Molecular mechanisms of lymphangiogenesis in health and disease

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Studies of the last decades have revealed the importance of angiogenesis for normal growth and for the pathogenesis of numerous diseases. Much less studied is lymphangiogenesis, the growth of lymphatic vessels, which drain extravasated fluid, proteins, and cells and transport them back to the venous circulation. Nonetheless, insufficient lymphangiogenesis causes incapacitating lymphedema, while lymphatic growth around tumors may facilitate metastatic spread of malignant cells that ultimately kill the patient. The recent discovery of the key lymphangiogenic factors VEGF-C and VEGF-D and their receptor VEGFR-3 has allowed novel insights into how the lymphatic vessels and blood vessels coordinately grow and affect human disease. In addition, these studies have opened novel diagnostic and therapeutic avenues for the treatment of lymphedema and metastasis. This overview highlights the recent insights and developments in the field of lymphatic vascular research.

Lymphatic vessels in health and disease

When blood circulates through the vascular system, fluid and proteins unavoidably leak out. A network of lymphatic vessels collects the extravasated bloodless fluid from the tissues and transfers it, as lymph, via the collecting lymphatic vessels and thoracic duct back into the venous circulation. Lymphatic vessels also serve an immune function by transporting white blood cells and antigen-presenting cells which patrol the tissues to the various lymphoid organs, where they elicit immune responses (Figure 1). The superficial lymphatics in the skin and intestine, which are in contact with foreign antigens, function as transport routes for antigen-presenting Langerhans cells and other lymphoid cells. Unfortunately, malignant cells that escape from their resident tumor can also traffic along the lymphatic tracts to the lymph nodes and, via entry into the circulation, cause metastatic spread to distant organs (Figure 1). In view of its important functions, is not surprising that derailed growth or function of the lymphatic system is implicated in numerous diseases, including lymphedema, inflammation/infection, immune diseases, and malignancy (reviewed in Karpanen and Alitalo, 2001; Pepper, 2001). Remarkably, however, even though lymphatic vessels were already discovered as "milky veins" in 1627 by Gasparo Asellius (Asellius, 1627), their study has remained relatively neglected until recently. This has been partly due to the difficulties in recognizing these vessels in tissues, due to a lack of specific markers. The recent identification of molecules that control the growth of lymphatic vessels therefore has important medical implications.

Are lymphatic and blood vessels alike?

The lymphatic vessels differ in many ways from the blood vessels, but they also share many properties. Both vascular systems are lined by an endothelium and surrounded by a smooth muscle framework, particularly around luminal valves in larger lymphatics (Witte et al., 1997). Although both vessel types are likely to share a common embryonic origin (see below), they also display several distinct molecular markers (Table 1 lists some lymphatic markers). Even certain factors that stimulate blood vessel growth also enhance lymphatic growth (Kubo et al., 2002). Unlike blood vessels, lymphatic vessels have a discontinuous or fenestrated basement membrane, lack tight interendothelial junctions, and are therefore permeable to interstitial fluid and cells (Leak, 1976). Through specialized anchoring filaments (e.g., fine strands of elastic fibers connecting lymphatic endothelial cells with their surrounding pericellular matrix [Gerli et al., 2000]), the lymphatic vessels stay open when the tissue pressure rises. Compared to the blood vessels, lymphatics are a low flow, low pressure system and much less coagulable due to lack of platelets and erythrocytes. They also send out fewer sprouts and are organized in a less complex network (Witte et al., 1997). Identification of lymphatic vessels therefore relies on the use of several markers (Table 1) and, ideally, on functional assays demonstrating uptake of dyes (lymphangiography) (Jain et al., 2002).

The genesis of lymphatic vessels in the embryo

Blood vessels initially arise via vasculogenesis, e.g., via the assembly of differentiated endothelial precursors into a primitive capillary network (Figure 2). Subsequently, the nascent vascular bed expands and remodels into a mature network, a process termed angiogenesis (Carmeliet, 2000) (Figure 2). Lymphatic vessels develop shortly after blood vessels and might share a common origin with the latter. Around the turn of the century, Sabin proposed that primitive lymph sacs originate by endothelial cell budding from embryonic veins (Huntington and McLure, 1908; Sabin, 1902) (Figure 2). The peripheral lymphatic system would then spread from these primary lymph sacs by sprouting and assembly into lymphatic capillaries. Now, a century later, genetic studies support a model whereby certain venous endothelial cells become responsive to (as yet unknown) lymphatic-inducing signals, differentiate to the lymphatic lineage, and send out lymphatic sprouts. *Prox-1*, a homeobox transcription factor, plays a critical role in the initial *trans*-differentiation and subsequent budding (Wigle and Oliver, 1999), while

