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Transgenic Induction of Vascular Endothelial Growth Factor-C Is Strongly Angiogenic in Mouse Embryos but Leads to Persistent Lymphatic Hyperplasia in Adult Tissues

Marja Lohela,* Hanna Heloterä,* Paula Haiko,*
Daniel J. Dumont,[†] and Kari Alitalo*

From the Molecular/Cancer Biology Laboratory,* Department of Pathology, Biomedicum Helsinki, Haartman Institute and Helsinki University Hospital, University of Helsinki, Helsinki, Finland; and the Division of Molecular and Cellular Biology Research,[†] Sunnybrook and Women's Research Institute, Toronto, Canada

Vascular endothelial growth factor-C (VEGF-C) is the quintessential lymphangiogenic growth factor that is required for the development of the lymphatic system and is capable of stimulating lymphangiogenesis in adults by activating its receptor, VEGFR-3. Although VEGF-C is a major candidate molecule for the development of prolymphangiogenic therapy for defective lymphatic vessels in lymphedema, the stability of lymph vessels generated by exogenous VEGF-C administration is not currently known. We studied VEGF-C-stimulated lymphangiogenesis in inducible transgenic mouse models in which growth factor expression can be spatially and temporally controlled without side effects, such as inflammation. VEGF-C induction in adult mouse skin for 1 to 2 weeks caused robust lymphatic hyperplasia that persisted for at least 6 months. VEGF-C induced lymphangiogenesis in numerous tissues and organs when expressed in the vascular endothelium in either neonates or adult mice. Very few or no effects were observed in either blood vessels or collecting lymph vessels. Additionally, VEGF-C stimulated lymphangiogenesis in embryos after the onset of lymphatic vessel development. Strikingly, a strong angiogenic effect was observed after VEGF-C induction in vascular endothelium at any point before embryonic day 16.5. Our results indicate that blood vessels can undergo VEGF-C-induced angiogenesis even after down-regulation of VEGFR-3 in embryos; however, transient VEGF-C expression in adults can induce long-lasting lymphatic

hyperplasia with no obvious side effects on the blood vasculature. (Am J Pathol 2008, 173:1891–1901; DOI: 10.2353/ajpath.2008.080378)

The formation of blood vasculature in embryos requires vascular endothelial growth factor (VEGF) or VEGF-A,^{1,2} whereas VEGF-C is indispensable for the development of lymph vessels.³ VEGF-C has been shown to induce lymph vessel sprouting and hyperplasia in various experimental settings, including transgenic overexpression in skin keratinocytes and islets of Langerhans in the pancreas, viral gene transfer in different tissues, and stably transfected tumor cells.^{4–9} In several tumor models, VEGF-C promoted metastasis,^{7,9–13} and also in human tumors VEGF-C expression correlates with lymph vessel involvement and metastasis.^{14,15} Importantly, VEGF-C has been successfully used in mouse models of lymphedema and wound healing to grow new, functional lymph vessels.^{3,16–19}

VEGF-C and its close homologue VEGF-D are the only known ligands for the tyrosine kinase receptor VEGF receptor (VEGFR)-3. Proteolytic processing of VEGF-C increases its affinity for VEGFR-3, and the mature form of VEGF-C also binds the major angiogenic receptor, VEGFR-2.²⁰ However, activation of VEGFR-3 by the re-

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Address reprint requests to Dr. Kari Alitalo, Molecular/Cancer Biology Laboratory, Biomedicum Helsinki, P.O.B. 63, (Haartmaninkatu 8), University of Helsinki, 00014 Finland. E-mail: kari.alitalo@helsinki.fi.

ceptor-specific engineered mutant VEGF-C156S or by mouse VEGF-D suffices for induction of lymphangiogenesis.^{17,21,22} In adults, VEGFR-3 expression is restricted to lymphatic endothelial cells, with the exception of some fenestrated or discontinuous blood vessel endothelia, small subsets of hematopoietic cells, and blood vessels in wounds and tumors.^{12,23–31}

In mouse embryos, VEGFR-3 is initially expressed in all endothelial cells, and is still detected in venous endothelia at embryonic day (E) 12.5, but primarily restricted to lymphatic endothelium by E14.5.^{23,32,33} Increasing evidence shows that VEGFR-3 is involved in angiogenesis as well as lymphangiogenesis, as first demonstrated by defective remodeling of the primary blood vascular plexus in *Vegfr3*-null embryos,³³ and by the more recent discovery of antiangiogenic effects of VEGFR-3 blocking molecules in the developing retina and in experimental tumors.^{27,28,34} Effects on blood vessels were also reported when VEGF-C was overexpressed via adenoviral gene transfer in skin, muscle, or mesenteric tissues,^{4,35,36} and angiogenic and immunomodulatory effects have been observed in some, but not all, tumor models.^{6,7,12,13}

To investigate the vascular effects of VEGF-C in a system in which its expression can be spatially and temporally controlled, without side effects such as inflammation that can result from viral delivery or the abnormal environment of a tumor, we overexpressed mouse VEGF-C via a tetracycline-inducible transgene in mice. Using the keratin-14 (K14) promoter for expression in the skin and Tie1 promoter for endothelial expression, we examined the vascular effects of VEGF-C during different developmental stages and in adults. Here we show that VEGF-C-induced lymphatic hyperplasia in adult skin persists for at least 6 months after a 2-week period of VEGF-C induction, with no obvious angiogenic side effects. We also show that endothelial production of VEGF-C causes angiogenesis as well as lymphangiogenesis in embryos, but stimulates predominantly lymphangiogenesis postnatally and in adult tissues.

