Tumor and lymph node lymphangiogenesis—impact on cancer metastasis

Nadja E. Tobler and Michael Detmar[1]

Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology, ETH Zurich, Switzerland

Abstract: The extent of lymph node (LN) metastasis is a major determinant for the staging and the prognosis of most human malignancies and often guides therapeutic decisions. Although the clinical significance of LN involvement is well documented, little has been known about the molecular mechanisms that promote tumor spread via lymphatic vessels to sentinel and distal LN and beyond. However, recent discoveries have identified novel lymphatic-specific markers, and the newly discovered lymphangiogenesis factors vascular endothelial growth factor-C (VEGF-C) and VEGF-D were found to promote tumor-associated lymphatic vessel growth in mouse tumor models, leading to enhanced tumor spread to sentinel LN. Our recent findings indicate that VEGF-A also acts as a potent tumor lymphangiogenesis factor that promotes lymphatic tumor spread. VEGF-A overexpressing primary tumors induced sentinel LN lymphangiogenesis even before metastasizing and maintained their lymphangiogenic activity after metastasis to draining LN. Our recent studies showed that primary human melanomas that later metastasized were characterized by increased lymphangiogenesis and that the degree of tumor lymphangiogenesis can serve as a novel predictor of LN metastasis and overall patient survival, independently of tumor thickness. Tumor lymphangiogenesis also significantly predicted the presence of sentinel LN metastases at the time of surgical excision of the primary melanoma. Together, these findings suggest that tumor lymphangiogenesis actively contributes to cancer dissemination, that blockade of lymphatic vessel growth might inhibit tumor metastasis to LN, and that the extent of tumor-associated lymphangiogenesis could serve as a novel, prognostic parameter for the metastatic risk of human cancers. *J. Leukoc. Biol.* **80:** 691–696; 2006.

Key Words: VEGF-A · VEGF-C

INTRODUCTION

Higher eukaryotes possess two vascular systems: the blood and the lymphatic system. The blood vasculature contains a basal membrane and smooth muscle cells and pericytes surrounding the blood vascular endothelial cells. Blood flow is organized in a circular manner, driven by the heart, and the blood vasculature is the main conduit for transporting oxygen, carbon dioxide, nutrients, and metabolic products, cells of the immune system, hormones, and other factors. In contrast, the lymphatic system consists of the lymphoid organs such as thymus, bone marrow, lymph nodes (LN), tonsils, Peyer's patches, spleen, and the lymphatic vessels, which are present in almost all tissues but are absent from avascular structures such as the epidermis, hair, nails, cartilage, and cornea and from some vascularized organs such as brain and retina. Lymphatic capillaries are blind-ending in the periphery and consist of a single layer of lymphatic endothelial cells (LECs), which are not surrounded by pericytes, smooth muscle cells, or a regular basement membrane [1]. LECs are anchored to the extracellular matrix by elastic anchoring filaments, which cause the lymphatic vessels to dilate rather than to collapse when hydrostatic pressure in the tissue increases.

The major function of lymphatic vessels is to collect and to transport protein-rich interstitial fluid via LN, larger collecting lymphatic vessels, and the thoracic duct to the subclavian vein and thereby, back to the blood vascular circulation. Lymphatic flow is activated mainly by contraction of smooth muscle cells covering larger collecting lymphatic vessels, by arterial pulsations, and by the action of neighboring skeletal muscles. In larger lymphatic vessels, valves prevent backflow. In addition to its role in tissue pressure homeostasis, the lymphatic system contributes to the immune surveillance of the body by attracting and transporting activated immune cells such as dendritic cells (DC) from the skin to the regional LN [2]. In addition, lacteal lymphatic vessels within the intestinal villi are involved in uptake of dietary fat and of the fat-soluble vitamins A, D, E, and K. The lymphatic system also plays a major role in tissue repair [3, 4], chronic inflammation [5], lymphedema [6], and tumor metastasis [1, 7].

TUMOR-INDUCED LYMPHANGIOGENESIS PROMOTES LYMPH NODE METASTASIS

Metastasis, the spread of cells from the primary neoplasm to LN and to distant organs, is the most fearsome aspect of cancer.

