Published in final edited form as:
Microvasc Res. 2007 ; 74(2-3): 145–158. doi:10.1016/j.mvr.2007.08.006.

POTENTIAL THERAPEUTIC STRATEGIES FOR LYMPHATIC METASTASIS

Bernadette M. M. Zwaans and **Diane R. Bielenberg**
Program in Vascular Biology, Department of Surgery, Children's Hospital, Harvard Medical School, Boston, Massachusetts

Abstract

Physiologically, the lymphatic system regulates fluid volume in the interstitium and provides a conduit for immune cells to travel to lymph nodes, but pathologically, the lymphatic system serves as a primary escape route for cancer cells. Lymphatic capillaries have a thin discontinuous basement membrane, lack pericyte coverage, and often contain endothelial cell gaps that can be invaded by immune cells (or tumor cells). In addition, tumor cells and stromal cells in the tumor microenvironment secrete factors that stimulate lymphangiogenesis, the growth of lymphatic endothelial cells and the sprouting of lymphatic capillaries. As a result, many tumors are surrounded by large, hyperplastic, peri-tumoral lymphatic vessels and less frequently are invaded by intra-tumoral lymphatic vessels. Carcinoma cells commonly metastasize through these lymphatic vessels to regional lymph nodes. The presence of metastatic cells in the sentinel lymph node is a prognostic indicator for many types of cancer, and the degree of dissemination determines the therapeutic course of action. Lymphangiogenesis is currently at the frontier of metastasis research. Recent strides in this field have uncovered numerous signaling pathways specific for lymphatic endothelial cells and vascular endothelial cells. This review will provide an overview of tumor lymphangiogenesis and current strategies aimed at inhibiting lymphatic metastasis. Novel therapeutic approaches that target the tumor cells as well as the vascular and lymphatic endothelial compartments are discussed.

INTRODUCTION

The Cutaneous Lymphatic System

Although the vascular system and the lymphatic system are both lined with endothelial cells, the two systems differ quite dramatically. The vascular system is a closed, circulatory system in which the heart pumps blood around the body through arteries, capillaries, and veins. In contrast, the lymphatic system is an open-ended, unidirectional system in which fluid flows from tissues back to the blood stream (Rusznyak, 1967). The cutaneous lymphatic system is depicted in Figure 1A. Initial lymphatics are blind-ended, finger-shaped vessels that protrude into the upper dermis near the epidermis. These lymphatic capillaries are lined with a thin, single layer of endothelial cells that form interdigitating, overlapping, and end-to-end-type junctions (Sauter et al., 1998). Terminal lymphatics drain the interstitial fluid and proteinous exudate that leaks from blood capillaries. Lymphatic endothelial cells (LEC) in the capillaries attach to collagen fibers in the dermal extracellular matrix via anchoring filaments composed

Correspondence: Dr. Diane R. Bielenberg, Department of Surgery, Children's Hospital, 12.211 Karp Family Research Laboratories, 1 Blackfan Circle, Boston, MA 02115, email: diane.bielenberg@childrens.harvard.edu, Tel: (617) 919-2428, Fax: (617) 730-0231.

of elastic fibers. These fibers are responsible for increasing luminal diameters of lymphatic vessels when interstitial fluid volumes are increased (Swartz and Skobe, 2001). In addition, lymphatic capillaries have an incomplete basement membrane, lack pericyte coverage, and contain frequent gaps between neighboring endothelial cells (Daróczy, 1988; Sauter et al., 1998; Schacht et al., 2004). Immune cells such as Langerhans cells in the skin can invade these interendothelial openings (Stoitzner et al., 2002).

The superficial lymphatic plexus is located in the upper dermis (near the arterial plexus) and includes a network of valve-less, lymphatic capillaries that interconnect to ensure adequate drainage even in the event that one becomes occluded (Haagensen et al., 1972). In general, veins outnumber lymphatics in the skin, but in certain regions such as the fingers, palms, soles, and pubic areas the density of lymphatic capillaries is abundant (Haagensen et al., 1972; Rusznyak, 1967). Lymphatic vessels are often found in close proximity to blood vessels, yet the two systems never intermix within the skin (Rafii and Skobe, 2003). In the dermis, lymphatic capillaries drain into larger lymphatic vessels called precollectors. The precollectors have a continuous basal lamina and contain endothelial cell protrusions into the vessel lumen that function as valves to maintain the unidirectional flow of lymph and to protect against reflux. In the skin, valves are present every 2-3 mm (Daróczy, 1988).

A deeper lymphatic plexus is found at the cutis-subcutis boundary, where precollectors drain into thicker lymphatic vessels of varying caliber called collectors (Figure 1A). Collectors have a continuous membrane, valves, and are surrounded by smooth muscle cells that contract to propel lymph toward afferent vessels of regional lymph nodes (Daróczy, 1988). The subcutaneous space contains no lymphatic capillaries, but the large collecting lymphatics in this region are found sparsely distributed and follow venous routes (Rusznyak, 1967). Lymphatic fluid enters the lymph node through several afferent vessels and usually exits via a single efferent vessel (Figure 1A). After the lymph is filtered through several lymph nodes, it is drained into larger lymphatic trunks that lead to the left lymphatic duct (thoracic duct) or right lymphatic duct and then into the subclavian veins. In this way, the lymphatic system helps to return approximately 10% of the fluid volume that escapes from the tissue capillary beds to the vascular system.

The Pathogenesis of a Lymphatic Metastasis

Metastasis, the spread of tumor cells from the primary site to distant organ environments, is the leading cause of death from cancer. Tumors are heterogeneous in that some tumor foci within the parent neoplasm have more invasive and metastatic properties than others (Fidler, 1990; Fidler, 2002). The metastatic cascade involving the vascular system has been well established (Fidler et al., 2000). But in many solid tumors, metastasis via the lymphatic system precedes metastasis via the vascular system.

Here, we outline our current understanding and the likely events in the pathogenesis of a lymphatic metastasis. Following transformation, a tumor cell continues to proliferate and receive its nutrients and oxygen by diffusion until the nodule reaches approximately 2 mm in diameter. In order to grow further, the tumor must recruit a new blood capillary network from the surrounding tissue, a process called angiogenesis (Folkman, 1971). Tumors promote angiogenesis by secreting molecules from tumor cells and stromal cells that attract vascular capillaries and stimulate their sprouting. Many of the same molecules that induce vascular endothelial cell (VEC) growth also stimulate LEC growth (Table 1) (McColl et al., 2005; Van der Auwera et al., 2006). In some patients, tumor-derived factors stimulate intra-tumoral lymphatic vessels (Dadras et al., 2003; Maula et al., 2003; Kyzas et al., 2005b; Sipos et al., 2004), while most tumors induce hyperplastic peri-tumoral lymphatic vessels (depicted in Figure 1B). The lymphatic capillaries surrounding the tumor can reach sizes of 10-50 times that of a normal lymphatic capillary. This increased lumen size may be the result of dilation,

LEC proliferation, high tumor-induced interstitial pressure, or the consequence of tumor-secreted extracellular matrix molecules that pull on the lymphatic focal adhesions keeping them in a constant state of openness.

