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REVIEW

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Lymphangiogenesis and tumor metastasis

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Abstract The lymphatic system transports interstitial fluid and macromolecules from tissues back to the blood circulation, and plays an important role in the immune response by directing the traffic of lymphocytes and antigen-presenting cells. The lymphatic system also constitutes one of the most important pathways of tumor dissemination. In many human cancers, increased expression of vascular endothelial growth factor-C (VEGF-C) is correlated with regional lymph node metastases. Experimental studies using transgenic mice overexpressing VEGF-C or xenotransplantation of VEGF-C-expressing tumor cells into immunodeficient mice have demonstrated a role for VEGF-C in tumor lymphangiogenesis and the subsequent formation of lymph node metastases. However, there is at present little evidence for lymphangiogenesis in human tumors and the relative importance of preexisting vs. newly formed lymphatics for metastasis in humans remains to be determined. Nonetheless, the striking correlation between the levels of VEGF-C in primary human tumors and lymph node metastases predicts its importance in cancer spread. Aside from promoting lymphangiogenesis, VEGF-C may also activate lymphatics to promote tumor cell chemotaxis, lymphatic intravasation and hence tumor cell dissemination.

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Introduction

The lymphatic system collects extravasated fluid, macromolecules and cells of the immune system within tissues and returns them to the blood circulation. This extensive drainage network is lined by a continuous layer of endothelial cells and is interspaced by lymph nodes. It begins in the tissues as a series of blind-ending capillaries which drain into collecting vessels that return lymph to the systemic blood circulation via the thoracic duct. When lymphatic circulation is compromised, lymphedema ensues. In addition to providing a means for interstitial fluid return to the circulation, the lymphatic system plays an important role in the immune response by directing lymphocytes and antigen presenting cells to lymph nodes. With regard to pathology, the lymphatic system constitutes one of the most important pathways for tumor cell dissemination (Pepper 2001; Stacker et al. 2002; Swartz and Skobe 2001).

Because the lymphatic system is optimally suited for the entry and transport of cells (e.g., immune cells) (Witte et al. 1997), it also has many advantages over the blood circulation as a transport route for metastasizing tumor cells. The smallest lymphatic vessels are much larger than blood capillaries, and flow velocities are orders of magnitude lower. Lymph is nearly identical to interstitial fluid and promotes cell viability. In contrast, tumor cells in the bloodstream experience serum toxicity, high shear stresses and mechanical deformation, leading to an extremely low success rate for metastasis formation (Liotta et al. 1991; Weiss and Schmid-Schonbein 1989). Metastasis via lymphatics might therefore promote survival of disseminating tumor cells and consequently increase their metastatic efficiency. Nearly all investigations of the mechanisms of metastasis, such as intravasation, survival, and extravasation, have focused on tumor cell behavior in the bloodstream (Liotta et al. 1991; Zetter

1993). There is therefore an urgent need to understand how tumor cells interact with lymphatics and to develop a paradigm for lymphatic metastasis analogous to that of hematogenous metastasis (Swartz and Skobe 2001).

Despite the longstanding recognition of the involvement of the lymphatic system in many clinical settings, formal experimental demonstration of its role (including lymphangiogenesis) in the pathogenesis of lymphedema or tumor cell dissemination has until recently been lacking. This was mainly due to the lack of lymphatic-specific markers as well as identification of lymphangiogenic growth factors and their receptors. However, much progress has been made in these areas in the past decade. It is now also possible to culture pure populations of blood and lymphatic vascular endothelial cells using the molecular tools which have become available. In this respect, one can truly speak of a renaissance in the field of lymphatic endothelial biology and pathophysiology.

Lymphatic endothelial cell biology: a renaissance

Lymphatic endothelial cell markers

Until the discovery of specific markers, lymphatic vessels were identified indirectly by lymphangiography, which is based on the ability of lymphatic capillaries to take up dyes and high-molecular weight molecules from the interstitium. Most commonly used are vital dyes—Evans blue, trypan blue and patent blue—and, more recently, fluorescently labeled tracers. The absence of basement membrane components such as laminin, collagen IV and collagen XVIII, the lack of PAL-E staining of CD31-positive endothelial cells, and 5'-nucleotidase activity have also been considered lymphatic endothelial-specific criteria (Sleeman et al. 2001).

