

Vascular Endothelial Growth Factor C Promotes Tumor Lymphangiogenesis and Intralymphatic Tumor Growth¹

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Abstract

Many solid tumors produce vascular endothelial growth factor C (VEGF-C), and its receptor, VEGFR-3, is expressed in tumor blood vessels. To study the role of VEGF-C in tumorigenesis, we implanted MCF-7 human breast carcinoma cells overexpressing recombinant VEGF-C orthotopically into severe combined immunodeficient mice. VEGF-C increased tumor growth, but unlike VEGF, it had little effect on tumor angiogenesis. Instead, VEGF-C strongly promoted the growth of tumor-associated lymphatic vessels, which in the tumor periphery were commonly infiltrated with the tumor cells. These effects of VEGF-C were inhibited by a soluble VEGFR-3 fusion protein. Our data suggest that VEGF-C facilitates tumor metastasis via the lymphatic vessels and that tumor spread can be inhibited by blocking the interaction between VEGF-C and its receptor.

Introduction

VEGF-C⁴ is a ligand for the lymphatic endothelial receptor VEGFR-3, but it binds also to VEGFR-2, which is the major mitogenic signal transducer for VEGF in blood vascular endothelial cells (1–3). VEGF-C stimulates almost exclusively lymphangiogenesis when applied to differentiated chick chorioallantoic membrane (4) or when overexpressed in the skin of transgenic mice (5). However, more recent studies report that VEGF-C also stimulates angiogenesis in mouse cornea (6), in developing chorioallantoic membrane of chick embryos (6), and in ischemic hind limbs of rabbits (7). Many tumors express VEGF-C, and the expression level has been suggested to correlate with tumor angiogenesis and metastasis via the lymphatic system (8–10). VEGFR-3 is normally expressed predominantly in the lymphatic vessels in adults (11–13), but this receptor is also induced in the angiogenic blood vascular endothelium of many tumors (9, 14, 15). To study the possible effects of VEGF-C on tumor growth, angiogenesis, and lymphangiogenesis, we overexpressed VEGF-C in human MCF-7 breast carcinoma cells, which otherwise produce minimal levels of this growth factor (16). The VEGF-C-overexpressing or

vector-transfected cells were then implanted orthotopically and grown as tumors in the mammary fat pads of SCID mice.

Materials and Methods

Plasmid Expression Vectors. The cDNAs coding for the human VEGF-C or VEGF₁₆₅ were introduced into the pEBS7 plasmid (17). The same vector was used for the expression of the soluble receptor chimeras VEGFR-3-Ig, containing the first three immunoglobulin homology domains of VEGFR-3 fused to the Fc-domain of human immunoglobulin γ chain and VEGFR-1-Ig, containing the first five immunoglobulin homology domains of VEGFR-1 in a similar construct (18).

Production and Analysis of Transfected Cells. The MCF-7S1 subclone of the human MCF-7 breast carcinoma cell line was transfected with plasmid DNA by electroporation, and stable cell pools were selected and cultured as described previously (19). The cells were metabolically labeled in methionine and cysteine free MEM (Life Technologies, Inc.) supplemented with 100 μ Ci/ml [³⁵S]methionine and [³⁵S]cysteine (Redivue Pro-Mix; Amersham Pharmacia Biotech). The labeled growth factors were immunoprecipitated from the conditioned medium using antibodies against VEGF-C (1) or VEGF (MAB293; R & D Systems). The immunocomplexes and the VEGFR-Ig fusion proteins were precipitated using protein A-Sepharose (Amersham Pharmacia Biotech), washed twice in 0.5% BSA, 0.02% Tween 20 in PBS, and once in PBS and analyzed in SDS-PAGE under reducing conditions.