Tumor cells capable of invading the host stroma may encounter these hyperplastic lymphatic capillaries as well as normal lymphatic capillaries. Terminal lymphatics do not have a continuous basement membrane and contain intercellular gaps that allow for fluid drainage and the infiltration of immune cells such as dendritic cells and Langerhans cells. These structural characteristics may make it easier for a tumor cell to enter the lymphatic system than the blood system (Sleeman, 2000). The basement membrane of tumor-associated lymphatic capillaries lacks laminin and collagen XVIII (Skobe et al., 2001b) and is completely lacking in some areas; therefore, tumor cells may not require as many invasive properties to invade the lymphatic system. In addition, LEC secrete chemotactic agents that attract malignant tumor cells toward areas of high lymphatic vessel density (Shields et al., 2007). Tumor cells must detach in order to be carried away in the lymph fluid, likely as small emboli, toward the afferent vessels of the sentinel lymph node. Fewer than 0.1% of tumor cells entering the blood circulation actually form metastases (Fidler, 1970), while dissemination through the lymphatic system appears much more efficient (Haagensen et al., 1972). The high flow rate and high serum concentrations in the blood stream are often toxic to tumor cells, whereas the relatively passive, low shear stress of the lymph fluid may allow for tumor cell survival (Sleeman, 2000).

Just as the lymph node acts as a filter for pathogens and immune cells, it also filters tumor cells. This filter function may initially protect the patient and prevent the early dissemination of tumor cells throughout the body. Hematogenous metastasis requires that the tumor cell extravasate through the vascular capillary out into the new organ environment, while lymphatic metastasis may not require such extravasation (Sleeman, 2000). The lymph node may simply concentrate all shed tumor cells into the same location in the reticular fibers of the marginal sinus where the emboli get trapped and subsequently proliferate in situ. The pooling of tumor cells in one site in the parenchyma of the node likely promotes their survival and further growth. Eventually, the entire node may become replaced by tumor. Tumor cells may escape from the efferent vessels of the draining lymph node to the next lymph node or to the thoracic duct and into the venous blood system for dissemination throughout the body. Alternatively, tumor cells may invade the blood vessels within the lymph node itself and metastasize to distant organs (Haagensen et al., 1972; Tobler and Detmar, 2006).

DISCUSSION

The Occurrence of Lymph Node Metastasis in Solid Tumors

Tumor cell metastasis to regional lymph nodes often marks the first step in tumor cell progression (Table 2). In general, carcinomas metastasize through the lymphatic system more often than sarcomas. In fact, carcinoma in the large intestine metastasizes almost exclusively via the lymphatics. Some carcinomas also metastasize via the vascular system. In breast cancer, the metastases usually result from early lymphatic dissemination followed by extensive spread into the vascular system in more advanced disease stages (Haagensen et al., 1972;Hess et al., 2006). Shortly after tumor formation, gastric carcinomas metastasize through both routes. Even within a certain type of cancer, location of the primary neoplasm can influence the route of metastasis. For instance, squamous cell carcinoma (SCC) in the lung metastasizes early through both hematogenous and lymphogenous paths (Haagensen et al., 1972), while SCC in the skin or cervix metastasizes late and mainly to sentinel lymph nodes (Weinberg et al., 2007).

Head and neck carcinomas frequently metastasize to lymph nodes (Table 2). Whether this is due to the fact that these tumors express high levels of lymphatic growth factors such as VEGF-

C or VEGF-D (discussed below) or whether this is partly due to structural considerations since this region contains 300 lymph nodes, is unclear. Conversely, liposarcoma, fibrosarcoma and osteosarcoma seldom metastasize to lymph nodes (Fong et al., 1993). Two possible explanations may account for the lack of lymphatic metastasis in sarcomas: their low expression of lymphatic stimulators such as VEGF-C or their secretion of endogenous lymphangiogenic inhibitors. In fact, T241 fibrosarcoma cells growing subcutaneously in a syngeneic mouse could prevent lymphangiogenesis at a distant site (cornea) in that mouse, presumably by the production of a systemic inhibitor (Chang et al., 2002). However, when T241 fibrosarcoma cells were transfected with VEGF-C, this inhibition was overridden as tumors readily metastasized to regional lymph nodes (Padera et al., 2002).

Predicting the metastatic potential of a patient's tumor is a challenging task. Evidence of tumor cells in a lymph node is often the first indicator of cancer spread. Lymph node metastasis is correlated with an increased risk of distant metastasis and a poor clinical outcome (Chen et al., 2006). For instance, 75% of prostate cancer patients with lymph node metastasis at diagnosis will possess bone metastasis in 5 years (Smith et al., 1983). According to the sentinel lymph node theory, tumor cells invade lymph nodes in sequence with the closest or draining lymph node (called the sentinel node) first, followed by the next node in line with the drainage flow, etc. (Schauer et al., 2005). Sentinel lymph node biopsy or lymphadenectomy is performed for many cancers, and the absence of tumor cells in the sentinel node predicts that other downstream nodes will likely be free of metastasis as well (Chen et al., 2006). Tumor foci in the lymph node are categorized as micrometastases (0.2 mm to ≤2 mm) or macrometastases (>2 mm). While isolated tumor cells (for example, single keratin-positive tumor cells) in the lymph node are not currently considered as micrometastases, and these lymph nodes are usually designated as “tumor-free” (Chen et al., 2006). Metastasis to any lymph node other than the regional node is considered a distant metastasis.

(Tumor-Derived) Stimulators of Lymphangiogenesis

Not all tumors metastasize to lymph nodes. Understanding the molecular cues that cause some tumors to induce lymphangiogenesis while others do not may result in the discovery and application of new strategies for disease prevention. For this reason, it is important to study the factors that regulate lymphatic metastasis (Table 1). The first proteins identified as stimulators of lymphangiogenesis were **VEGF-C** and **VEGF-D** (Joukov et al., 1996;Achen et al., 1998). Both are members of the VEGF family and bind VEGFR-3, inducing proliferation and migration of LECs. Knock-out models for VEGF-C fail to form initial lymphatic vessels during embryogenesis, indicating that VEGF-C is a vital factor in lymphatic development (Karkkainen et al., 2004). VEGF-D, on the other hand, is not required for lymphatic vessel formation during embryogenesis (Baldwin et al., 2005), but is the strongest inducer of lymphangiogenesis in the adult when given via adenoviral delivery into mouse skeletal muscle (Rissanen et al., 2003).

VEGF-C or VEGF-D can stimulate tumor lymphangiogenesis (Skobe et al., 2001a; Skobe et al., 2001b; Stacker et al., 2001; Karpanen et al., 2001; Mattila et al., 2002; He et al., 2002) and increase the metastatic spread of tumor cells to sentinel lymph nodes (Skobe et al., 2001b; Stacker et al., 2001; Mandriota et al., 2001; Mattila et al., 2002; Krishnan et al., 2003). VEGF-D expression correlates with lymph node metastasis in many human cancers including invasive breast, ovarian, cervical, undifferentiated gastric, and lung adenocarinoma (Renyi-Vamos et al., 2005; Tammela et al., 2005a; Hess et al., 2006). VEGF-C expression correlates with lymphatic vessel invasion or lymph node metastasis in human breast, cervical, colon, prostate, endometrial, esophageal, gall bladder, ovarian, pancreatic, and non-small cell lung carcinomas (Ristimaki et al., 1998; Swartz and Skobe, 2001; Stacker et al., 2002; Tammela et al., 2005a). On the other hand, VEGF-C expression did not correlate with lymph node metastasis in

neuroblastoma tumors, which typically lack lymphatic vessels (Tammela et al., 2005a). VEGF-C expression also correlates with poor prognosis or poor patient survival in many tumors, especially breast, cervical, esophageal and non-small cell lung cancer (Tammela et al., 2005a).