In the past few years, several positive markers specific for lymphatic endothelium have been discovered. These include vascular endothelial growth factor receptor-3 (VEGFR-3), which is predominantly expressed by lymphatic endothelial cells in normal adult tissues, but can also be expressed by blood vascular endothelial cells in tumors or during wound healing. Other transmembrane proteins such as LYVE-1 (Banerji et al. 1999) and podoplanin (Breiteneder-Geleff et al. 1999) and a transcription factor Prox-1 (Wigle and Oliver 1999) have been shown to be reliable in distinguishing lymphatic from blood endothelium, although none is strictly endothelial-specific (Sleeman et al. 2001).

Lymphangiogenic growth factors

Two lymphangiogenic factors, which are members of the VEGF family, have been identified to date: VEGF-C and VEGF-D. Other molecules which play an important role in the development of the lymphatic system include angiopoietin-2 (Gale et al. 2002), neuropilin-2 (Yuan et al. 2002) and Prox-1 (Wigle and Oliver 1999).

The VEGF family comprises several secreted glycoproteins that play a prominent role in the formation of blood and lymphatic vessels. The mammalian VEGF family consists of VEGFs-A, -B, -C and -D as well as placental growh factor (PlGF). VEGF signaling in endothelial cells occurs through three tyrosine kinase receptors (VEGFRs): VEGFRs-1, -2 and -3. VEGFR-1 binds to VEGFs-A and -B and PlGF, VEGFR-2 binds VEGFs-A, -C and -D, whereas VEGFR-3 binds only VEGFs-C and -D. In adult tissues VEGFRs-1 and -2 are predominantly expressed by blood vascular endothelial cells and signal to promote cell proliferation, migration, and angiogenesis. VEGFR-2 has also been detected on lymphatic endothelium in vivo (Nagy et al. 2002; Saaristo et al. 2002) and in vitro (Kriehuber et al. 2001; Makinen et al. 2001b; Podgrabinska et al. 2002), yet its role in lymphangiogenesis is less clear. VEGFR-3, which is widely expressed in the early embryonic vasculature, becomes restricted to lymphatic endothelial cells in the later stages of embryogenesis and in the adult (Dumont et al. 1998) and is also expressed in some fenestrated blood vessels (Partanen et al. 2000). VEGFs-C and -D are secreted as homodimers that undergo extensive proteolytic processing of their N- and C-terminal domains following secretion (Joukov et al. 1997; Stacker et al. 1999). Processing of VEGFs-C and -D alters their receptor binding affinities, thereby modulating the biological effects of these growth factors. The secreted 31-kDa form of VEGF-C/-D predominantly activates VEGFR-3, whereas the mature, fully processed 21-kDa form activates both VEGFRs-2 and -3.

VEGFs-C and -D have the dual capacity to induce lymphangiogenesis and angiogenesis, as demonstrated in a number of experimental systems including the chick chorioallantoic membrane (CAM), the rabbit cornea assay and transgenic mice (Table 1). Application of recombinant VEGF-C protein to the differentiated avian CAM or the mouse cornea (Kubo et al. 2002; Oh et al. 1997) as well as application of tumor cells onto the differentiated avian CAM (Papoutsi et al. 2000, 2001) (Table 5) leads to lymphatic endothelial proliferation and the formation of new lymphatic capillaries. Local transfer of naked plasmid DNA encoding human VEGF-C in two animal models of secondary lymphedema (one in the rabbit ear and one in the mouse tail) promoted selective proliferation of functional lymphatics associated with increased lymphatic drainage and decreased skin thickening (Yoon et al. 2003). Similar effects were obtained by administration of a single dose of recombinant VEGF-C in the same model of acquired lymphedema in the rabbit ear (Szuba et al. 2002). Viral gene delivery of fully processed VEGF-D in rat skin induced a significant lymphangiogenic effect together with a (less robust) angiogenic response, whereas viral gene delivery of VEGF-A in the same setting only induced angiogenesis (Byzova et al. 2002). In transgenic mice expressing VEGF-D or VEGF-C, or a VEGF-C mutant (VEGF-C-156S) that binds VEGFR-3 but not VEGFR-2, VEGFR-3 signaling was shown to be sufficient to mediate selective lymphangio-