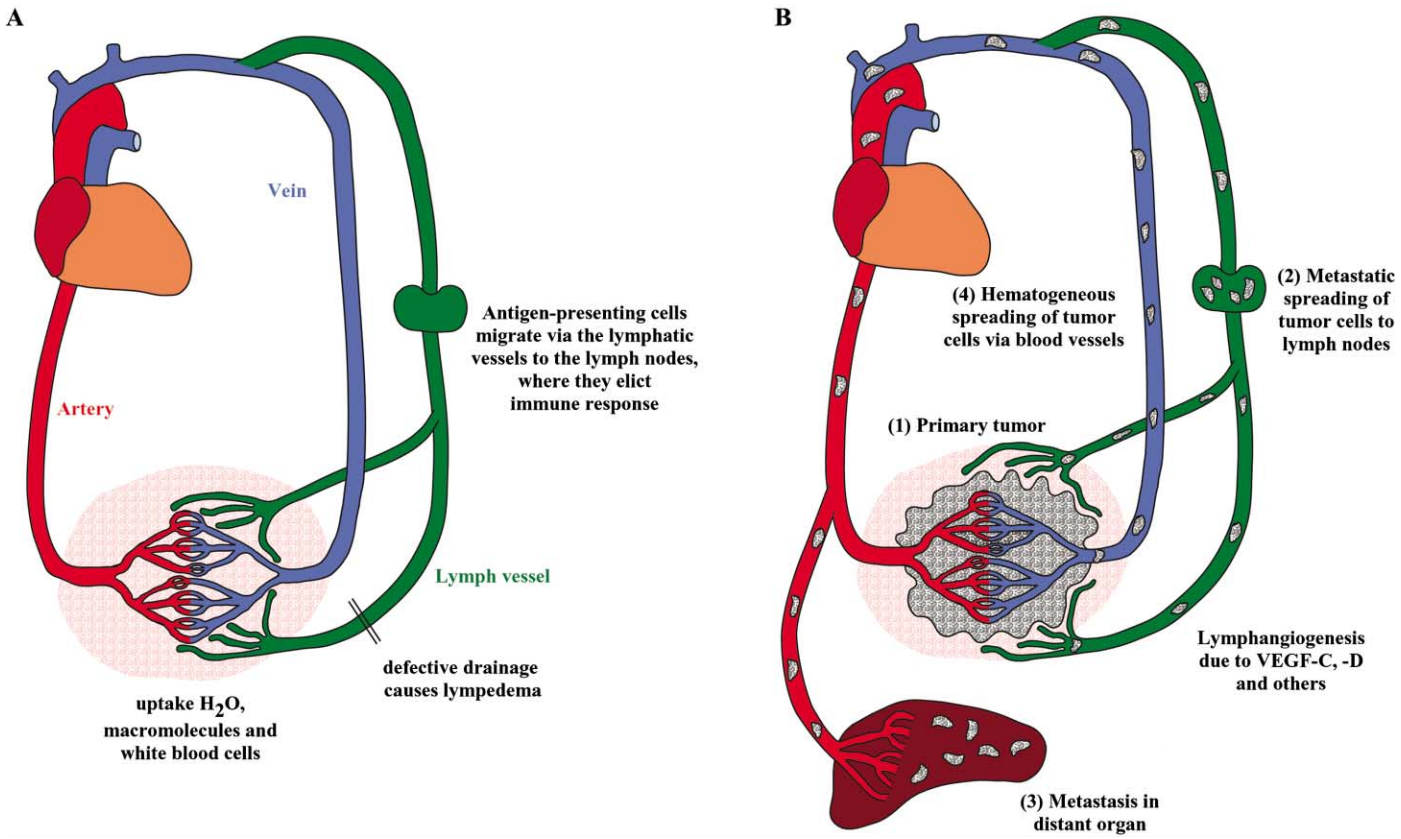

Figure 1. Scheme illustrating the relationship between blood vascular and lymphatic system in normal health and in cancer

A: In a healthy individual, lymphatics drain extravasated fluid, proteins, and cells to the lymph nodes and, via the thoracic duct, to the venous circulation. Immune cells such as antigen-presenting cells, patrolling through the body for foreign antigens, traffic to the lymph nodes, where they elicit an immune response. Lymphedema develops when lymphatic drainage is insufficient as a result of primary hypoplasia, surgical resection, radiation, infection (such as filariasis), etc. **B:** In cancer, as a result of lymphangiogenesis around and possibly also within (controversial) the primary tumor (1), dislodged tumor cells drain to the lymph nodes, which represents a poor prognosis (2). Tumor cells then traffic to the vascular circulation, through which they metastasize to distant organs and kill the patient (3). Since tumors are highly angiogenic, tumor cells may also metastasize via an hematogeneous route to distant organs (4), which may explain why inhibition of lymphangiogenesis blocks lymph node but not lung metastasis in an experimental tumor model (see text).

VEGFR-3, a receptor of the lymphangiogenic factor vascular endothelial growth factor-C (VEGF-C; Joukov et al., 1996, see below), is initially expressed in embryonic blood vessels, but subsequently restricted to lymphatic vessels once they are committed to this lineage and express additional lymphatic markers (see Table 1) (Kaipainen et al., 1995). According to an alternative model, the initial lymph sacs would arise in the mesenchyme from precursor cells (lymphangioblasts), independent of the veins, and only later establish venous connections (the equivalent of "vasculogenesis"; Figure 2). The existence of primitive lymphangioblasts, which can be recruited by the developing lymphatic vessels, has been shown at least in the avian species (Schneider et al., 1999). A combination of both mechanisms is therefore likely to contribute to the formation of the lymphatic system.