Materials and Methods

Mouse Models

All animal experiments were approved by the Committee for Animal Experiments of the District of Southern Finland. The K14-rtTA (tet-On) mouse line was a kind gift from Dr. Jeffrey Kudlow (University of Alabama),³⁷ and the VE-Cadherin-tTA (tet-Off) driver mice were generously provided by Dr. Laura Benjamin (Harvard Medical School).³⁸ The Tie1-tTA (tet-Off) driver mouse line³⁹ and the K14-VEGF-C mouse line⁶ were generated as described.

The tet-VEGF-C responder construct was made by cloning the full-length mouse VEGF-C cDNA (GenBank NM_009506) downstream of the tet-responsive promoter in the pTET^{OS}-vector described previously.³⁹ Transgenic mouse lines were generated by injection of a 2.7-kb expression cassette from the vector into fertilized mouse oocytes of the strain FVB/NIH (schematic representations

of the constructs are depicted in Figure 1). Two independent transgenic founder lines with a lymphangiogenic phenotype inducible by the K14-rtTA driver were obtained. The one with the stronger phenotype in the initial screen was chosen for further analysis.

The driver and responder transgenic mouse lines were bred together to obtain double-transgenic K14-rtTA/TET-VEGF-C, Tie1-tTA/TET-VEGF-C, and VE-Cadherin/TET-VEGF-C offspring. In the case of K14-rtTA/TET-VEGF-C mice, VEGF-C expression was induced by administering 2 mg/ml of doxycycline (Sigma-Aldrich, St. Louis, MO) with 5% sucrose in the drinking water of double-transgenic mice for the desired length of time. For Tie1-tTA/TET-VEGF-C and VE-Cadherin-tTA/TET-VEGF-C mice, VEGF-C expression in double-transgenic embryos was repressed by 2 mg/ml of doxycycline or tetracycline (Sigma-Aldrich) with 5% sucrose in the drinking water of pregnant females starting within 3 days *post coitum*, until VEGF-C expression was induced by discontinuing the administration of doxycycline or tetracycline. The embryonic time points used for these experiments were estimated from the days when vaginal plugs were first observed. In all experiments, the doxycycline- or tetracycline-containing water was changed every 2 to 3 days. Single-transgenic or wild-type littermates were used as controls for double-transgenic mice, and in the experiments with K14-rtTA/TET-VEGF-C mice, double-transgenic mice with no doxycycline induction were also used. None of these control mice displayed any obvious phenotype.

Analysis of Lymph Vessel Function

Functionality of lymph vessels was analyzed by microlymphangiography after intradermal injection of ~3 μ l of fluorescein isothiocyanate (FITC)-conjugated dextran (molecular weight, 2000 kDa; Sigma-Aldrich), 10 mg/ml in phosphate-buffered saline (PBS), into the tip of the ear and monitoring its progress along the collecting lymph vessels by fluorescence microscopy.

Immunohistochemistry

For analysis of the vasculature, mice were sacrificed; organs or embryos were collected and processed for paraffin sectioning, cryosectioning, or whole-mount preparations. Some mice were perfused with 1% paraformaldehyde through the left cardiac ventricle for improved fixation. For paraffin sectioning, tissue samples were fixed in 4% paraformaldehyde overnight at +4°C, dehydrated, and embedded in paraffin. After deparaffination, rehydration, and antigen retrieval (0.25 mg/ml Trypsin, 9 mmol/L CaCl₂, 0.05 mol/L Tris, pH 7.8, 30 minutes at +37°C), 6- μ m sections were stained with rabbit polyclonal anti-mouse LYVE-1 40 followed by biotinylated anti-rabbit secondary antibodies (Vector Laboratories, Burlingame, CA) and the tyramide signal amplification system (NEL700; NEN Life Sciences, Boston, MA). Peroxidase activity was visualized with 3,3'-diaminobenzi-

dine tetrahydrochloride (Sigma-Aldrich) and sections were counterstained with hematoxylin.

