Lymphangiogenesis in Cancer: Current Perspectives

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Abstract Although the lymphatic system has been initially described in the sixteenth century, basic research has been limited. Despite its importance for the maintenance of tissue fluid homeostasis and for the afferent immune response, research of the molecular mechanisms of lymphatic vessel formation and function has for a long time been hampered. One reason could be because of the difficulties of visibility due to the lack of lymphatic markers. But since the discovery of several molecules specifically expressed in lymphatic endothelial cells, a rediscovery of the lymphatic vasculature has taken place. New scientific insights has facilitated detailed analysis of the nature and organization of the lymphatic system in physiological and pathophysiological conditions, such as in chronic inflammation and metastatic cancer spread. Knowledge about the molecules that control lymphangiogenesis and tumor-associated lymphangiogenesis is now expanding, allowing better opportunities for the development of drugs interfering with the relevant signaling pathways. Advances in our understanding of the mechanisms have translated into a number of novel therapeutic studies.

8.1 Introduction

The lymphatic vasculature develops separately, but is functionally related to the blood vascular system. While the blood vascular system is a closed circulatory system, the lymphatic system is open-ended. It collects the interstitial fluid in the periphery and drains the absorbed lymph in the nuchal region into the subclavian veins. This loop controls the balance of various factors and 10% of the body fluid volume. Next to the transport of interstitial fluid, the lymphatic system plays an essential role in the circulation of macromolecules, dietary fats, lymphocytes, and antigen-presenting cells. In the immune-regulatory network, the lymphatic system directs the trafficking of cytokines and immune cells. However, the lymphatic system is also a common pathway for lymphatic metastasis, and therefore plays an essential role for overall survival of cancer patients.

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R. Liersch et al. (eds.), *Angiogenesis Inhibition,* Recent Results in Cancer Research,
DOI: 10.1007/978-3-540-78281-0_8, © Springer Verlag Berlin Heidelberg 2010

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8.2 Embryonic Lymphatic Development

The lymphatic system develops in parallel with the blood vascular system, but although major progress has been made, it remains controversial as to whether the lymphatic vasculature is developing from embryonic veins, from lymphangioblasts, or from both (Wilting et al. 1999). In 1902, Florence Sabin proposed the most widely accepted theory that the lymphatic vasculature develops from embryonic veins (Sabin 1902; Sabin 1904) and that the peripheral lymphatic system expands from the primary lymph sacs, originates from vascular endothelial cells, and then spreads by endothelial sprouting, forming capillaries. Upon the formation of the vascular system, Lyve-1 (lymphatic vessel endothelial hyaluron receptor) starts to be expressed in venous endothelial cells of the cardinal vein, and endothelial cells become competent to respond to lymphatic signals (lymphatic competence). Induced by a so far unknown signal almost at the same time, Prox-1 expression occurs in restricted areas of the cardinal vein, determining the lymphatic fate (lymphatic bias) of budding endothelial cells. Homebox gene Prox-1 is a transcription factor related to the Drosophila gene prospero, and expressing endothelial cells are detected in a polarized manner in a subset of cells of the cardinal vein, leading to budding of endothelial cells, initially in the jugular and mesonephric regions (Wigle and Oliver 1999). The analysis of Prox-1 null mice revealed that Prox-1 is required to promote lymphangiogenesis in a specific subpopulation in the embryonic vein. The importance becomes evident by the fact that in Prox-1 null mice the lymphatics do not develop, whereas the blood vessels seem to be unaffected (Wigle and Oliver 1999). Prox-1 promotes the lymphatic differentiation and leads to the downregulation of blood vessel markers (Wigle et al. 2002).

The vascular endothelial growth factor (VEGF)-C plays another essential role during lymphatic development. Binding of its receptor, the VEGF-Receptor-3 (VEGFR-3), expressed on early blood vessels and on lymphatic endothelium is required for migration and budding. In VEGF-C knockout mice, endothelial cells commit to the lymphatic lineage but do not sprout to form lymph vessels (Karkkainen et al. 2004). Xenopus tadpoles with VEGF-C knockdown had lymphatic commitment but impaired the directional migration and budding (Ny et al. 2005). Taken together, these results suggest that Prox-1 activity is required for the commitment of the venous endothelial cells to lymphatic differentiation, whereas VEGF-C/VEGFR-3 signaling provides essential signals for sprouting (Karkkainen et al. 2004; Wigle and Oliver 1999). The development of the lymphatic vasculature during embryogenesis lags behind that of the blood vessels, and these vessels at a later point in time develop Prox-1, Lyve-1, and CD31 positive vessel structures. Vascular endothelial growth factor–A and –C, but not basic FGF-2 (basic FGF), hepatocyte growth factor (HGF), and hypoxia, stimulate the development of early lymphatics (Kreuger et al. 2006; Liersch et al. 2006). Additional molecules, including the mucin-type glycoprotein podoplanin, Neuropilin-2 (Nrp-2), and angiopoietin-2 (Ang2) play major roles in the further maturation of the developing lymphatic system. Integrin $\alpha 9\beta 1$ is required for the development of the fully functional lymphatic system and is involved in mediating the effects of VEGF-C and VEGF-D via VEGFR-3. Mice deficient in the integrin $\alpha 9$-subunit show edema and chylothorax, and die shortly after birth.