Molecular regulation of lymphangiogenesis in disease

Blood vessels in the adult are normally quiescent and require signals to grow ("angiogenic switch"). It is now widely accepted that the switch is off when the effect of proangiogenic molecules is balanced by that of antiangiogenic molecules, and is on when the net balance is tipped in favor of angiogenesis (Carmeliet and Jain, 2000; Hanahan and Folkman, 1996). Several molecules have been implicated, but members of the VEGF family of growth factors (VEGF, VEGF-B, -C, -D, and PlGF) and their receptors (VEGFR-1, -2, and -3) play critical roles in mediating angiogenic processes (Carmeliet, 2000; Carmeliet et al., 2001; Dvorak, 2000; Ferrara, 2001; Karkkainen and Petrova, 2000). Of those, activation of VEGFR-2 by VEGF provides crucial angiogenic signals. Much less is known, however, about the signals switching on lymphangiogenesis in pathological disorders. Since lymphatic vessels arose from blood vessels, it is not surprising that some of the prototype angiogenic mechanisms are also employed in lymphangiogenesis. This is the case for VEGF-C and VEGF-D, which interact with VEGFR-3 in lymphatic endothelial cells (Joukov et al., 1997; Kaipainen et al., 1995; Achen et al., 1998). VEGF-C and VEGFR-3 are usually coexpressed at sites where lymphatic vessels sprout, in the embryo (Dumont et al., 1998; Kukk et al., 1996), and in disease (see below). VEGF-C induces growth, migration, and survival of primary lymphatic endothelial cells (Makinen et al., 2001b), stimulates lymphatic sprouting in the chorioallantoic membrane (Oh et al., 1997), and, when overexpressed in transgenic mice, lymphatic vessel hyperplasia (Jeltsch et al., 1997). Signaling via VEGFR-3 alone was sufficient for the lymphangiogenic signals, since VEGF-C^{C156S}, which only activates VEGFR-3 but not

Table 1. Lymphatic markers, their expression pattern and putative function

Marker	Comments
VEGFR-3	Receptor of VEGF-C and VEGF-D in lymphatic endothelium (Jussila et al., 1998; Kaipainen et al., 1995); also present in a subset of blood vessels and reactivated in angiogenic vessels during pathological conditions (Partanen et al., 2000; Valtola et al., 1999)
Podoplanin	Glomerular podocyte membrane mucoprotein, coexpressed with VEGFR-3 in small lymphatic vessels, but also in vascular tumors and in osteoblasts, kidney podocytes and lung alveolar type I cells (Breiteneder-Geleff et al., 1999)
Prox-1	Homeobox transcription factor involved in the growth and differentiation of lymphatic vessels during development; also expressed in the lens, heart, liver, pancreas, and nervous system. Prox-1-deficient neonates die of defective lymphatic sprouting and differentiation (Wigle and Oliver, 1999)
LYVE-1	Lymphatic Vessel Endothelial HA-receptor-1 (homolog of CD44), involved in transport of hyaluronan by lymphatic endothelial cells. Also present in normal kidney, pancreas, adrenal, and thyroid epithelia and in hepatic blood sinusoidal endothelial cells (Carreira et al., 2001; Banerji et al., 1999)
Nrp-2	Neuropilin-2, a receptor for VEGF on venous endothelium and neurorepellant semaphorins on neural cells, is also a coreceptor of VEGF-C in lymphatic vessels (Karkkainen et al., 2001)

VEGFR-2, induced a similar phenotype (Veikkola et al., 2001). VEGF-D is also lymphangiogenic when overexpressed in skin keratinocytes (Veikkola et al., 2001). Little is known about the expression of VEGF-D in physiological conditions, but it is expressed in tumors (Achen et al., 2001) (see below). It remains to be determined to what extent VEGF, via binding to VEGFR-2 on lymphatic endothelial cells (Makinen et al., 2001b), directly stimulates lymphangiogenesis or stimulates it indirectly by inducing leakage and edema or other lymphangiogenic signals.

Lymphangiogenesis appears to often accompany angiogenesis (Figure 3). This is understandable since nascent blood vessels are leaky, and lack of accompanying lymphatic growth would result in increasing tissue edema. For instance, during wound healing, VEGFR-3 positive lymphatic vessels sprout from preexisting lymphatics into the granulation tissue in parallel with angiogenesis (Clark and Clark, 1932; Oden, 1960; Paavonen et al., 2000). When there is an imbalance in the growth of both vascular systems (as can, for instance, occur in tumors), the interstitial pressure may rise (see below). The molecular properties of VEGF-C and VEGF-D link angiogenesis with lymphangiogenesis, as they stimulate both processes. Indeed, VEGF-C and VEGF-D are synthesized as preproproteins, and proteolytic processing enhances their receptor binding to VEGFR-3 (predominantly a lymphangiogenic receptor), while only the fully processed forms bind to and activate VEGFR-2 (primarily an angiogenic receptor) (Joukov et al., 1996, 1997; Achen et al., 1998). Thus, both angiogenic and lymphangiogenic signals can be coordinately generated from a single molecule, depending on the degree of processing and the relative expression of the receptors. This may explain why VEGFR-3 deficient embryos succumb due to vascular defects (Dumont et al., 1998), why VEGF-C affects early vascular development in the chorioallantoic membrane before the emergence of the lymphatics, and why angiogenesis is enhanced in the ischemic limb by VEGF-C, and in the cornea by VEGF-C and VEGF-D (Cao et al., 1998; Marconcini et al., 1999; Witzenbichler et al., 1998).