Cell Proliferation and Tumorigenesis Assays. Cells (20,000/well) were plated in quadruplicate in 24-well plates, trypsinized on replicate plates after 1, 4, 6, or 8 days, and counted using a hemocytometer. Fresh medium was provided after 4 and 6 days. For the tumorigenesis assay, subconfluent cultures were harvested by trypsinization and washed twice, and 10⁷ cells in PBS were inoculated into the fat pads of the second (axillar) mammary gland of ovariectomized SCID mice, carrying s.c. 60-day slow-release pellets containing 0.72 mg of 17 β -estradiol (Innovative Research of America). The ovariectomy and implantation of the pellets were done 4–8 days before tumor cell inoculation. Tumor length and width were measured twice weekly in a blinded manner, and the tumor volume was calculated as the length \times width \times depth \times 0.5, assuming that the tumor is a hemi-ellipsoid and the depth is the same as the width (20).

Histology and Quantitation of the Blood Vessels. The tumors were excised, fixed in 4% paraformaldehyde (pH 7.0) for 24 h, and embedded in paraffin. Sections (7 μ m) were immunostained with monoclonal antibodies against PECAM-1 (PharMingen), VEGFR-3 (21), PCNA (Zymed Laboratories), or polyclonal antibodies against LYVE-1 (a kind gift from Dr. David G. Jackson, University of Oxford, Oxford, United Kingdom; Ref. 22), VEGF-C (1) or laminin (a kind gift from Dr. Karl Tryggvason, Karolinska Institute, Stockholm, Sweden) according to published protocols (14). The average of the number of the PECAM-1-positive vessels was determined from three areas ($\times 60$) of the highest vascular density (vascular hot spots) in a section. All histological analysis was done using blinded tumor samples.

Adenoviral Expression of Soluble VEGFR-3 and Evan's Blue Draining Assay. The cDNA coding for the VEGFR-3-Ig fusion protein was subcloned into the pAdCMV plasmid, constructed by subcloning the human cytomegalovirus immediate-early promoter, the multiple cloning site, and the bovine growth hormone gene polyadenylation signal from the pcDNA3 (Invitrogen) into the pAdBgIII vector, and the adenoviruses were produced as described previously (23). The VEGFR-3-Ig or LacZ control (23) adenoviruses, 10⁹

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⁴ The abbreviations used are: VEGF-C, vascular endothelial growth factor C; VEGFR, VEGF receptor; SCID, severe combined immunodeficient; PCNA, proliferating cell nuclear antigen; Ig, immunoglobulin.

pfu/mouse, were injected i.v. into the SCID mice 3 h before the tumor cell inoculation. After 3 weeks, four mice from each group were narcotized, the ventral skin was opened, and 5–10 μ l of 3% Evan's blue dye (Sigma) in PBS were injected into the tumor. The drainage of the dye from the tumor was followed macroscopically.

Results

Expression of VEGF-C or VEGFR-3-Ig Does Not Affect MCF-7 Cell Growth *in Vitro*. The MCF-7 human breast carcinoma cells were transfected with expression plasmids coding for full-length human VEGF-C or a soluble VEGFR-3 fusion protein (VEGFR-3-Ig), and stable cell pools were selected. For comparison, human VEGF₁₆₅ or VEGFR-1-Ig was expressed in the same cells. The efficient production and secretion of the proteins was verified by immunoprecipitation from the conditioned medium (Fig. 1A). However, the growth rate of the transfected cells was not affected (Fig. 1B).

VEGF-C Increases Tumor Growth without Affecting Tumor Angiogenesis. The MCF-7 cell pools were implanted into the mammary fat pads of ovariectomized SCID mice carrying slow-release estrogen pellets to provide a constant level of the hormone needed to support the growth of the MCF-7 tumors. Overexpression of VEGF-C increased tumor growth significantly (VEGF-C: 545 mm³ \pm 110 mm³, control: 268 mm³ \pm 69 mm³, at 13 days, $n = 8$, $P < 0.0001$, Student's t test; Fig. 2A). However, the effect of VEGF-C overexpression on tumor growth was much less dramatic than that of VEGF (VEGF: 1136 mm³ \pm 339 mm³, control: 189 mm³ \pm 57 mm³, at 15 days, $n = 6$, $P < 0.0001$, Student's t test; Fig. 2C). The increased tumor growth was neutralized by mixing the VEGF-C- or VEGF-overexpressing MCF-7 cells with cells expressing the soluble VEGFR-3 or VEGFR-1 fusion proteins, respectively (Fig. 2, B and