Table 1 Experimental models of lymphangiogenesis

Growth factors/receptors expressed ectopically	Lymphatic marker	Animal model	References
VEGF-C	VEGFR-3	Transgenic expression in mouse skin	Jeltsch et al. 1997
	LYVE-1	Transgenic expression in mouse pancreas	Mandriota et al. 2001
	LYVE-1	Viral gene delivery in mouse skin	Enholm et al. 2001
	LYVE-1, VEGFR-3	Recombinant protein pellets implanted into mouse cornea	Kubo et al. 2002
	VEGFR-3	Application of recombinant protein to chick CAM	Oh et al. 1997
	LYVE-1, podoplanin	Viral gene delivery or skin-specific transgenic expression in Chy mice	Karkkainen et al. 2001; Saaristo et al. 2002
	LYVE-1, VEGFR-3	Transfer of naked VEGF-C plasmid in two models of lymphedema (in the rabbit ear and in mouse skin in the tail)	Yoon et al. 2003
	PAL-E/CD31	Recombinant protein injection in a lymphedema model in the rabbit ear	Szuba et al. 2002
VEGF-C156S	LYVE-1, VEGFR-3	Transgenic expression in mouse skin	Veikkola et al. 2001
	LYVE-1, podoplanin	Viral gene delivery or skin-specific transgenic expression in Chy mice	Karkkainen et al. 2001; Saaristo et al. 2002
VEGF-D	VEGFR-3	Viral gene delivery in rat skin	Byzova et al. 2002
	LYVE-1, VEGFR-3	Transgenic expression in mouse skin	Veikkola et al. 2001
Soluble VEGFR-3	LYVE-1, VEGFR-3	Transgenic expression of VEGFR-3-Ig in mouse skin	Makinen et al. 2001a

genesis without affecting angiogenesis (Jeltsch et al. 1997; Veikkola et al. 2001). During early embryogenesis, lack of VEGFR-3 in VEGFR-3-null mice affects neither vasculogenesis nor angiogenesis. Instead, remodeling and maturation of blood vessels are impaired, leading to embryonic death at day 9.5, well before the emergence of the lymphatic vasculature (Dumont et al. 1998). Selective expression of a soluble VEGFR-3-Ig in mouse skin demonstrated that sequestering VEGFs-C and -D during embryogenesis prevents formation of lymphatic capillaries as well as regression of previously-formed fetal lymphatics, without affecting the blood vasculature. However, lymphatics appeared spontaneously as the mice got older, suggesting that other factors can compensate for the loss of VEGF-C or -D in postnatal life (Makinen et al. 2001a). Further evidence for the importance of these ligand-receptor interactions for lymphangiogenesis has come from studies in Chy mice, which carry a spontaneous mutation in VEGFR-3 (Karkkainen et al. 2001; Saaristo et al. 2002) and which display features of lymphedema, similar to VEGFR-3-Ig transgenic mice.

In summary, in vivo and in vitro studies have demonstrated that VEGFR-2 signaling regulates angiogenesis whereas VEGFR-3 signaling mediates lymphangiogenesis. However, the role of VEGFR-2 in lymphangiogenesis and the role of VEGFR-3 in angiogenesis remain to be established.

Cultured lymphatic endothelial cells

Very few differentially expressed molecules have been identified to date that distinguish lymphatic from blood vascular endothelium, and the extent to which the two cell types are related remains unclear. This scenario has changed very recently with the first reports which describe the isolation of lymphatic endothelial cells (LECs) using specific molecular markers (Kriehuber et al. 2001; Makinen et al. 2001b; Podgrabinska et al. 2002). Kriehuber et al. isolated LECs and BECs from adult human skin by flow cytometry using antibodies to the lymphatic marker podoplanin (Breiteneder-Geleff et al. 1999; Kriehuber et al. 2001). Makinen et al. employed antibodies to the extracellular domain of VEGFR-3 to purify LECs from commercially available human dermal microvascular endothelial cells (Joukov et al. 1996; Makinen et al. 2001b). Finally, Podgrabinska et al. employed antibodies to LYVE-1, a lymphatic specific receptor for hyaluronan (Banerji et al. 1999), to separate LECs and BECs from human neonatal foreskin tissue (Podgrabinska et al. 2002). LECs were characterized by their homotypic association, selective responsiveness to VEGF-C in terms of growth, survival and morphogenesis, and by the differential extracellular matrix requirements. Microarray analyses revealed important differences between the two cell types at the molecular level (Petrova et al. 2002; Podgrabinska et al. 2002). The molecular signature of LECs appears to reflect their unique functional characteristics. These studies should facilitate the discovery of novel lymphatic vessel markers, and provide a basis for the analysis of the molecular mechanisms that account for the characteristic functions of lymphatic capillaries.