An alternative model suggested that the primary lymphatics develop in the mesenchyme from precursor cells, so-called lymphangioblasts, independent from veins, and only later establish connections with the venous system (Huntington and McClure 1910). This was supported by the findings obtained in birds, where the lymph sacs develop by sprouting and form the embryonic mesenchyme (Schneider et al. 1999). Recently it has been shown in the tadpole model that both mechanisms can also contribute to lymph vessel development (Ny et al. 2005). Evidence for both models has been recently found in murine embroid bodies. In these

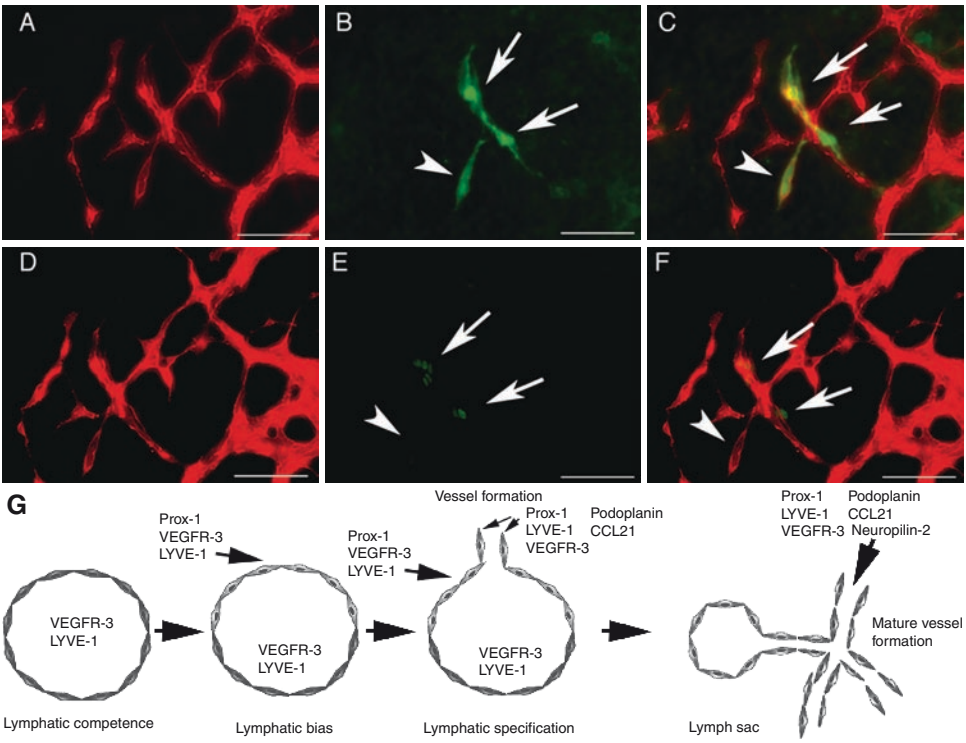

Fig. 8.1 Embryonic development of the lymph system. Lymphatic competence of vascular structures in embryoid bodies (EB) show differential expression of Lyve-1 and Prox-1. Double immunofluorescence stains of 21 days old EBs for CD31 (*red*; **a**, **d**) and Prox-1 (*green*, **e**) revealed CD31-positive blood vessels and CD31+/Prox-1+ (**e**; *arrow*) positive lymphatic vessels (f; merged image). Differential immunofluorescence stains for CD31 (**a**, **d**; *red*) and LYVE-1 (**b**; *green*) revealed that vascular structures are CD31+/Lyve-1 positive (b; *arrow/arrowhead*) with no expression of Prox-1 (e; *arrowhead*). (**c**, **f**) Merged images. Scale bars: 100 µm. (**g**) At early embryonic development endothelial cells of the cardinal vein express LYVE-1 and VEGFR-3 (lymphatic competence). Upon stimulation a subset of endothelial cells express the transcription factor Prox-1, a master regulator of lymphatic differentiation (lymphatic bias). These Prox-1 cells bud off and migrate out to form the primitive lymph sacs and then the mature lymphatic network. During this process, they upregulate the expression of additional lymphatic lineage markers