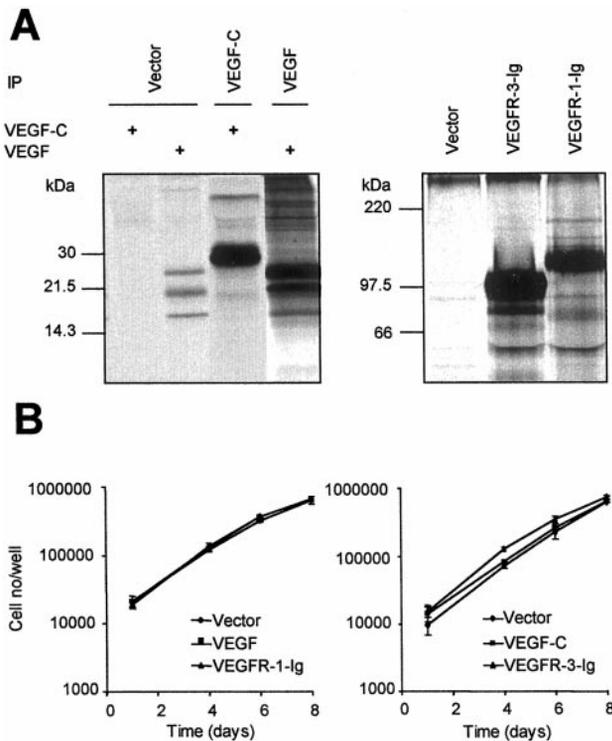


Fig. 1. Overexpression of VEGF-C, VEGF, or the soluble VEGFR-3 or VEGFR-1 fusion proteins does not affect the proliferation of the MCF-7 breast carcinoma cells *in vitro*. A, immunoprecipitates of VEGF-C, VEGF, or the soluble receptor proteins from metabolically labeled MCF-7 cells were analyzed in PAGE under reducing conditions. The three polypeptide bands in Lane 2 represent endogenous VEGF. B, cells were seeded in 24-well plates, and after the indicated growth periods, the cells were counted in a hemacytometer. Bars, \pm SD.