Lymphatic metastasis: controversies

To enter the lymphatic circulation, tumor cells must traverse the lymphatic vessel wall. This is believed to occur at the level of lymphatic capillaries which are devoid of a continuous basement membrane and pericytes, both of which typically invest blood capillaries. In addition, while the junctions in blood vessels connect adjacent endothelial cells over entire cell boundaries, in lymphatics the junctions are generally more sparse. Based on these differences, it has been suggested that the entry

Table 2 Relationship between VEGF-C levels in primary human tumors and lymph node metastases

Tumor type	VEGF-C detection	Relationship between VEGF-C and metastases	Comment	References
Thyroid carcinoma	IHC	LN	–	Tanaka et al. 2002a
	RT-PCR	LN	–	Tanaka et al. 2002b
	RT-PCR, IHC	LN	–	Bunone et al. 1999
Esophageal SCC	IHC	LVI, LN	Increased MVD	Kitadai et al. 2001
Gastric carcinoma	IHC	LVI, LN	–	Ishikawa et al. 2003
	IHC	LVI	Poor DFS 5 years	Ichikura et al. 2001
	RT-PCR, IHC,WB	LVI, LN	Poor DFS 5 years	Yonemura et al. 1999
	IHC	LVI, none LN	–	Kabashima et al. 2001
	IHC	LVI, LN	Poor DFS 6 years	Takahashi et al. 2002
	IHC	LVI, LN	Increased MVD	Amioka et al. 2002
Breast carcinoma	IHC	None LN	Poor DFS 8 years	Yang et al. 2002
	RT-PCR, IHC	LVI	Poor DFS 5 years	Kinoshita et al. 2001
	RT-PCR, IHC	None LN	–	Gunningham et al. 2000
	RT-PCR	LN	–	Kurebayashi et al. 1999
	IHC	None LN	Inflammation with increased LVD, LVI with LN	Schoppmann et al. 2002
Cervical carcinoma	RT-PCR	LN	Poor DFS 5 years	Hashimoto et al. 2001
	IHC	LN	Increased MVD, poor DFS 8 years	Ueda et al. 2002
Lung carcinoma	IHC	None LN	Correlation VEGF-C level and VEGFR-3 level in tumor, poor DFS 5 years	Arinaga et al. 2003
	IHC	LVI, LN	Poor DFS 3 years	Kajita et al. 2001
	IHC	LN	–	Ohta et al. 2000
	RT-PCR	LVI	–	Niki et al. 2000
Mesothelioma	RT-PCR 5'-Nase	LVI, none LN	–	Ohta et al. 1999
Pancreatic carcinoma	IHC	LVI, LN	No relationship to DFS 5 years	Tang et al. 2001
Endometrial carcinoma	IHC	LVI, LN	Poor DFS 5–10 years	Hirai et al. 2001
Ovarian carcinoma	IHC	LN, peritoneal	Poor DFS 10 years	Yokoyama et al. 2003
Gallbladder cancer	IHC	LVI, LN	Poor DFS 5 years	Nakashima et al. 2003
Neuroblastoma	RT-PCR	None LN	–	Komuro et al. 2001
Prostatic carcinoma	ISH	LN	–	Tsurusaki et al. 1999
Colorectal carcinoma	IHC	LVI, LN, liver	Poor DFS 5 years	Kaio et al. 2003
	RT-PCR	None LN	–	George et al. 2001
	RT-PCR, IHC	LVI, LNs	–	Akagi et al. 2000
	IHC	LVI, LN	Increased MVD, poor DFS 5 years	Furudoi et al. 2002
Head and neck SCC	RT-PCR, WB	LN	–	O-Charoenrat et al. 2001
	RT-PCR	–	No relationship between VEGF-C levels and intratumoral lymphatic vessel proliferation	Beasley et al. 2002

LVI lymphatic vessel invasion, *LN* lymph node, *none LN* no metastases detected in lymph nodes—metastatic status of other organs not specified, *LVD* lymphatic vascular density, *MVD* microvascular density, *DFS* disease-free survival, *SCC* squamous cell carcinoma, *ISH* in situ hybridization, *IHC* immunohistochemistry, *RT-PCR* reverse transcription polymerase chain reaction, *WB* Western blot, *5'-Nase* 5'-nucleotidase/alkaline phosphatase

of cells into lymphatic vessels is easier than into blood vessels. This concept, however, still lacks proof, and it has yet to be demonstrated that the nature of the lymphatic endothelial junctions facilitates tumor cell entry. Another dogma has been that the entry of tumor cells into lymphatics is a passive process. Thus, tumor cells are believed to be washed into lymphatics along with interstitial fluid and proteins (Hartveit 1990), or simply to grow into an adjacent vessel. To date there is no convincing evidence in the literature which would support or oppose these concepts. Once tumor cells are in the lymphatics, they are presumably carried to lymph nodes by the tide of lymph flow. However, once again nothing is known about tumor cell behavior within the lymphatic system. Finally, the prevailing view has been that lymphatic vessels are absent from tumors and that lymphangiogenesis does not occur in cancer (Carmeliet and Jain 2000; Jain and Padera 2002; Leu et al. 2000). The recent identification of molecular markers of lymphatic vessels has made it possible to reexamine these established views.

