Lymphangiogenesis in development and disease

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Summary
The lymphatic vascular system plays an important role in the maintenance of fluid homeostasis, in the afferent immune response, in the intestinal lipid uptake and in the metastatic spread of malignant cells. The recent discovery of specific markers and growth factors for lymphatic endothelium and the establishment of genetic mouse models with impairment of lymphatic function have provided novel insights into the molecular control of the lymphatic system in physiology and in embryonic development. They have also identified molecular pathways whose mutation inactivation leads to human diseases associated with lymphedema. Moreover, the lymphatic system plays a major role in chronic inflammatory diseases and in transplant rejection. Importantly, malignant tumors can directly promote lymphangiogenesis within the primary tumor and in draining lymph nodes, leading to enhanced cancer metastasis to lymph nodes and beyond. Based upon these findings, novel therapeutic strategies are currently being developed that aim at inhibiting or promoting the formation and function of lymphatic vessels in disease.

Keywords
Lymphatic endothelium, lymphangiogenesis, lymphedema, inflammation, cancer metastasis

Introduction
The lymphatic vascular system was first described in 1627 by Gasparo Aselli (1). Despite its importance for the maintenance of tissue fluid homeostasis and for the afferent immune response, research into the molecular mechanisms of lymphatic vessel formation and function has for a long time been hampered by the lack of lymphatic-specific markers and growth factors. However, the recent discovery of several specific markers for lymphatic endothelium, together with a plethora of genetic mouse studies which identified novel mediators of lymphatic vessel formation, differentiation and function, have provided novel insights into the role of the lymphatic system in physiology and in disease. In particular, the emerging active role of lymphatic vessels in chronic inflammation and in metastatic cancer spread have drawn considerable attention to the previously rather neglected field of lymphangiogenesis. Moreover, the characterization of pathways critical for lymphatic function has led to the identification of potential drug targets for enhancing or inhibiting lymphatic vessel formation (2).

Organization and function of the lymphatic system
In contrast to the blood vascular system, the lymphatic system is open ended and serves as a drainage system by collecting interstitial fluid, proteins and macromolecules in the periphery and by transporting them back to the subclavian veins in the nuchal region. In the intestine, lymphatic vessels play an important role in the uptake of dietary fats. Lymphatic vessels also attract antigen-presenting and other immune cells from peripheral tissues to the draining lymph nodes.

The lymphatic vascular system is composed of peripheral capillaries, collecting vessels, lymph nodes, larger trunks and the thoracic duct (3). Peripheral capillary walls consist of a single layer of overlapping lymphatic endothelial cells that are connected to the surrounding tissue by anchoring filaments. Due to the lack of a continuous basement membrane, the absence of pericyte or smooth muscle cell coverage, and the lack of tight junctions (4–6), these anchoring filaments represent the major means of vessel stabilization (7). Under physiological conditions, most lymphatic capillaries remain collapsed; however, when the interstitial pressure increases, the anchoring filaments...
"pull open" the capillaries to ensure enhanced drainage and increased luminal volume. The collecting vessels possess valves and are surrounded by smooth muscle cells. The intrinsic pump activity, nitric oxide-responsiveness (8, 9) and valves enable efficient unidirectional lymph flow (9). All collecting lymphatic vessels pass through lymph nodes that are organized in clusters throughout the lymphatic system. Theafferent lymphatics empty into the subcapsular sinus of draining lymph nodes. Lymph nodes function as filters and reservoirs where both T and B lymphocytes are activated. Their capsule is perforated at various points by afferent lymphatics. Lymph fluid, macromolecules and cells travel through the trabecular and marginal sinuses to reach the efferent lymphatics. Sinuses are lined by endothelial cells that express typical lymphatic markers (10), and they are possibly connected with the surrounding nodal parenchyma and with blood vessels by lymphaticovenous shunts (10).

