Minireview

Lymphangiogenesis and Tumor Metastasis: Myth or Reality? 1

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Abstract
The metastatic spread of tumor cells is responsible for the majority of cancer deaths, and with few exceptions, all cancers can metastasize. Clinical findings have long suggested that by providing a pathway for tumor cell dissemination, tumor-associated lymphatics are a key component of metastatic spread. It is not known, however, whether preexisting vessels are sufficient to serve this function, or whether tumor cell dissemination requires de novo lymphatic formation (lymphangiogenesis) or an increase in lymphatic size. Lymphangiogenesis has traditionally been overshadowed by the greater emphasis placed on the blood vascular system (angiogenesis). This is due in part to the lack of identification of lymphangiogenic factors, as well as suitable markers that distinguish blood from lymphatic vascular endothelium. This scenario is changing rapidly after the identification of the first lymphangiogenic factor, vascular endothelial growth factor C (VEGF-C). Increased expression of VEGF-C in primary tumors correlates with increased dissemination of tumor cells to regional lymph nodes in a variety of human carcinomas. Here I will review what is known about the mechanisms of lymphangiogenesis, particularly in the context of metastatic tumor spread, and will critically examine the role of VEGF-C in this process. However, despite recent progress in this field, it remains to be determined whether inhibition of lymphangiogenesis is a realistic therapeutic strategy for inhibiting tumor cell dissemination and the formation of metastasis.

Introduction

With few exceptions, all cancers can metastasize. Metastasis unequivocally signifies that a tumor is malignant, and the metastatic spread of tumor cells is responsible for the majority of cancer deaths. Tumor dissemination may occur through a number of pathways: (a) local tissue invasion; (b) direct seeding of body cavities or surfaces; (c) hematogenous spread; and (d) lymphatic spread. Clinical and pathological observations suggest that for many carcinomas, transport of tumor cells via lymphatics is the most common pathway of initial dissemination, with patterns of spread via afferent lymphatics following routes of natural drainage. Sentinel lymph nodes are a variable but limited set of nodes that are the first to receive drainage from any given location. As a rule, carcinomas preferentially metastasize to these lymph nodes, although intralymphatic tumor cells can pass directly into the blood vascular system through venolymphatic communications, and vice versa. Sentinel lymph node biopsy and histopathological examination improve tumor staging and facilitate the planning of therapeutic strategies (reviewed in Refs. 1–3).

Despite these well-established principles, many key questions regarding the mechanisms of lymphatic tumor spread still remain unanswered:

(a) Does the de novo formation of lymphatic capillaries (lymphangiogenesis) and/or lymphatic enlargement dilatation increase the probability of lymphatic tumor dissemination beyond that which would occur exclusively through preexisting vessels?

(b) What are the molecular mechanisms of lymphangiogenesis and lymphatic enlargement?

(c) Is the process of tumor cell intravasation into lymphatics analogous to that which occurs in the blood vascular system?

(d) Is inhibition of lymphangiogenesis a realistic therapeutic strategy for inhibiting tumor cell dissemination and the formation of metastasis?

It has long been suggested that “A lymphatic system as an anatomical entity is not demonstrable in tumors” (4), and studies in human and animal tumors involving injection of tracers into lymphatics revealed that tumors do not have an intrinsic lymphatic vascular supply (see, for example, Refs. 5 and 6). However, it has been proposed that this reflects the collapse of lymphatics within the tumor due to mechanical stress generated by proliferating cancer cells, and that this in turn contributes to increased pressure within the tumor interstitium (7). Although the lack of intratumoral lymphatics appears to be a consistent feature, dilated and engorged lymphatics in peritumoral stroma, which occasionally penetrate into the tumor periphery, are features that are observed with equal frequency (7, 8). It has been suggested that these lymphatics are likely to be existing lymphatics that have enlarged in response to VEGF-C 3 , rather than new lymphatic vessels (lymphangiogenesis; Ref. 7).

