Commentary

Lymphatic Vessels as Targets of Tumor Therapy?

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Recent evidence on the association of lymphangiogenic growth factors with intralymphatic growth and metastasis of cancers (1–4) has raised hopes that lymphatic vessels could be used as an additional target for tumor therapy. Cancer cells spread within the body by direct invasion to surrounding tissues, spreading to body cavities, invasion into the blood vascular system (hematogenous metastasis), as well as spread via the lymphatic system (lymphatic metastasis). Regional lymph node dissemination is the first step in the metastasis of several common cancers and correlates highly to the prognosis of the disease. The lymph nodes that are involved in draining tissue fluid from the tumor area are called sentinel nodes, and diagnostic measures are in place to find these nodes and to remove them in cases of suspected metastasis. However, in spite of its clinical relevance, little is known about the mechanisms leading to metastasis via the bloodstream or via the lymphatics.

Until recently, the lymphatic vessels have received much less attention than blood vessels, despite their importance in medicine. Lymphatic vessels collect protein-rich fluid and white blood cells from the interstitial space of most tissues and transport them as a whitish opaque fluid, the lymph, into the blood circulation. Small lymphatic vessels coalesce into larger vessels, which drain the lymph through the thoracic duct into large veins in the neck region. Lymph nodes serve as filtering stations along the lymphatic vessels and lymph movement is propelled by the contraction of smooth muscles surrounding collecting lymphatic vessels and by bodily movements, the direction of flow being secured by valves as it is in veins. The lymphatic capillaries are lined by endothelial cells, which have distinct junctions with frequent large interendothelial gaps. The lymphatic capillaries also lack a continuous basement membrane, and are devoid of pericytes. Anchoring filaments connect the abluminal surfaces of lymphatic endothelial cells to the perivascular extracellular matrix and pull to maintain vessel patency in the presence of tissue edema. The absence or obstruction of lymphatic vessels, which is usually the result of an infection, surgery, or radiotherapy and in rare cases, a genetic defect, causes accumulation of a protein-rich fluid in tissues, lymphedema. The lymphatic system is also critical in fat absorption from the gut and in immune responses. Bacteria, viruses, and other foreign materials are taken up by the lymphatic vessels and transported to the lymph nodes, where the foreign material is presented to immune cells and where dendritic cells traverse via the lymphatics. There has been slow progress in the understanding of and ability to manipulate the lymphatic vessels during the past several decades.

Two members of the vascular endothelial growth factor (VEGF) family, VEGF-C and VEGF-D, have been associated with lymphangiogenesis (5–7). These factors are ligands for the lymphatic endothelial VEGF receptor 3 (VEGFR-3), but upon proteolytic processing they gain the ability to bind and activate also VEGFR-2 (8, 9). VEGFR-2 is the main angiogenic signal transducer for VEGF while VEGFR-3 is specific for VEGF-C and VEGF-D and necessary and sufficient for lymphangiogenic signaling (for a review, see reference 10). However, both VEGF-C and VEGF-D can also be angiogenic (11, 12), provided they undergo enough proteolytic processing, and that their receptors are expressed on the target blood vessels. In normal adult tissues VEGFR-3 is expressed almost exclusively in lymphatic endothelia, but for example in tumors it is also expressed in endothelial cells of blood vessels, where it is thought to contribute to tumor angiogenesis (13, 14). VEGF-C can also enhance blood vascular permeability via VEGFR-2 (15).

VEGF-C expression has been detected in about half of human cancers analyzed (16). In breast cancer VEGF-C expression seems to correlate with lymph node positive tumors whereas VEGF-D may be expressed predominantly in inflammatory breast carcinoma (17). Increased VEGF-C levels have also been reported to correlate with lymph node metastases in thyroid, prostate, gastric, colorectal, and lung cancers (18–23). In one study VEGF-C expression correlated with lymphatic vessel density, but not metastasis (24). Such highly provocative clinical correlations between lymphangiogenic growth factor expression and metastasis should be extended to larger sets of patients and tumor types. In addition, animal models are needed to elucidate the mechanisms by which such correlation occurs. Clarijs et al. (25) attributed the strictly hematogenous metastasis of primary uveal melanomas to the absence of lymphatics in

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and around the tumor. Their data suggests that, although VEGFR-3 is expressed in tumor blood vessels, VEGF-C expression is not sufficient to induce lymphangiogenesis from preexisting blood vessels in human cancer. This is consistent with the conclusion of Kriehuber et al. (26) and Makinen et al. (27) that in adults differentiated lymphatic and blood vascular endothelial cells form separate and stable cell lineages. This reinforces the view that angiogenesis and lymphangiogenesis represent coordinated but distinct processes that can be separately targeted in human diseases.