Embryonic development of the lymphatic system

There has been a century-old controversy about the origin of lymphatic vessels during embryonic development. In 1902, Florence Sabin proposed that the lymphatic vasculature develops from embryonic veins (11, 12), and that the peripheral lymphatic system develops from primary lymph sacs by endothelial sprouting. This concept has been recently confirmed by genetic mouse studies of the home gene Prox-1 (13). At a certain stage of embryonic development, the lymphatic vascular endothelium hyaluronan receptor LYVE-1 is expressed by endothelial cells of the cardinal vein, representing a stage of lymphatic competence (responsiveness to lymphatic signals) (14). Upon a yet unidentified signal, Prox-1 expression occurs in some endothelial cells of the cardinal vein (Fig. 1) (lymphatic bias), leading to budding of endothelial cells, initially in the jugular and mesonephric regions. Prox-1 is required for lymphatic development, since Prox-1 null mice do not develop a lymphatic vascular system, whereas blood vessels seem to be unaffected (13). Moreover, Prox-1 also promotes the lymphatic-specific differentiation of the budding endothelial cells, and leads to downregulation of blood vessel markers (15). Indeed, induction of lymphatic differentiation has been observed after ectopic overexpression of Prox1 in cultured vascular endothelial cells (16, 17), and the lymphatic reprogramming of vascular endothelium by the Kaposi's sarcoma-associated herpes virus (18, 19) is partially dependent on Prox1 activity (18).

Vascular endothelial growth factor (VEGF)-C also plays an essential role during early lymphatic development. VEGF-C activates the VEGF receptor-3 (VEGFR-3) that is expressed on early embryonic blood vessels and on lymphatic endothelium (15). In VEGF-C null mice, the endothelial cells that bud off the cardinal veins are committed to the lymphatic lineage, but they are unable to sprout or to form lymphatic vessels (20). Moreover, VEGF-C knockdown in Xenopus tadpoles impairs the directional migration and budding of lymphatic precursors (21). The distinct role of VEGFR-3 during embryonic lymphatic development is currently difficult to assess because VEGFR3 null mice die during early development due to cardiovascular failure (22). Taken together, these results suggest that Prox-1 activity is required for the lymphatic differentiation of embryonic venous endothelium, whereas VEGF-C/VEGFR-3 provide essential signals for sprouting (13, 20). Additional molecules, including the mucin-type glycoprotein podoplanin (23), neuropilin-2 (24) and angiopoietin-2 (Ang2) (25) play major roles in the further maturation of the developing lymphatic system. Mice lacking Ang2 develop subcutaneous edema, chylous ascites, and die shortly after birth due to defective lymphatic vessels (25). Angiopoietin-1 (Ang1) rescues these effects, while the impaired angiogenesis is not affected (25). An important role of ephrin B2, a cell surface tyrosine kinase, in lymphatic maturation has been found in mice expressing a mutant form of ephrin B2. These mice display normal blood vessel development but disturbed postnatal maturation of the lymphatic system (26). The integrin α9β1 is also required for proper lymphatic development and is involved
in mediating the effects of VEGF-C and VEGF-D via VEGFR-3 (27). Mice deficient in the integrin α9 subunit show edema and chylothorax, and die shortly after birth (28). Likely, additional molecules are essential for both the early and late stages of lymphangiogenesis.

It has also been proposed that the primary lymphatic endothelial cells develop directly from the mesenchyme, independently from veins, and that they only later establish connections with the venous system (29). There is evidence that in birds the lymph sacs develop by sprouting of progenitor cells from the embryonic mesenchyme (30). Moreover, during tadpole development, both budding of lymphatic precursors from preexisting veins and development of lymphatics from mesenchymal precursors has been reported (21). Recently, we found that embryoid bodies, obtained from murine embryonic stem cells, spontaneously show formation of lymphatic endothelium and lymphatic vascular structures (31). The development of these structures lagged behind that of the blood vessels, and LYVE-1-positive endothelial cells appeared earlier than Prox-1-expressing cells. It is of interest that in embryoid bodies, lymphatic structures were observed that apparently sprouted from blood vessels, as well as independent accumulations of lymphatically differentiated cells (31, 32). Overall, the major mechanism for embryonic formation of the lymphatic vasculature appears to be the budding from preexisting veins, with a possible contribution from mesenchymal progenitors.