Studies using injection techniques have reported the absence of a functional lymphatic network in tumors, and this has been ascribed to the solid pressure generated within tumors as a result of rapidly dividing tumor cells (Jain 1987; Leu et al. 2000). However, lack of lymphan-

Table 3 Relationship between VEGF-D levels in primary human tumors and lymph node metastases

Tumor type	VEGF-D detection	Relationship between VEGF-D and metastases	Comment	References
Breast carcinoma	RT-PCR	None LN	Inflammatory response	Kurebayashi et al. 1999
	IHC	LN	Poor DFS 10 years, c-erbB-2 overexpression	Nakamura et al. 2003
Gastric carcinoma	IHC	LVI, LN	–	Ishikawa et al. 2003
Thyroid carcinoma	RT-PCR	None LN	–	Tanaka et al. 2002b
Lung carcinoma	RT-PCR	LN	VEGF-D level in tumor lower than in normal tissue	Niki et al. 2000
Head and NECK SCC	RT-PCR, WB	None LN	VEGF-D level in tumor lower than in normal tissue	O-Charoenrat et al. 2001
Ovarian carcinoma	IHC	LN, peritoneal	Poor DFS 10 years	Yokoyama et al. 2003
Colorectal carcinoma	RT-PCR	None LN	VEGF-D level in tumor lower than in normal tissue	George et al. 2001
	IHC	LN	Poor DFS 7 years	White et al. 2002

IHC immunohistochemistry, *RT-PCR* reverse transcription polymerase chain reaction, *WB* Western blot, *LN* lymph node, *none LN* no metastases detected in lymph nodes—metastatic status of other organs not specified, *DFS* disease-free survival, *SCC* squamous cell carcinoma

giogenesis and/or impairment of fluid and macromolecular transport are not the only possible explanations for the absence of functional tumor lymphatics. Fluid and macromolecules travel through tissues according to hydrostatic and oncotic pressure gradients, following the pathways of least resistance to transport (Schmid-Schonbein 1990). In normal tissues, extracellular matrix fibers are ideally arranged for directing fluid into lymphatic vessels (Ryan 1989). In tumors, the extracellular matrix composition and organization are frequently altered, and it is possible that fluid channels in tumor stroma do not direct fluid into tumor lymphatics in an organized manner. Furthermore, the elevated interstitial fluid pressure in tumors (Jain 1990) may result in steep hydrostatic pressure gradients at the tumor edge that may primarily force fluid out of the tumor and not laterally into tumor lymphatics. Moreover, the conclusion that tumor cells cannot utilize lymphatic vessels which are undetectable by lymphangiography, for transport to lymph nodes, is based on the assumption that the transport of fluid and cells in tissues and their uptake into the lymphatics is governed by the same principles. This is unlikely, as cell migration in tissues is a tightly controlled process involving a defined set of cell-cell and cell-matrix interactions. In contrast, the major forces controlling uptake of fluid and macromolecules into lymphatics are pressure gradients in tissues (Schmid-Schonbein 1990). Whether these forces have any effect on cell transport into the lymphatics remains an open question.

Lymphangiogenic factors and lymphangiogenesis in human tumors

Expression of lymphangiogenic factors in human tumors

The discovery of the lymphangiogenic factors raised the question as to whether they are expressed in human cancers and if so whether this contributes to the ability of tumors to metastasize. Expression of VEGF-C has indeed been demonstrated in many human tumors (Salven et al. 1998; Stacker et al. 2002). Although expression of VEGF-D in human tumors has been less well studied, it has been detected in melanoma and colorectal carcinoma (Achen et al. 2001; White et al. 2002).