For cryosectioning, samples were infiltrated with 25% sucrose and frozen in OCT compound. Sections were cut 7 to 10 μm in thickness, dried, and fixed with acetone for 10 minutes at -20°C . For whole-mount staining, dissected whole-mount preparations of ears, tracheas, diaphragms, mesenteria, embryonic skin, or whole embryos were fixed with 4% paraformaldehyde for 1 to 2 hours at room temperature or overnight at $+4^{\circ}\text{C}$ and washed several times with PBS. Cryosections and whole-mount preparations were blocked for at least 1 hour with 5% normal goat or donkey serum in 0.3% Triton X-100 (Fluka Biochemika, Steinheim, Switzerland). Samples were then incubated overnight with primary antibodies in the blocking buffer. Antibodies used for cryosections and whole-mount preparations were rabbit antiserum against LYVE-1 (dilution, 1:1500),⁴⁰ rabbit antiserum no. 6 to full-length human VEGF-C (dilution, 1:500),⁴¹ goat polyclonal anti-mouse VEGFR-3 antibody 1 $\mu\text{g}/\text{ml}$ (AF743; R&D Systems, Minneapolis, MN), goat polyclonal anti-mouse VEGFR-2 antibody (1 $\mu\text{g}/\text{ml}$, AF644; R&D Systems), hamster monoclonal anti-mouse CD31 (PECAM-1) antibody (1 $\mu\text{g}/\text{ml}$, clone 2H8, MAB-13982Z; Chemicon, Temecula, CA), and rat monoclonal anti-mouse CD31 (PECAM-1) antibody (1.25 $\mu\text{g}/\text{ml}$; BD Biosciences Pharmingen, San Diego, CA). Secondary antibodies used for immunofluorescence were Alexa488 and Alexa594 conjugates (Invitrogen Molecular Probes, Eugene, OR) or FITC conjugates (Jackson ImmunoResearch, West Grove, PA), all in 1:500 dilution. All fluorescently labeled samples were mounted with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (H-1200; Vector Laboratories). Fluorescently labeled samples were imaged with confocal microscopes (LSM 510 Meta and LSM 5 Duo; Carl Zeiss, Göttingen, Germany) in multichannel mode. Three-dimensional projections were digitally reconstructed from confocal z-stacks using the LSM software. Images in Figure 3B were acquired by a compound fluorescence microscope (Zeiss 2, Carl Zeiss).

Quantification of Endothelial Cells

Cryosections from the jugular region of E10.5 embryos induced since E7.5 were stained for PECAM-1 and 4,6-diamidino-2-phenylindole as described above. From three Tie1-tTA/TET-VEGF-C and four control embryos, nuclei of PECAM-1-positive cells from six to eight close but not consecutive sections were counted, and the average number of endothelial cells per section was calculated for each embryo. Unpaired Student's *t*-test was used to determine the statistical difference between the average number of endothelial cells in the embryos Tie1-tTA/TET-VEGF-C and control embryos.

β -Galactosidase Staining

Dissected embryos were fixed in 0.2% glutaraldehyde, 2 mmol/L MgCl_2 , 5 mmol/L EGTA in 100 mmol/L phosphate

buffer at room temperature for 15 to 30 minutes, washed in 100 mmol/L phosphate buffer containing 2 mmol/L MgCl_2 , 0.02% Nonidet P-40, and 0.01% deoxycholate, stained overnight at $+37^{\circ}\text{C}$ in 1 mg/ml X-gal, 5 mmol/L $\text{K}_3\text{Fe}(\text{CN})_6$, 5 mmol/L $\text{K}_4\text{Fe}(\text{CN})_6$, 2 mmol/L MgCl_2 , 0.02% Nonidet P-40, and 0.01% deoxycholate in 100 mmol/L phosphate buffer, and washed in 100 mmol/L phosphate buffer.

Immunoprecipitation and Immunoblot Analysis

For immunoprecipitation and immunoblot analysis, either whole E10.5 embryos or lungs from E16.5 embryos were lysed on ice with cold PBS containing 1% Triton X-100, 1 mmol/L Na_3VO_4 , 2 mmol/L phenylmethyl sulfonyl fluoride, 0.076 U/ml aprotinin, and 10 $\mu\text{g}/\text{ml}$ leupeptin. Equal amounts of protein in pooled lysates were used for immunoprecipitation and Western blotting. VEGFR-3 was immunoprecipitated using goat polyclonal anti-mouse VEGFR-3 antibody (1 μg , AF743; R&D Systems). The immunocomplexes were captured using protein-G Sepharose (GE Health Care Bio-Sciences, Uppsala, Sweden), separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and detected using mouse monoclonal anti-phosphotyrosine (67 ng/ml, 4G10; Upstate Biotechnology, Lake Placid, NY) and goat polyclonal anti-mouse VEGFR-3 0.1 $\mu\text{g}/\text{ml}$, (AF743; R&D Systems) antibodies, biotinylated anti-goat (0.64 $\mu\text{g}/\text{ml}$) or anti-mouse (0.3 $\mu\text{g}/\text{ml}$) secondary antibodies (DAKO, Glostrup, Denmark), and streptavidin-biotinylated horseradish peroxidase complex in 1:5000 dilution (GE Health Care, Little Chalfont, UK) followed by enhanced chemiluminescence detection with the SuperSignal West Femto maximum sensitivity substrate (Pierce, Rockford, IL). For reprobing, membranes were stripped for 15 minutes at room temperature using Re-Blot Plus strong solution (Chemicon). For β -actin detection, 40 μg of protein from each sample was separated in 7.5% SDS-PAGE, Western blotted, and detected with rabbit polyclonal β -actin antibody in 1:1000 dilution (4967; Cell Signaling Technology, Danvers, MA) and horseradish peroxidase-conjugated swine anti-rabbit secondary antibody (0.13 $\mu\text{g}/\text{ml}$, DAKO).