Molecular regulation of lymphangiogenesis

The first specific lymphangiogenesis factors identified were VEGF-C and VEGF-D (Fig. 2) (33–35). They bind to and activate VEGFR-3, also known as Flt4, the first known lymphatic-specific growth factor receptor (36). VEGF-3 is expressed in venous and lymphatic endothelium during early embryonic development (36). In adults, the expression of VEGF-3 becomes confined to the lymphatic endothelium (36), as well as to activated macrophages and dendritic cells (DC) (37–39). VEGF-3 can be reexpressed by tumor-associated blood vascular endothelium and thus might contribute to tumor angiogenesis and growth (40). Inactivation of VEGFR-3 results in embryonic lethality due to a failure of remodeling of the capillary networks before the emergence of lymphatic vessels (22).

Recent studies have revealed that VEGF-A can also support lymphangiogenesis through interaction with VEGFR-2 (41–43). VEGF-A predominantly binds to VEGFR-1 and VEGFR-2 on blood vascular endothelium, but VEGF-2 is also expressed on lymphatic endothelial cells (41, 44, 45). VEGF-A potently induces proliferation and migration of lymphatic endothelial cells in vitro, and overexpression of VEGF-A in vivo induces lymphangiogenesis in tissue repair and inflammation (41–43).

The VEGF-coreceptor neuropilin-2 (Nrp-2), a semaphorin receptor in the nervous system, mediates axonal guidance during neuronal development. It is also a receptor for VEGF-C and VEGF-D (46). Neuropilin-2 is expressed by lymphatic endothelium, and neuropilin-2-deficient mice show a reduction of small lymphatic vessels (24). This raises the question whether VEGF-C signaling may be enhanced by neuropilin, similar to neuropilin-1 promotion of VEGF-A signaling via VEGFR-2 (47).

Additional lymphangiogenic factors have recently been identified (2), including fibroblast growth factor-2 (bFGF), hepatocyte growth factor (HGF), angiopoietin-1 (Ang1), platelet-derived growth factor (PDGF)-BB and insulin-like growth factors (Fig. 2). Fibroblast growth factor-2 promotes lymphangiogenesis in the mouse cornea, likely via induction of VEGF-C (48, 49), but direct effects on lymphatic endothelial cells have also been observed (50). HGF promotes lymphatic endothelial cell proliferation, migration and tube formation in vitro, and transgenic overexpression of HGF in mice results in increased numbers of enlarged lymphatic vessels (51). Ang1 activates the endothelial-specific receptor tyrosine kinase Tie2 (52) that is expressed by LECs in vitro and in vivo. Ang1 induces lymphangiogenesis in the cornea and other tissues, and its skin-specific overexpression in transgenic mice results in cutaneous lymphatic hyperplasia (53, 54). Ang1 might also act indirectly via the VEGF-C/VEGFR-3 pathway because the effects of virally delivered Ang1 in mice are inhibited by treatment with a soluble
VEGFR-3, and because VEGFR-3 is up-regulated after Ang1 stimulation of LECs in vitro and in vivo (53, 54).

The successful isolation, culture and transcriptional analysis of lymphatic endothelial cells obtained from human tissues have provided additional candidate molecules that might modulate lymphatic growth and function in development and disease (17, 44, 45).

The role of the lymphatic vasculature in disease

Lymphedema

Lymphedemas are characterized by interstitial fluid accumulation, most frequently in the limbs, due to impaired formation or function of lymphatic vessels. They are either primary, likely caused by genetic alterations, or – most frequently – secondary to trauma or infection. Primary lymphedemas are divided into early-onset Milroy’s disease and Meige disease with a preferential onset at puberty (55). In Milroy’s disease, a mutation leading to inhibition of the phosphorylation of the VEGFR-3 receptor has been found in several cases (56, 57). Similarly, an inactivating VEGFR-3 mutation in the germ line of Chy mice leads to chylous ascites and peripheral lymphedema (47). Importantly, expression of a soluble VEGFR-3 receptor – that neutralizes the VEGFR-3 ligands VEGF-C and -D – resulted in lymphedema in transgenic mice (58). In addition, inactivating mutations of the forkhead transcription factor FOXC2 have been found in the autosomal-dominant lymphedema-distichiasis syndrome that is characterized by late onset of lymphedema and by a double row of eyelashes (distichiasis) (59). Additional mutations implicated in primary lymphedemas are those of the transcription factor SOX18 and of reelin (60, 61).