Several pathways may contribute to the dissemination of primary malignant cancer cells: local invasion into the surrounding tissue, systemic metastasis via tumor-associated blood vessels to distant organs, and lymphatic metastasis via tumor-associated lymphatic vessels to draining (sentinel) LN, distal LN, and from there to distal organs (**Fig. 1**). The extent of LN metastasis is a major determinant for the staging and the prognosis of most human malignancies and often guides therapeutic decisions. Although the clinical significance of LN involvement is well documented, little is known about the molecular mechanisms that promote tumor spread via lymphatic vessels to sentinel and distal LN and beyond. A traditional view has assigned a rather passive role to lymphatic vessels during the process of LN metastasis, assuming that tumor cells are passively taken up by lymphatic vessels along with the protein-rich interstitial fluid [8]. It has also been proposed that entry of cancer cells into the lymphatic vasculature might be facilitated by the higher permeability of lymphatic vessels, as compared with blood vessels, and by the absence of a regular basement membrane barrier [9].

Research into the role of the lymphatic system in cancer metastasis has been hampered by the lack of specific markers that distinguish lymphatic vessels from blood vessels and by the lack of identified lymphatic-specific growth factors. However, recent discoveries have identified novel lymphatic-specific markers, including podoplanin [10], lymphatic vascular endothelial cell hyaluronan receptor-1 (LYVE-1) [11], and Prox1, a homeobox transcription factor that induces lymphatic, lineage-specific differentiation and that is essential for the embryonic development of the lymphatic system from the blood vascular system [12, 13]. Furthermore, vascular endothelial growth factor-C (VEGF-C) and VEGF-D have been discovered as novel members of the VEGF family of angiogenic factors, which specifically activate VEGF receptor-3 (VEGFR-3) expressed on lymphatic endothelium [14, 15]. Indeed, transgenic overexpression of VEGF-C in the skin promotes cutaneous lymphangiogenesis [16], whereas the targeted disruption of VEGF-C in mice leads to a failure of early lymphatic endothelial cells to migrate away from cardinal veins and to form lymphatic vessels [17].

Based on these findings, we and others [18–20] have recently discovered that VEGF-C (and also the related factor VEGF-D) promotes tumor-associated lymphatic vessel growth (tumor lymphangiogenesis) in xenotransplant and transgenic mouse tumor models and that this promotes sentinel LN metastasis. We found that in an orthotopic breast cancer model in immunodeficient mice, overexpression of VEGF-C by tumor cells induced growth and enlargement of tumor-associated lymphatic vessels within and surrounding these tumors [19]. These tumor-induced lymphatic vessels were actively proliferating and occasionally contained tumor cells. It is important that VEGF-C expression and increased tumor lymphangiogenesis were associated with enhanced tumor metastasis to sentinel LN. Together, these findings suggest that tumor lymphangiogenesis actively contributes to cancer dissemination, that blockade of lymphatic vessel growth might inhibit tumor metastasis to LN, and that the extent of tumor-associated lymphangiogenesis could serve as a novel prognostic parameter for the metastatic risk of human cancers.

The biological relevance of intratumoral lymphatic vessels has remained unclear. Proliferating intratumoral lymphatics have been observed in tumor xenotransplants and in slowly growing, chemically induced, orthotopic squamous cell carcinomas (SCC) in mice, as well as in primary human malignant melanomas of the skin, which metastasized to LN [19, 21–23]. Conversely, intratumoral lymphatic vessels within experimental tumors in mice might not be functional with regard to fluid transport [24], and a recent study has observed efficient experimental prostate cancer metastasis to LN in the absence of intratumoral lymphatics [25]. Although the functionality of lymphatics with regard to fluid transport does not necessarily reflect their ability to guide tumor cells toward the draining LN, e.g., via secretion of chemokines such as CC chemokine ligand 21, which has been shown to attract DC and CC chemokine receptor 7-expressing tumor cells toward LN [2, 26], the majority of studies indicates that peritumoral lymphatic vessels