In some tumors there appears to be a lymphangiogenic switch. Van Trappen and colleagues report that pre-cancerous cells in the cervix (CIN-1 and CIN-2) seldom express VEGF-C and VEGF-D, but pre-invasive carcinoma in situ lesions (CIN-3) upregulate VEGF-C/D expression (Van Trappen et al., 2003). Presumably, following this switch, tumor cells recruit and invade lymphatic capillaries and travel to lymph nodes. The increase in lymph node metastasis caused by VEGF-C may be due in part to its ability to stimulate LEC proliferation thereby increasing the size of tumor-associated lymphatic vessels and increasing the chance that a tumor cell will come in contact with a LEC. Other mechanisms may include the upregulation of chemokines by VEGF-C that attract immune cells and tumor cells toward the lymphatic capillary (Shields et al., 2007). In addition, VEGF-C is a permeability factor that causes blood vessels to leak fluid thereby raising the interstitial pressure, dilating lymphatic capillaries, and forcing a greater flow of fluid to lymph nodes (Hoshida et al., 2006). Tumor-secreted VEGF-C has recently been shown to act systemically by inducing lymphangiogenesis in the sentinel lymph node even prior to tumor cell invasion (Hirakawa et al., 2007). This may prepare the lymph node for disseminated cells to come and may facilitate the further spread of cells to other nodes (Tobler and Detmar, 2006; Harrell et al., 2007).

When fully processed, VEGF-C and VEGF-D, can also bind to VEGFR-2. VEGFR-2 is expressed on both VECs and LECs (Saaristo et al., 2002). In fact, high expression of VEGF-C or VEGF-D results in increased angiogenesis in addition to increased lymphangiogenesis in some models (Skobe et al., 2001a; Roberts et al., 2006; Achen et al., 2002;). VEGF-C and VEGF-D also bind to a non-tyrosine kinase receptor called Neuropilin 2 (NRP2) (Karpanen et al., 2006a). NRP2 acts as a co-receptor with VEGFR-2 and VEGFR-3 (Soker et al., 1998; Favier et al., 2006). NRP2 knock-out mice have decreased numbers of lymphatic capillaries and veins, but surprisingly do not show edema (Yuan et al., 2002).

VEGF-A is a potent inducer of tumor angiogenesis. Recent studies demonstrate that tumor-derived VEGF-A can also increase intra- and peri-tumoral lymphangiogenesis and increase lymphatic metastasis (Bjorndahl et al., 2005b; Hirakawa et al., 2005). However, in 293EBNA tumors VEGF-A over-expression was insufficient to induce lymphangiogenesis whereas VEGF-D effectively stimulated lymphangiogenesis and lymph node metastasis (Stacker et al., 2001). Dramatic lymphangiogenesis is found when VEGF-A (165 isoform) is delivered to mice using adenoviral strategies. Surprisingly, the newly formed lymphatics do not regress for >1 year although the adenoviral VEGF-A is depleted in approximately 2 weeks (Nagy et al., 2002). VEGF-A also induces lymphangiogenesis in other non-tumor murine models including adenoviral delivery to skeletal muscle, K14-VEGF-A transgenic skin, UV-B irradiation, and corneal pellet models (Rissanen et al., 2003; Kunstfeld et al., 2004; Kajiya et al., 2006; Rogers et al., 2003). The effects of VEGF-A on lymphangiogenesis may be direct through VEGFR-2 and NRP2 expression on LECs or indirect as VEGF-A causes upregulation of VEGF-C/D in inflammatory cells (Cursiefen et al., 2004).

Most tumors express fibroblast growth factor-2 (FGF-2), also called basic FGF (for review Abuharbeid et al., 2006). **FGF-2** is a powerful mitogen for VECs and also stimulates the growth of LECs (Chang et al., 2004). Although there are currently no reports linking tumor-derived FGF-2 with lymphangiogenesis and lymph node metastasis, injection of FGF-2 protein during the early phases of tumor progression stimulated neovascularization and metastasis (Tsunoda et al., 2007). In addition, mouse corneal lymphangiogenesis is strongly stimulated with FGF-2 (Kubo et al., 2002; Chang et al., 2004). Interestingly, low-dose FGF-2 pellets can induce

lymphatic vessel sprouting in the absence of blood vessel sprouting (Chang et al., 2004; Chang et al., 2002). FGF-2 may directly affect the growth of lymphatic vessels since LEC express FGFR-3 (Shin et al., 2006). Alternately, FGF-2 upregulates VEGF-C and VEGF-D in the cornea (Kubo et al., 2002; Chang et al., 2004) and upregulates VEGF-A in tumor stroma (Tsunoda et al., 2007).

The angiopoietins (Ang) may also stimulate lymphatics partly through VEGFR-3. Two angiopoietins are known, **Ang-1** and **Ang-2**, and both play a role in vascular development in combination with VEGF-A (Sato et al., 1995). Ang-1 (adenovirus) promotes LEC proliferation, lymphatic vessel enlargement, and lymphatic vessel sprouting in vivo in adult mouse ears (Tammela et al., 2005b). Inhibitors of the VEGF-C/VEGFR-3 pathway block Ang-1-stimulated lymphangiogenesis (Tammela et al., 2005b). Alternatively, Ang may affect lymphangiogenesis directly since embryonic and adult LECs express the tyrosine kinase angiopoietin receptor, Tie2 (Morisada et al., 2005; Tammela et al., 2005b). Ang-1 protein stimulates the growth of LEC in vitro and in vivo in a mouse corneal pocket model (Morisada et al., 2005), and genetically engineered mice expressing Ang-1 in basal keratinocytes have hyperplastic dermal lymphatic vessels (Tammela et al., 2005b). Transgenic mice deficient in Ang-2 expression fail to form large collecting lymphatic vessels and have defective patterning of small lymphatic capillaries, possibly indicating that Ang-2 functions in the maturation of lymphatic vessels (Gale et al., 2002). Although the majority of tumors show an increase in Ang-1 and Ang-2 expression (for review Tait and Jones, 2004), a direct correlation between either of these factors and tumor lymphangiogenesis is lacking.

Pathways other than the classical VEGF-C/VEGFR-3 signal transduction can stimulate lymphangiogenesis as well. These include the platelet-derived growth factor (PDGF)/PDGFR pathway, the hepatocyte growth factor (HGF)/c-met pathway, and the insulin-like growth factor (IGF)/IGFR pathway. **PDGF-AA**, **PDGF-AB** and **PDGF-BB** induce mouse corneal lymphangiogenesis, with PDGF-AA being the least effective stimulator (Cao et al., 2004). Mouse fibrosarcoma-derived PDGF-BB induced peri- and intra-tumoral lymphangiogenesis, possibly through activation of the PDGFR, and promoted lymphatic metastatasis (Cao et al., 2004). Recently, **HGF** was identified as a lymphangiogenic factor. In vitro, HGF stimulates LEC to proliferate, migrate and form tube-like structures (Kajiya et al., 2005). HGF did not upregulate VEGF-C or VEGF-D in LEC, and therefore the effect of HGF was assumed to be directly through its receptor, c-met. On the other hand, corneal mouse models demonstrate lymphangiogenesis toward HGF-releasing pellets, but the neo-lymphatic vessels did not express c-met. This corneal HGF-stimulated lymphangiogenesis could be partially blocked with soluble VEGFR-3, indicating an indirect effect of HGF (Cao et al., 2006). HGF was associated with tumor lymphangiogenesis in transgenic mammary tumors (Cao et al., 2006). Infusion of rhHGF into tumors or co-injection of a fibroblast cell line producing HGF with tumor cells resulted in increased tumor lymphangiogenesis (Jiang et al., 2005). **IGF-1** and **IGF-2** stimulate the proliferation and migration of primary LECs that express IGFR-1 and IGFR-2 (Bjorndahl et al., 2005a). In vivo, IGF-1 and IGF-2 could both stimulate corneal lymphangiogenesis independently (Bjorndahl et al., 2005a). Although the effect of IGF-1 on tumor lymphangiogenesis has yet to be shown, tumor secreted-IGF-1 may indirectly affect LEC by the upregulation of VEGF-A and activation of VEGFR-2 similarly to the way it affects VEC (Akagi et al., 1998).