The most common cause of secondary lymphedema is filariasis, a parasitic worm infection (Brugia malayi or Wuchereria bancrofti) associated with massive fibrosis of the lymph nodes and lymphatic vessels in the inguinal region. Within the Western countries, secondary lymphedema is most often observed after cancer surgery and lymph node resection, and after radiotherapy. The resulting fluid accumulation in the extremities can lead to chronic and disabling swelling, tissue fibrosis, impaired wound healing, reduced immune function and susceptibility to infections (62). Recent studies of experimental wound healing in diabetic mice indicate that overexpression of VEGF-C in the wounded area may induce accelerated wound healing and tissue repair (63). Experimentally, both primary and postsurgical lymphedemas can be ameliorated by VEGF-C-based therapies, either by adenoviral delivery of VEGF-C or by direct delivery of VEGF-C protein (47, 64, 65). These findings open up new avenues for the future treatment of lymphedema patients.

Inflammation and transplant rejection

There is increasing evidence that lymphatic vessels have an active role in acute and chronic inflammation. Skin lesions in the chronic inflammatory disease psoriasis show lymphatic hyperplasia (43), and lymphatic endothelial cell proliferation and lymphatic hyperplasia are also observed in chronic skin inflammation in mice (43). Moreover, inflammation induced by acute UVB irradiation of the skin results in edema and hypermeable, leaky lymphatic vessels that are functionally impaired (66). It is of interest that inhibition of VEGFR-3 signaling leads to prolonged edema and inflammation after UVB irradiation (67). Thus, in addition to removing inflammation-associated tissue edema, lymphatic vessels might also actively participate in the maintenance of chronic inflammatory diseases.

Data from human and rodent corneal transplantation suggest that alloimmune responses are significantly enhanced when the graft bed is vascularized and lymphatic-rich (38, 68). The generation of immunity to ocular surface antigens requires a functional eye-lymphatic axis (69). The normal lack of functional lymphatics in the cornea has been related to its ‘immune privileged’ status since it hinders efficient delivery of antigens and antigen-presenting cells to T-cell reservoirs (70). The induction of lymphatic vessels in the cornea thereby facilitates the delivery of antigen presenting cells to lymph nodes, which may contribute to inflammation and rejection of corneal grafts. Lymphangiogenesis has also been found to be associated with kidney transplant rejection, where the LEC-derived chemokine CCL21 might further enhance the inflammatory process (71). Thus, lymphatic neoangiogenesis – mediated in part by VEGF-C producing macrophages (72) – might not only contribute to the export of the rejection infiltrate but might also be involved in the maintenance of an alloreactive immune response in renal transplants (71). These results indicate that anti-lymphangiogenic strategies may improve transplant survival (73, 74).

Tumor lymphangiogenesis and metastasis

The metastatic spread of tumor cells is responsible for the majority of cancer deaths. Lymph nodes are the most common sites of metastasis. Once tumor cells are established in the sentinel lymph node (SLN), they can further metastasize to distant lymph nodes and beyond. Depending on the type of cancer, haematogenous metastasis can occur without SLN metastasis, but likely in a minority of cases (75). The mechanisms of lymphatic metastasis have remained unclear for decades, mainly due to the lack of reliable markers for lymphatic vessels, and to the absence of identified lymphangiogenic growth factors. Recent studies, however, have convincingly shown that tumors can actively induce the formation of lymphatic vessels – via release of VEGF-C or VEGF-D – and thereby promote metastasis to draining lymph nodes (76–78). Although these results were obtained in mouse tumor models, an increasing number of studies have shown a strong correlation between the expression of VEGF-C (or VEGF-D), tumor lymphangiogenesis, cancer metastasis and patient survival (79). In human malignant melanomas of the skin, an enhanced degree of lymphangiogenesis in the primary tumor was significantly correlated with reduced disease-free and overall survival (80). Importantly, tumor lymphangiogenesis was the most significant prognostic parameter to predict the presence of melanoma metastasis in SLN at the time of surgery (81).