three-dimensional structures lymphatic endothelial cells (LEC) seem to develop not only from blood vessels. In agreement with earlier observation, LYVE-1/CD31 positive vessels develop much earlier than Prox-1 expression occurs. But Prox-1 was partially expressed not only in a subpopulation of LYVE-1/CD31 positive blood endothelial vessels (Fig. 8.1), but also in additional areas of newly formed lymphatic vessels not associated to any blood vessel.

In summary, until now published data suggest that the lymphatic vasculature is budding of from pre-existing veins, with a contribution from mesenchymal progenitors.

8.3 The Lymphatic Function

The lymphatic system consists of capillaries, collecting vessels, lymph nodes, trunks, and ducts. In the periphery, the blind-ended, finger shaped capillaries consist of a single layer of

overlapping cells, connected to the surrounding tissue by fibrillin-containing anchoring filaments (Gerli et al. 2000). Due to an absent basal membrane, no smooth muscle cells, and lack of tight cell–cell junctions (Barsky et al. 1983; Leak and Burke 1968; Sauter et al. 1998), only these filaments stabilize the lymphatic capillaries and facilitate lymphatic flow and drainage (Leak and Burke 1966). Under physiological conditions, lymphatic capillaries remain collapsed, but especially in the case of increased interstitial pressure the anchoring filaments provide a better drainage by increasing their luminal volume. After the capillaries merge into collecting vessels, they consist of valves and are surrounded by smooth muscle cells. Intrinsic pump activity, nitric oxide–responsiveness (Shirasawa et al. 2000; von der Weid 2001), skeletal muscle action and valves regulate the unidirectional lymph flow (von der Weid 2001). Collecting vessels become the afferent lymphatics of lymph nodes, emptying into the subcapsular sinus. Lymph nodes are discrete structures surrounded by a capsule composed of connective tissue. Lymph nodes function as filters and reservoirs and exist for the activation of T-lymphocytes and B-lymphocytes. The capsule is perforated at various points by afferent lymphatics. Lymph fluid, macromolecules, and cells travel through the subcapsular, the trabecular, and marginal sinuses to reach the efferent lymphatic. The lining endothelium of the sinuses is lymphatic endothelium, expressing the typical lymphatic markers. Lymph node sinuses have an irregular surface with many reticular cells and fibers protruding into or crossing the lumen and, equivalent to the anchoring filaments of the peripheral capillaries, these fibers support the intranodal vessel lumen (Okada et al. 2002). Casts of these sinuses are connected with the surrounded nodal parenchyma and blood vessels by lymphaticovenous shunts (Okada et al. 2002). All collecting lymphatic vessels pass through lymph nodes, which are organized in clusters through the lymphatic systems. After leaving the lymph node, the efferent lymphatic vessels merge to thoracic ducts and drain the collected fluids, proteins, and cells back into the blood vascular circulation. Reflecting this specialized function in drainage, transport, and dissemination the lymphatic vasculature is crucially involved in the pathogenesis of various diseases or inflammatory conditions.

8.3.1 Molecular Players in the Regulation of Lymphangiogenesis

The lymphatic endothelium expresses most of the common endothelial cell markers and shares various biological similarities with the blood endothelium (Sauter et al. 1998; Wissmann and Detmar 2006). The main regulator of lymphatic differentiation is the homebox transcription factor *Prox-1* (Drosophila prospero related homeobox gene) (Alitalo and Carmeliet 2002; Wigle et al. 2002). Essential for lymph vessel growth are growth factors like *VEGF-C* and *VEGF-D* (Jeltsch et al. 1997; Oh et al. 1997; Veikkola et al. 2001). These were the first described stimulators of lymphangiogenesis (Fig. 8.2). Both are members of the VEGF-family, and they bind and activate the vascular endothelial growth factor receptor (VEGFR)-3 (Achen et al. 1998; Cao et al. 2004; Joukov et al. 1996; Lee et al. 1996; Makinen et al. 2001a; Veikkola et al. 2001), but after stepwise proteolytic processing by enzymes such as plasmin and proprotein convertases, they also bind VEGFR-2 (Joukov et al. 1997; Stacker et al. 1999) influencing angiogenesis as well (Cao et al. 1998; Marconcini et al. 1999; Witzenbichler et al. 1998). VEGFR-3, also known as FLT-4, was the first lymphangiogenic specific growth factor receptor (Kaipainen et al. 1995). It is expressed in early embryonic development in venous and lymphatic endothelium (Kaipainen et al. 1995) and synthesis is in parts controlled via activation of the p42/p44 MAPK signaling cascade, in protein C kinase dependent fashion, and via AKT phosphorylation (Makinen