The presence of tumor cells in peri- or juxtatumoral lymphatics is not an uncommon feature for many primary tumors. Does lymphatic dissemination of tumor cells require active tumor cell intravasation into lymphatic vessels? In a detailed study on breast carcinoma, Hartveit (8) has described the presence of an open lymphatic labyrinth in close association with the primary tumor and suggests that “Tumor cells lying free in the

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; bFGF, basic fibroblast growth factor; TNF, tumor necrosis factor.
Mechanisms of Lymphangiogenesis in Vivo

Before considering the mechanisms of lymphangiogenesis, it is necessary to recall the morphological features that distinguish lymphatic capillaries from capillaries of the blood vascular system. Like the latter, lymphatic capillaries are lined by a single layer of endothelial cells. However, unlike blood capillary endothelium, lymphatic endothelial cells have poorly developed junctions with frequent large interendothelial gaps. Lymphatic endothelial cells are also devoid of fenestrations. Because pressure within lymphatic capillaries is only slightly higher than the interstitium, lumen patency is maintained by anchoring filaments that connect the abluminal surfaces of endothelial cells to the perivascular extracellular matrix. In contrast to capillary blood vessels, lymphatic capillaries do not possess a continuous basement membrane, and they are devoid of pericytes. However, like blood vascular endothelium, lymphatic endothelial cells contain Weibel-Palade bodies, and they are immunoreactive for von Willebrand factor and CD31 (platelet/endothelial cell adhesion molecule 1).

During early development of the blood vascular system (vasculogenesis), new capillaries arise either de novo from mesoderm-derived angioblasts or by sprouting from newly formed vessels. How do new lymphatic capillaries arise during embryogenesis? Studies on lymphatic ontogeny performed almost 100 years ago, in which injection techniques were combined with serial sectioning, led to the hypothesis that lymphatics arise through progressive sprouting from veins (see, for example, Refs. 9 and 10). However, recent studies have revealed that lymphatic capillaries also develop de novo from putative lymphangioblasts (11), as originally proposed at the beginning of the last century (12, 13). Two theories have therefore emerged regarding the mechanisms of vasculogenesis (formation of the blood vascular tree during development) and embryonic lymphangiogenesis: (a) local de novo differentiation of blood or lymphatic endothelium from angioblasts or lymphangioblasts, respectively; and (b) sprouting from preexisting blood capillaries (vasculogenesis/angiogenesis) or veins [lymphangiogenesis (reviewed in Refs. 14 and 15)].

Exquisitely detailed descriptive studies have revealed the mechanisms of lymphangiogenesis in adult tissues, although very little is known about the molecules involved. Thus, during wound healing, lymphatic capillaries grow by sprouting as extensions from preexisting lymphatics, much in the same way as new blood capillaries arise by sprouting from preexisting capillaries or postcapillary venules during angiogenesis. The appearance of new lymphatic capillaries is always secondary to that of blood capillaries. Regeneration of severed lymphatics is therefore slower than that of blood capillaries, although linear growth occurs at a comparable speed (16, 17). Lymphatic capillaries are less labile than blood capillaries: they send out fewer sprouts, anastomose less frequently, and show much less tendency to retract or undergo changes in size or form. Although temporary lymphedema occurs after lymphatic disruption, this usually resolves due to spontaneous regeneration or reconnection of lymphatics (18–20). Therefore, despite microsurgical advances in the repair of severed arteries, veins, and nerves, disrupted lymphatics are usually not identified or reconnected during surgical intervention. In a study on incisional and punch biopsy skin wounds, Paavonen et al. (21) have confirmed that new lymphatic vessels appear a few days after blood vessels in the peripheral granulation tissue. They also found that in resolving inflammation at the end of the wound-healing process, lymphatic capillaries tended to involute more rapidly than blood capillaries. Extensive lymphatic proliferation has also been observed in acute and chronic inflammation outside of the wound-healing process (22). Newly formed lymphatics regress on resolution of the inflammatory process.

Lymphangiogenesis has traditionally been overshadowed by the greater emphasis placed on the blood vascular system (angiogenesis). This is due in part to the lack of identification of lymphangiogenic factors, as well as suitable markers with which to distinguish blood from lymphatic vascular endothelium. However, this scenario is changing rapidly following the discovery of the first lymphangiogenic factor, VEGF-C (23, 24).