A number of studies have investigated the relationship between levels of VEGFs-C and -D and clinicopathological features related to the ability of tumors to spread (i.e., lymphatic vessel invasion, lymph node involvement and disease-free survival). With respect to VEGF-C, a significant correlation between levels in the primary tumor and lymphatic vessel invasion (LVI) or lymph node metastasis has been observed in most of the studies (Table 2). Its expression is often detected preferentially at invasive sites (Amioka et al. 2002; Furudoi et al. 2002). With respect to VEGF-D, a correlation with metastasis is less clear (Table 3). In fact, some studies have suggested that the expression of VEGF-D in human tumors is reduced relative to normal tissue (George et al. 2001; O-Charoenrat et al. 2001). It remains to be seen whether there is any relationship between VEGF-C and VEGF-D levels in tumors (Niki et al. 2000; O-Charoenrat et al. 2001). Another avenue thus far unexplored is the role of VEGFs-C and -D as potential survival factors for tumor cells in certain cancer types (Fielder et al. 1997; Orpana and Salven 2002).

Table 4 Relationship between lymphatic density in primary human tumors and lymph node metastases

Tumor type	Lymphatics detection	Relation with metastases	Comment	References
Lung carcinoma	IHC (VEGFR-3)	LN	Ki67 in lymphatic vessels	Niki et al. 2001
Endometrial carcinoma	IHC (VEGFR-3)	LN	Poor DFS 5 years	Yokoyama et al. 2000
Gastric carcinoma	IHC (VEGFR-3)	LVI, LN	Correlation VEGF-C level and VEGFR-3 level in tumor	Yonemura et al. 2001
Cutaneous melanoma	IHC (LYVE-1, podoplanin)	None LN	Ki67 in lymphatic vessels, peritumoral LVD with poor DFS 5 years but not intratumoral LVD	Straume et al. 2003
Tongue SCC	IHC (VEGFR-3)	LN	–	Okamoto et al. 2002
Head and neck SCC	IHC (LYVE-1/CD34)	LN (only in oropharyngeal carcinoma)	Intratumoral lymphatic vessels	Beasley et al. 2002
Cervical carcinoma	IHC (podoplanin)	LN	LVI	Schoppmann et al. 2001b
	IHC (podoplanin)	LN	LVI with poor DFS 10 years	Birner et al. 2001
Breast carcinoma	IHC (VEGFR-3)	LN	–	Nathanson et al. 2000
	IHC (podoplanin)	LN	LVI with LN but not LVD with LN	Schoppmann et al. 2001a

LVI lymphatic vessel invasion, *LN* lymph node, *none LN* no metastases detected in lymph nodes—metastatic status of other organs not specified, *LVD* lymphatic vessel density, *DFS* disease-free survival, *SCC* squamous cell carcinoma, *IHC* immunohistochemistry, *n.d.* not determined

Lymphatic vessels in human tumors

The discovery of lymphatic endothelial markers has for the first time allowed the unambiguous characterization of tumor lymphatics and the assessment of lymphangiogenesis during tumor progression. In several studies, a strong correlation was found between the presence of lymphatic markers (e.g., podoplanin, VEGFR-3, LYVE-1) and lymph node metastases. Tumor cells are frequently found in juxtatumoral lymphatics and many studies have reported a strong correlation between LVI and lymph node involvement (Table 4). LVI has long been considered to be an important prognostic indicator in cancer. However, in these studies, lymphatic vessels were identified by morphological criteria alone; recent studies using molecular markers are in agreement with this notion. Intratumoral lymphatics have so far been observed only in human head and neck cancers and in melanoma (Beasley et al. 2002; Dadras et al. 2003). On the contrary, enlarged, dilated lymphatic vessels, in which endothelial proliferation is often observed, are very frequently present in peritumoral areas of many tumor types (Beasley et al. 2002; Niki et al. 2001; Padera et al. 2002; Straume et al. 2003).

It is not known to what extent tumor cell-secreted factors are directly responsible for the formation of the large lymphatic vessels that are detected around human tumors. Inflammatory cells could, for example, also contribute to lymphatic enlargement and lymphangiogenesis. A recent study has demonstrated that in breast cancer, tumor-associated macrophages express VEGFs-C and -D, thereby indicating an additional source of lymphangiogenic factors (Schoppmann et al. 2002). VEGF-C is also chemotactic for macrophages (Skobe et al. 2001b) and is readily induced by proinflammatory cytokines (Narko et al. 1999). In this regard, a significant correlation between the tumor inflammatory response and lymphangiogenic factor expression has been observed in breast and cervical cancer (Kurebayashi et al. 1999; Schoppmann et al. 2002).

