The role of tumor lymphangiogenesis in metastatic spread

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ABSTRACT **The high mortality rates associated with cancer can be attributed to the metastatic spread of tumor cells from the site of their origin. Tumor cells invade either the blood or lymphatic vessels to access the general circulation and then establish themselves in other tissues. Clinicopathological data suggest that the lymphatics are an initial route for the spread of solid tumors. Detection of sentinel lymph nodes by biopsy provides significant information for staging and designing therapeutic regimens. The role of angiogenesis in facilitating the growth of solid tumors has been well established, but the presence of lymphatic vessels and the relevance of lymphangiogenesis to tumor spread are less clear. Recently, the molecular pathway that signals for lymphangiogenesis and relatively specific markers for lymphatic endothelium have been described allowing analyses of tumor lymphangiogenesis to be performed in animal models. These studies demonstrate that tumor lymphangiogenesis is a major component of the metastatic process and implicate members of the VEGF family of growth factors as key mediators of lymphangiogenesis in both normal biology and tumors.—Stacker, S. A., Baldwin, M. E., Achen, M. G. The role of tumor lymphangiogenesis in metastatic spread. *FASEB J.* 16, 922–934 (2002)**

Key Words: metastasis · VEGF-C · VEGF-D · VEGF receptors

EACH YEAR IN the United States more than 500,000 people die principally as a result of the metastatic spread of cancer (1, 2). Cells from malignant primary tumors spread from their sites of origin to invade local tissue and enter the systemic circulation (3, 4). This spread can occur directly into the local tissue or via blood vessels (hematogenous spread) and lymphatics (lymphogenous spread) or by invasion of body cavities such as the pleura or peritoneum. Cells must first invade either blood or lymphatic vessels to enter the circulation. In the case of blood vessels, this requires penetration of the basement membrane and migration through the cellular layers of the vessel (**Fig. 1**). In contrast, it has been proposed that the entry of tumor cells into the lymphatic circulation may be easier due to the nature of lymphatic vessels and that the thin and discontinuous basement membrane of lymphatics (5–7) might not provide a significant barrier to entry of tumor cells.

Tumor cells can escape from the primary site by entering existing vessels or new vessels actively recruited into the primary tumor (4). The relative importance of the established vessels vs. the active invasion of a tumor by new blood and lymphatic vessels for the initial metastatic spread of tumor cells is still unclear. Previous studies have established the role of angiogenesis in solid tumor growth (8–14), and there is some evidence indicating a direct role for angiogenesis in hematogenous tumor spread (15). The onset of angiogenesis within small clusters of tumors cells, known as the 'angiogenic-switch', is important for blood vessel formation in further development of malignant cells that have already spread from the primary tumor (16, 17). However, little is known about the role of tumor lymphangiogenesis (the growth and production of new lymphatic vessels) in the spread of tumors and whether this process is important in the overall context of lymphatic spread (18, 19).

Clinical and pathological data point to the spread of solid tumors via the lymphatics as an important early event in metastatic disease (1, 3). Detection of tumor cells in lymphatic vessels and regional lymph nodes is a key factor in the staging of human tumors and forms the basis for treatment of regional lymph nodes by surgery and radiation therapy (1, 3, 20, 21). Although a large body of clinical and pathological evidence points to a major role for the lymphatics in the initial spread of malignant tumors, the exact mechanism whereby tumor cells enter the lymphatic system is uncertain (18, 22, 23).

The role of lymphangiogenesis in promoting the metastatic spread of tumor cells via the lymphatics has been an area that has achieved little publicity in the past decade. This has in part been due to difficulty in studying lymphatic vessels because of their morphology and a lack of lymphatic-specific markers (24). In reviewing this topic, we will emphasize the progress made in the past 2 years in identifying novel lymphatic markers, as well as lymphangiogenic factors and receptors responsible for the generation and maintenance of the lymphatic system. This will include a summary of the recent clinical and experimental data that support the notion that lymphangiogenic factors influence the spread

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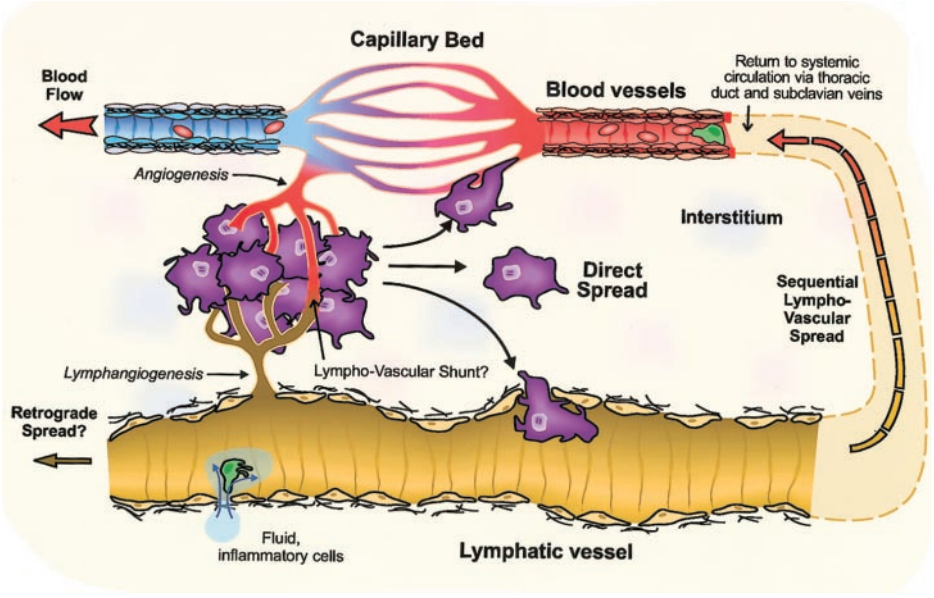