Potential Therapies and New Possibilities

The tumor-associated lymphatic endothelium represents a novel target for cancer therapy. Metastasis to lymph nodes results from tumor cell invasion into lymphatic capillaries. Lymphangiogenesis is documented in numerous animal models and in many human cancers as well. For many neoplasms, the lymphatic system is the primary and only route of metastasis.

The existence and functionality of intra-tumoral lymphatic vessels has been vehemently debated (Padera et al., 2002; Schneider et al., 2006), but the fact that many tumors are surrounded with grossly enlarged lymphatic vessels cannot be denied (Figure 2). Large peri-tumoral lymphatic vessels may result from: 1) pre-existing capillaries that become highly dilated due to increased interstitial pressure, 2) pre-existing capillaries that have increased their luminal diameter by proliferation of LECs (hyperplasia), and 3) neovascularization or sprouting of existing vessels toward a stimulus (tumor) that results in the increased size or density of vessels in the vicinity. Regardless of the origin, these vessels represent an increased opportunity for tumor cells to enter the lymphatic system and the increased chance of systemic dissemination. Therapies targeted toward the tumor associated-lymphatic compartment may inhibit metastasis if given early and may prevent further spread of tumor cells even if given at later time points.

Angiogenesis inhibitors have been intensely studied over the last decades. Currently, there are more than twenty-five anti-angiogenic drugs in clinical trials (Folkman, 2004; Morabito et al., 2006). The recent FDA-approval of the first exclusively anti-angiogenic agent, bevacizumab (Avastin™), a humanized monoclonal antibody to VEGF-A to be used in combination with chemotherapy for the treatment of advanced stage colon cancer, has proven beneficial to patients and pivotal for the field of vascular biology (Slevin and Payne, 2004). This clinical "proof of concept" has paved the way for other novel anti-vascular strategies and opened a new frontier in cancer therapy (Folkman, 2004). Lymphangiogenesis research has lagged behind angiogenesis, but the discovery of new lymphatic markers and an increased understanding of the growth factor signaling pathways in LEC have poised scientists to begin testing many potential anti-lymphangiogenic therapeutic strategies (Table 3). New trials will be needed to validate these pre-clinical studies and determine their medicinal significance.

Targeting Lymphangiogenic Ligands

VEGF-A is a major tumor-derived angiogenic factor, while VEGF-C is a major tumor-derived lymphangiogenic factor. Indeed, many other growth factors (FGF-2, Ang-1, VEGF-A, IGF-1, HGF) stimulate lymphangiogenesis indirectly through VEGF-C. VEGF-C is secreted by other cells in the tumor microenvironment besides neoplastic cells including fibroblasts, macrophages, platelets, and keratinocytes (Skobe and Detmar, 2000; Sleeman, 2006). VEGF-C not only induces the hyperplasia of peri-tumoral vessels, but also increases the volumetric flow rate of fluid in lymphatics that results in the increased delivery of tumor cells to the lymph node (Hoshida et al., 2006). In fact, tumors over-expressing VEGF-C were found to deliver 200-fold more tumor cells to the regional lymph node and increase lymph node metastasis 4-fold (Hoshida et al., 2006). Since angiogenic inhibitors targeting VEGF-A (ie., antibodies, kinase inhibitors, and soluble receptors) have proven effective (for review Manley et al., 2002), strategies targeting the VEGF-C pathway may prove equally effective against tumor lymphangiogenesis.

Most anti-lymphangiogenic pre-clinical studies to date have targeted the VEGF-C/VEGF-D/VEGFR-3 signaling pathway (Table 3). These strategies have included antibodies to VEGF-D, knock-down of VEGF-C using RNAi, and soluble receptors, all aimed to neutralize the effects of these ligands. VD1, a monoclonal **VEGF-D antibody**, inhibited binding of VEGF-D to both VEGFR-2 and VEGFR-3 (Achen et al., 2000). This antibody blocked lymphatic spread of VEGF-D over-expressing human 293EBNA cells in immunocompromised mice (Stacker et al., 2001). The down-modulation of VEGF-C with stable RNAi transfection in murine breast cancer cells results in reduced tumor lymphangiogenesis and lymph node metastasis (Chen et al., 2005). In a prostate cancer model, VEGF-C silencing completely abolished intra-tumoral lymphatic vessels when cells were implanted subcutaneously, but reduced lymphatic vessel density by only 50% when implanted orthotopically (Wong et al.,

2005). The reduction of lymphatic vessels in the orthotopic model was insufficient to inhibit the incidence of lymph node metastasis. These results may indicate that stromal cells of different organ environments may differ in their VEGF-C expression. Additionally, peri-tumoral vessels may be sufficient for tumor cell invasion and metastasis in some cases. It is important to note that RNAi strategies have not yet been approved for clinical use in human patients.

Inhibiting Tumor Lymphangiogenesis with Soluble Receptors

Targeting an individual ligand (eg., VEGF-C) can neutralize its effects on several receptors (eg., VEGFR-3, VEGFR-2, and NRP2), but will not affect the ability of another ligand (eg., VEGF-D) to stimulate these same receptors. Targeting a specific receptor (eg., VEGFR-2) with a kinase inhibitor or blocking antibody will inhibit its activation by multiple ligands (eg., VEGF-C, VEGF-D, or VEGF-A), but will not affect the ability of these ligands to stimulate another receptor such as NRP2 or VEGFR-3. Soluble receptors may prove more effective since they bind multiple ligands (for example, sVEGFR-2 binds VEGF-A, C, and D) and compete for binding with multiple receptors (for example, sVEGFR-2 may sequester VEGF-C away from VEGFR-2, VEGFR-3, and NRP2).

sVEGFR-3 proteins and adenoviral constructs have been the most widely used strategy for targeting tumor lymphangiogenesis. sVEGFR-3 globulin protein inhibited lymphangiogenesis and lymph node metastasis in rat and human breast cancer models (Karpanen et al., 2001; Krishnan et al., 2003). sVEGFR-3 protein was effective even when the tumor cells were engineered to express high levels of VEGF-C (Karpanen et al., 2001). Blocking lymph node metastasis in breast tumors with sVEGFR-3 protein inhibited subsequent distant lung metastases as well (Krishnan et al., 2003). The same strategy did not inhibit distant metastases in a lung carcinoma model (He et al., 2002). These results may indicate that sVEGFR-3 strategies may be more useful in cancers like breast and prostate carcinomas that initially metastasize to lymph nodes and later to distant sites.