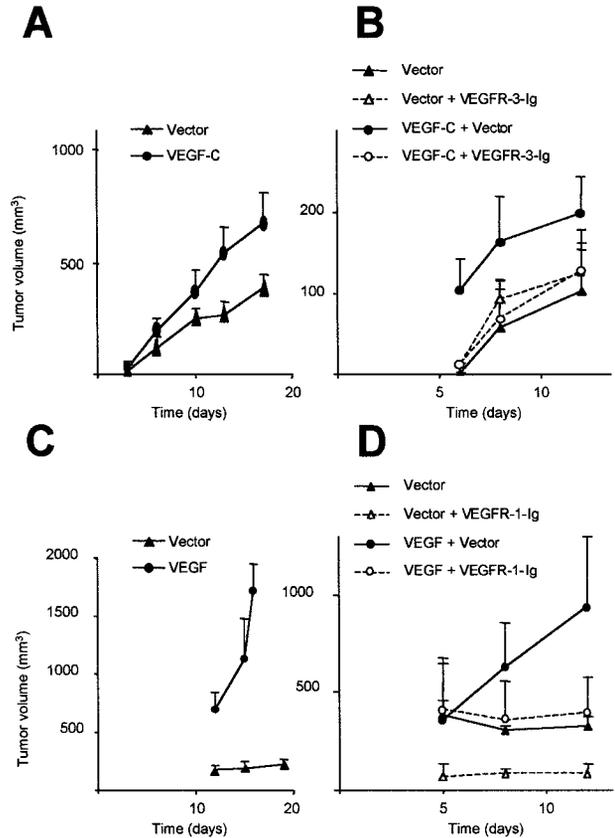


Fig. 2. Effect of VEGF-C, VEGF, and their soluble receptors on tumor growth. A and C, 10^7 MCF-7 cells stably transfected with VEGF-C (A), VEGF (C), or empty vector were injected into the mammary fat pads of ovariectomized SCID mice carrying slow-release estrogen pellets. B, equal numbers of MCF-7 cells stably transfected with VEGF-C, VEGFR-3-Ig, or empty vector were mixed and injected into the mammary fat pads. D, equal numbers of MCF-7 cells stably transfected with VEGF, VEGFR-1-Ig, or empty vector were mixed and injected into the mammary fat pads. The diameters of the formed tumors were measured at the indicated times, and the tumor volumes were calculated. Bars, \pm SD.

D). The increased growth of the VEGF-C-overexpressing tumors was inhibited also by a circulating soluble VEGFR-3-Ig expressed in the liver by an i.v.-injected recombinant adenovirus (data not shown).

To study the effect of VEGF-C on tumor angiogenesis, sections of the tumors were stained for PECAM-1, an endothelial antigen expressed primarily in blood vessels and only weakly in lymphatic vessels. Quantitation of the PECAM-1-positive vessels in the tumors revealed that overexpression of VEGF-C had very little effect on the density of the tumor blood vessels (40.2 ± 12.2 vessels/microscopic field for VEGF-C tumors, $n = 18$, and 36.6 ± 11.6 for control tumors, $n = 23$; average of three different experiments; Fig. 3). In contrast, overexpression of VEGF increased the vascular density ~ 2 -fold (Fig. 3).

VEGF-C Overexpression Is Associated with Lymphangiogenesis and Intralymphatic Growth of Tumor Cells. The effect of VEGF-C on tumor-associated lymphatic vessels was analyzed by immunostaining for the lymphatic specific marker LYVE-1 (22). This marker revealed highly hyperplastic lymphatic vessels in the periphery of the VEGF-C-overexpressing tumors (Fig. 4A). The proliferating cell nuclear antigen was detected in many of the LYVE-1-positive endothelial cells (Fig. 4A, inset), showing that these lymphatic vessels were actively proliferating. Confirmation of the lymphatic identity of the vessels was obtained by staining for VEGFR-3 (Fig. 4C) and by the lack of staining for the basal lamina component laminin (data not shown). Thin lymphatic vessels were also present inside some of the VEGF-C-overexpressing tumors (Fig. 4B).