Fig. 1. Pathways of malignant tumor cell dissemination. Several pathways can contribute to cancer cell dissemination. Metastatic cells enter the intra- or peritumoral lymphatic vessels and form metastases within the sentinel LN, which is commonly used as a prognostic marker. Further metastatic spread from sentinel LN occurs to distant LN and via the thoracic duct and the left subclavian vein to distant organs. Tumor cells likely also spread directly to distant organs via tumor-associated blood vessels or possibly, via blood vessels within the metastatic sentinel LN.
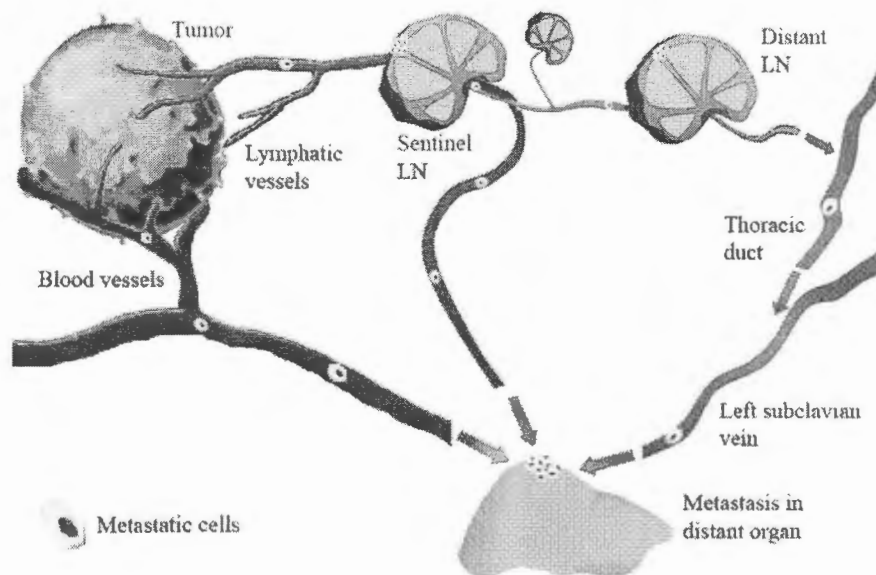
are predominantly responsible for promoting lymphatic cancer metastasis. Dilated, peritumoral lymphatic vessels are observed frequently [21, 24, 27], and they have been shown to actively proliferate [28]. It has been our own experience that quantitation of lymphatic "hot spots" in the peritumoral area of melanomas of the skin more accurately predicted LN metastasis than the evaluation of intratumoral lymphatics, and diverging results observed in different studies might be caused by different analysis methods used for the quantitation of tumor lymphangiogenesis. These include the analysis of peri- versus intratumoral vessels, of hot spots versus total tumor area, and of specific lymphatic stains (LYVE-1, Podoplanin, Prox1) versus indirect detection of lymphatic vessels (e.g., CD31-positive/pathologische anatomie Leiden-endothelium (PAL-E)-negative vessels).

In human cancers, a strong correlation between the expression levels of the lymphangiogenic factor VEGF-C (and less often, VEGF-D) and LN metastasis was found in more than 30 retrospective studies (for review, see refs. [9, 29, 30]). Our own recent studies in human cutaneous, malignant melanomas demonstrated the presence of tumor-associated lymphangiogenesis [22]. They also showed that metastatic melanomas were characterized by increased lymphangiogenesis as compared with nonmetastatic tumors and that the degree of tumor lymphangiogenesis can serve as a novel predictor of LN metastasis and overall patient survival, independently of tumor thickness. Tumor lymphangiogenesis and levels of tumor-expressed VEGF-C also significantly predicted the presence of sentinel LN metastases at the time of surgical excision of the primary melanoma [31]. Further studies involving larger numbers of cases are needed to confirm these findings. It is tempting to speculate that lymphangiogenic factors, in addition to increasing the mass of tumor-associated lymphatic vessels, might activate the lymphatic endothelium to express increased amounts of chemokines or adhesion molecules and receptors, which are involved in tumor cell-LEC interactions, thereby actively contributing to cancer dissemination.

Likely, leukocytes also play an important role in promoting tumor-associated lymphatic vessel growth and activation. Activated macrophages express VEGFR-3, and the lymphangiogenic factor VEGF-C has been shown to enhance macrophage chemotaxis [32, 33]. In turn, activated macrophages secrete lymphangiogenic factors including VEGF-C [33]. Recently, VEGF-A secreted by follicular B cells has been implicated in the mediation of LN lymphangiogenesis [34]. The relative contribution of leukocyte-derived lymphangiogenic factors remains unclear at present. It has been proposed that bone marrow-derived progenitor cells and macrophages might be physically incorporated into newly formed lymphatic vessels [35–37]. However, this has not been observed in tumor-associated lymphangiogenesis [38].

VEGF-A PROMOTES TUMOR LYMPHANGIOGENESIS AND LN METASTASIS

VEGF-A has been seen traditionally as a blood vessel-specific growth factor. However, its major signaling receptor, VEGFR-2, is also expressed by LECs in vitro and in vivo [4,

39], and treatment of cultured LECs with VEGF-A potently promotes LEC proliferation [40, 41]. Moreover, adenoviral delivery of murine VEGF-A164 into mouse ears resulted in the new formation of enlarged and tortuous lymphatic vessels, which persisted over several months [42], and transgenic delivery of VEGF-A to the skin promoted lymphangiogenesis associated with tissue repair [4] and with chronic inflammation [5]. As most human and mouse cancers strongly express VEGF-A [43] and as an association between VEGF-A production and LN metastasis has been found in several cancer types including gastric cancer [44], we hypothesized that VEGF-A might also promote tumor lymphangiogenesis and LN metastasis.