Mandriota et al. (1) established transgenic mice in which VEGF-C expression, driven by the rat insulin promoter, is targeted to the β-cells of the endocrine pancreas. The transgenics developed an extensive network of lymphatic vessels around the islets of Langerhans, which normally do not have associated lymphatic vessels. The VEGF-C overexpressing mice were crossed with mice that develop pancreatic β-cell tumors, which are neither lymphangiogenic nor metastatic. Double transgenic mice formed tumors surrounded by well-developed lymphatics, which frequently contained tumor cell masses of β-cell origin. These mice also frequently developed metastases in the lymph nodes draining the pancreas. Similarly, human breast cancer cells expressing ectopic VEGF-C were recently shown to induce lymphangiogenesis in and around the orthotopically implanted tumors (2, 4) and to spread to the regional lymph nodes more frequently than the control cells (2). Stacker et al. (3) overexpressed VEGF-D in transformed human kidney cell xenografts in mice and obtained tumor lymphangiogenesis but also angiogenesis, probably because of increased proteolytic processing of VEGF-D. All groups used monoclonal antibodies against a lymphatic-specific hyaluronan receptor called LYVE-1 (28, 29) to demonstrate an increase of peritumoral lymph vessels when they compared VEGF-C or VEGF-D overexpressing tumors to control tumors. Intratumoral lymphatic vessels were also observed in the tumor xenografts, but not in the transgenic tumors, which may be at least partially explained by the trapping of vessels in between growing tumor foci in the xenografts that start from an injected cell suspension. Thus, tumor vascularization can be dissected into pathways that preferentially activate angiogenesis (driven by VEGF) and pathways that preferentially activate lymphangiogenesis (driven by VEGF-C and VEGF-D; Fig. 1), although there now is evidence that both processes can occur simultaneously in response to certain growth factors (unpublished data).

Although it seems evident that VEGF-C can induce new lymphatic vessels as well as hyperplasia of preexisting lymphatic vessels (5, 6), we need to understand more about the relationship between VEGF-C and VEGF-D expression and lymphatic metastasis and its possible mechanisms. For example, is it sufficient for preexisting lymphatic vessels to expand by circumferential growth or does one need additional new vessels for the enhancement of metastasis? Do lymphatic vessels penetrate into existing tumors or are they just trapped in between expanding tumor foci, which appears likely in experimental tumor xe-