Considering the complexity of the molecular regulation of angiogenesis, regulation of lymphangiogenesis is likely to be as complex. Initial insights into the role of additional lymphangiogenic signals have been recently derived from gene targeting studies. For instance, loss of angiopoietin-2, another vascular-specific growth factor that binds the Tie-2 receptor tyrosine kinase, results in abnormal lymphatic patterning and function (N. Gale, G. Yancopoulos, et al., submitted). In addition, mice with deficient DNA binding of *Net*, a member of the Ets-domain transcription factor that is coexpressed with VEGFR-3 in lymphatic vessels, succumb neonatally of insufficient lymph drainage (Ayadi et al., 2001). Furthermore, integrin $\alpha_9\beta_1$ seems required for proper lymphatic development, as α_9 deficient neonates succumb due to similar lymph drainage problems (Huang et al., 2000). This may relate to a cooperation between $\alpha_9\beta_1$ and VEGFR-3 signaling (Wang et al., 2001). A major advance in the field has been the discovery of additional lymphatic markers (Table 1), and a challenge for the future is now to unravel their function in vivo.

Lymphedema, a failure of lymph transport

An imbalance between lymph formation and absorption into lymphatic vessels results in edema. Lymphedema due to impaired lymphatic drainage can be caused by inflammatory or neoplastic obstruction of the lymphatic vessels (Figure 3). For instance, ascites fluid may accumulate due to lymphatic obstruction in peritoneal carcinomatosis, or edema of the arm may follow surgery or radiotherapy for breast cancer, or in non-healing wounds. Lymphatic filariasis, the second leading cause of permanent and long-term disability worldwide, is a parasitic infection in the lymphatic vessels, which leads to abnormal transport function, massive edema, and deformation of the limbs (Witte et al., 1997). Primary lymphedema due to a transport failure of the cutaneous lymphatic vessels gradually results in thickening of the skin, accumulation of adipose tissue, and dermal fibrosis of the affected area. Congenital lymphedema (Milroy's disease) is linked to the VEGFR-3 gene with autosomal dominant inheritance (Ferrell et al., 1998; Witte et al., 1998). This mutation reduces the VEGFR-3 tyrosine kinase activity with subsequent failure in transducing VEGF-C/VEGF-D signals (Karkkainen et al., 2000). Experimentally, transgenic overexpression of a soluble VEGFR-3 (a "VEGF-C/D trap") in the skin also causes lymphedema (Makinen et al., 2001a). Besides VEGFR-3, *FOXC2* gene mutations cause primary lymphedema (Fang et al., 2000), and other genes are likely involved in this disease. Identification of genetic markers and high-risk members of lymphedema families would facilitate the identification and management of lymphedema.

Lymphangiomas and Kaposi's sarcoma

Derailed growth of VEGFR-3/podoplanin-positive lymphatic vessels results in lymphangioma formation with secondary lymphedema due to impaired lymph fluid drainage. Lymphangiogomas affect most organs, although they are most commonly found in the soft tissues of the head, neck, and axilla,

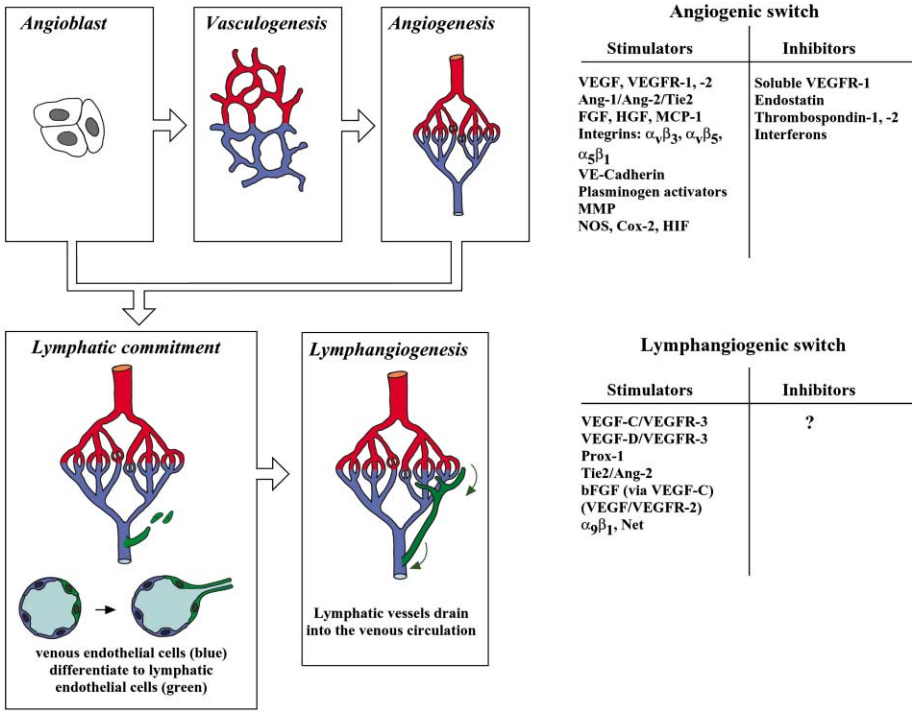

Figure 2. Scheme illustrating the formation of vascular blood and lymphatic vessels