Results

VEGF-C-Induced Lymphatic Hyperplasia Persists after Growth Factor Removal

We induced VEGF-C in the skin under the control of the keratin-14 promoter, using the Tet-On system in which K14-rtTA driver mice and TET-VEGF-C responder mice were crossed to obtain double-transgenic K14-rtTA/TET-VEGF-C offspring and transgene expression was induced by doxycycline (Figure 1A). The K14-rtTA driver has previously been shown to induce rapid and reversible transgene expression in the skin on doxycycline administration.³⁷ We confirmed by reverse transcriptase-polymerase chain reaction that VEGF-C expression

Figure 1. Schematic representation of the transgenic Tet-on and Tet-off VEGF-C expression models. For inducible VEGF-C expression, mice carrying the tissue-specific driver and VEGF-C responder transgenes are mated to obtain double-transgenic offspring. **A:** The K14-rtTA driver construct produces a transcriptional activator fusion protein that binds to the Tet operator sequence in the responder construct only in the presence of tetracycline antibiotics. In double-transgenic K14-rtTA/TET-VEGF-C mice, the VEGF-C responder construct is silent, and expression can be activated by administration of tetracycline or doxycycline. **B:** Tie1-tTA and VEG-tTA driver constructs produce a transcriptional activator fusion protein that binds to the Tet operator sequence in the responder construct only in the absence of tetracycline antibiotics. In double-transgenic Tie1-tTA/TET-VEGF-C or VEG-tTA/TET-VEGF-C mice, the VEGF-C responder construct is constitutively active. VEGF-C expression can be suppressed by administration of tetracycline or doxycycline, and induced again by withdrawal of the antibiotic.

reached peak levels within 24 hours of doxycycline administration, and was maintained until doxycycline was removed, returning to almost basal levels 1 week after doxycycline withdrawal (data not shown). As expected, VEGF-C induced robust lymph vessel sprouting and hyperplasia in the skin (Figure 2, B and H). The lymphatic plexus was disorganized, variable in morphology, and often appeared to fill all available space around the hair follicles, resembling the phenotype of constitutive K14-VEGF-C transgenic mice (Figure 2, B and H; Supplementary Figure S1 at <http://ajp.amjpathol.org>). In single transgenic TET-VEGF-C mice (not shown) or double-transgenic K14-rtTA/TET-VEGF-C mice that did not receive doxycycline, the skin vasculature appeared normal (Figure 2, A, G, and J). To investigate the stability of new lymph vessels generated by VEGF-C induction, doxycycline was administered for 2 weeks, after which some of the mice were sacrificed, and the remaining littermates were kept without doxycycline. Six months after the cessation of VEGF-C induction, lymphatic hyperplasia persisted, although the lymph vessel endothelium appeared

Figure 2. Hyperplastic lymph vasculature induced by VEGF-C is maintained after removal of stimulation. Double-transgenic K14-rtTA/TET-VEGF-C mice were given doxycycline for 2 weeks, after which some mice were sacrificed and analyzed (**B, E, H, K**), and some were maintained without doxycycline for 6 months (**C, F, I, L**) before analysis of ear skin by fluorescent dextran lymphangiography and whole-mount immunostaining of lymphatic (LYVE-1, red) and blood vessels (PECAM-1, green). Compared to double-transgenic control littermates that did not receive doxycycline (**A** and **G**), 2-week stimulation resulted in robust lymphangiogenesis, lymphatic sprouting, and hyperplasia (**B** and **H**, inset in **B** shows lymphatic sprouts at higher magnification). **Arrows** point to the locations of some hair follicles, around which the hyperplastic plexus has formed. **C** and **I:** Six months after transgene activation was stopped, the lymphatic vasculature was still very hyperplastic. The collecting lymph vessels (**double arrows**) appeared to function normally, as demonstrated by fluorescent dextran lymphangiography (**K** and **L** compared to **J**). In the constitutive transgenic K14-VEGF-C mice (**M**), the dextran spread into the hyperplastic lymph capillary plexus (**arrowhead**) before it was taken up by the collecting vessels (**double arrow**). No blood vessel hyperplasia was observed in any of these mice (**D–F**). Original magnifications: $\times 100$ (**A–F**); $\times 400$ (**G–I**); $\times 10$ (**J–M**); $\times 200$ (**B**, inset).

smoother and very few sprouts were visible (Figure 2, C and I). Thus, the vessels appeared more quiescent than acutely after the induction. In this setting, VEGF-C did not have obvious effects on blood vessels at any point (Figure 2, D–F). FITC-dextran microlymphangiography did not show any differences in the functionality of collecting lymph vessels after the 2-week induction or after the 6-month follow-up (Figure 2, K and L; arrows). In contrast, in constitutive transgenic K14-VEGF-C mice in which transgene expression begins after midgestation, FITC-dextran spread into the hyperplastic superficial lymph capillary network in the ear before it was taken up by the collecting lymph vessels

(Figure 2M), indicating that lateral backflow was taking place.