VEGF-C

VEGF-C is a member of the VEGF family of growth factors, which are highly conserved secreted glycoproteins that regulate vasculogenesis, hematopoiesis, angiogenesis, lymphangiogenesis, and vascular permeability and are implicated in many physiological and pathological processes. To date, the VEGF family is comprised of VEGF-A, -B, -C, and -D and Orf virus VEGFs (also called VEGF-E). Of the three VEGF tyrosine kinase receptors identified thus far (VEGFR-1, -2, and -3), VEGFR-1 binds VEGF-A and –B, VEGFR-2 binds VEGF-A, -C, -D, and -E, and VEGFR-3 binds VEGF-C and -D. VEGFRs differ with respect to mechanisms of regulation and patterns of expression. For example, VEGFR-1 and -2 are expressed almost exclusively by vascular endothelial cells and hematopoietic precursors, whereas VEGFR-3 is widely expressed in the early embryonic vasculature but becomes restricted to lymphatic endothelium at later stages of development and in postnatal life (reviewed in Refs. 25 and 26).

VEGF-C displays a high degree of similarity to VEGF-A, including conservation of the eight cysteine residues involved in intra- and intermolecular disulfide bonding. The cysteine-rich COOH-terminal half increases the length of the VEGF-C polypeptide relative to other members of this family. Like VEGF-A, both human and murine VEGF-C are alternatively spliced (24). In addition, VEGF-C mRNA is first translated into a precursor from which the mature ligand is derived by cell-associated proteolytic processing after secretion (27). Unprocessed VEGF-C binds to VEGFR-3. Removal of its NH₂-terminal extensions increases the affinity of VEGF-C for VEGFR-3 by approximately 400-fold. Processing also allows VEGF-C to bind to VEGFR-2. Processed VEGF-C induces endothelial cell proliferation and migration, as well as increased vascular permeability in the Miles assay. However, the respective roles of VEGFR-2 and -3 in mediating the biological effects
of VEGF-C are incompletely understood. Unlike VEGF-A, VEGF-C expression does not appear to be regulated by hypoxia (28).

Gene deletion studies on VEGF-C have not been published to date. However, in a manner that is unprecedented for a gene that does not undergo imprinting, heterozygous inactivation of the VEGF-A gene is embryonic lethal (29, 30). The observation that the phenotype of VEGF-A+/− mice is more severe than that of VEGF-A−/− mice demonstrates the existence of a dose-dependent requirement for VEGF-A during embryogenesis. VEGFR-2-deficient mice die at an earlier stage in embryonic development than mice deficient in VEGF-A (31). This suggests that other VEGFR-2 ligands may compensate for the loss of VEGF-A. It remains to be determined whether this ligand is VEGF-C, or whether VEGF-A and -C are redundant in early vasculogenesis.

Based on its expression profile and its binding to VEGFR-3, VEGF-C has been implicated in the development of the lymphatic system (32). In addition, transgenic overexpression of VEGF-C using the keratin 14 promoter induces lymphatic vessel enlargement in the skin (33), and recombinant VEGF-C induces lymphangiogenesis in the chick chorioallantoic membrane (34). However, VEGF-C also stimulates angiogenesis under certain experimental conditions (35, 36). Furthermore, studies in VEGFR-3-null mice have revealed that this receptor plays an important role in the development of the early blood vascular system, before the emergence of lymphatic vessels (37). VEGF-3 is also expressed in endothelial cells of tumor blood vessels, where it is thought to play a role in tumor angiogenesis (7, 38, 39). In wound healing, however, VEGF-3 is confined to lymphatic endothelium (quiescent and regenerating) and is not expressed in blood vessels (21).

VEGF-D, an additional member of the VEGF family, contains the eight conserved cysteine residues characteristic of the VEGF family and has a cysteine-rich COOH-terminal extension similar to VEGF-C. Like VEGF-C, VEGF-D is proteolytically processed after secretion, and it binds to and activates VEGFR-2 and -3 (40–42). Clearly, this VEGFR-3 ligand could also play a role in (tumor) lymphangiogenesis.

In summary, in the normal adult organism, VEGF-C appears to be a lymphangiogenic factor, and VEGF-3 is restricted to lymphatic endothelium. VEGF-2, in contrast, is expressed by both blood vascular and lymphatic endothelium. However, VEGF-C also induces the formation of new blood vessels, but this appears to be restricted to early development and certain pathological settings such as tumorigenesis; in both of these settings, VEGF-3 is also expressed by blood vascular endothelium. Although there is currently no explanation for the selective effects of VEGF-C on lymphangiogenesis versus angiogenesis, this may depend on the repertoire of VEGFRs expressed (including heterodimers between the different VEGFRs) or on the extent of VEGF-C proteolytic processing.