With respect to lymphatic vessel density (LVD), a correlation exists between the number of tumor-associated lymphatics and the presence of lymph node metastases for a given tumor type (Table 4). However, a comparison of tumor-associated LVD with LVD of the tissue in which the tumor arose has only been undertaken in a limited number of studies. In a single study of colorectal carcinoma, LVD was increased in tumors relative to normal colonic mucosa (White et al. 2002). In two other studies on cervical and gastric cancers, although there was a trend towards an increase in tumor associated LVD, this did not reach statistical significance (Schoppmann et al. 2002; Yonemura et al. 2001). A melanoma study reported that LVD associated with tumors was comparable to LVD in normal skin (de Waal et al. 1997).

Existing lymphatic vessels in the surrounding tissue provide a readily accessible avenue for tumor cell dissemination. Tumor spread via the lymphatic vascular bed may therefore be facilitated by the high intrinsic lymphatic density in the tissue in which the tumor arises. Although preexisting peritumoral lymphatics are likely to be sufficient for tumor spread, recruitment of lymphatic vessels into the close proximity of a tumor may increase the propensity of the tumor to metastasize. Therefore, increased lymphatic vessel density as well as the presence of intratumoral lymphatics should be regarded as an additional pathway rather than a necessity for metastasis. Whereas a relative increase in LVD has been correlated with a more metastatic phenotype, future studies are required to determine whether intratumoral lymphatics are restricted only to certain cancer types and whether their presence in tumors has prognostic significance.

Table 5 Experimental models of tumor lymphangiogenesis and related metastasis

Cytokine/receptor	Transfected tumor cell line	Animal model	Lymphatic marker	Tumor-associated lymphatics		Angiogenesis	Lymph node metastasis	Reference
				Peritumoral	Intratumoral			
VEGF-C endogenous	A375 human melanomas	Avian CAM	Prox-1	Yes, inhibited by VEGFR-3-Ig	Yes, inhibited by VEGFR-3-Ig	Not inhibited by VEGFR-3 Ig	n.d.	Papoutsi et al. 2000
VEGF-C endogenous	10AS rat pancreatic carcinoma	Avian CAM	VEGFR-3	Yes	Yes	n.d.	n.d.	Papoutsi et al. 2001
VEGF-C overexpression	Rip1Tag2 pancreatic β-cells transgenic mice	Rip1Tag2 transgenic mice	LYVE-1	Yes	No	No	Yes	Mandriota et al. 2001
VEGF-C overexpression	MeWo human melanoma cell line	Nude mice	LYVE-1, VEGFR-3	Yes	Yes	Yes	n.d.	Skobe et al. 2001a
VEGF-C overexpression	MDA-MB-435 human melanoma cell line	Nude mice	LYVE-1, VEGFR-3	Yes	Yes	No	Yes	Skobe et al. 2001b
VEGF-C overexpression	MCF7 human breast cancer cells	SCID mice	LYVE-1, VEGFR-3	Yes, inhibited by VEGFR-3-Ig	Yes, inhibited by VEGFR-3-Ig	No	No	Karpanen et al. 2001
VEGF-C overexpression	MCF-7 human breast cancer	Nude mice	LYVE-1, VEGFR3	Yes	Yes	Yes	Yes	Mattila et al. 2002
VEGF-C overexpression	Murine T241 fibrosarcoma or B16-F10 melanoma	Nude mice	LYVE-1, Prox-1	Yes	Yes	Yes	Yes	Padera et al. 2002
VEGFC-C152S overexpression	NM-081 rat mammary carcinoma cells	Wistar Furth rat	Prox-1	Yes	No	No	Yes	Krishnan et al. 2003
VEGF-D overexpression	293EBNA cells	SCID mice	LYVE-1	Yes, inhibited by VEGF-D neutralizing antibody	Yes, inhibited by VEGF-D neutralizing antibody	Yes, inhibited by VEGF-D neutralizing antibody	Yes, inhibited by VEGF-D neutralizing antibody	Stacker et al. 2001
VEGFR-3-Ig overexpression	LNM35 human cancer cell line expressing endogenous VEGF-C	SCID mice	LYVE-1	Yes, inhibited by VEGFR-3-Ig	Yes, inhibited by VEGFR-3-Ig	Not affected by VEGFR-3-Ig	Yes, inhibited by VEGFR-3-Ig	He et al. 2002
VEGFR-3-Ig overexpression	MT-450 rat mammary tumor cell line expressing endogenous VEGF-C and -D	Wistar Furth rat	Prox-1	Yes, inhibited by VEGFR-3-Ig	No	Not affected by VEGFR-3-Ig	Yes, inhibited by VEGFR-3-Ig	Krishnan et al. 2003

n.d. not determined

Finally, the relative importance of preexisting versus newly formed lymphatic vessels to lymphogenous metastasis remains poorly understood.