Endothelial VEGF-C Expression Induces Lymphangiogenesis but Not Angiogenesis

To investigate how different vessel types in different tissues respond to VEGF-C stimulation, we overexpressed VEGF-C under the endothelial Tie1 promoter postnatally and in adult mice using the TET-Off system (Figure 1B). Tie1-tTA driver mice were crossed with TET-VEGF-C responder mice, and tetracycline or doxycycline was administered in drinking water to repress expression from the transgene until the desired time point. To further validate our model system, some analyses were also repeated using another endothelial-specific driver mouse line, the VE-Cadherin-tTA (VEC-tTA).

When tetracycline was removed at E17.5-P0, Tie1-tTA/TET-VEGF-C double-transgenic pups died or had to be sacrificed at ~3 to 5 weeks of age because of the presence of clear or cloudy liquid in the thoracic cavity. At this point, we could also detect VEGF-C protein by immunofluorescence, localizing mainly in lymph vessels (Supplementary Figure S2 at <http://ajp.amjpathol.org>). It should be noted that this staining might detect VEGF-C protein bound to VEGFR-3 on the surface of lymphatic endothelial cells, and thus may not faithfully reflect the cells producing VEGF-C from the transgene. Robust lymphangiogenesis was observed in almost every tissue examined (Figure 3, A–F; and Supplementary Figure S3 at <http://ajp.amjpathol.org>, LYVE-1 staining), whereas no obvious angiogenic effects were observed even in the fenestrated capillaries in kidney glomeruli and pancreatic islets that are known to express VEGFR-3 (Figure 3, A'–D'; and Supplementary Figure S4 at <http://ajp.amjpathol.org>, PECAM-1 staining).^{42,43} Similar results were obtained when VEC-tTA/TET-VEGF-C mice were induced at P0 and sacrificed at P15 (Supplementary Figure S5 at <http://ajp.amjpathol.org>, bottom). Despite the hyperplastic lymphatic capillary network in the skin, collecting lymph vessels of Tie1-tTA/TET-VEGF-C mice induced at P0 functioned normally as assessed by FITC-dextran microlymphangiography in the ear, with no evidence of backflow (Figure 3, G and H). When VEGF-C expression was suppressed until adulthood, and the Tie1 promoter-driven VEGF-C was then induced for 2 months, lymphangiogenesis was seen in most organs, with the exception of some such as the skin, where lymph vessels showed no detectable response (Figure 3, I–N). This was presumably because of the previously reported down-regulation of the Tie1 promoter in some adult tissues.⁴³ Also in adult mice, the blood vasculature appeared unaffected (Figure 3, I'–L').

VEGF-C Induces Both Angiogenesis and Lymphangiogenesis in Embryos

We were surprised to note that VEGF-C produced by endothelial cells did not induce obvious angiogenesis in

several postnatal or adult tissues where a strong lymphangiogenic response was seen. We next examined VEGF-C effects in early embryonic blood vessels that still express VEGFR-3.³¹ Pregnant females were given tetracycline to repress the transgene until E6.5 to E7.5, at which point tetracycline was removed, and Tie1-tTA/TET-VEGF-C embryos were analyzed at approximately E10.5. At this stage, peripheral lymph vessels have not yet developed, and most blood vessels still express VEGFR-3 (Figure 4A). Embryos expressing the VEGF-C transgene in endothelial cells showed a varying degree of peripheral blood vessel hyperplasia with disorganized and fused vascular plexi and significantly increased numbers of endothelial cells (Figure 4, A and B); some double-transgenic embryos were growth retarded or already dead at this point. During later embryonic development, VEGF-C overexpression affected both blood and lymphatic vessels. When pregnant females were taken off tetracycline at E12.5 to E15.5, double-transgenic embryos displayed extensive hemorrhage (Figure 5A) and were often found dead within 3 days, never surviving beyond P0. The lymphatic vasculature in the skin was hyperplastic and in many cases integrity of the endothelium had been lost, with individual lymphatic endothelial cells no longer tightly attached to each other to form tubes (Figure 5A). Also the blood vessel network appeared to have lost endothelial integrity, and hierarchical organization of the vascular tree was greatly reduced (Figure 5A). Immunostaining showed VEGFR-3 and VEGFR-2 within the lymphatic endothelial cells in the VEGF-C induced embryos before the loss of endothelial integrity (Figure 5B), apparently because of ligand-induced internalization. Expression of the VEGFR-3 knock-in β -galactosidase reporter had already been down-regulated in blood vessels at this time point (Supplementary Figure S6 at <http://ajp.amjpathol.org>). The embryonic phenotypes could also be replicated using the VEC-tTA driver mouse line (Supplementary Figure S5 at <http://ajp.amjpathol.org>, top and middle). When induction was started at approximately E16.5 or later, double-transgenic pups were born and no obvious angiogenic effects were observed although lymphangiogenesis was seen in a wide range of tissues analyzed after 2 to 4 weeks (Figure 3; Supplementary Figures S3, S4, and S5 at <http://ajp.amjpathol.org>; and data not shown).