The mode of delivery of sVEGFR-3 may influence its therapeutic potential. Tumor cells transfected with sVEGFR-3 suppressed lymph node metastasis by 66% (from 12/12 lymph node metastases in controls to 4/12 lymph node metastases in transfected mice), while sVEGFR-3 given systemically in an adenovirus completely inhibited lymph node metastasis (from 11/14 metastases in Adeno-LacZ mice to 0/28 lymph node metastases in Adeno-sVEGFR-3 mice) (He et al., 2002). At this time, transfection or viral therapies may not be desirable for human patients, but sVEGFR-3 given as a protein may be a viable strategy. sVEGFR-3 protein inhibits lymphangiogenesis in a dose-dependent manner with the most effective dose depending on the VEGF-C levels in the tumor (Karpanen et al., 2001; Krishnan et al., 2003; He et al., 2005; Karpanen et al., 2006b). sVEGFR-3 therapy can inhibit multiple stimulators of lymphangiogenesis including HGF, FGF-2, IGF-1, and Ang-1, which may prove useful considering that tumors do not always express VEGF-C directly (Bjorndahl et al., 2005a; Cao et al., 2006; Chang et al., 2004; Tammela et al., 2005b).

The timing of sVEGFR-3 delivery is also quite important. When sVEGFR-3-Fc adenovirus or adeno-associated virus was given prior to tumor cell inoculation or early in tumor progression, lymphangiogenesis and lymph node metastasis could be suppressed in several tumor models including melanoma, prostate, renal cell, and lung carcinoma (He et al., 2005; Lin et al., 2005). When systemic sVEGFR-3 therapy was started too late, tumor cells had already reached regional lymph nodes and metastasis was not affected (He et al., 2005).

Targeting Receptors on Lymphatic Endothelium

To date, nearly all attempts to target receptors on the lymphatic endothelium of tumors have been via VEGFR-3. VEGFR-3 was the first lymphatic-specific marker identified (Kaipainen et al., 1995). Blocking antibodies that target the ligand-binding domain of murine VEGFR-3 inhibit tumor lymphangiogenesis (Shimizu et al., 2004; Hoshida et al., 2006; Pytowski et al., 2005; Roberts et al., 2006; Laakkonen et al., 2007), FGF-2 induced corneal lymphangiogenesis (Kubo et al., 2002), spontaneous corneal lymphangiogenesis in *corn1* mice (Cursiefen et al., 2005), and UV-B-induced (VEGF-A-mediated) lymphangiogenesis (Kajiya et al., 2006). Early intervention (7 days after tumor cell injection) with **VEGFR-3 antibodies** reduced lymphatic vessel density and inhibited lymph node metastasis in highly metastatic gastric carcinomas (Shimizu et al., 2004). Peri-tumoral lymphatic vessel hyperplasia was also reduced with VEGFR-3 antibodies. The smaller vessel area resulted in fewer GFP-labeled tumor cells delivered to regional lymph nodes (Hoshida et al., 2006; Roberts et al., 2006).

VEGFR-3 is expressed by tumor-associated blood vessels as well as lymphatic blood vessels (Clarijs et al., 2002; Partanen et al., 1999). Therefore, VEGFR-3 antibodies inhibited tumor angiogenesis and primary tumor growth in some models (Kubo et al., 2000; Roberts et al., 2006; Laakkonen et al., 2007). Treated tumors showed reduced microvessel density and in some cases micro-hemorrhaging throughout the tumor. VEGF-C can induce tumor angiogenesis via VEGFR-2 as well as VEGFR-3 on tumor endothelial cells and a blocking antibody to VEGFR-2, called DC101, was also effective at inhibiting VEGF-C-induced tumor angiogenesis (Kadambi et al., 2001; Roberts et al., 2006). LECs express VEGFR-2, although preferentially on peri-tumoral lymphatics and less so on intra-tumoral lymphatics; and accordingly, DC101 was able to decrease tumor lymphangiogenesis (Roberts et al., 2006). In addition, DC101 was found to inhibit lymphatic vessel regeneration in cutaneous wounds in K14-VEGF-A transgenic mice (Hong et al., 2004). Lymphangiogenesis in cutaneous delayed-type hypersensitivity reactions, however, could not be inhibited with VEGFR-2 antibodies alone and required the combination of both VEGFR-2 and VEGFR-1 antibodies (Kunstfeld et al., 2004).

When comparing VEGFR-3 versus **VEGFR-2 antibodies**, VEGFR-3 antibodies were more effective at inhibiting regional lymphatic metastases and distant metastases than VEGFR-2 antibodies (Roberts et al., 2006). VEGFR-2 antibodies were more effective at inhibiting tumor growth and angiogenesis. Interestingly, even though tumor volume was reduced by 84% and tumor blood vessel area was decreased by 71% in the anti-VEGFR-2 treated group, these small weakly vascularized tumors still managed to metastasize to regional lymph nodes in 8/10 mice compared with 10/10 mice in the controls (Roberts et al., 2006). These results underscore the importance of blocking lymphangiogenesis in addition to angiogenesis. The combination of both antibodies was even more effective at inhibiting metastasis than either antibody alone (Roberts et al., 2006). A diabody, a bi-specific antibody that recognizes both VEGFR-2 and VEGFR-3 and can block the interaction of VEGF-C/VEGFR-2, VEGF-C/VEGFR-3, and VEGF-A/VEGFR-2 was recently produced by Imclone (Jimenez et al., 2005), but the efficacy of this antibody at inhibiting metastasis or prolonging survival has not been reported yet. Strategies such as these that utilize both anti-angiogenic and anti-lymphangiogenic approaches may be the most potent methods for blocking the deadly spread of cancer cells (see Table 3).

Using RNA microarray technology, investigators have compared the expression profiles of VECs and LECs (Hirakawa et al., 2003; Podgrabinska et al., 2002). VECs express α5 and αv integrins, whereas cultured LECs express **α1 and α2 integrins** (which partner with β1 subunits). Lymphatic capillaries have an incomplete basement membrane and lie in close contact to interstitial collagen. VEGF-A enhances the expression of collagen type I binding-subunits, α1 and α2, on cultured LECs (Hong et al., 2004; Senger et al., 1997). Monoclonal antibodies targeting α1 and α2, used in combination, inhibited physiologic lymphangiogenesis

in a wound healing model and inhibited pathologic lymphangiogenesis in a skin tumor model (Hong et al., 2004).

Small compounds have been used to block the kinase domains of cell surface receptors. These **kinase inhibitors** generally target one or more receptors specifically, but can have non-specific effects on other receptors. Many kinase inhibitors are currently in clinical trials. Several compounds inhibit the activities of VEGF receptors including: Semaxanib (SU5416), Sumitnib (SU11248), Vatalanib (PTK/ZK), Sorafenib (BAY 43-9006), Recentin (AZD2171), SU6668, AG-013736, MAZ51, CEP-7055 (for review Ahmed et al., 2004; Baka et al., 2006; Morabito et al., 2006). The relative effects of these inhibitors on tumor lymphangiogenesis versus tumor angiogenesis are unclear. Kinase inhibitors targeting the c-met receptor, the PDGFR, and the IGFR may target LECs in addition to tumor cells (Table 3).

Other Strategies to Suppress Lymphangiogenesis

Many different strategies are being explored to target tumor lymphangiogenesis and prevent lymphatic metastases. These potential therapies include using competive ligands of lymphangiogenic stimulators, inflammatory regulators, and endogenous inhibitors of angiogenesis/lymphangiogenesis. A novel approach may be to select those inhibitors that target both vascular and lymphatic endothelial cells as well as tumor cells (Table 3).