Upper panels: endothelial precursors ("Angioblasts") differentiate to a primitive capillary network ("Vasculogenesis"), which subsequently remodels and expands to a mature vascular network ("Angiogenesis") of arteries (red) and veins (blue). Lower panels: during embryonic development, venous endothelial cells respond to putative lymphangiogenic signals and differentiate to lymphatic endothelial cells (green) ("Lymphatic commitment"). Lymphatic vessels further sprout, expand, and remodel and establish an open-ended vessel system that connects to the venous circulation ("Lymphangiogenesis"). Alternatively, lymphatic angioblasts may differentiate to lymphatic endothelium and form new vessels. Some essential molecules defining the (lymph)-angiogenic switch being on or off are indicated. Abbreviations: VEGFR, VEGF receptors; Ang, angiopoietin; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase; HIF, hypoxia inducible factor. Synthetic inhibitors such as VEGF receptor tyrosine kinase inhibitors and blocking VEGFR-3 antibodies can also block lymphangiogenesis, etc. (see text).

where they consist of a benign multicystic mass of networks of dilated lymphatic channels. Findings that spindle cells and cells lining the irregular vascular spaces in Kaposi's sarcoma in AIDS patients are positive for VEGFR-3 and podoplanin suggest that these cells could be of lymphatic endothelial origin (Jussila et al., 1998; Skobe et al., 1999; Weninger et al., 1999).

Blood vessels and lymphatic vessels in tumors

Like in normal tissues, both vascular and lymphatic vessels are present in malignant tissues, but some remarkable differences with normal vessels have been observed. Unraveling how angiogenesis and lymphangiogenesis are derailed in tumors is of major importance for understanding tumor biology and designing future cancer preventive measures. Since both vascular systems probably crosstalk to each other and share common signals, their structural and functional aspects are compared. Tumor vascular vessels are highly disorganized, tortuous, and dilated with uneven diameter, excessive branching, shunts, and incomplete or absent muscular coverage (Carmeliet and Jain, 2000). Their walls have numerous "openings," widened interendothelial junctions, and a discontinuous or absent basement membrane. In addition, the endothelial cells are abnormal in shape, growing on top of each other and projecting into the lumen. Consequently, tumor vessels are leaky and blood flow is chaotic and variable, all contributing to increased interstitial tumor pressure and impairing delivery of therapeutic agents in tumors (Jain and Carmeliet, 2001).

Tumor lymphatic vessels have been less studied, in part due to lack of appropriate molecular markers. In addition, because of technical challenges involved in performing functional lymphangiography, a highly debated and unresolved question nowadays is whether there are functional lymphatic vessels inside tumors (see below) (Jain and Fenton, 2002). Indeed, depending on the molecular markers used to label putative lymphatic vessels and the tumor model or type used to study tumor lymphangiogenesis, intratumoral lymphatic vessels were documented in tumor xenografts overexpressing VEGF-C or VEGF-D (Karpanen et al., 2001; Skobe et al., 2001; Stacker et al., 2001) or in head and neck squamous cell carcinomas (Jackson et al., 2001). However, no such intratumoral lymphatic vessels were detectable in other tumors, such as in invasive breast cancer (Jackson et al., 2001), or in VEGF-C transgenic mouse tumors (Mandriota et al., 2001). This discrepancy may be at least partially explained by the trapping of vessels in between the rapidly growing tumor foci in the xenografts. An outstanding, yet critical, question is whether these intratumoral VEGFR-3/LYVE-1 positive vessels are functional lymphatics. Indeed, lymphangiography by intravital microscopy after injection of dyes in experimental animal models of implanted and spontaneously arising tumors reveals that lymphatic vessels in the periphery of the tumors are enlarged and perfused, but compressed and nonfunctional in the inside of the tumor (Jain and Fenton, 2002). One explanation may be that neoplastic cells grown in a confined space generate mechanical stress, which compresses the newly formed lymphatic channels inside the tumor, while at the periphery, excess VEGF-C causes lymphatics to enlarge. These enlarged lymphatics may collect interstitial fluid and metastatic cancer cells "oozing" from the tumor surface, and thus facilitate lymphatic metastasis. Absence of functional lymphatics within tumors may contribute to interstitial hypertension and interfere with the delivery of therapeutic agents (Carmeliet and Jain, 2000).

