}$,[18,37] as both factors potently stimulate endothelial cell proliferation but fail to provide the cells with sufficient guidance cues, which leads to formation of endothelial sheets instead of functional vessels.

Processing of heparan sulfate–bound VEGF by plasmin matrix metalloproteinases regulates its bioavailability and vascular patterning.[37] Interestingly, VEGF signaling in endothelial cells is fully supported by heparan sulfate expressed in trans by adjacent perivascular smooth muscle cells.[38] Such ability of the HBD to regulate vessel patterning was also reflected in the lymphangiogenic activity of the corresponding chimeras CA65 and CA89. Importantly, we did not observe any changes in lymphatic vessels after gene transfer of $VEGF\text{-}B_{167}$, which contains similar HBD and neuropilin-binding domains as $VEGF_{189}$ and CA89,[15] indicating that heparin and neuropilin binding capacities alone are not sufficient to stimulate either lymphangiogenesis or angiogenesis unless the factor is able to activate VEGFR-2 and/or VEGFR-3.

Some of the distinct biological activities of CA65 and CA89 may also be attributable to the observed increased binding to NP-1 or NP-2, which regulate vessel patterning. It has been shown that NP-1 enhances $VEGF_{165}$ binding to VEGFR-2 by forming a ternary complex on endothelial cell surfaces,[39] whereas NP-2, a possible coreceptor for VEGF-C, is required for the normal development of lymphatic vessels.[40,41] NP-1–signaling activity has been shown to regulate tip cell guidance and the fusion of sprouts of adjacent vessels.[42] Furthermore, recent experiments have indicated that NP-1 is essential for VEGF-induced vascular remodeling.[43]

VEGF-C was shown to induce angiogenesis in mouse corneas,[22] and a dose-dependent angiogenic response was also observed with adenoviral expression in normal mouse skin,[44] as well as in wounds of diabetic mice.[45] Consistent with this, all tested adenoviral vectors induced a modest increase in endothelial cell proliferation. The CA89 growth factor chimera that binds very tightly to the pericellular matrix showed a trend toward highest angiogenic activity. Furthermore, we also investigated the vascular effects in a

rabbit nonischemic limb model, in which AdCA89 and AdCA65 were injected into the semimembranosus muscle, and animals were euthanized 6 days later. Consistent with the mouse data, a slight increase in capillary diameter was observed.

In summary, the heparin-binding chimeric VEGF-C forms induced a distinct pattern of lymphatic vessel growth longitudinally along the basement membranes and muscle fibers. This suggests that by using a heparin-binding growth factor, one can achieve a more defined localization of growth factor expression in a given tissue and, therefore, minimize the danger of obtaining aberrant side effects at other sites. More generally, we envision that such growth factor domain swap combinations should have a great potential for building vascular networks in tissue-engineering and other therapeutic applications.

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Disclosures

Kari Alitalo is a minority shareholder and board member of Lymphatix Ltd.

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