Competitive Ligands—Semaphorin 3F (SEMA3F) is a competitive ligand of VEGF-A and VEGF-C for Neuropilin 2 (NRP2). NRP2 is a co-receptor for VEGF-A with VEGFR-2 and a co-receptor for VEGF-C with VEGFR-3. NRP2 is also a co-receptor for SEMA3F with plexins, a family of transmembrane receptors involved in neuronal guidance. In addition to competing for VEGF binding, SEMA3F induces repulsion of NRP2-expressing VECs and LECs (Bielenberg et al., 2004). Human melanoma cells over-expressing SEMA3F have reduced microvessel density and a complete inhibition of lymph node metastasis and lung metastasis. SEMA3F-expressing tumors have no intra-tumoral lymphatic vessels and reduced peri-tumoral lymphatic vessel area as compared to control A375SM tumors that have large peri-tumoral lymphatic vessels (Figure 2) and intra-tumoral lymphatic vessels (manuscript submitted, Bielenberg and Klagsbrun). The SEMA3F chromosomal loci, 3p21.3, is often deleted in lung cancer (Roche et al., 1996; Sekido et al., 1996; Xiang et al., 1996), therefore SEMA3F has been suggested to act as a tumor suppressor. SEMA3F protein may be a novel therapy capable of inhibiting angiogenesis, lymphangiogenesis, tumorigenicity, and metastasis (Bielenberg et al., 2006).

NK4 is a competitive inhibitor of HGF binding to c-met and was effective at inhibiting HGF-induced tumor angiogenesis and tumor lymphangiogenesis (Jiang et al., 2005; Kuba et al., 2000). NK4 may also reduce neovascularization independently of HGF as it decreased VEGF- and FGF-stimulated proliferation and migration of EC in vitro as well (Kuba et al., 2000).

Endothelial Inhibitors—Surprisingly few studies have compared the endogenous or classical angiogenesis inhibitors in lymphangiogenesis models. Some in vitro studies do suggest that these compounds may affect both endothelial cell compartments. For example, Shao and colleges reported that **angiostatin**, **endostatin**, and **platelet-factor 4** could inhibit LEC proliferation (MTT assay) and migration (scratch assay) in a dose-dependent manner (Shao and Chi, 2005; Shao and Xie, 2005). On the other hand, these compounds need to be examined in vivo to truly establish their potency. For instance, thalidomide inhibited LEC growth in vitro (Shao and Xie, 2005), but was inefficient at inhibiting FGF-2 induced corneal lymphangiogenesis (Chang et al., 2004).

Interferon (IFN) α and β have been shown to regress cutaneous hemangiomas in children and to block tumor angiogenesis and metastasis in murine models by down-regulating FGF-2 and

matrix metalloproteinases (Ezekowitz et al., 1992; Ezekowitz et al., 1995; Dinney et al., 1998; Bielenberg et al., 1999; Dong et al., 1999). FGF-2 injection during the initial phase of tumorigenesis increases tumor growth and metastasis (Tsunoda et al., 2007). Tumor-secreted FGF-2 may also stimulate tumor lymphangiogenesis. IFNα inhibits LEC growth and induces apoptosis in vitro (Shao and Liu, 2006), but its effect on tumor lymphangiogenesis remains to be seen.

Thrombospondin-1 (TSP-1) is a competent angiogenesis inhibitor, and tumors resulting from chemical-induced carcinogenesis in K14-TSP-1 mice have reduced microvessel density (Hawighorst et al., 2002). Lymphangiogenesis and lymph node metastasis, on the other hand, was not diminished in tumors from TSP-1 transgenic mice possibly due to the fact that cutaneous lymphatic capillaries lack CD36, one of the TSP-1 receptors.

Inflammatory Regulators—Cyclooxygenase-2 (COX-2) expression correlates with VEGF-C expression and lymph node metastasis in numerous human cancers including lung adenocarcinoma, head and neck squamous cell carcinoma, esophageal carcinoma, gastric carcinoma and colon carcinoma (Su et al., 2004; Kyzas et al., 2005a; von Rahden et al., 2005; Zhang et al., 2005; Soumaoro et al., 2006). COX-2 over-expression induces VEGF-C but not VEGF-D in human lung adenocarcinomas and correlates with lymphatic vessel density (Su et al., 2004). **COX-2 inhibitors** decrease VEGF-C levels (Timoshenko et al., 2006). When three esophageal cell lines were treated in vitro with COX-2 inhibitors such as diclofenac, rofecoxib, and SC560, VEGF-A and VEGF-C levels decreased (von Rahden et al., 2005). The COX-2 inhibitor, celecoxib (Celebrex, Pfizer), powerfully blocked FGF-2 induced corneal lymphangiogenesis (Chang et al., 2004). Celecoxib inhibited tumor angiogenesis and microvessel density by causing apoptosis in tumor endothelial cells (Raut et al., 2004). Celecoxib also inhibits tumor lymphangiogenesis as assessed by Q-PCR and immunohistochemistry in MCF7 and MDAMB231 breast cancers (Barnes et al., 2007).

Glucocorticoids, including prednisone, hydrocortisone, and dexamethasone, inhibit tumor angiogenesis by down-regulating VEGF-A and IL-8 (Yano et al., 2006). Dexamethasone reduced VEGF-C expression in Du145 prostate tumors by 50% and inhibited tumor lymphangiogenesis (Yano et al., 2006).

Summary and Perspective

Two safety concerns that arise when considering any therapy against lymphangiogenesis is whether the therapy will specifically target abnormal tumor lymphatic vessels and spare normal lymphatic vessels or blood vessels and whether such therapy will result in lymphedema by reducing lymphatic flow and accumulating interstitial fluid (Gershenwald and Fidler, 2002). Several recent studies have indirectly addressed these concerns. sVEGFR-3 in an adenoviral construct (which results in high expression) did not appear to affect normal lymphatic vessels once mice reached adolescence, approximately 4 weeks of age (Karpanen et al., 2006b). In addition, tumor studies in adult animals using sVEGFR-3 therapy have not reported normal lymphatic vessel damage or non-specific effects on normal blood vessels (Karpanen et al., 2001; Krishnan et al., 2003; He et al., 2005; Karpanen et al., 2006b). Edema formation was not reported in any of the anti-tumor lymphangiogenic studies using sVEGFR-3, but was found in newborn transgenic mice. When mice were engineered to express sVEGFR-3-Ig under the keratin 14-promoter, which results in transgene expression in all basal keratinocytes of the skin, normal lymphatic vessels regressed and edema resulted (Makinen et al., 2001). Using locally administered protein strategies (such as soluble receptors, antibodies, or competitive inhibitors) instead of adenoviral or transgenic strategies and appropriate dosing schedules may alleviate these unwanted edema reactions.

Angiogenesis and lymphangiogenesis are both important processes contributing to tumor progression and metastasis. In order to prevent the deadly spread of cancer cells, therapeutic strategies combining inhibitors of both these endothelial compartments may be necessary. Alternatively, single compounds that target both tumor-associated blood vessels and tumor-associated lymphatic vessels may decrease tumor size and decrease the incidence of local and distant metastases (Table 3). Other strategies such as “low-dose chemotherapy” or “metronomic chemotherapy” may target the lymphatic endothelium as well as the vascular endothelium (Gately and Kerbel, 2001;Kerbel and Kamen, 2004;Kieran et al., 2005). Future studies will require the use of metastatic tumor models to elucidate the most effective strategies of inhibiting tumor progression.