Figure 1. Lymphatic vessel structure and potential modes of tumor cell dissemination. Lymphatics are thin-walled, low-pressure vessels that collect fluid and cells from the interstitium and return it to the circulation via the thoracic duct. In contrast, blood vessels are subject to high pressure and have a more robust structure with a well-defined basement membrane and supporting cells. Tumor cells leave the primary tumor and spread directly into the surrounding tissue, from which they subsequently invade preexisting blood vessels or lymphatic vessels. Alternatively, the formation of new blood vessels (angiogenesis) or new lymphatic vessels (lymphangiogenesis), induced by tumor-associated angiogenic and/or lymphangiogenic factors, could promote the growth of new vessels into the tumor, providing an escape route for tumor cells (diagram shows single tumor cells leaving the primary mass and entering the lymphatic or vascular system). Cells that invade the lymphatics could find their way into the bloodstream via the thoracic duct (sequential lymphovascular spread) or by the formation of shunts, speculated to exist in tumors, between the vascular system and the lymphatic system. Retrograde spread from blocked lymphatic vessels could be speculated as a means for tumor cells to travel upstream and into the venous system (18).

of tumors and a discussion of the potential therapeutic options for anti-lymphangiogenic treatment of cancer.

STRUCTURE AND FUNCTION OF THE LYMPHATICS

The lymphatic system consists of thin-walled, low-pressure vessels, nodes that occur along the course of lymphatic vessels, aggregations of lymphoid tissue, such as the spleen and thymus, and circulating lymphocytes (5, 6, 25). By regulating fluid absorption from the interstitium, harboring macrophages, and providing a passage for lymphocyte trafficking, the lymphatic system maintains plasma volume, prevents increases in tissue pressure, and has an important role in immune system function (26). Lymphatic vessels are distinct functionally and ultrastructurally from their blood vessel counterparts (27). Compared to blood vessels, the walls of lymphatic vessels are thinner. This is in part due to the highly attenuated cytoplasm of lymphatic endothelial cells (Fig. 1) (5, 6, 27, 28). The endothelium of lymphatic vessels contains fewer tight junctions than that of blood vessels and it has been speculated that this may be the cause of the greater permeability of the lymphatic vessels (25). Lymphatic capillaries have a poorly developed or absent basal lamina and lack associated pericytes (5, 6). Their lumens are ~ threefold wider than the lumens of blood capillaries and are more irregularly shaped, appearing collapsed in tissue section (5, 28, 29). Lymphatic vessels are connected to the extracellular matrix by reticular fibers and collagen (5, 6, 30). Upon increases in interstitial fluid and pressure, the connecting tissue fibers become stretched, thereby opening the lymphatic lumen (27, 29, 31). As the lumen widens, the endothelial cells, which overlap under normal conditions, move apart, effectively opening intercellular channels to aid fluid and macromolecular uptake into the lymphatic vessel (5, 6, 29, 30) (Fig. 1).

LYMPHANGIOGENESIS

The growth of lymphatic vessels, lymphangiogenesis, has received considerable attention in the last 2 years, due to the identification of proteins specifically expressed on lymphatic vessels and the discovery of molecules that can drive lymphatic vessel growth (32–38). Vascular remodeling associated with lymphangiogenesis and angiogenesis is likely to involve similar processes, although formal evidence of this assertion has yet to be published. In response to molecular mediators, both lymphatic and vascular endothelial cells proliferate and migrate toward a stimulus as the extracellular matrix is degraded, followed by association of the endothelial cells into tube-like structures (31, 39). New production and realignment of the extracellular matrix and controlled apoptosis at appropriate sites are required for blood vascular and lymphatic system formation. Besides using similar processes of remodeling, blood and lymphatic vessels are closely associated in vivo. Blood vascular plexuses often accompany lymphatic vessels (27, 40), although the ratio of lymphatic to blood vessels varies depending on tissue type and function (29). An abundant and neighboring blood supply provides essential nourishment for lymphatic vessels that is needed for adequate function,

intrinsic contractility of lymphatic endothelial cells, and an ability to regenerate rapidly when required, processes essential for maintaining fluid balance within an organism (31). The close association of blood and lymphatic vessels and their coordinated development in vivo suggest that some molecules may control both angiogenesis and lymphangiogenesis (41).

LYMPHATIC MARKERS

Progress in understanding lymphangiogenesis has been hampered by the very similar characteristics of blood and lymphatic vessels in tissue section and is confounded by the lack of lymphatic-specific markers (24). Consequently, visualization of lymphatic vessels in the past was restricted to imaging techniques involving the injection of dyes that are specifically taken up by the lymphatics (reviewed in refs 29, 31). Vital dyes, such as Evans blue, trypan blue, and Patent blue, which are readily taken up by lymphatic but not blood vessels, are less toxic than the materials that were previously used (29). These dyes and fluorescent conjugates of high molecular weight material such as rhodamine-dextran are now used routinely in animal experiments (37, 42). Until recently, immunohistochemical identification of lymphatic vessels was achieved, somewhat unreliably, by comparing staining of pan-endothelial markers with markers of the basal lamina. The pan-endothelial marker PECAM-1/CD31, which is expressed on both blood and lymphatic vessels (43, 44), has been used in combination with the basement membrane markers laminin and collagen type IV (45, 46). Vessels that reacted with PECAM-1 antibodies but lacked basement membrane staining and red blood cells in their lumens were deemed lymphatic (44, 47). Use of the blood vessel-specific marker PAL-E in combination with PECAM-1 has also been useful for identifying lymphatic vessels in human tissue sections (44, 47).