VEGF-C Induces Tyrosine Phosphorylation as well as Increased VEGFR-3 Synthesis and Degradation in Embryos

To examine the effects of VEGF-C expressed by endothelial cells at the molecular level, we used immunoprecipitation and Western blotting to detect tyrosine phosphorylation of VEGFR-3 in the Tie1-tTA/TET-VEGF-C mouse embryos. Lysates of E10.5 embryos and of lungs from E16.5 embryos showed increased tyrosine phosphorylation of VEGFR-3 after a 2-day induction (Figure 6, top). Increased phosphorylation in response to VEGF-C induction could be detected in both the 195-kDa form of the receptor and for its proteolytically cleaved mem-

Figure 3. VEGF-C expressed in the endothelium induces lymphangiogenesis but no angiogenesis. Whole-mount staining of lymph (LYVE-1, red) and blood vessels (PECAM-1, green) in ear skin, trachea, and diaphragm of double-transgenic Tie1-tTA/TET-VEGF-C and littermate control pups (**A–F**, **A'–D'**) and adult mice (**I–N**, **I'–L'**) after VEGF-C induction; fluorescent dextran lymphangiography in the ear of Tie1-tTA/TET-VEGF-C and littermate control pups after VEGF-C induction (**G**, **H**). **A–H** and **A'–D'**: Pregnant females were maintained on doxycycline until pups were born, at which point doxycycline was removed. VEGF-C expression was induced by maintaining double-transgenic Tie1-tTA/TET-VEGF-C pups without doxycycline, and Tie1-tTA/TET-VEGF-C pups and littermate controls were sacrificed when ~4 weeks old. Function of the collecting lymph vessels was analyzed by FITC-dextran lymphangiography. **I–N** and **I'–L'**: Pregnant females were maintained on doxycycline until pups were born, and pups were maintained on doxycycline until they reached adulthood. VEGF-C expression was induced in adult double-transgenic Tie1-tTA/TET-VEGF-C mice by removing doxycycline, and mice were then maintained without doxycycline for 2 months and analyzed together with littermate controls. Original magnifications: $\times 10$ (**G**, **H**); $\times 100$ (**A–F**, **I–N**, **A'–D'**, **I'–L'**).

Figure 4. Endothelial VEGF-C induces embryonic angiogenesis. Whole-mount staining of blood vessels (**A**) and quantification of endothelial cells in cryosections (**B**) from E10.5 Tie1-tTA/TET-VEGF-C and littermate control embryos after VEGF-C induction. **A:** Pregnant females were maintained on tetracycline until approximately E6.5, at which point tetracycline was removed to induce VEGF-C expression in double-transgenic embryos. Tie1-tTA/TET-VEGF-C and control embryos were analyzed at approximately E10.5 by whole-mount staining for PECAM-1 (green) and VEGFR-3 (red). **Arrows** point to large vessels in control embryos in which VEGFR-3 expression has been down-regulated. **B:** A pregnant female was maintained on tetracycline until approximately E7.5, at which point tetracycline was removed to induce VEGF-C expression in double-transgenic embryos, and embryos were collected at approximately E10.5. Cryosections of Tie1-tTA/TET-VEGF-C and control embryos were stained for the blood vascular marker PECAM-1 and 4,6-diamidino-2-phenylindole for nuclei. PECAM-1-positive endothelial cells were counted from equivalent vessel cross-sections from the jugular area in Tie1-tTA/TET-VEGF-C and control embryos. **Arrowheads** point to regions with vessel hyperplasia. Error bars represent standard deviations between the average endothelial cell numbers in individual embryos; ****P* < 0.001. Original magnifications: $\times 100$ (**A**, **top**); $\times 400$ (**B**, **bottom**).

brane-bound 125-kDa form. In addition, we observed increased protein levels of the major intracellular, poorly phosphorylated precursor form (175 kDa) of VEGFR-3, and decreased levels of the 125-kDa processed form in VEGF-C-overexpressing embryos (Figure 6, middle panel), suggesting increased rates of VEGFR-3 synthesis, internalization, and degradation on VEGF-C stimulation (see also VEGFR stainings in Figure 5). Direct Western blotting of β -actin confirmed equal amounts of protein in the lysates used for the above immunoprecipitations (Figure 6, bottom).

Discussion

In this study we have addressed the activities of VEGF-C in a model system in which the specific effects of growth factor overexpression in the chosen tissue can be analyzed without side effects from gene delivery vectors, and in which the timing of expression can be controlled. We have shown that VEGF-C has profound effects on blood vessels only during embryonic development, and most importantly, that VEGF-C can induce the growth of new lymph vessels that become independent of the growth factor and remain stable when stimulation is removed. Encouragingly, in this setting we did not observe the horizontal lymph backflow phenomenon in the superficial dermal lymphatic plexus that was observed in the constitutive K14-VEGF-C model and in adult skin where VEGF-C was expressed via an adenoviral vector.⁴⁴ These results should be important for the development of effective therapy for various forms of lymphatic vessel damage and lymphedema.