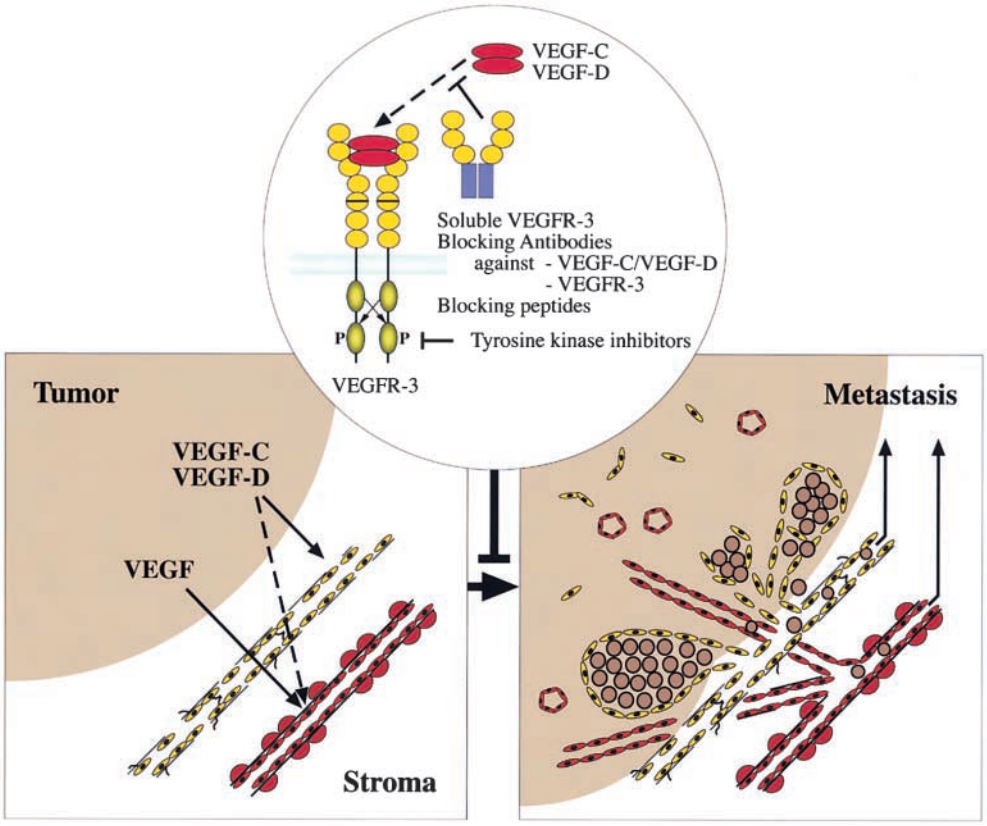

Figure 1. Tumor lymphangiogenesis and its inhibition. VEGF-C and VEGF-D produced by tumor cells or associated inflammatory cells induce the growth of lymphatic vessels in the tumor periphery. These factors may also contribute to angiogenesis induced by VEGF. Tumor cells grow inside of the lymphatic vessels, which provide a route for tumor metastasis. Shown in the top part are various approaches to inhibit tumor lymphangiogenesis and lymphatic metastasis by blocking VEGFR-3 signaling.

nografts? Does lymphatic sprouting occur into tumors, or do lymphatics grow by peritumoral hyperplasia and by vessel splitting and fusion? Are the intratumoral lymphatic vessels seen in occasional tumors collapsed and nonfunctional because of the high interstitial pressure in solid tumors? Are all lymphatic endothelial cells derived from preexisting ones or is there contribution by some sort of precursor cells (lymphangioblasts) similar to what has been described for the blood vascular endothelium (hemangioblasts and endothelial precursor cells; for a review, see reference 30) and for avian embryonic tissues (31). Do the blood vascular and lymphatic endothelia become irreversibly committed to their differentiated phenotypes, as suggested by the recent data (26, 27), and at what stage in their differentiation program does this occur? Does lymphangiogenesis follow tumor angiogenesis as it does in several developmental processes, where VEGF-C may act as a coupling factor between the two?

In human tumors, most of the lymphatic vessels occur peritumorally (32), but detection of occasional intratumoral lymphatic vessels can have prognostic significance, for example in melanomas of the skin (Michael Detmar, personal communication). It is not known to what extent tumor cell secreted factors are directly responsible for the large lymphatics detected around the tumor and to what extent for example inflammatory cells, such as macrophages contribute to lymphangiogenesis. VEGF-C is chemotactic for macrophages (33) and readily induced by proinflammatory cytokines (34, 35). The dilated and engorged peritumoral lymphatics may function poorly because intralymphatic growth of tumor cells clogs such vessels. Could this cause a backwash effect diverting invading cells into the venous circulation?

The simplest explanation for the metastasis-enhancing effects of VEGF-C and VEGF-D is that they eliminate one rate-limiting step by increasing the surface contact area between the invading tumor cells and the hyperplastic lymphatic endothelium. However, VEGF-C could also facilitate metastasis by increasing vascular permeability, or by changing the adhesive properties or cytokine or chemokine expression patterns of the lymphatic endothelium. For example, there is evidence that the lymphatic expression of the secondary lymphoid tissue chemokine (SLC; reference 26) not only attracts dendritic cells, but also certain tumor cells expressing the corresponding receptors (36). VEGF-C and VEGF-D secreted by tumor cells could also have important effects on the tumor interstitial pressure. Both can increase vascular leak, but not as efficiently as VEGF (15), and a possible parallel increase in lymphangiogenesis could alleviate this effect. The increased interstitial pressure could be a major determinant of tumor cell seeding into the blood vascular and lymphatic circulation, especially as recent studies have shown that a significant proportion of the surface of tumor blood vessels is covered by the tumor cells (37, 38).