More accurate and simplified lymphatic vessel identification has recently been made possible by the discovery of molecules that are specifically expressed by lymphatic endothelium (**Table 1**). Vascular endothelial growth factor receptor-3 (VEGFR-3) is predominantly expressed on lymphatic endothelium in normal adult tissues (48, 49); it is also up-regulated on blood vessel endothelium in tumors (44, 50) and in wound healing (51). The lymphatic receptor for hyaluronan, LYVE-1, has been reported to be a specific marker of lymphatic vessels (52, 53) and is thought to function in transporting hyaluronan from the tissue to the lymph (53, 54). Antibodies to LYVE-1 have been used to localize the receptor to lymphatic endothelium in normal and tumor tissue (38, 52). Although appearing relatively specific for lymphatic endothelium, staining of blood vessels in normal lung tissue has been observed (R. A. Williams, S. A. Stacker, and M. G. Achen, unpublished observations) as has staining of blood vessels in normal hepatic blood sinusoidal endothelial cells (55). The transcription factor Prox1, although required for lymphatic vessel development and expressed on lymphatic endothelium (56), is also expressed in other cell types and tissues, including hepatocytes of the liver (57) and lens tissue (58), and is therefore of limited use immunohistochemically to identify lymphatic vessels. Podoplanin and desmoplakin have been reported to be markers for lymphatic endothelium, but also react with other cell types (59–61). In summary, a more extensive range of markers for lymphatic endothelium is now available that should aid in defining the role of lymphatic vessels in tumor biology.

TABLE 1. *Markers for discrimination of lymphatics and blood vessels*

Name-marker	Function	Lymphatics	Blood vessels	Reference
LYVE-1	Hyaluronan receptor on lymphatic endothelium	+++	+[a]	(52, 54, 123)
Prox-1	Homeobox gene	+++	+	(56, 129)
Podoplanin	38 kDa membrane glycoprotein of podocytes	+++	−	(60, 130, 131)
Desmoplakin	Protein located at the inner plaque of desmosomes and associates with desmosomal cadherins to form a cell adhesion complex	++	−	(61)
5′-Nucleotidase	Enzyme	+++	−	(132)
VEGFR-3	Growth factor receptor; ligands are VEGF-C and VEGF-D	+++	(+++)[b]	(44, 92, 133, 134)
VEGFR-2	Growth factor receptor; ligands are VEGF, VEGF-C, and VEGF-D	+	+++	(32, 135–137)
PAL-E	Molecule associated with endothelial vesicles	−	+++	(138, 139)
Factor VIII	Also referred to as von Willebrand factor (vWF), clotting factor	+[c]	+++	(29, 46, 140)
Laminin	Basement membrane component of enothelial cells. Involved in cell attachment and growth promotion	−	+++	(45, 141–143)
PECAM-1	CD31, a vascular cell–cell adhesion molecule	++	+++	(43, 139)

[a] Some expression has been observed on blood vessel endothelium of the normal lung (R. A. Williams, S. A. Stacker, and M. G. Achen unpublished observations). [b] VEGFR-3 is expressed on blood vessels in tumors but not in blood vessels of the normal adult. [c] See ref 29 for discussion of vWF in lymphatic endothelium.

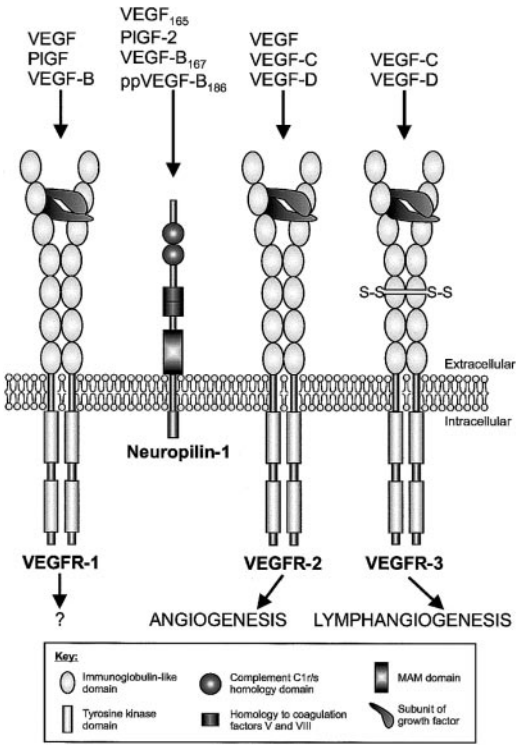

Figure 2. Receptor binding specificities of VEGF family members. VEGF receptors are shown spanning the plasma membrane. VEGFR-1, VEGFR-2, and VEGFR-3 are structurally homologous and consist of seven immunoglobulin homology domains in the extracellular region and a tyrosine kinase domain in the intracellular portion that is interrupted by a tyrosine kinase insert domain. A soluble form of VEGFR-1 exists (125) but is not shown. The extracellular domain of VEGFR-3 is proteolytically cleaved in the fifth immunoglobulin-like domain and the fragments remain associated by disulfide bonds (S-S) (126, 127). Neuropilin 1 consists of a short intracellular domain and an extracellular domain containing two complement C1r/s homology domains, two domains with homology to coagulation factors V and VIII, and a single MAM domain (128). Neuropillin-2, which also binds VEGF, is not shown here. The VEGF family members (represented as dimers) that interact with each receptor are indicated at the top of the figure and are represented in the diagram as dimers bound to the receptors. The biological consequence of signaling through VEGFR-1 is not fully understood whereas activation of VEGFR-2 and VEGFR-3 signals predominately for angiogenesis and lymphangiogenesis, respectively. Nonetheless, it is apparent that VEGFR-2 is also present on lymphatic endothelium and that VEGFR-3 can be expressed on the endothelium of tumor blood vessels. pp = proteolytically processed