Metastasis via lymphatic vessels

In most instances, metastatic spreading of tumors to distant organs—not the local growth of the primary tumor—kills the patient. Tumor metastasis involves a series of complex processes that include the detachment of tumor cells from the primary tumor mass, microinvasion into stromal tissues, intravasation into the lymphatic or blood vessels, and extravasation and

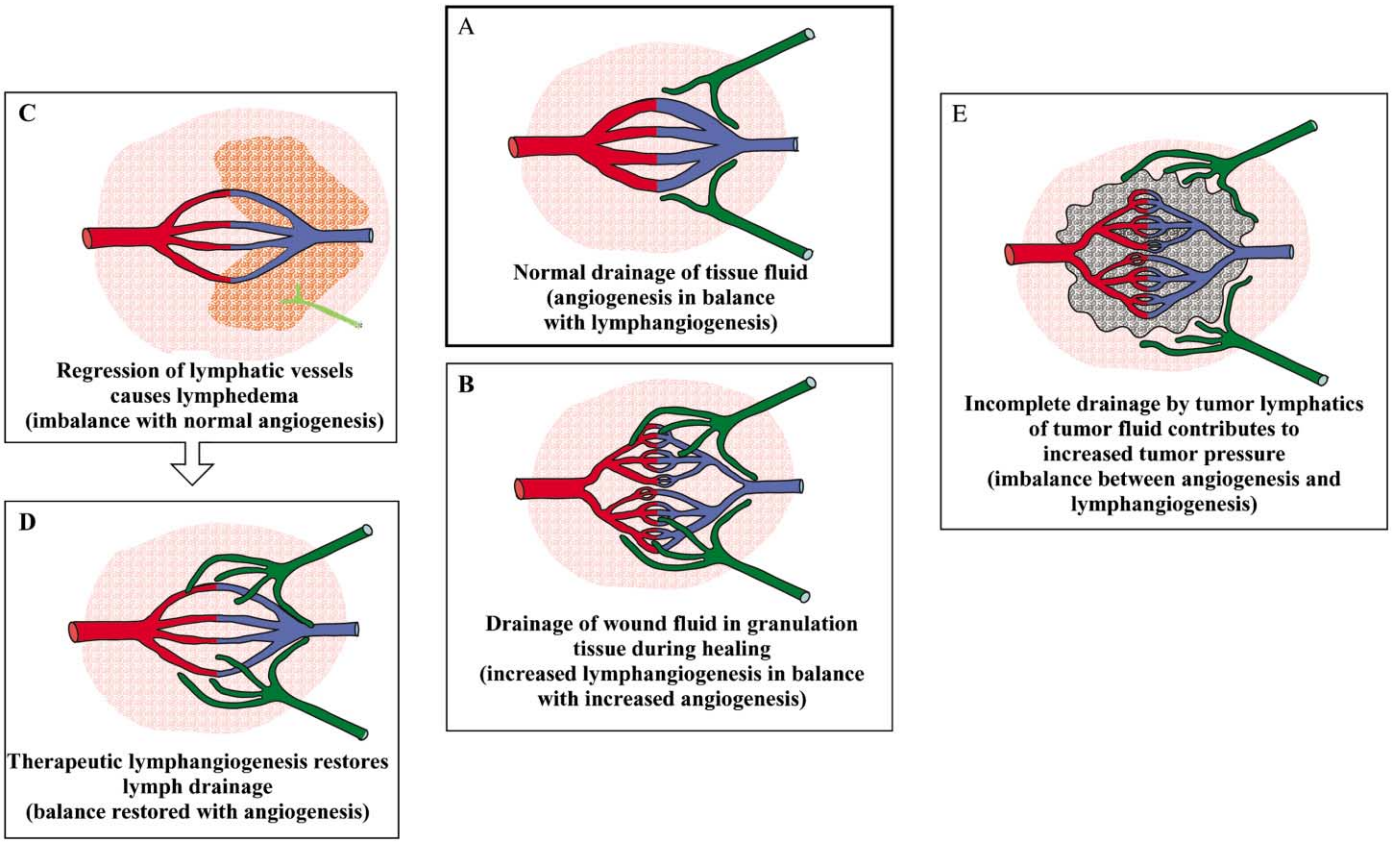

Figure 3. Scheme illustrating the interrelationship between angiogenesis and lymphangiogenesis

A: In normal tissues, lymphatic vessels balance blood vessels by draining extravasated fluid, macromolecules, and white blood cells so that the interstitial pressure does not increase due to edema. **B:** In wounds, lymphangiogenesis increases coincidently with angiogenesis to drain extravasated fluid from leaky neovessels, thereby preventing excessive lymphedema. **C:** When lymphatic vessels are damaged (radiation therapy) or are hypoplastic, obstructed (due to infection and inflammation), or removed (surgery), lymph drainage is insufficient, resulting in lymphedema and increased interstitial tissue pressure. **D:** Therapeutic lymphangiogenesis with VEGF-C (or perhaps VEGF-D) improves tissue fluid drainage by stimulating the growth of new lymphatic vessels (1). Whether collateral lymphatic vessels can also be stimulated to grow (as in blood vascular vessels) remains to be determined (2). **E:** In tumors, lymphangiogenesis and angiogenesis are not (necessarily) balanced. Blood vessels grow excessively inside tumors but, due to their grossly abnormal structure and function, excessive fluid leaks out and increases the interstitial tumor pressure, thereby compressing intratumor lymphatic vessels. Lymphatic vessels around tumors become hyperplastic and facilitate metastatic spreading of tumor cells to the lymph nodes. Tumor cells can, however, also metastasize via an hematogeneous route, which is facilitated by tumor angiogenesis.