To directly investigate the biological role of VEGF-A during tumor progression, we recently created transgenic mice that overexpress VEGF-A and green fluorescent protein specifically in the skin and subjected them to a standard, chemically induced skin carcinogenesis regimen. We found that VEGF-A not only strongly promoted multistep skin carcinogenesis but also induced active proliferation of VEGFR-2 expressing tumor-associated lymphatic vessels as well as tumor metastasis to the sentinel and distant LN [28]. The lymphangiogenic activity of VEGF-A-expressing tumor cells was maintained even after their metastasis to draining sentinel LN. The most surprising finding of this study was that even before metastasizing, VEGF-A-overexpressing primary tumors induced sentinel LN lymphangiogenesis (**Fig. 2**). This suggests that primary tumors might begin preparing their future metastatic site by producing lymphangiogenic factors that mediate their efficient transport to sentinel LN. The newly identified mechanism of inducing LN lymphangiogenesis likely contributes to tumor metastasis and might represent a new therapeutic target for advanced cancer and/or for the prevention of metastasis.

The relative contribution of direct (via activation of VEGFR-2 on LECs) versus indirect effects toward the lymphangiogenic activity of VEGF-A remains to be explored. VEGF-A has been shown to induce VEGF-C expression in cultured blood vascular endothelial cells, and VEGF-A-producing transgenic tumors indeed showed higher VEGF-C protein levels than wild-type tumors [28]. In a recent study, VEGF-A-expressing tumors also contained higher numbers of macrophages [27], which might have contributed lymphangiogenic mediators. However, lymphatic vessels associated with VEGF-A-expressing tumors strongly expressed VEGFR-2, and VEGF-A-induced LEC migration in culture involves activation of specific integrin ligands, which are independent of VEGFR-3 activation [4]. As VEGF-A regulates the expression of adhesion molecules in blood vessels [45, 46], this might also be the case in lymphatic vessels. Further studies are needed to investigate the direct effects of VEGF-A on LEC gene expression, with a particular focus on adhesion molecules and chemokines, which might facilitate cancer cell and lymphatic vessel interactions.

PERSPECTIVES

In many human cancers, the lymphatic vasculature represents the most important pathway for tumor cell dissemination, and
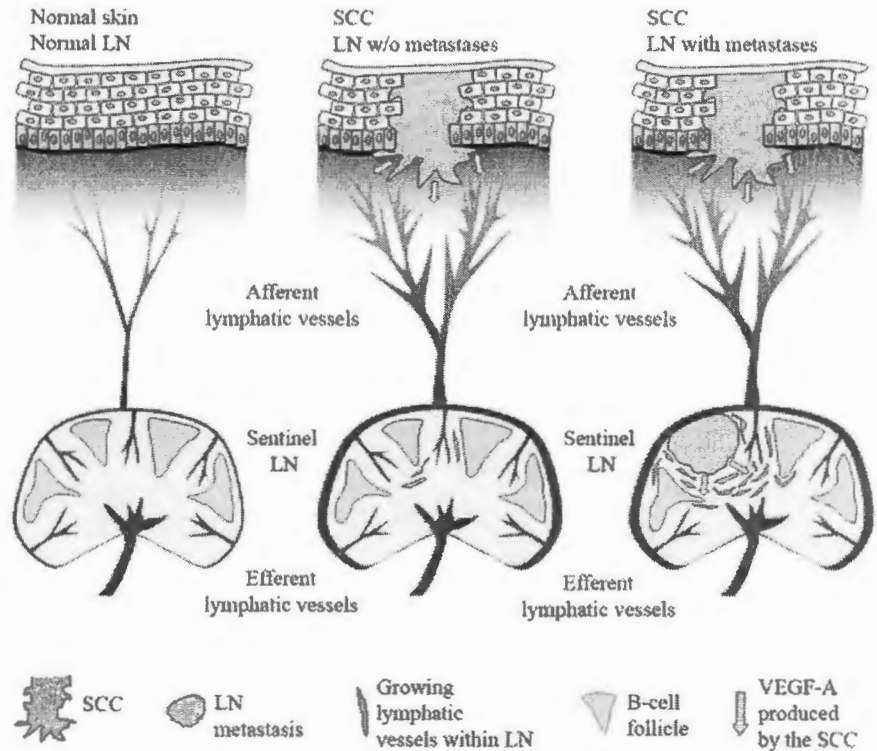
Fig. 2. VEGF-A-expressing cancer cells induce tumor and LN lymphangiogenesis. In normal skin, lymphatic vessels are present in the dermis and maintain tissue fluid homeostasis. There is no detectable lymphangiogenesis within draining LN. SCC of K14/VEGF-A-transgenic mice induce primary, tumor-associated, lymphatic vessel growth but also lymphangiogenesis within sentinel LN, even before they metastasize, possibly preparing the LN for their later arrival. Metastatic, VEGF-A-expressing SCC maintain their lymphangiogenic activity after metastasis to sentinel LN.