LYMPHANGIOGENIC GROWTH FACTORS

Recently VEGF-C and VEGF-D, members of the VEGF family of secreted glycoproteins that are ligands for VEGFR-3 (Flt4) (34, 62–64) (**Fig. 2**, **Table 2**), have been identified as regulators of lymphangiogenesis in mammals (32, 42). Structurally, these protein growth factors differ from the angiogenic growth factor VEGF (65) because of the presence of amino- and carboxyl-terminal propeptides, but retain the central VEGF homology domain (VHD) containing the cystine knot motif that is conserved in all VEGF family members (66, 67). Biosynthetic processing of the VEGF-C and VEGF-D polypeptides, by as yet uncharacterized extracellular proteases, results in mature growth factor consisting of dimers of the VHD that bind VEGFR-3 and VEGFR-2 (KDR/Flk-1) with high affinity (68, 69). The unprocessed and partially processed forms of VEGF-C and VEGF-D have reduced affinity for both receptors, indicating that processing is important for receptor binding. VEGF-C and VEGF-D are mitogenic for lymphatic and vascular endothelial cells in vitro (34, 68, 70), and VEGF-C (62, 71), but not VEGF-D (72), can induce vascular permeability. Analysis of VEGF-C and VEGF-D function in vivo and in vitro using a range of animal-based assay systems, including the chick chorioallantoic membrane, rabbit cornea assays, and transgenic mouse models, has demonstrated the ability of these factors to drive angiogenesis and lymphangiogenesis (Table 2) (32–34, 37, 38, 73–75).

VEGF-C gene expression is induced by a range of growth factors, including platelet-derived growth factor and epidermal growth factor (76), and by numerous proinflammatory cytokines (77). Such mediators may be responsible for the induction of *VEGF-C* gene expression observed in a wide range of human tumors. In contrast to VEGF, expression of VEGF-C is not induced by hypoxia (76). Expression of the *X*-linked *VEGF-D* gene (78) is induced by the transcription factor c-fos (79) and by signaling resulting from cell–cell contact that is dependent on cadherin 11 (80). As c-fos is induced in a range of human tumors and many tumors are characterized by high cell density, these forms of regulation could induce expression of *VEGF-D* in tumor cells. VEGF-D has been reported to be regulated by the

TABLE 2. *Growth factors and receptors involved in lymphangiogenesis*

Growth factors/ receptors	Receptor/factor specificity	Evidence for role in lymphangiogenesis	Reference
VEGF-C	VEGFR-2, VEGFR-3	Ectopic expression of VEGF-C in transgenic animal	(32, 33, 62)
		In vitro analysis of recombinant protein (CAM assay)	(32)
		Adenoviral delivery to mouse skin	(108)
		Expression in tumor models	(35, 36, 104, 105)
VEGF-D[a]	VEGFR-2, VEGFR-3	Transgenic expression of VEGF-D in mouse skin	(34, 79)
		Expression in tumor model	(38)
VEGFR-3	VEGF-C and VEGF-D	Transgenic expression of VEGFR-3-Ig	(37, 42, 144)
		Activation by VEGFR-3-specific mutant	(37)

[a] Human VEGF-D binds both VEGFR-2 and VEGFR-3 whereas mouse VEGF-D binds only VEGFR-3 (145).

transcription factor AP-1 in human glioblastoma multiforme (81).

The receptor for VEGF-C/VEGF-D with specificity for lymphatic endothelium is VEGFR-3 (34, 62). This receptor is expressed on venous endothelium at sites of lymphatic vessel sprouting during embryogenesis and, in adults, becomes restricted to lymphatic endothelium (48, 49). VEGFR-3 has been shown to play an important role in remodeling and maturation of the primary capillary plexus in the early embryo (82), and VEGFR-3 mutations have been associated with hereditary lymphedema (83). Recent studies using a VEGF-C mutant that binds VEGFR-3 but not VEGFR-2 demonstrated that activation of VEGFR-3 is sufficient to induce growth of lymphatics (37). Studies using a soluble form of the VEGFR-3 extracellular domain expressed as a transgene under the control of the *keratin-14* (skin) promotor have demonstrated the requirement for VEGF-C and VEGF-D and, by implication, VEGFR-3 signaling, in lymphangiogenesis (42, 84), although the blood vasculature was unaffected (42). Evidence is now emerging from a range of such studies which suggests that VEGFR-2 is the primary receptor for angiogenesis whereas VEGFR-3 regulates lymphangiogenesis.

LYMPHATIC INVOLVEMENT IN CANCER

The metastatic spread of tumor cells is the underlying cause of most cancer-related deaths (2, 3). Clinical and pathological evidence confirms that the metastatic spread of tumors via lymphatic vessels to local/regional lymph nodes is an early event in metastatic disease for many solid human tumors (3, 20, 21). The presence of tumor cells in local lymph nodes is a significant factor in the staging of human tumors and forms the basis for surgical and radiation treatment of regional lymph nodes (3, 20, 21, 84). More recently, the use of sentinel lymph nodes has developed as a promising method for the diagnosis and staging of such diseases as breast cancer and melanoma (85–87).