growth in secondary sites. Tumor cells spread via the lymphatic system to regional lymph nodes and, because the large lymphatic vessels reenter into the blood vascular system, to distant organs (Figure 1). Sentinel lymphnodectomy is used in daily hospital practice to diagnose metastasis of primary tumor cells to regional lymph nodes and to evaluate the prognosis and appropriate treatment schedules. Even though most disseminated tumor cells have a limited life span and only a few develop into clinically detectable metastases, identification of those occult tumor cells and prevention of their growth and spread would be of great clinical significance. Few data are available on the influence of lymphatic microvessel density on survival in cancer, because until recently there was no reliable immunohistological marker for the lymphatic endothelium. In ovarian cancer, lymphatic vessel density was not correlated to the progression of the disease (Birner et al., 2000), while in cervical cancer, an increased amount of lymphatic vessels even appeared to be associated with a favorable prognosis (Birner et al., 2001). It is likely that human tumors demonstrate heterogeneity with regard to the presence or absence of intratumoral lymphatics. Considering the lack of specificity of currently used markers to identify lymphatic vessels, multiple markers should be used in elucidating any correlation between lymphatic vessel density and tumor growth, metastases, and prognosis, at least until the specificity of these markers in lymphatic vessels during tumor progression and metastases has been clarified.

VEGF-C and VEGF-D in experimental tumor lymphangiogenesis

Recent experimental models have highlighted the role of VEGF-C and VEGF-D in tumor biology. Transgenic mice overexpressing VEGF-C in β cells of the endocrine pancreas developed extensive lymphangiogenesis around the endocrine islets of Langerhans (Mandriota et al., 2001). When tumors were induced in these VEGF-C overexpressing islets by intercrossing with mice expressing the SV40 T-antigen oncogene in the β cells, metastatic tumor cell aggregates of β cell origin were observed in the surrounding lymphatic vessels. These mice also frequently developed metastases in the lymph nodes, which drain the pancreas, whereas tumors in mice lacking the VEGF-

C transgene never metastasized, nor were tumor cells observed inside the lymphatic vessels (Mandriota et al., 2001). VEGF-C overexpression by the tumors did not significantly alter tumor volume, transition from adenoma to carcinoma, or tumor angiogenesis, but, intriguingly, tumor incidence was increased—for as yet unknown reasons. Ectopic expression of VEGF-C in human breast cancer cells induced lymphangiogenesis in and around the orthotopically implanted tumors (Karpanen et al., 2001; Skobe et al., 2001) and enhanced tumor growth and spreading to regional lymph nodes, and, notably, the degree of tumor lymphangiogenesis correlated with lymph node metastases (Karpanen et al., 2001; Skobe et al., 2001; Mattila et al., 2002).

VEGF-D also promotes the metastatic spread of tumor cells via the lymphatics (Stacker et al., 2001). In addition to lymphangiogenesis and increased metastases, tumors secreting VEGF-D had an increased growth rate and tumor angiogenesis. Growth of the tumor, angiogenesis, and formation of metastases were inhibited by anti-VEGF-D antibodies. The differences between the tumor angiogenic properties of VEGF-C and VEGF-D may be due to differences in their proteolytic processing in different tumors or the variable expression of VEGFR-2 and VEGFR-3 on blood vascular and lymphatic endothelia. In particular, in the above case, enhanced tumor angiogenesis by VEGF-D was probably attributable to its increased proteolytic processing, resulting in a VEGF-D form with an increased affinity to VEGFR-2 (Stacker et al., 2001). Consistent with this model, the vascular effects of VEGF-C in experimental tumors were reduced by anti-VEGFR-2 antibodies (Kadambi et al., 2001). On the basis of these observations, tumor vessel formation can be dissected into pathways that preferentially activate angiogenesis (driven by VEGFR-2) or lymphangiogenesis (driven by VEGFR-3), although there is evidence that the receptors share overlapping expression patterns.

In theory, VEGF-C and VEGF-D could facilitate metastasis by increasing the surface area of tumor cells in contact with the lymphatic endothelium, by increasing vascular permeability, and/or by changing the adhesive properties or cytokine/chemokine expression of the lymphatic endothelium. These factors could also alter the tumor interstitial fluid pressure, which might determine tumor cell seeding, especially as recent studies revealed that a proportion of the lumen of tumor blood vessels themselves consists of tumor cells (Chang et al., 2000). Other molecules are likely to contribute to lymphatic metastasis. Indeed, attraction of CCR7-positive tumor cells to SLC-expressing lymphatic endothelium enhanced lymph node metastasis (Muller et al., 2001).

VEGF-C and VEGF-D in human tumors

It is still unknown whether VEGF-C or VEGF-D expression also promotes lymphangiogenesis in human tumors, and, if so, whether this increases the rate of metastasis to the lymph nodes. VEGF-C expression is detectable in about half of human cancers analyzed (Salven et al., 1998). A number of reports have described a correlation between VEGF-C expression in human tumors and the formation of metastases in regional lymph nodes. So far, VEGF-C levels in primary tumors have been shown to correlate significantly with lymph node metastases in thyroid, prostate, gastric, colorectal, lung, and esophageal carcinomas (reviewed in Karpanen and Alitalo, 2001; Pepper, 2001). Less is known about the expression of VEGF-D in human tumors: it is upregulated in human melanoma cells and in vessels adjacent to immunopositive tumor cells, but not in vessels distant from the tumors (Achen et al., 2001), suggesting that VEGF-D binds to the endothelial cells of nearby vessels and contributes in a paracrine manner to the regulation of tumor angiogenesis.