Although tumor cells often are the prime source of lymphangiogenic factors, tumor-associated leukocytes can release a number of mediators and proteinases that directly affect lymphatic vessels. In human cervical cancer, lymphangiogenesis was found to be correlated with the degree of inflammation and with VEGF-C expressing macrophages (39, 82). Tumor-secreted VEGF-C can recruit VEGFR-3-expressing monocytes to the tumor site, and the number of tumor-associated macrophages has
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been found to be correlated with breast cancer lymphangiogenesis, leading to enhanced metastasis to sentinel lymph nodes (SLN). Tumor-derived lymphangiogenic factors such as VEGF-C or VEGF-A are drained to the SLN where they induce expansion of the lymphatic network (lymph node lymphangiogenesis; A, B), likely preparing the lymph node for the latter arrival of the metastatic cells. Metastatic cells then further enhance SLN (C) and distant lymph node lymphangiogenesis, enhancing further cancer spread to distant lymph nodes and – via the thoracic duct and the venous system – to organs. The relative contribution of lymphatic versus direct haematogenous metastasis is different in distinct cancer types.

**Lymph node lymphangiogenesis promotes cancer metastasis**

A new concept of tumor metastasis has been recently developed by Hirakawa et al. (86, 87). Using a multistep chemical skin carcinogenesis model in transgenic mice overexpressing either VEGF-A or VEGF-C in the epidermis, we found that skin cancers can induce expansion of the lymphatic network in the sentinel (draining) lymph node even before they metastasize (86, 87). Lymph node lymphangiogenesis was even further enhanced after arrival of metastatic cells (86, 87). These findings give a new spin to the "seed-and-soil" hypothesis and suggest that the seed (cancer) can modify the soil (lymph node) to prepare it for its later arrival (Fig. 3). Thus, the expanded subcapsular sinus of the sentinel lymph nodes might represent a metastatic niche that facilitates metastatic cancer growth. Likely, expansion of the lymphatic network in the lymph nodes is mediated by remote control from the distant tumor site, via secretion of VEGF-C and/or VEGF-A which are transported via lymphatic vessels to the sentinel lymph node (86, 87, 98). Cancers might exploit a mechanism originally developed for promoting the afferent immune response, since lymph node lymphangiogenesis has also been observed after skin sensitization and inflammation, possibly facilitating dendritic cell migration to lymph nodes (99, 100).

Importantly, we recently found that induction of lymph node lymphangiogenesis strongly promotes the further cancer metastasis to distant lymph nodes and to organs (86). Thus, lymph node lymphangiogenesis represents a novel target for therapies aimed at inhibiting and/or treating cancer metastasis. Moreover, imaging of activated lymphatic vessels within (pre)metastatic lymph nodes might enable detection of tumor micrometastases – possibly even before they occur.

**Concluding remarks**

The field of lymphangiogenesis is currently receiving tremendous scientific and clinical interest. The identification of novel mediators of lymphangiogenesis, together with the discovery of genetic defects associated with diseases of the lymphatic system, will likely lead to new advances in our understanding of the mechanisms underlying many diseases, including chronic inflammation and metastatic cancer. Based upon the results of preclinical studies, it can be expected that modulation of lymphatic vessel growth and activation will find its way into the clinic within the near future.

**Figure 3: Pathways of malignant tumor cell dissemination.**

In many solid tumors, cancer cells promote tumor-associated lymphangiogenesis, leading to enhanced metastasis to sentinel lymph nodes (SLN). Tumor-derived lymphangiogenic factors such as VEGF-C or VEGF-A are drained to the SLN where they induce expansion of the lymphatic network (lymph node lymphangiogenesis; A, B), likely preparing the lymph node for the latter arrival of the metastatic cells. Metastatic cells then further enhance SLN (C) and distant lymph node lymphangiogenesis, enhancing further cancer spread to distant lymph nodes and – via the thoracic duct and the venous system – to organs. The relative contribution of lymphatic versus direct haematogenous metastasis is different in distinct cancer types.
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