Much conjecture exists in the literature regarding the existence of lymphatics within tumors (18, 22, 31, 88). Until recently, evidence linking the presence of lymphatic vessels in solid tumors with the spread of cancer was not compelling (18). This was due to the lack of suitable markers to distinguish blood vessels from lymphatic vessels, the difficulty in identifying these vessels by injection techniques, and the poorly defined structure of these vessels. Various reports of the high interstitial pressure in tumors have been used as a theoretical basis for assuming there is a lack of functional lymphatic vessels within the tumor mass (88). Recent studies using a sarcoma model demonstrated the lack of functional lymphatic vessels in a tumor expressing the lymphangiogenic factor VEGF-C and its receptor, VEGFR-3. The conclusion of that study was that the physical stress exerted by the growing tumor cells caused the collapse of the lymphatic vessels (88). In contrast, there are many historical reports of lymphatic vessels in solid tumors and anecdotal evidence that many tumors are not edematous in nature, suggesting the existence of functional lymphatic vessels in tumors (31, 89). The recent explosion of interest in the development of blood vessels within tumors did for a time overshadow the need for further study of tumor lymphangiogenesis.

LYMPHANGIOGENESIS IN CANCER

The discovery of the lymphangiogenic factors VEGF-C and VEGF-D raises the question as to whether these factors are expressed in human cancers and whether this expression is responsible for the ability of tumors to metastasize. It is already clear from a number of studies that members of the VEGF family are expressed in a variety of human tumors (90–94). VEGF-C and VEGF-D expression has been detected in a range of human tumors including malignant melanoma and lung, breast, colorectal, and gastric carcinomas (see **Table 3**) (91, 92, 94). These studies have used either immunohistochemistry or reverse transcription polymerase chain reaction (RT-PCR) to detect expression of the genes, and these techniques do not take into account the need for proteolytic cleavage to activate the polypeptides. The detection of mRNA or full-length protein may not in all circumstances reflect fully active/mature growth factor. Expression of VEGFR-3 is also an important factor in determining the potential for a lymphangiogenic response. Some studies have shown the coexpression of VEGF-C/D with VEGFR-3 in malignant melanoma (94) and lung cancer (95).

Tumor expression studies (Table 3) have allowed a direct comparison of VEGF-C and VEGF-D expression with clinicopathological factors that relate directly to the ability of primary tumors to spread (e.g., lymph node involvement, lymphatic invasion, secondary metastases, and disease-free survival) (95–98). In many instances, these studies demonstrate a statistical correlation between the expression of lymphangiogenic factors and the ability of a primary solid tumor to spread. For example, levels of VEGF-C mRNA in adenocarcinoma of the lung are associated with lymph node metastasis (99) and in breast cancer correlate with lymphatic vessel invasion and shorter disease-free survival (100). Non-small cell lung cancer showed a significantly increased survival of patients with tumors lacking VEGF-C compared to VEGF-C-positive tumors (101). Studies by Hashimoto et al. examining samples of cervical cancer demonstrated that the level of VEGF-C mRNA detected by RT-PCR was the sole independent factor influencing pelvic lymph node metastases (102). The majority of these studies showed significant correlation between VEGF-C levels and the clinical parameters of tumor spread (see Table 3), indicating the close association between expression of this lymphangiogenic factor and tumor metastasis. Some studies have suggested that expression of VEGF-D in human tumors is reduced relative to VEGF-C (99,

TABLE 3. *Clinical data showing a relationship between expression of the lymphangiogenic growth factors VEGF-C and VEGF-D and the metastatic spread of tumors*

Tumor studied	Markers examined	Technique	Comments	Reference
Thyroid tumors	VEGF, VEGF-C	RT-PCR[a]	VEGF-C expression correlates with lymph node invasive tumors	(96)
Gastric cancer	VEGF, VEGF-C	RT-PCR	Correlation between grade of VEGF-C expression pattern and the lymph node status	(97)
	VEGF, VEGF-C	IHC[b]	VEGF-C expression correlated with lymphatic and venous invasion. VEGF-C expression had a significant negative impact on prognosis in patients which did not express VEGF.	(146)
Prostate cancer	VEGF-C	ISH[c]	Higher expression of VEGF-C mRNA in lymph node positive patients	(147)
Adenocarcinoma of the lung	VEGF-A, B, C, and D	RT-PCR	Lymph node metastasis associated with high VEGF-C expression levels and low VEGF-D expression levels	(99)
Non-small cell lung cancer (NSCLC)	VEGF and VEGF-C	RT-PCR	Expression of both VEGF and VEGF-C in the primary tumor was significantly associated with nodal microdissemination	(101)
	VEGF-C	IHC	VEGF-C expression was significantly associated with lymph node metastasis, lymphatic vessel invasion and poor outcome for the patient	(95)
Colorectal cancer	VEGF-C	RT-PCR	Expression of VEGF-C mRNA correlated with lymph node metastasis, lymphatic involvement and depth of invasion	(98)
	VEGF, VEGF-C, VEGF-D	RT-PCR, IHC	Expression of VEGF but not VEGF-C and VEGF-D mRNA correlated with lymphatic metastases	(148)
Cervical cancer	VEGF-C	RT-PCR	VEGF-C expression was found to be the sole independent factor influencing pelvic lymph node metastases	(102)
Esophageal cancer	VEGF-C	IHC	VEGF-C expression correlated with depth of tumor invasion, tumor stage, venous invasion, lymphatic invasion and lymph node metastasis	(149)
Head and neck squamous cell carcinoma	VEGF, VEGF-C, VEGF-D	RT-PCR	Increased expression of all four isoforms of VEGF, increased expression of VEGF-C, but decreased expression of VEGF-D. Levels of VEGF-C had predictive value for cervical node metastases.	(103)
Breast cancer	VEGF-C	RT-PCR, IHC	VEGF-C expression correlated with lymphatic vessel invasion. Disease-free survival of the VEGF-C positive group was significantly poorer	(100)