Lymphatic metastasis versus distant organ metastasis

Overexpression of VEGF-C in orthotopic breast tumor cells enhanced tumor metastasis to regional lymph nodes and the lungs (Skobe et al., 2001). However, the presence of a metastasis in a lymph node does not necessary mean that the tumor cells have been arrived via the lymphatic vessels. Intralymphatic tumor cells may pass directly into the blood vascular system through venolymphatic communications, which have been observed in certain organs. An increase in circulating tumor cells in the peripheral blood, resulting from enhanced tumor angiogenesis, could also facilitate lymph node metastases. In a recent study, a highly metastatic human lung cancer cell line LNM35 and its parental line N15 with low metastatic capacity were used (He et al., 2002). LNM35 tumor cells, expressing the highest amounts of VEGF-C, showed intratumoral lymphatic vessels and metastases in the draining lymph nodes. Even though lymphangiogenesis and lymphatic metastasis were suppressed in LNM35 tumors expressing a soluble form of VEGFR-3, tumors still metastasized to the lungs, suggesting that LNM35 cells can spread via other mechanisms and routes, for instance the blood (Figure 1). These data demonstrate that blockage of VEGFR-3 signaling can suppress tumor lymphangiogenesis and lymphatic metastasis, but not necessarily lung metastasis, indicating that the mechanisms of lymphatic and lung metastasis may differ (He et al., 2002). Thus, overexpression of VEGF-C and the associated de novo formation of lymphatic vessels are necessary, but not sufficient, for the metastatic dissemination of tumor cells to the lymph nodes. Factors in addition to VEGF-C are thus apparently required for metastatic spread.

Antilymphangiogenic therapy

VEGF-C-induced tumor growth, lymphangiogenesis, and intralymphatic tumor growth were inhibited by adenoviral expression of the soluble VEGFR-3 receptor, which "traps" available VEGF-C and VEGF-D (Karpanen et al., 2001) (Figure 3). Inhibition of VEGFR-3 by neutralizing antibodies also suppressed tumor growth by destabilizing large vessels in tumor xenografts in mice (Kubo et al., 2000), without affecting the established blood and lymphatic vasculature. Furthermore, lymphatic spread induced by VEGF-D was blocked with an antibody specific for VEGF-D (Stacker et al., 2001). In theory, anticancer drugs specifically targeted to peritumoral lymphatic vessels might inhibit lymphatic metastasis. However, caution is warranted, since destruction of these vessels could further elevate the already increased interstitial fluid pressure inside the tumors, thereby further impairing the delivery of other anticancer drugs. As VEGF-D expression can be upregulated by direct cell-cell contacts, one can also speculate that an increased intratumoral pressure could increase close contacts between the tumor cells and lead to a compensatory increase of the lymphangiogenic growth factor levels (Orlandini and Oliviero, 2001). Increased intratumoral fluid pressure could also enhance the likelihood of hematogeneous metastasis (Carmeliet and Jain, 2000; Stohrer et al., 2000).

Therapeutic lymphangiogenesis

At present, lymphedema is treated by manual lymphatic drainage and by compressive garments. The discovery of specific genes involved in the regulation of lymphatic vessels and in the pathology of lymphedema should make the design of more targeted treatments for this disease possible. Indeed, subcutaneous adenoviral gene transfer of VEGF-C in normal mice induced lymphangiogenesis (Enholm et al., 2001). More importantly, however, overexpression of VEGF-C in the skin of a mouse model (Chy) of lymphedema (due to an inherited partial loss of VEGFR-3 activity) also enhanced growth of functional cutaneous lymphatic vessels (Karkkainen et al., 2001), suggesting that VEGF-C/D gene therapy may be applicable to human lymphedema (Figure 3). It remains to be determined whether such therapy could also be used in nonhereditary, regional forms of lymphedema resulting from trauma, surgery, or lymphatic vessel destruction after filariasis. Future studies will need to address the differences in the molecular "lymphangiogenic switch" between lymphatic vessels in the skin versus the visceral organs or the larger connecting lymphatic vessels, as those vessels are differently regulated by VEGFR-3 signals (Karkkainen et al., 2001). In addition, it remains to be examined whether lymphangiogenesis in adults is only mediated by endothelial cell sprouting from or splitting of preexisting lymphatic or blood vessels or in situ differentiation of endothelial cells—or, alternatively, whether new lymphatic vessel growth can be stimulated by enhancing or recruitment and lymphatic differentiation of endothelial precursor cells, as has been described in other models (Schneider et al., 1999). The latter would be attractive, as it would allow cell transplantation for regeneration of damaged or insufficient lymphatic vessels.

Conclusion

The recent discovery of the key molecules VEGF-C and VEGF-D and the isolation of lymphatic endothelial cells have allowed studies of lymphangiogenesis at the molecular level. Similarities between the regulation of blood and lymphatic vessels have been observed, and these two vessel systems appear to work in a tightly regulated manner. Thus far, results on therapeutic lymphangiogenesis with VEGF-C for lymphedema and inhibition of metastatic spread of tumor cells via the lymphatic vasculature by blocking VEGFR-3 signaling have been most encouraging. Future clinical trials will show the therapeutic potential of these molecules in man.

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