Markers of Lymphatic Endothelium in Vivo

The second major advance in the field of lymphangiogenesis has come with the discovery of lymphatic endothelium-specific markers. These include: (a) podoplanin, a glomerular podocyte membrane mucoprotein (43); (b) Prox-1, a homeobox gene product involved in regulating early lymphatic development (44); and (c) LYVE-1, a lymphatic endothelial receptor for the extracellular matrix/lymphatic fluid glycosaminoglycan, hyaluronan (45). To date, none of these markers have been shown to retain their lymphatic specificity in the tumor setting.

With regard to VEGFR-3, although it is expressed exclusively by lymphatics in normal adult tissues, the fact that it is widely expressed in embryonic blood vascular endothelium and reexpressed in tumor blood vessels (39, 46, 47) complicates its use in studies on tumor lymphangiogenesis.

5′-Nucleotidase has also been used successfully to distinguish lymphatic from blood vascular endothelium (48, 49), and several apparently lymphatic-specific monoclonal antibodies have been reported (50, 51), although these antibodies do not appear to have found widespread use. Finally, because lymphatic capillaries lack a continuous basement membrane, immunohistochemistry for extracellular matrix components has also been used to distinguish them from capillaries of the blood vascular system (52, 53). However, this is unlikely to be reliable in tumors because angiogenic blood vessels appear to be partially or completely devoid of a basement membrane.
When segments of rat thoracic duct were cultured in a plasma clot or collagen gel, lymphatic-like capillary channels arose spontaneously from ductal endothelium (73). Newly formed channels containing patent lumina were clearly visible after 10–14 days and were surrounded by highly attenuated endothelium with abluminal anchoring filaments.

Angiogenesis (and presumably lymphangiogenesis) is characterized by the triad of endothelial proliferation, migration, and protease activity. With respect to proliferation, this was regulated positively in bovine large vessel lymphatic endothelial cells by bFGF, epidermal growth factor, and transforming growth factor-α, whereas TNF-α and interleukin-1 were inhibitory (68). With respect to migration, bFGF stimulated migration of bovine large vessel lymphatic endothelial cells, whereas TNF-α inhibited it (68). Finally, with respect to protease activity, VEGF, VEGF-C, and bFGF stimulate expression of urokinase-type plasminogen activator, urokinase-type plasminogen activator receptor, tissue-type plasminogen activator, and plasminogen activator inhibitor 1 in bovine large vessel lymphatic endothelial cells (65, 68, 71). Tissue-type plasminogen activator and plasminogen activator inhibitor 1 were also stimulated by TNF-α (60, 68).

As indicated above, to date all in vitro studies have been performed on large vessel lymphatic endothelial cells. However, postnatal lymphangiogenesis occurs by sprouting from preexisting lymphatic capillaries (see above). In the future, working with primary endothelial cells from lymphatic capillaries will be of paramount importance. In addition to in vitro studies similar to those described above, lymphatic capillary endothelial cells would be useful (a) for the identification of molecules involved in adhesive interactions with other cells (e.g., lymphocytes and tumor cells) and (b) for application of techniques of differential gene expression to identify molecular differences between blood and lymphatic capillary endothelial cells. The utility of these techniques in identifying gene expression profiles in normal versus tumor endothelial cells has recently been demonstrated (74).

**VEGF-C, Lymphangiogenesis, and Tumor Metastasis**

A number of reports have recently described a correlation between VEGF-C expression, tumor lymphangiogenesis, and the formation of lymphatic vessels in region lymphatic nodes. Thus, a significant correlation has been described in a variety of carcinomas (thyroid, prostate, gastric, colorectal, and lung carcinomas) between VEGF-C levels in primary tumors and lymph node metastases (75–80). One study has described a strong correlation between lymphatic vessel density and VEGF-C expression (81). However, in this study, no correlation was observed between lymphatic vessel density and lymph node metastases. Despite these highly suggestive correlative clinical findings, a direct role for VEGF-C in tumor lymphangiogenesis and subsequent metastasis has yet to be demonstrated, and to date there is no animal model in which these phenomena can be explored. In addition, these observations raise a number of questions regarding the mechanisms by which increased expression of VEGF-C in primary tumors results in an increase in lymph node metastases:

(a) Does increased VEGF-C expression promote an increase in lymphatic vessel density (lymphangiogenesis) and/or size?

(b) If so, is this sufficient to increase the rate of metastasis to lymph nodes?