[a] Reverse transcription polymerase chain reaction used to detect mRNA in tissue samples. [b] Immunohistochemistry used to detect protein in tissue sections. [c] In situ hybridization used to detect mRNA in tissue sections.

103); further studies are required to examine this potential difference.

Although studies showing a correlation between expression levels of *VEGF-C* mRNA and clinical parameters are suggestive of a role for lymphangiogenic factors in promoting tumor spread, no direct demonstration of VEGF-C or VEGF-D involvement had been documented until recently. Studies using expression of VEGF-C and VEGF-D polypeptides in various tumor backgrounds in animal models have provided direct evidence that these factors can indeed promote tumor lymphangiogenesis and spread (**Table 4**). Three studies in which full-length VEGF-C was overexpressed in tumor cells showed that the presence of VEGF-C polypeptide induced the formation of lymphatic metastases in regional lymph nodes (see Table 4). A study by Skobe et al. showed that expression of VEGF-C in the breast cancer cell line MBA-MD-435 induced increased lymphangiogenesis, but not angiogenesis, in tumors grown in immunocompromised mice (36). These tumors spread to local lymph nodes and the lung whereas control tumors lacking VEGF-C did not, demonstrating that VEGF-C could drive metastatic spread. Others have used Rip1Tag2 transgenic mice to analyze the activity of overexpressed VEGF-C in a pancreatic β cell tumor model (35). In these tumors, VEGF-C promoted the development of peri-tumoral lymphatics and this correlated with an increased rate of metastatic spread to the draining pancreatic lymph nodes. Studies in which the breast tumor cell line MCF-7 expressing recombinant VEGF-C was implanted orthotopically in SCID mice have shown the role played by VEGF-C in promoting tumor growth (104). Unlike lines expressing VEGF, which showed increased angiogenesis, the VEGF-C expressing lines promoted growth of only tumor-associated lymphatics. Inhibition of this growth by soluble VEGFR-3 protein showed the potential for tumor growth and metastasis to be inhibited with reagents that

TABLE 4. *Experimental data showing a relationship between expression of lymphangiogenic growth factors VEGF-C and VEGF-D and the metastatic spread of tumors*

Tumor type[a]	Lymphangiogenic growth factor studied[b]	Method of protein production[c]	Results/observations	Reference
MBA-MD-435	VEGF-C	Overexpression	Increased incidence of metastases from the primary tumor expressing full-length VEGF-C to both the regional lymph node and the lung	(36)
Pancreatic β cell tumors	VEGF-C	Rip1Tag2 model	Mice overexpressing VEGF-C produced tumors that spread to the pancreatic lymph nodes via peritumoral lymphatics	(35)
293	VEGF-D	Overexpression	Increased lymphangiogenesis and angiogenesis resulting in increased tumor growth and metastatic spread of primary tumors overexpressing VEGF-D to local lymph nodes	(38)
MCF-7	VEGF-C	Overexpression	VEGF-C promoted the growth of tumor-associated lymphatics, which were infiltrated with tumor cells	(104)
MeWo	VEGF-C	Overexpression	Enhanced tumor angiogenesis, intratumoral lymphatics, and recruitment of macrophages	(105)
AZ521	VEGF-C	Overexpression	VEGF-C induced an increase in the number of mice with lymph node metastases and an increase in the number of nodes involved	(150)

[a] Represents the cell line or tumor type in which the lymphangiogenic growth factor is expressed. MBA-MD-435 human carcinoma of the breast; tumors of the β cells of the pancreatic islets; 293 cells form an epitheliod tumor; MCF-7 carcinoma of the breast; MeWo malignant melanoma; AZ521 human gastric carcinoma. [b] In these studies either full-length VEGF-C or VEGF-D were analyzed. [c] In general, the genes were overexpressed using a range of mammalian expression vectors. The Rip1 Tag2 mice are transgenic animals in which expression of VEGF-C is driven by the rat insulin promoter and targeted to the β cells of the endocrine pancreas.

block VEGFR-3 signaling (104). Other studies have used the melanoma cell line MeWo to show the effects of VEGF-C overexpression on the metastatic behavior of tumors grown in vivo (105). These studies showed that MeWo cells expressing VEGF-C induced increased levels of both lymphangiogenesis and angiogenesis, which is in contrast to previous experiments in which VEGF-C was expressed in tumor models. This may reflect the degree of proteolytic processing of the growth factor, and was also seen in an earlier study analyzing the effect of VEGF-D (19, 38). This study also reported the recruitment of macrophages into the tumors as a result of overexpression of VEGF-C. This reveals a potential function of VEGF-C and VEGF-D as immune modulators, a role that has already been demonstrated for the related angiogenic factor VEGF (106).