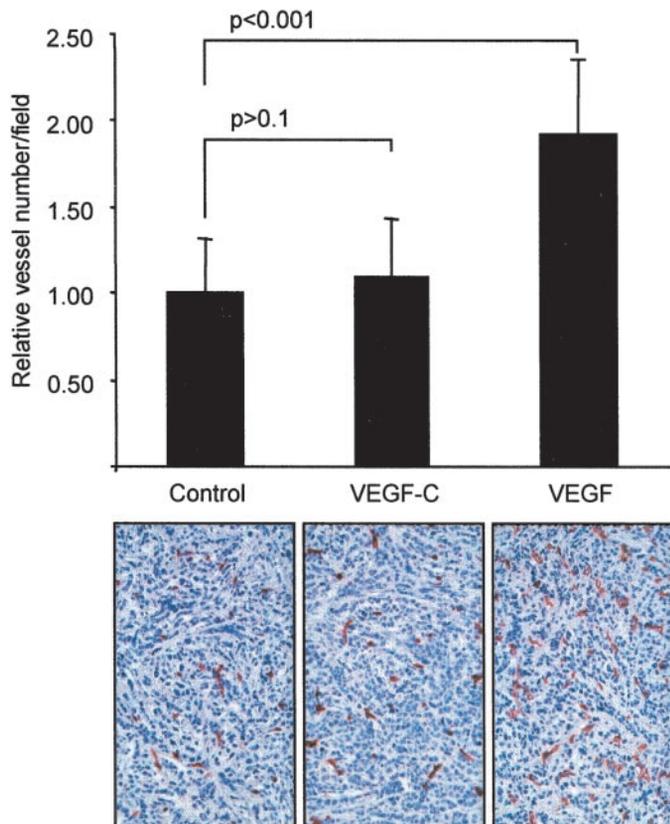


Fig. 3. Tumor angiogenesis is not affected by overexpression of VEGF-C. Sections of VEGF-C- or VEGF-overexpressing tumors or of control tumors were stained for the endothelial cell marker PECAM-1 (*bottom panel*). The positive vessels in three microscopic fields of the highest vascular density were counted and normalized to the number of vessels in the control tumors (*top panel*). Note that whereas VEGF induced an ~2-fold increase in vascular density, VEGF-C had no significant effect on tumor blood vessels. The relative vessel numbers were compared by the Student's *t* test with two-tailed distribution and two-sample equal variance. Bars, \pm SD.

The lymphatic vessels in the tumor periphery were commonly infiltrated by the VEGF-C-positive tumor cells (Fig. 4, A, C, and D). In a striking contrast, the VEGF-overexpressing and control tumors contained no or only few lymphatic vessels (Fig. 4, E and F).

VEGF-C-induced Lymphangiogenesis Is Inhibited by a Circulating Soluble VEGFR-3 Fusion Protein. In human breast cancer, the sentinel node method is used to trace lymphatic drainage and metastatic spread (reviewed in Ref. 24). To trace lymphatic drainage of the MCF-7 tumors, Evan's blue dye was injected into VEGF-C-overexpressing or control tumors in mice infected with VEGFR-3-Ig or control adenovirus. Control experiments indicated that infection of cultured human embryonic kidney cells with the VEGFR-3-Ig adenovirus resulted in the secretion of high amounts of the soluble VEGFR-3-Ig fusion protein, and i.v. infection of mice led to high systemic levels of the VEGFR-3-Ig fusion protein in the serum.⁵ Injection of Evan's blue dye into the tumors resulted in the staining of lymphatic but not blood vessels and revealed an increased number of enlarged lymphatic vessels surrounding the VEGF-C-overexpressing tumors (Fig. 4G) when compared with control tumors (Fig. 4H). Most of the enlarged lymphatic vessels were absent from VEGF-C-overexpressing tumors in mice treated with the VEGFR-3-Ig adenovirus (Fig. 4I). These results were confirmed by immunohistochemical analysis of the tumor samples (data not shown).

⁵ T. Karpanen and T. Makinen, unpublished data.

Discussion

This study shows that VEGF-C overexpression in MCF-7 mammary tumors strongly and specifically induces the growth of tumor-associated lymphatic vessels but does not have major effects on tumor angiogenesis. However, tumor growth was significantly increased by VEGF-C overexpression. Furthermore, increased tumor growth and tumor-associated lymphangiogenesis were inhibited by a soluble VEGFR-3 fusion protein. On the other hand, VEGF-overexpressing and control tumors almost completely lacked lymphatic vessels.

Because of the lack of specific markers, it has been questioned whether tumors can actively induce lymphangiogenesis or if solid tumors just encompass by overgrowth the already existing lymphatic vessels and compress them because of the high interstitial fluid pressure inside the tumor. In various experimental models, the latter seems to be the case (25, 26). Here, for the first time, we show that overexpression of VEGF-C can induce the growth of lymphatic vessels in association with experimental tumors. The VEGF-C-induced lymphatic vessels in the tumor periphery were highly hyperplastic and mostly filled with tumor cells, whereas the lymphatic vessels inside the tumor were flattened and in general without a lumen. These intratumoral lymphatic vessels may be trapped by multiple expanding tumor cell islets in tumor xenografts, but they may be rare in naturally occurring tumors. Unlike lymphatic endothelial cells in normal adult tissues, the lymphatic endothelial cells associated with the MCF-7 tumors were actively proliferating. On the basis of this information, we speculated that most of the peri- and intratumoral lymphatic vessels were generated by proliferation of the endothelial cells of preexisting lymphatic vessels.