Adenoviral delivery of VEGF-C into adult mouse skin has been shown to cause blood vessel dilation, tortuosity, and leakage.³⁵ However, the inflammatory response generated by the adenoviral capsid proteins and the very high expression levels obtained with these vectors are likely to affect the angiogenic response. The present study shows that when transgenic VEGF-C expression is induced in adult skin, no obvious angiogenic effects can be observed in the absence of inflammatory stimuli. This is a very promising finding for the possible use of VEGF-C therapy in treatment of lymphedema. Even when VEGF-C was produced directly from the endothelium, it did not have detectable blood vascular effects in neonates or adults, while retaining strong lymphangiogenic activity.

VEGF-C, VEGF-D, and the engineered specific VEGFR-3 ligand VEGF-C156S have been shown to induce lymphangiogenesis and lymphatic hyperplasia without detectable effects on blood vessels when overexpressed under the keratin 14 promoter.^{6,17,21} In this model, the growth factors are expressed at high levels during embryonic development from approximately E13 onwards. The lymph vessels in K14-VEGF-C mice are thus formed under a continuous stimulation by an excess of VEGF-C during the sprouting of the superficial lymphatic capillary plexus, differentiation of the collecting vessels, and valve morphogenesis, and they display dysfunctional changes affecting fluid distribution in the superficial plexus. In contrast, collecting lymph vessels appeared to be minimally affected by VEGF-C overexpression by both keratinocytes and by the endothelium itself in our inducible transgenic models, and remained functional although lymphatic capillaries were actively

Figure 5. Endothelial VEGF-C affects both lymph and blood vessels in late embryogenesis. Representative photographs and whole-mount staining of vascular markers from skins of E16.5 Tie1-tTA/TET-VEGF-C and control embryos after VEGF-C induction. **A:** Pregnant females were maintained on tetracycline until approximately E12.5, at which point tetracycline was removed to induce VEGF-C expression in double-transgenic embryos. Photos were taken and skins from Tie1-tTA/TET-VEGF-C and control embryos were analyzed at approximately E16.5 by whole-mount stainings of lymphatic (LYVE-1, red) and blood vessels (PECAM-1, green). **B:** Pregnant females were taken off tetracycline at E14.5 to induce VEGF-C expression in double-transgenic embryos, and skins from Tie1-tTA/TET-VEGF-C and control embryos were analyzed at approximately E16.5 by whole-mount stainings of VEGFR-3 (red) and PECAM-1 (green) or LYVE-1 (red) and VEGFR-2 (green). Original magnifications: $\times 100$ (**A**); $\times 400$ (**B**).

sprouting and hyperplastic. Smooth muscle cell contact is known to decrease VEGFR-3 expression by endothelial cells, and accordingly, collecting lymph vessels express lower levels of VEGFR-3 than lymphatic capillaries *in vivo*.^{17,45} Therefore, after collecting vessels mature and acquire smooth muscle coating, they are likely to be less responsive to VEGF-C stimulation. Furthermore, experiments with transgenic or viral overexpression of a soluble VEGFR-3 ligand trap protein, or with anti-VEGFR-3 antibodies, have demonstrated that some large collecting lymph vessels can be disrupted by blockage of VEGFR-3 signaling during late embryonic development, but not postnatally^{46,47}; another indication that the mature collecting vessels may no longer require VEGFR-3 signaling.

The inducible transgenic models used in this study gave us a unique opportunity to investigate the dependency of VEGF-C-induced lymphatic vasculature on the continuous presence of the growth factor. Interestingly, newly generated lymphatic capillaries acquired a quiescent, relatively stable phenotype when VEGF-C stimulation was removed. Six months after a 2-week VEGF-C pulse in the skin, the longest time point analyzed in our experiments, the lymphatic vasculature was still clearly hyperplastic, but there were very few visible sprouts or filopodia, and the endothelium was smooth and continuous in appearance. These findings are in agreement with previous observations in a model of airway inflammation, in which infection-induced lymphatic hyperplasia persisted after resolution of inflammation and associated blood vessel remodeling by antibiotic treatment, and

smoother lymphatic endothelium with dramatically reduced sprouting was also observed after removal of the stimulus.⁴¹ After the first 2 weeks of postnatal mouse development, not only collecting vessels but also lymphatic capillaries in many tissues apparently become independent of VEGFR-3 signaling, because inhibiting concentrations of soluble VEGFR-3 ligand trap in the circulation no longer caused lymph vessel regression, and lymph vessels that had previously regressed were able to grow back.^{46,47} A similar maturation event or switch, leading to lymph vessel independence from VEGF-C/VEGFR-3 survival signals, may take place in the newly formed hyperplastic capillary plexi in our inducible transgenic model and in the setting of airway inflammation.⁴¹

VEGF-C therapy has previously been successfully used for lymphangiogenesis in various preclinical models of lymphedema,^{16,18,44,48,49} and our results now suggest that the therapeutic effect achieved can persist after cessation of growth factor expression. Together with a recent study of Tammela and co-workers, in which the initial capillary network generated by adenoviral VEGF-C administration could also mature into collecting lymph vessels in a model of lymph node dissection,⁴⁴ these data further emphasize the potential of VEGF-C therapy for a condition in which the treatment options are currently limited and often ineffective.