Our study in which cells overexpressing VEGF-D were grown as tumors in SCID/NOD mice showed that VEGF-D was capable of inducing both lymphangiogenesis and the spread of tumor cells to local lymph nodes (38). This was not seen in tumors expressing the angiogenic factor VEGF, which only induced the formation of additional blood vessels and did not induce spread of the tumor to local lymph nodes. The dependence of these effects on VEGF-D was conclusively demonstrated by the use of a monoclonal antibody (mAb) that blocked the binding of VEGF-D to both VEGFR-2 and VEGFR-3. This antibody inhibited the growth and lymphogenous spread of tumors expressing VEGF-D. In contrast to some of the studies with VEGF-C (36), overexpression of VEGF-D resulted in an increased angiogenic response in the tumors, presumably via stimulation of VEGFR-2. The difference between the studies could be due to the degree of processing of the full-length forms of the polypeptides in the various models, which may in turn influence the receptor specificity of the growth factors. Identification of the proteases that cleave VEGF-C and VEGF-D will be an important step forward in understanding how these growth factors induce tumor angiogenesis and lymphangiogenesis. It will be important to elucidate the biological function of the propeptides of VEGF-C and VEGF-D and their role in influencing the balance between angiogenesis and lymphangiogenesis. The experimental studies carried out so far do show that the expression of growth factors such as VEGF-C and VEGF-D can directly influence the development of lymphatic vessels within tumors and the rate of metastatic spread. In combination with the expression data derived from various human tumors (Table 3), these studies allow us to postulate that expression of these growth factors and the proteases that activate them may be critical for determining the metastatic potential of a tumor. However, molecules other than those of the VEGF family are likely to play a role in the development and growth of lymphatic endothelium.

THERAPEUTIC MANIPULATION OF LYMPHANGIOGENESIS

Recent experimental approaches with animal models and analysis of genetic lesions causing hereditary

lymphedema in humans have indicated that the VEGF-C/VEGF-D/VEGFR-3 signaling system drives lymphatic hyperplasia and/or lymphangiogenesis during embryonic development (32, 33, 37, 42, 83, 107) in adult tissues (108) and in or around tumors (35, 36, 38, 104). Therefore, manipulation of this pathway offers the opportunity for therapeutic strategies designed to inhibit or stimulate growth of lymphatic vessels in conditions such as lymphedema, cancer and infectious diseases.

Inhibition of lymphangiogenesis

In the context of cancer, it may be beneficial to inhibit lymphangiogenesis so as to reduce the occurrence of lymphogenous metastatic spread. Potential inhibitors of the VEGFR-3 lymphangiogenic signaling pathway include mAbs that block the binding of VEGF-C and VEGF-D to VEGFR-3. A neutralizing VEGF-D mAb that blocks binding to both VEGFR-2 and VEGFR-3 (109) inhibited angiogenesis, lymphangiogenesis, and metastatic spread via the lymphatics in a mouse tumor model that secreted recombinant VEGF-D (38). Similar studies using neutralizing VEGF-C mAbs have not yet been reported. A mAb against mouse VEGFR-3 was found to block the binding of VEGF-C and presumably VEGF-D. This mAb induced microhemorrhage from tumor blood vessels in a mouse tumor model, although the effects on tumor lymphatics were not analyzed (110).

An alternative approach to antibodies would be to sequester VEGF-C and VEGF-D with a soluble version of the extracellular domain of VEGFR-3. The potential of this approach is illustrated by a transgenic mouse study in which a soluble form of the ligand binding region of the VEGFR-3 extracellular domain was expressed in the epidermis of the skin (42). This protein construct consisted of the first three immunoglobulin homology domains of VEGFR-3 fused to the Fc-domain of the human immunoglobulin γ chain. This soluble form of VEGFR-3 inhibited fetal lymphangiogenesis; consequently, these mice developed a lymphedema-like phenotype involving swelling of the feet, edema, and dermal fibrosis (42). When delivered via an adenovirus, this soluble form of VEGFR-3 blocked the growth of peritumoral lymphatic vessels in a mouse breast cancer model (104).

An attractive approach for inhibiting the VEGFR-3 signaling pathway would involve identification of orally active small molecules that interfere with the binding of VEGF-C/D to this receptor. Rational design of such compounds will be aided by defining the structure of the complex consisting of the VEGFR-3 ligand binding domain bound to VEGF-C/D. However, peptidomimetic approaches based on the structures of the ligands or receptor, as modeled from the known structure of the VEGF/VEGFR-1 complex (111), could also be pursued to generate such inhibitors. Small molecule inhibitors of the tyrosine kinase catalytic domain of VEGFR-3 could be useful for blocking this signaling pathway. Tyrosine kinase inhibitors of the closely related VEGFR-2 have shown promise for inhibition of tumor angiogenesis, at least in animal models (112, 113). As the catalytic domains of VEGFR-2 and VEGFR-3 are closely related in structure, it is important to test all known VEGFR-2 tyrosine kinase inhibitors for activity against VEGFR-3, although it is already known that one such inhibitor, PTK787/ZK 222584, inhibits both receptors (113). A series of indolinones has recently been reported that inhibit the kinase activity of VEGFR-3 but not VEGFR-2 (114).

Inhibitors of the VEGFR-3 signaling pathway may be useful anti-cancer therapeutics via mechanisms other than blocking lymphangiogenesis. For example, Kaposi's sarcoma (KS) is characterized by the presence of a core of spindle-shaped cells that may be derived from lymphatic endothelium. VEGF-C potently induces proliferation of these cells in vitro (115) and may play a critical role in controlling KS cell growth, migration, or invasion (116). If this is the case, inhibition of VEGFR-3 signaling may be useful for inhibiting KS progression by acting on tumor cells directly. This may also be true for lymphangiomas and lymphangiosarcomas.