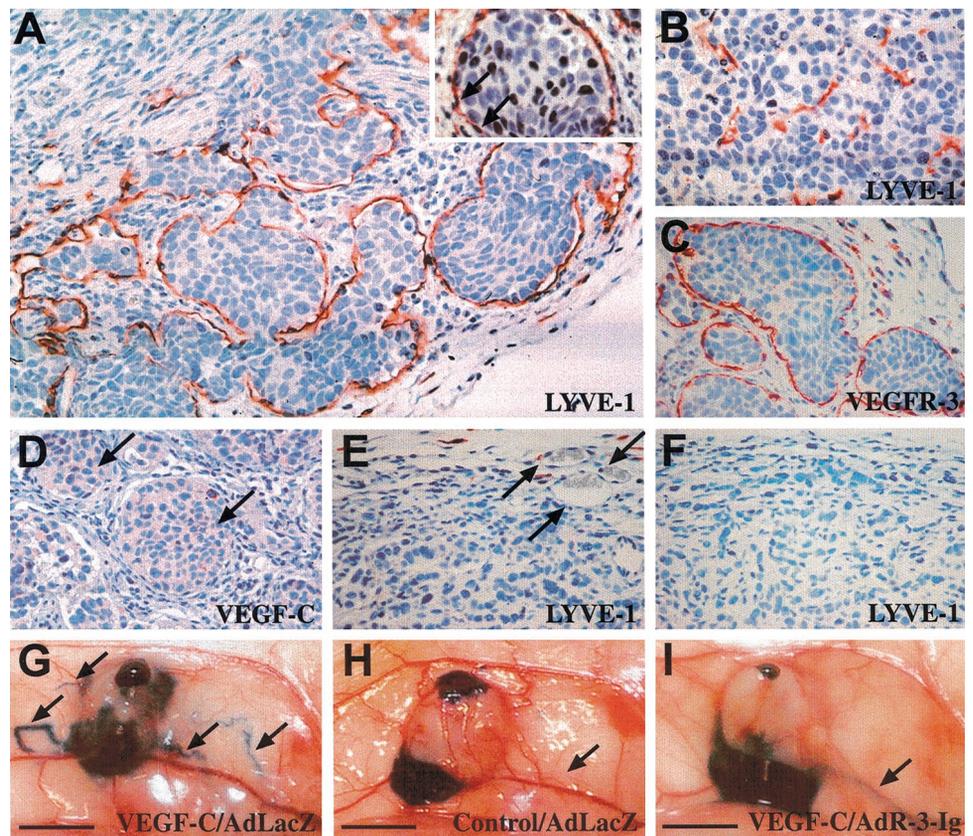
Although the spread of cancer through the lymphatics into the regional lymph nodes has long been an important prognostic indicator in clinical use, tumor metastasis at the mechanistic level is still poorly understood. The growth of tumor cells inside the enlarged lymphatic vessels associated with the VEGF-C-overexpressing tumors in this study resembles the peritumoral lymphatic invasion, which correlates with metastatic spread to the lymph nodes and poor survival in human breast cancer (27). This suggests that expression of VEGF-C can promote tumor metastasis via the lymphatic system. Despite this, we did not detect macroscopic metastases in the lymph nodes of mice bearing the VEGF-C-overexpressing tumors (data not shown). This may be attributable to the facts that MCF-7 tumors rarely form macrometastasis (28) and that the duration of our experiments was relatively short. However, lymph node micrometastases were promoted by VEGF-C overexpression in the MCF-7 tumors.⁶

VEGF and its receptor VEGFR-2 are considered to be the main regulators of tumor angiogenesis (2, 3). Also VEGFR-3, although normally restricted to the lymphatic endothelial cells in adults, is up-regulated in the blood vessels of many kinds of solid tumors (9, 14). A previous report suggested that VEGFR-3 could be involved in the maintenance of the integrity of the endothelial cell lining during tumor angiogenesis (21). Therefore, we speculated that VEGF-C may influence tumor neovascularization. However, in the present tumor model, overexpression of VEGF-C, in comparison with VEGF, did not significantly increase tumor angiogenesis. Instead, its effects were mainly lymphangiogenic.