The switch on of tumor angiogenesis occurs at least partially due to activation of matrix metalloproteases upon tumor progression (39). Tumor or associated inflammatory cell secreted proteases can also cleave VEGF-C and VEGF-D and thus regulate their angiogenic versus lymphangiogenic properties. Furthermore, at least some specific genetic events in tumor progression correlate with lymphatic metastasis and a lymphangiogenic switch mechanism could operate upon such oncogenic insults. In a recent study Cavallaro et al. (40) found that in transgenic mice deficient

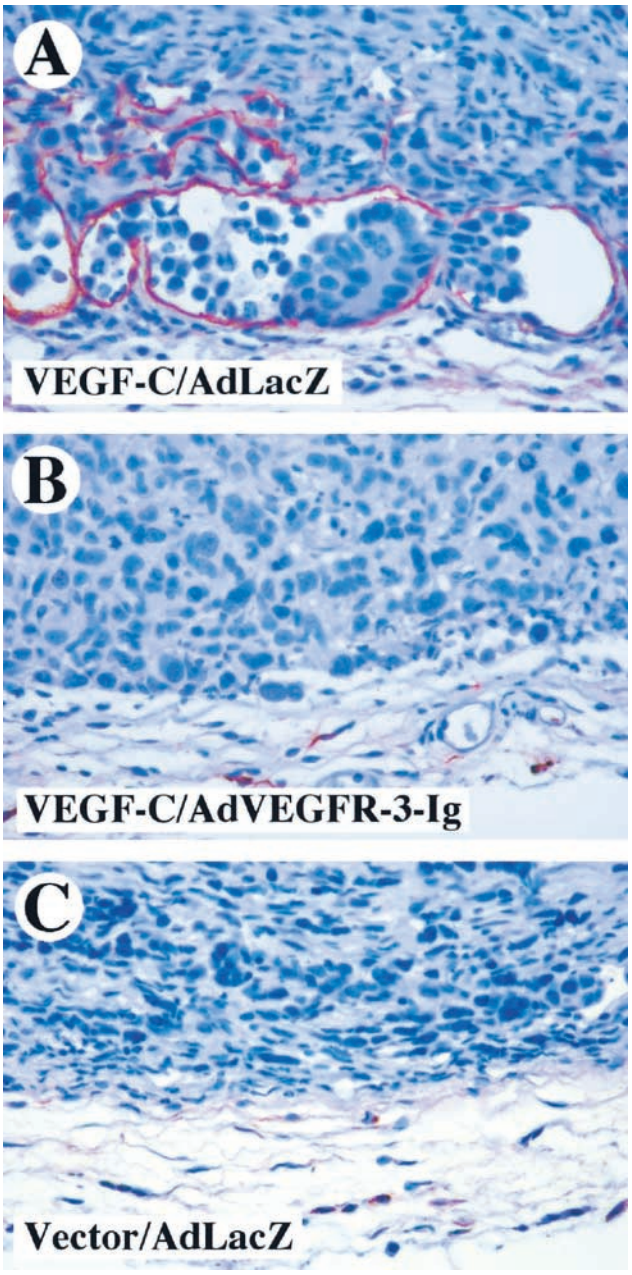

Figure 2. Inhibition of tumor lymphangiogenesis by adenovirally encoded soluble VEGFR-3. MCF-7 breast carcinoma cells were stably transfected with a VEGF-C expression plasmid (A and B) or an empty vector (C) and 10^7 cells were injected into the mammary fad pads of ovariectomized SCID mice carrying slow-release estrogen pellets. The mice were injected intravenously with 10^9 PFU of an adenovirus encoding soluble VEGFR-3 (B) or control adenovirus (A and C). See reference 4 for further details.

of the neural cell adhesion molecule, pancreatic tumors show disaggregation and detachment of cells and disperse cell clumps that can be found in blood and lymphatic vessels and which metastasize to nearby lymph nodes. It would be interesting to know if VEGF-C or VEGF-D are upregulated in such tumors.