Acknowledgements

This work was supported by a Howard Temin Award (CA118732-01A1) from the National Cancer Institute and by the Robert Leet and Clara Guthrie Patterson Trust Award. The authors would like to acknowledge Ethan Bickford and Kristin Johnson for illustrations and Ricardo Sanchez for histology.

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Abbreviations

Ang
: angiopoietins

COX-2
: cyclooxygenase-2

EGF
: epidermal growth factor

FGF-2
: fibroblast growth factor-2, also called basic fibroblast growth factor

FGFR
: fibroblast growth factor receptor

HGF
: hepatocyte growth factor

IGF

insulin-like growth factor

IGFR
insulin-like growth factor receptor

IL-8
interleukin-8

LEC
lymphatic endothelial cell

NRP
neuropilin

PDGF
platelet-derived growth factor

PDGFR
platelet-derived growth factor receptor

SEMA3F
semaphorin 3F

TSP-1
thrombospondin-1

VEC
vascular endothelial cell

VEGF
vascular endothelial growth factor

VEGFR
vascular endothelial growth factor receptor

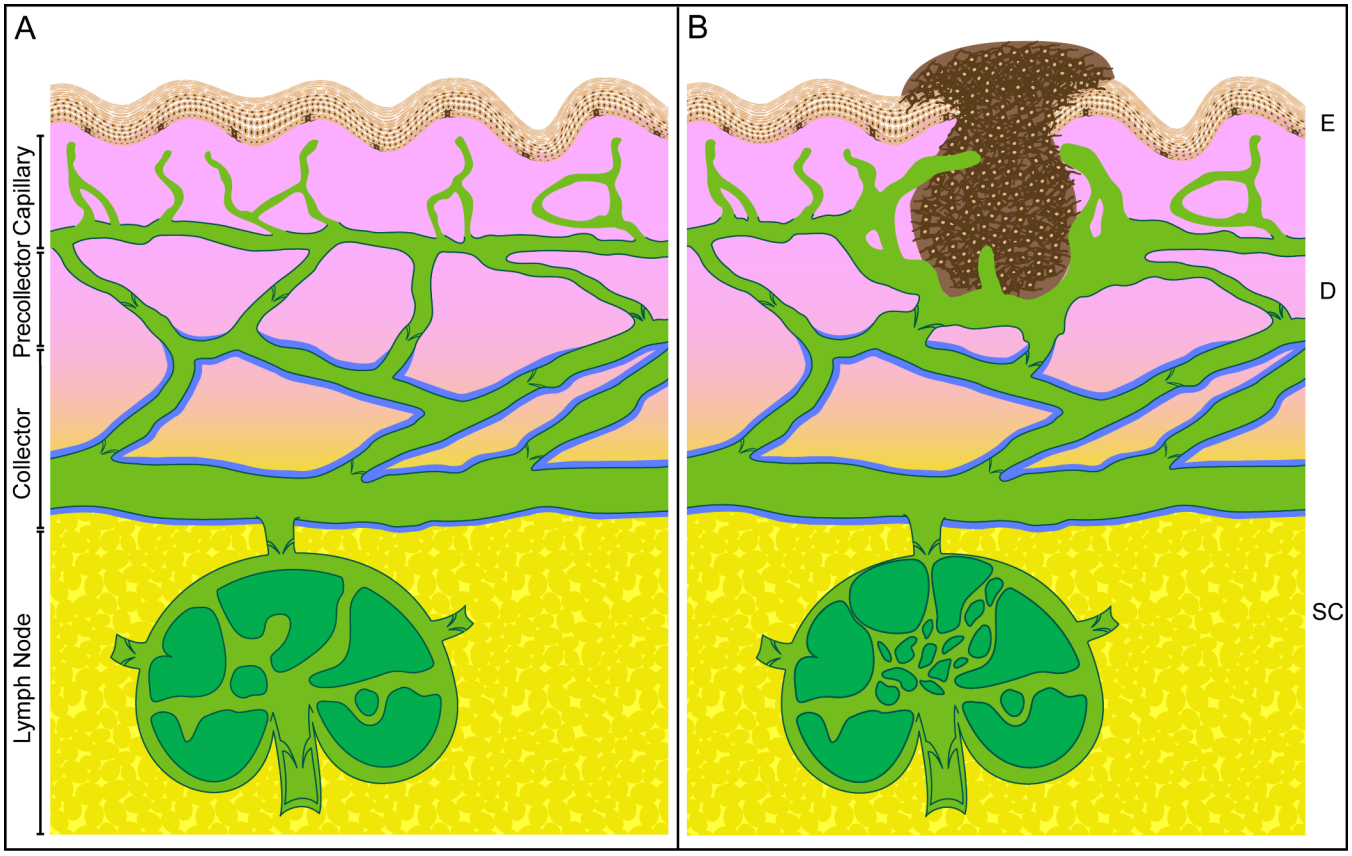

Figure 1.

Structural diagram of the cutaneous lymphatic system under physiological and pathological (tumor-bearing) conditions. **A**. Interstitial fluid is drained through wide luminal capillaries (green color) that extend up near the epidermis (E, peach color). Capillaries are composed of thin layers of endothelial cells connecting to the extracellular matrix through anchoring filaments. Capillaries possess inter-endothelial cell gaps, discontinuous basement membrane, no valves, and no pericyte coverage. In the dermis (D, pink color), capillaries drain into lymphatic vessels called precollectors that have a continuous basement membrane (denoted by dark green line) and valves that prevent the reflux of lymph. At the border to the subcutis (SC, yellow color), precollectors drain into collecting lymphatic vessels that are surrounded with smooth muscle cells or pericytes (denoted by blue line) that constrict to propel the lymph along to regional lymph nodes. **B**. An invasive melanoma (dark brown color) is shown. Tumor cells metastasize through peri-tumoral and intratumor lymphatic capillaries. Lymphatic capillary density around the tumor is increased and tumor-associated lymphatic capillaries are dilated and hyperplastic. A few lymphatic capillaries have sprouted into the tumor. The sentinel lymph node is shown with lymphangiogenic vessels as well. Note: this diagram is not drawn exactly to scale.