The increased growth of the primary tumors overexpressing VEGF-C was unexpected, given that VEGF-C had no effect on tumor cell proliferation in cell culture or on tumor angiogenesis. The effect of VEGF-C on tumor growth was not simply attributable to variation between the cell pools, as shown by the ability of the VEGFR-3 fusion protein to inhibit the growth of VEGF-C-overexpressing tumors. By

⁶ M. Mattila, J. Ruohola, and C. P. Härkönen, unpublished data.

Fig. 4. Overexpression of VEGF-C, but not VEGF, induces tumor-associated lymphangiogenesis. **A**, hyperplastic LYVE-1-positive (red) lymphatic vessels in the periphery of VEGF-C-overexpressing tumors are frequently infiltrated by tumor cells. *Inset* in **A**, double staining for LYVE-1 (red) and PCNA (brown). Note that some LYVE-1-positive cells are also labeled for PCNA (arrows). **B**, intratumoral lymphatic vessels appear flattened and without a lumen. **C**, VEGFR-3 (red) and **D**, VEGF-C (red) staining of sections from VEGF-C-overexpressing tumors. Note that the tumor cells growing inside the lymphatic vessels produce more VEGF-C than the rest of the tumor (arrows in **D**). **E** and **F**, LYVE-1 staining of sections from VEGF-C-overexpressing and control tumors, respectively. Arrows in **E**, LYVE-1-negative blood vessels. All sections were counterstained with hematoxylin (blue). **G** and **H**, drainage of Evan's blue dye from a VEGF-C-overexpressing tumor and from a control tumor in mice treated with LacZ adenovirus, respectively. **I**, drainage of Evan's blue dye from a VEGF-C-overexpressing tumor in a mouse treated with VEGFR-3-Ig adenovirus. The tumors shown in **G-I** were selected to be of similar size and were photographed 6 min after injection of Evan's blue. **A** and **B**, $\times 200$; **C-F**, $\times 120$. Bars in **G-I**, 0.5 cm.



injecting Evan's blue dye into the tumors, we observed that an increased number of large draining lymphatic vessels were associated with the VEGF-C-overexpressing tumors. One could speculate that the higher number of functional lymphatic vessels may result in a better lymphatic drainage and thus a lower interstitial pressure and enhanced blood perfusion of the VEGF-C-overexpressing tumors.

In conclusion, our results show that VEGF-C produced by tumor cells can induce the growth of lymphatic vessels around tumors and thus facilitate the intralymphatic spread of cancer. Because of the specific lymphangiogenic response and the lack of significant effects on tumor angiogenesis, the VEGF-C-overexpressing MCF-7 breast carcinoma represents a useful model to study the development of tumor-associated lymphatic vessels. Furthermore, the data suggest that inhibition of tumor-associated lymphangiogenesis, for example by gene therapy using soluble VEGFR-3 proteins, could be a valuable way of inhibiting tumor metastasis.

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Note Added in Proof

While this paper was being processed, three other papers have also reported increased tumor lymphangiogenesis in different VEGF-C or VEGF-D overexpressing tumor models (Stacker *et al.* *Nature Medicine*, 7: 186–191, Skobe *et al.* *Nature Med.*, 7: 192–198, Mandriotta *et al.* *EMBO J*, in press). In these models, also lymphatic metastasis was enhanced. Furthermore, Kadambi *et al.*

(*Cancer Res.*, in press, 2001) report that in the early stages of tumorigenesis, VEGF-C can increase tumor angiogenesis as well.

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