Recent reports have indicated that at least newly formed lymphatic vessels are dependent on molecular signals that can be interrupted (41), and these findings form a basis for the inhibition of lymphangiogenesis. In transgenic mice with targeted expression of a soluble form of VEGFR-3 in the skin, lymphatic vessels initially formed normally, but the onset of transgene expression led to regression of lymphatic vessels in embryos and to mice that completely lack a dermal lymphatic system (41). Such mice now serve as a model for studies of for example skin tumor metastasis in the absence of the lymphatic vessels. Furthermore, neutralizing antibodies against VEGF-D decreased the number of lymphatic metastases of the VEGF-D producing tumors (3) and soluble VEGFR-3 produced via an adenovirus vector could inhibit tumor-associated lymphangiogenesis in a transplantable human breast carcinoma model in SCID mice (4; Fig. 2). Microhemorrhage and the subsequent collapse of large tumor vessels was also reported in mice injected with blocking monoclonal antibodies against VEGFR-3 (42); thus, VEGFR-3 targeted therapy has the possibility of destabilizing tumor blood vessels as well. It is now essential to find out if such anti-lymphangiogenic treatments have side effects on normal vascular function.

Besides the recently identified lymphatic endothelial cell surface proteins, the lymphatic vessels are also likely to express other tissue- and even tumor-specific molecules. Methods such as cDNA microarray analysis and phage display screening are being used to identify relevant markers and use them for selective drug targeting to the lymphatic vessels. Targeted imaging of lymphatic vessels could also be possible in cancer patients. The current targeting technologies make it possible to develop almost any drug into a targeted compound, thereby increasing the potency of the drug at the intended target tissue, while reducing side effects elsewhere in the body (43, 44). An attractive possibility would also be to target anti-cancer drugs into the tumor lymphatics and to specifically destroy peritumoral lymphatic vessels, which could inhibit lymphatic metastasis. However, we do not know yet whether lymphatic destruction would further elevate the tumor interstitial pressure, which is a severe problem for drug delivery into tumors and presumably a risk factor for hematogenous metastasis (45, 46). One consideration is that there could be a compensatory increase of lymphangiogenic growth factor levels; at least VEGF-D expression is regulated by cell–cell contacts (47).

For the discovery of drug targets in the lymphatic endothelium, direct in vivo screening poses a problem, because of the complexity of tissues within which the slender lymphatic vessels are embedded. An alternative is to isolate differentiated lymphatic endothelial cells. Previous studies have reported on the isolation of lymphatic endothelium from lymphangiomas or from large mesenteric or thoracic collecting ducts (for a review, see reference 48). In the study of Kriehuber et al. (26) human skin was used for the isolation of the endothelial cells derived from lymphatic capillaries. This advance comes with the discovery of the lymphatic endothelium-specific marker podoplanin, a glomerular podocyte membrane mucoprotein (49). Both podoplanin and LYVE-1 appear useful for the isolation of lymphatic endothelial cells, although neither is absolutely specific for these cells. Monoclonal antibodies against VEGFR-3 also allowed the isolation of lymphatic endothelial cells, which grew in culture in the presence of VEGF-C and a suitable pericellular matrix without losing their differentiated properties (27). Specific VEGFR-3 ligands could induce cell migration and protected serum-deprived lymphatic endothelial cells from apoptosis. In normal adult tissues VEGFR-3 antibodies are useful for the isolation of lymphatic endothelial cells because the expression of VEGFR-3 occurs almost exclusively by lymphatic vessels (50). However, as already mentioned, in tumors it tends to lose its specificity due to its upregulation in the angiogenic blood vessels (13, 14).

There has been very little progress during the past several decades in the ability to manipulate lymphatic vessel growth or function. This may have changed now. The discovery of the VEGF-C and VEGF-D growth factors for lymphatic vessels as well as their receptor VEGFR-3 has revived the lymphatic vascular field. Isolation and culture of lymphatic endothelial cells is another milestone that facilitates the screening of useful drug targets in this system. One important task for further research is to analyze the effects of various inhibitors of VEGFR-3 on tumor growth, angiogenesis, interstitial pressure, lymphangiogenesis, and metastasis (see Fig. 1). Another important one is to study how to reconstitute lymphatic vessels of patients with lymphedema after surgery or radiotherapy. This research provides a unique opportunity for the development of new and innovative medical technology for cancer and other human diseases.

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