Experimental models implicating lymphangiogenesis in tumor spread

In addition to clinicopathological studies which have provided a correlation between VEGF-C expression in primary tumors and tumor spread via lymphatics, several recent experimental studies have provided direct evidence for the importance of VEGF-C in tumor lymphangiogenesis and metastasis (Table 5).

In our own studies, transgenic mouse lines were established in which VEGF-C expression, driven by the rat insulin promoter (Rip), was targeted to beta cells of the endocrine pancreas. Transgenic RipVEGF-C mice developed an extensive network of lymphatics around islets of Langerhans, as shown by a number of criteria including staining with the lymphatic endothelial cell-specific marker, LYVE-1 (Mandriota et al. 2001). As a model of tumor progression in the same tissue, Rip1Tag2 mice (Hanahan 1985) were employed. In these mice, expression of the SV40 oncogene is driven in islet beta cells by the rat insulin promoter. These mice predictably and reproducibly develop pancreatic beta-cell tumors which are not metastatic. When RipVEGF-C and Rip1Tag2 mice were crossed, it was found that double transgenic mice formed tumors surrounded by well-developed lymphatics, and that this was accompanied by the formation of metastases in regional pancreatic lymph nodes (Mandriota et al. 2001). Of importance was the finding that in the same model, tumor cells overexpressing VEGF-A promoted angiogenesis and tumor growth, but did not promote either lymphangiogenesis or the formation of lymph node metastasis (Gannon et al. 2002).

Skobe et al. demonstrated that overexpression of VEGF-C in MDA-MB-435 human breast cancer cells increased the incidence of metastases to regional lymph nodes following orthotopic transplantation of the cells into immunocompromised mice (Skobe et al. 2001b). Interestingly, this study showed that VEGF-C-induced lymphangiogenesis not only correlated with lymph node metastasis, but also with lung metastases (Skobe et al. 2001b). Induction of tumor-associated lymphangiogenesis by VEGF-C and a subsequent increase in metastases has also been reported using MCF-7 human breast cancer cells (Mattila et al. 2002), in a mouse sarcoma model (Leu et al. 2000; Padera et al. 2002) and in an immunocompetent rat model using the weakly metastatic NM-081 rat mammary carcinoma cell line overexpressing VEGF-C-C152S (Krishnan et al. 2003). Stacker et al. demonstrated a role for VEGF-D in lymphatic-dependent tumor cell dissemination using a 293EBNA xenotransplantation model (Stacker et al. 2001). Importantly, when tumor cells overexpressing VEGF-A were assessed in the same setting, angiogenesis and tumor growth were increased with no effect on either lymphangiogenesis or the formation of lymph node metastases. Targeting of VEGF-C/VEGFR-3 in two mouse and in one rat tumor model expressing high levels of VEGF-C resulted in decreased lymphangiogenesis and reduction of lymph node metastases (He et al. 2002; Karpanen et al. 2001; Krishnan et al. 2003).

Conclusion and perspectives

As with the blood vascular system, tumor cell dissemination via lymphatics requires their intravasation into lymphatic capillaries. Recent studies on human and mouse tumors have convincingly demonstrated the importance of VEGF-C in lymphogenous metastasis. Although VEGF-C may increase the propensity of tumors to metastasize simply by increasing tumor cell access to lymphatic vessels via induction of lymphangiogenesis, it is equally conceivable that lymphatic endothelial cells and tumor cells enter into a reciprocal dialogue. VEGF-C may alter the function of preexisting lymphatics which may then become actively involved in tumor cell chemotaxis, lymphatic intravasation and dissemination. For example, chemokines may be the mediators of directional tumor cell migration. VEGF-C may also alter adhesion molecule expression in lymphatic endothelial cells.

Understanding the molecular and cellular mechanisms of metastasis is essential for the development of new forms of cancer therapy. In preclinical studies, molecules which have been shown to be effective in inhibiting tumor lymphangiogenesis and lymph node metastasis include a soluble VEGFR-3-IgG fusion protein and neutralizing anti-VEGF-D antibodies. Furthermore, indolinones have been synthesized and characterized that differentially block VEGF-C- and -D-induced VEGFR-3 kinase activity but not that of VEGFR-2 (Kirkin et al. 2001). These tools provide a glimpse of what could potentially be a novel therapeutic opportunity for preventing or halting tumor cell dissemination and the formation of metastasis.

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