tumor cell metastasis to LN is an early event in their metastatic spread. Most frequently, tumor thickness is used as a prognostic parameter to evaluate the risk for future metastasis of primary tumors, but additional markers for metastatic spread are urgently needed. Our recent findings in human malignant melanomas, that increased lymphatic vessel density was the single-most significant, independent prognostic indicator to predict whether the tumors had already metastasized to the sentinel LN [22], indicate that beyond mouse models of cancer, the quantitation of tumor lymphangiogenesis might help patients and their physicians to better evaluate the disease prognosis and therapeutic options. Based on our identification of VEGF-A as a potent tumor lymphangiogenesis factor, it will be of great interest to see whether treatment with bevacizumab, a neutralizing monoclonal anti-VEGF-A antibody approved for treating patients with colon cancer, might also inhibit further metastastic spread. However, the recent identification of novel lymphangiogenesis factors (Fig. 3), including hepatocyte growth factor [47] and angiopoietin-1 [48], indicates that efficient, antilymphangiogenic therapies might need to target additional lymphangiogenic molecules (or their receptors), which are not members of the VEGF family of growth factors.

Although impressive, new knowledge about the role of lymphatic vessels in cancer progression has been acquired over the last few years, there still remain a number of open questions: What are the molecular mechanisms that control the interaction of cancer cells with lymphatic endothelium? Are there organ-specific differences between tumor lymphatics? Are there molecules that are specifically expressed by tumor-activated lymphatic endothelial cells and that might promote lymphatic metastasis? Can we identify additional molecules not related to the VEGF/VEGFR system, which mediate lymphatic vessel development and function, and if so, could these serve as targets for the development of novel, anticancer treatment strategies? Finally, is there a "lymphangiogenetic" disposition that predisposes some of us to enhanced lymphatic vessel growth upon stimulation and thus to an enhanced risk
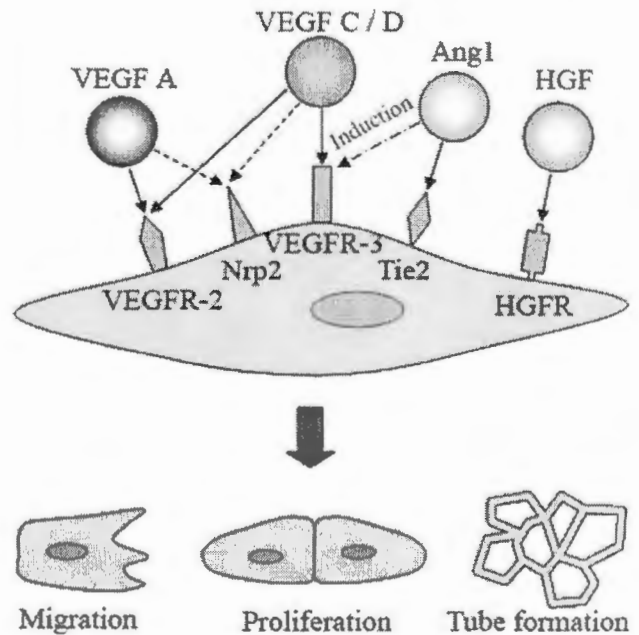
Fig. 3. Molecular control of lymphangiogenesis. Schematic representation of the major currently identified lymphangiogenesis factors and their receptors on lymphatic endothelium. Ang1, Angiopoietin-1; HGF, hepatocyte growth factor; HGFR, HGF receptor; Nrp2, neuropilin-2.

for cancer metastasis? As a result of the tremendous recent scientific interest in lymphatic research and to the development of a number of novel research tools, we might soon be able to provide an answer to some of these questions.

ACKNOWLEDGMENTS

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