(c) Are newly formed lymphatic vessels phenotypically different from established lymphatics, and if so, does this make new lymphatic vessels more prone to promoting metastases?

(d) Are there other functions of VEGF-C that account for the increase in lymph node metastasis? These might include the production of trophic, mitogenic, or chemotactic factors for tumor cells by VEGF-C-stimulated lymphatic endothelium.

(e) Are lymphatics passive conduits for tumor cells or can their adhesive and transport capacities be modulated?

It has recently been reported that lymphatics surrounding a VEGF-C-overexpressing tumor are enlarged, and it has been suggested that the increase in lymphatic diameter may be sufficient to increase metastasis (82). However, the respective roles of lymphangiogenesis and lymphatic hyperplasia in tumor dissemination remain to be determined. An important function of VEGF-A, besides those listed above, is to up-regulate expression of adhesion molecules in the vasculature (83, 84). Whether other members of the VEGF family affect adhesion is not known.

**Lymphangiogenesis and Other Human Pathologies**

With respect to human pathology, the importance of the lymphatic system is not restricted to tumor metastasis. Other clinical settings in which the lymphatic system is involved/perturbed include: (a) congenital hypoplasia leading to lymphedema [inactivating VEGFR-3 mutations (85, 86)]; (b) lymphedema due to impaired lymphatic drainage caused by inflammatory or neoplastic obstruction [this includes ascites due to lymphatic obstruction in peritoneal carcinomatosis (ovarian carcinoma), edema of the arm after surgery or radiotherapy for breast cancer; elephantiasis due to massive fibrosis in the inguinal region of patients with the parasitic infection filariasis]; and (c) abnormal proliferation in lymphangioma, lymphangiosarcoma, and Kaposi’s sarcoma.

Much attention is currently being devoted to the regulation of angiogenesis in acute and chronic ischemia and to the regulation of angiogenic growth factor gene expression by hypoxia. In addition, therapeutic angiogenesis is currently being tested as an alternative therapeutic strategy for chronic cardiac or peripheral ischemia. However, lymphangiogenesis has never been studied in setting of ischemia. Likewise, no evidence exists at present concerning the possible role of hypoxia in the regulation of this process.

**Summary and Perspectives**

Clinicopathological findings have long suggested that by providing a pathway for tumor cell dissemination, lymphatics are a key component of metastatic spread. Tumor cell metastasis to regional lymph nodes is thus an early event in metastatic spread, and this parameter is frequently used to predict disease outcome or to determine therapeutic strategies. However, as Wilting *et al.* (15) recently pointed out: “A completely open question is that of the development of lymphatics in tumors and...
the(ri) relation to the formation of lymphogenic metastases.” This opinion has also been expressed by others (87, 88).

Increased expression of VEGF-C in spontaneously arising human tumors has recently been reported to correlate with increased lymphangiogenesis and dissemination of tumor cells to regional lymph nodes. However, it is not known whether preexisting lymphatics are sufficient to serve this function, or whether metastasis requires the de novo formation of lymphatic capillaries (lymphangiogenesis) or lymphatic enlargement. Some authors have suggested that it is not necessary to invoke lymphangiogenesis in this setting and that preexisting peritumoral lymphatics that enlarge in response to VEGF-C in tumors will suffice (7). If this hypothesis is correct, it may be necessary to invoke nonlymphangiogenic functions of VEGF-C to account for the increase in lymph node metastasis. This might include the production of trophic, mitogenic, or chemotactic factors for tumor cells by VEGF-C-stimulated lymphatic endothelium or alterations in lymphatic endothelial-tumor cell adhesion. To date, nothing is known about these potential interactions.

Finally, in contrast to its sister field, angiogenesis (reviewed in Refs. 89–92), very little is known about the mechanisms and mediators of lymphangiogenesis. With regard to angiogenesis, an extensive effort is currently being directed worldwide to identify antiangiogenic agents, particularly for use in anticancer therapy, and many potentially useful compounds have progressed beyond preclinical studies into the early phases of clinical trials. This is largely due to the identification of key molecular mediators. The recent, almost explosive interest in lymphangiogenesis, after many decades of dormancy, will undoubtedly ensure that we move rapidly to attain similar objectives in the lymphatic system. However, it still remains to be determined whether inhibition of lymphangiogenesis is a realistic therapeutic strategy for inhibiting tumor cell dissemination and the formation of metastases.

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