In this study we show that VEGF-C overexpressed in the endothelium activates VEGFR-3 tyrosine phosphorylation, increases receptor synthesis, internalization and

Figure 6. Endothelial VEGF-C overexpression induces tyrosine phosphorylation, synthesis, and degradation of VEGFR-3 in embryos. Analysis of VEGFR-3 tyrosine phosphorylation in Tie1-tTA/TET-VEGF-C and control embryos after VEGF-C induction. Pregnant females were maintained on tetracycline until it was removed to induce VEGF-C expression in double-transgenic embryos at approximately E8.5 or E14.5. Tie1-tTA/TET-VEGF-C and control embryos were harvested after 2 days of induction. VEGFR-3 was immunoprecipitated from pooled lysates of whole embryos at E10.5 and of the lungs of E16.5 embryos. The immunocomplexes were separated on SDS-PAGE, Western blotted, and probed with anti-phosphotyrosine antibody (**top**). The blots were then stripped and reprobed with anti-VEGFR-3 antibodies (**middle**). Aliquots from the same lysates were separated on SDS-PAGE, Western blotted, and probed for β -actin to confirm equal amounts of protein in the lysates (**bottom**). **Arrows** in the **top** and **middle** panels point to the 195-kDa, 175-kDa (**open arrow**), and 125-kDa forms of VEGFR-3. Note the decreased amount but increased relative phosphorylation of the 125-kDa VEGFR-3 receptor band. IP, immunoprecipitation; WB, Western blot; TL, total lysate.

turnover, and leads to lymphangiogenesis in late embryos. However, VEGF-C can also induce a strong angiogenic response in midgestation embryos, in which VEGFR-3 is still expressed widely in the developing blood vasculature. The phenotype of VEGFR-3-null embryos demonstrates that VEGFR-3 has a crucial role in early embryonic angiogenesis, and very recently, the important angiogenic role of VEGFR-3 in tumors and in the developing blood vasculature has started to emerge.^{26,34}

Unexpectedly, profound blood vascular effects were still seen when VEGF-C expression was induced during late embryogenesis. The fact that we can induce angiogenesis when VEGFR-3 expression levels have already been down-regulated in the blood vessels is a paradoxical finding. At this point, we can only speculate on the reasons for the surprising persistence of the sensitivity of blood vessels to VEGF-C when VEGFR-3 levels have been down-regulated, and for the abrupt decrease of this sensitivity before birth. This finding may reflect insufficient sensitivity of our methods to detect low VEGFR-3 levels that are still functionally significant. However, mature VEGF-C can also bind to and activate

VEGFR-2, and we did observe a staining pattern that suggests increased internalization of VEGFR-2 in endothelial cells in embryos overexpressing VEGF-C in the endothelium, indicating that the receptor has been activated by a ligand. It is possible that less proteolytic processing of VEGF-C occurs toward the end of embryogenesis, decreasing VEGF-C binding to VEGFR-2 and thus also its angiogenic activity. Additional possibilities for loss of the blood vessel responses include changes in the expression or activity of other modulators of angiogenesis or lymphangiogenesis, such as the neuropilin co-receptors for VEGFs.⁵⁰

Blood vessel or pericellular matrix maturation might also be the reason behind the decreased sensitivity of blood vessel endothelium to VEGF-C. At E16.5, the large vessels have already acquired smooth muscle cell coverage. However, it has been shown that the plasticity of blood vasculature is not only dependent on pericyte coverage, and blood vessel dependence on VEGF first decreases ~4 weeks postnatally.⁵¹ The levels of bioavailable VEGF might also affect VEGF-C-induced angiogenesis via up-regulation of VEGFR-3.³⁴ In addition, recent evidence indicates that VEGF-C stimulates blood vascular angiogenesis in mesenteric tissues best in locations further away from the lymphatic vessels.⁴ This interesting finding raises the possibility that changes in lymph vessel density, and perhaps other factors such as maturation status of the lymphatic plexus, might affect tissue responses to VEGF-C, a possibility that deserves to be analyzed in more detail in various tissues. Careful research into the expression and activation of VEGF receptors and co-receptors, and characterization of the maturation process of vasculature at different time points in late embryonic development, is needed to elucidate the mechanisms behind this switch in the sensitivity of the blood vessels to VEGF-C.

After some years of intensive study, the lymphangiogenic potential of VEGF-C is well known. With our new findings, we unravel an interesting switch in the angiogenic property of VEGF-C, and further define its efficiency and safety when used in a wide variety of adult tissues. The interesting and surprising effects that we have found during development should open up possibilities to develop new concepts on VEGF/VEGFR signaling *in vivo* in the complexity of vascular development.

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