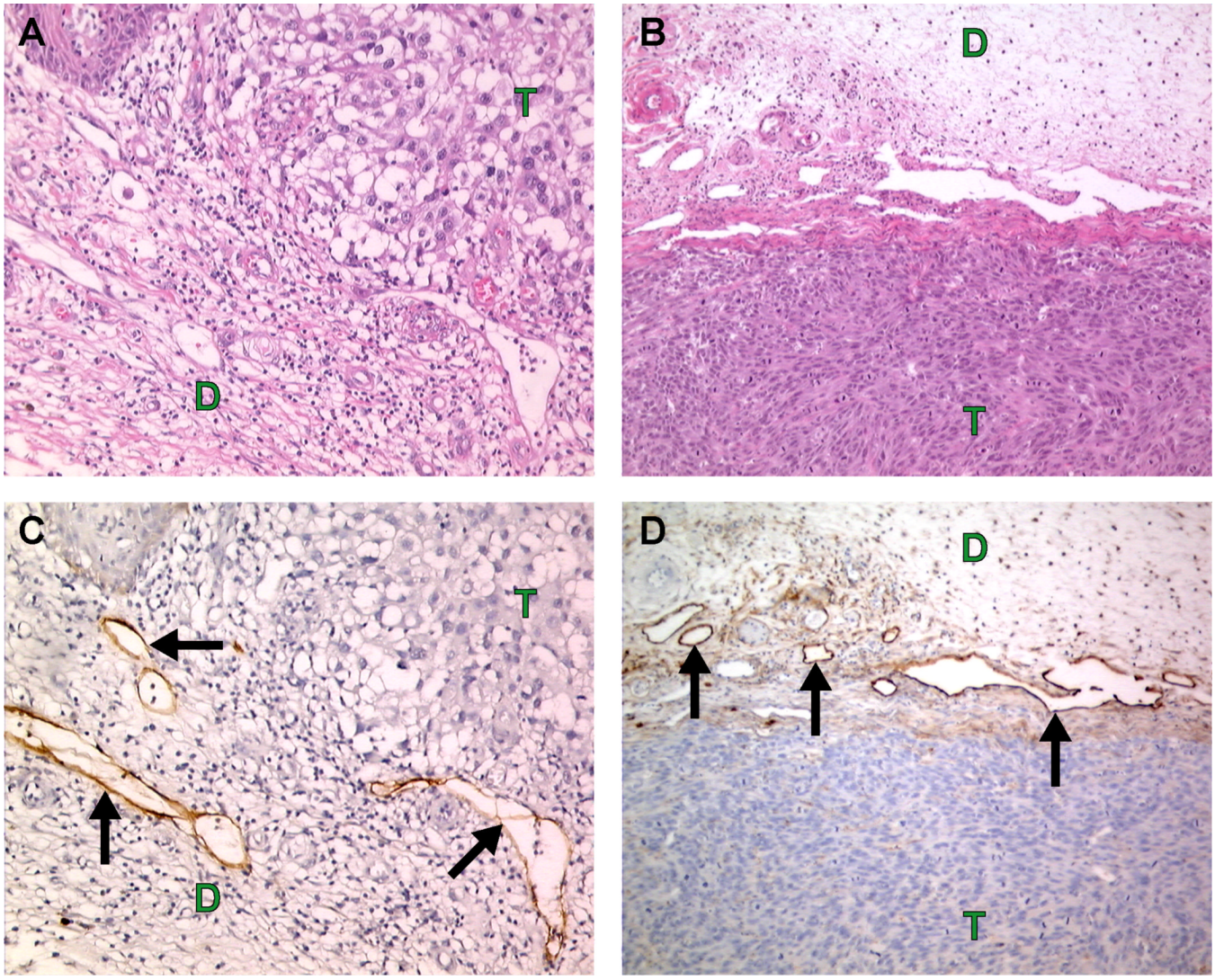

Figure 2.
Human melanoma (**A**, **C**) and human melanoma (A375SM) xenograft (**B**, **D**) are surrounded with large peri-tumoral lymphatics (arrows). **A**, **B**. Hematoxylin and eosin. **C**. Human podoplanin (D2-40 antibody, Signet Laboratories) staining (brown color). **D**. Murine podoplanin (Reliatech) staining (brown color). T = tumor, D = dermis.

Table 1

Lymphangiogenic factors.

Growth factor/cytokine	Selected references
VEGF	
• VEGF-A	(Nagy et al., 2002; Bjorndahl et al., 2005b; Hirakawa et al., 2005)
• VEGF-C	(Joukov et al., 1996; Jeltsch et al., 1997; Skobe et al., 2001b)
• VEGF-D	(Achen et al., 1998; Stacker et al., 2001; Rissanen et al., 2003)
PDGF	(Cao et al., 2004)
HGF	(Jiang et al., 2005; Kajiya et al., 2005; Cao et al., 2006)
Ang-1	(Morisada et al., 2005; Tammela et al., 2005b)
Ang-2	(Gale et al., 2002)
IGF-1/2	(Akagi et al., 1998; Bjorndahl et al., 2005a)
FGF-2 (bFGF)	(Kubo et al., 2002; Chang et al., 2004)

Table 2

Solid tumors that metastasize to lymph nodes.

Tumor type	Selected references
Common:	
Bladder carcinoma	(Shariat et al., 2006; Jones et al., 2005)
Breast carcinoma	(Chen et al., 2006; Hess et al., 2006)
Cervical carcinoma	(Pandit-Taskar, 2005)
Colorectal carcinoma	(Chen et al., 2006; Hess et al., 2006)
Endometrial carcinoma	(Sohaib et al., 2007)
Esophageal carcinoma	(Hatakeyama et al., 2006)
Head and Neck carcinomas	(Chen et al., 2006)
Hepatocellular carcinoma	(Uka et al., 2007)
Melanoma	(Chen et al., 2006; Lin et al., 2005)
Non-small cell lung carcinoma	(Hess et al., 2006; Renyi-Vamos et al., 2005)
Ovarian carcinoma	(Onda et al., 1996)
Pancreatic ductual adenocarcinoma	(Sipos et al., 2005; Von Marschall et al., 2005)
Prostate carcinoma	(Morisawa et al., 2006)
Thyroid carcinoma*	(Rodriguez et al., 2000; Scollo et al., 2003)
Less common:	
Gastric Carcinoma	(Saito et al., 2007)
Renal cell carcinoma	(Denzinger et al., 2007)
Testicular carcinoma**	(Jones et al., 2000)

* Papillary thyroid carcinoma, medullary thyroid carcinoma and anaplastic thyroid carcinoma metastasize to sentinel lymph nodes.

** Testicular carcinoma has a near 100% cure rate, but the primary site of relapse is the para-aortic lymph node.

Table 3

Potential targets and therapeutic strategies for anti-lymphangiogenesis.

Target	Strategy	Anti-		
		Angiogenesis	Lymphangiogenesis	Tumor
Ligands				
HGF	Antibody to HGF *	+	+	+
	NK4	+	+	+
VEGF-A	Antibody to VEGF-A	++	+	
	Soluble VEGFR-2	++	+	+/-
	VEGF trap (Soluble R1/R2)	++	+	
	Soluble NRP1	++	+	
	Soluble NRP2*	+	++	
	Semaphorin 3F	+	+	+
VEGF-C	Antibody to VEGF-C *	+	++	
	Soluble VEGFR-3	+	++	
	Soluble NRP2*	+	++	
	Semaphorin 3F	+	+	+
VEGF-D	Antibody to VEGF-D	+	++	
	Soluble VEGFR-3	+	++	
	Soluble NRP2*	+	++	
Receptors				
c-met	Antibody to c-met *		+	++
	c-met kinase inhibitors		+	++
Neuropilin 2	Antibody to NRP2 *	+	+	
	Semophorin 3F	+	+	+
PDGFRα/β	Antibody to PDGFR *	+	+	++
	PDGFR kinase inhibitors	+	+	++
VEGFR-3	Antibody to VEGFR-3	+	++	
	VEGFR-3 kinase inhibitors	+	++	
VEGFR-2	Antibody to VEGFR-2	++	+	+
	VEGFR-2 kinase inhibitors	++	+	+
Integrins	Antibody to integrin-α1/α2		+	
Others				
	Angiostatin	++	+	
	COX-2 inhibitors	+	+	+
	Endostatin	++	+	
	Glucocorticoids	+	+	
	Interferon α/β	++	+	++
	Platelet-factor 4	++	+	
	Semaphorin 3F	+	+	+

* These strategies have not yet been reported for anti-lymphangiogenesis.