One potential problem that could arise from targeting the VEGFR-3 signaling pathway in cancer is that lymphatic vessel function in normal tissues could be compromised if VEGFR-3 signaling is required for the integrity/function of mature lymphatics. The role of VEGFR-3 in mature lymphatics, which express this marker, is unknown.

Therapeutic lymphangiogenesis

Lymphedema is an impairment of lymph flow from an extremity that may be caused by lymphatic vessel obstruction, ablation, lymphatic insufficiency or dysfunction, or parasitic (*filariasis*) infection (31, 117). Affected areas become swollen and fibrotic as a consequence of accumulation of fluid and insufficient protein and macromolecular uptake by lymph node macrophages (118). Another common cause of lymphedema is the removal of the entire breast (mastectomy) and axillary lymph nodes (radical mastectomy) for breast cancer treatment (119). Removal of the axillary lymph nodes impairs fluid clearance from the upper region of the chest and arm (117). Significant edema of the arm occurs in ~20% of patients who undergo mastectomy and axillary dissection, and is more common in patients who undergo radiotherapy or suffer postoperative infection (117). Late-onset or secondary edema may occur in radical mastectomy patients as a consequence of infection that affects lymphatic drainage. The use of limbs can be severely affected by lymphedema (117). It has been proposed that mastectomy patients may benefit from stimulation of lymphangiogenesis in the region of lymph node removal to aid fluid drainage and prevent side effects associated with breast cancer. Therapeutic approaches to achieve this could be based on gene therapy or direct protein application to administer VEGF-C or VEGF-D to affected sites. Alternatively,

lymphatic tissue could be transplanted from internal sites to affected skin and VEGF-C/D administered to facilitate lymphangiogenesis from the transplanted tissue. One potential danger associated with such approaches, at least in patients who have had surgery to remove primary tumors and affected lymphatics, is that lymphangiogenesis occurring near the site of tumor removal could facilitate metastatic spread from small islands of remaining tumor. It will be important to test these approaches in appropriate animal models of lymphedema and metastatic spread before proceeding to clinical trials.

CONCLUDING REMARKS

The metastatic spread of tumor cells causes the vast majority of cancer deaths. Clinicopathological analyses long ago indicated that lymphatic vessels play a very important role in the metastatic spread of cancer. Therefore, metastatic spread to lymph nodes is considered a prognostic indicator and in part determines the therapeutic approaches used for treatment. Nevertheless, it has not been clear whether tumors induce lymphangiogenesis, which facilitates metastatic spread, or whether such spread occurs via preexisting lymphatics.

Recent studies using mouse tumor models expressing VEGF-C and VEGF-D indicate that these growth factors can induce hyperplasia of peritumoral lymphatics, as well as formation of intratumoral lymphatics (35, 36, 38, 104), and that these lymphatics facilitate metastatic spread to lymph nodes (35, 36, 38). VEGF-C expression in some human tumors correlates with lymphangiogenesis and dissemination of tumor cells to lymph nodes (120–122). VEGFR-3 can be up-regulated on tumor blood vessels (92); a study using a neutralizing VEGFR-3 mAb indicated that signaling via this receptor may be critical for blood vessel integrity in cancer (110). Therefore, the VEGF-C/VEGF-D/VEGFR-3 signaling system for lymphangiogenesis constitutes a potential new target for development of anti-cancer therapeutics.

The development of approaches to block tumor lymphangiogenesis and treat lymphedema would benefit from the availability of markers specific for lymphatic endothelium. The absence of such markers has been a major problem in the field until recently. However, the advent of lymphatic markers such as VEGFR-3 (44, 49), podoplanin (59), prox-1 (56), and LYVE-1 (52, 123) will give researchers a much better opportunity to monitor the effects of potential therapeutics on lymphangiogenesis in tumors and normal tissues. Additional requirements for progress in this field are animal models of lymphangiogenesis, lymphogenous metastatic spread, and lymphedema. Fortunately, progress has been made. A recombinant adenovirus encoding VEGF-C that induces lymphangiogenesis in a rodent model was recently reported (108) and mouse models of lymphedema are now available: the so-called 'Chy' mouse, which has an inactivating *Vegfr3* mutation in its germline (124), and another mouse model in which expression of a soluble form of VEGFR-3 in skin blocks fetal lymphangiogenesis (42). Thus, many of the requisite tools for analysis of therapeutics designed to inhibit or stimulate lymphangiogenesis are now available. Clearly this is a field that will experience rapid progress in the near future. FJ

Note added in proof: Very recent findings have further illustrated the relevance of lymphangiogenesis and VEGF-D to human cancer. Beasley et al. (*Cancer Res.* **62,** 1315–1320, 2002) identified lymphangiogenesis occurring in human head and neck cancer and White et al. (*Cancer Res.* **62,** 1669–1675, 2002) reported that VEGF-D is an independent prognostic indicator for survival in colorectal cancer.

This work was supported by the National Health and Medical Research Council of Australia and the Anti-Cancer Council of Victoria. M.B. is a recipient of the Australian Post-Graduate Research Award and the Anti-Cancer Council of Victoria Post-Doctoral Fellowship. We thank Prof. Tony Burgess for critical analysis of this manuscript and Janna Stickland for assistance with figures.

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