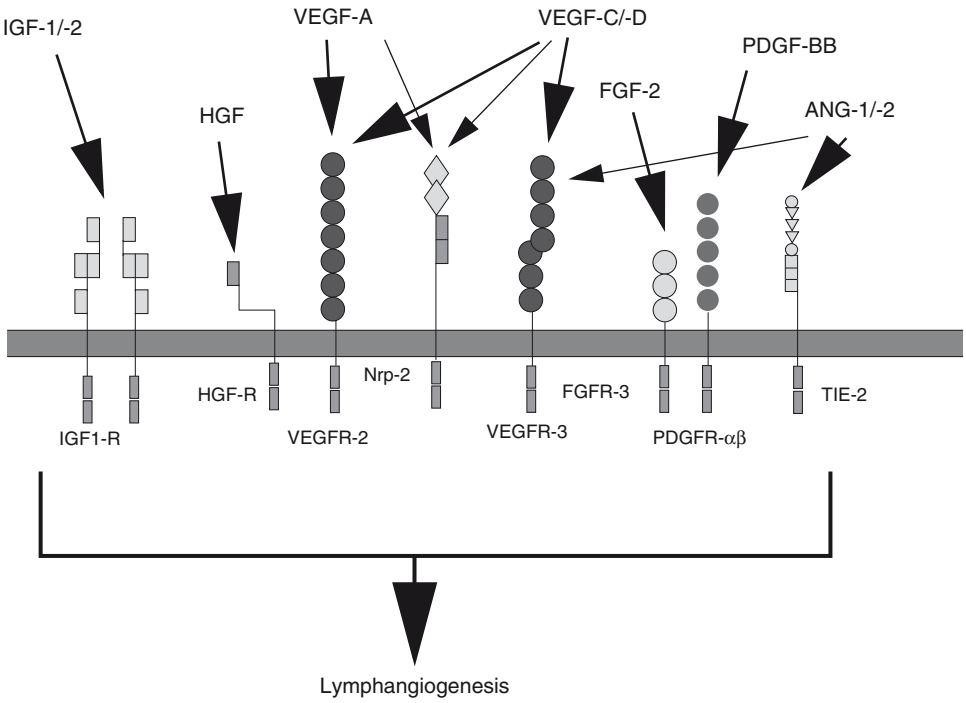

Fig. 8.2 Lymphangiogenic growth factors and their receptors. *VEGFR* vascular endothelial growth factor receptor; *HGFR* hepatocyte growth factor receptor; *IGFR* insulin-like growth factor receptor; *PDGFR* platelet derived growth factor receptor, *FGF* fibroblast growth factor, *TIE* Tyrosine kinase with immunoglobulin-like and EGF-like domains

et al. 2001b). However, in adults the expression of VEGFR-3 becomes confined to the lymphatic endothelium (Kaipainen et al. 1995), but in addition also monocytes, macrophages, dendritic cells, and fenestrated capillaries and veins express VEGFR-3 (Hamrah et al. 2003; Partanen et al. 2000; Schoppmann et al. 2002). Interestingly, VEGFR-3 is reexpressed on capillary endothelium in tumor tissue and is even involved in tumor-angiogenesis and tumor growth (Laakkonen et al. 2007). Signaling via VEGFR-3 is also important for the remodeling of primary vascular networks into larger blood vessels, a function essential for the development of the cardiovascular system in embryos (Dumont et al. 1998). Targeted inactivation of VEGFR-3 results in embryonic lethality as a result of failure to remodel the capillary network before the emergence of lymphatic vessels (Dumont et al. 1998). VEGF-C induces lymphangiogenesis both in embryos and tumors mainly by its interaction with VEGFR-3 (Jeltsch et al. 2003). VEGF-C knockouts fail to form initial lymphatic vessels indicating the pivotal role in embryogenesis (Karkkainen et al. 2004). In contrast, VEGF-D is not required for embryogenesis (Baldwin et al. 2005), but is the strongest inducer of lymphangiogenesis in the adult when given via adenoviral delivery (Rissanen et al. 2003). Exogenous VEGF-D can rescue the phenotype of VEGF-C deficient mice (Karkkainen et al. 2004). Recent studies revealed that VEGF-A also supports lymphangiogenesis through interaction with VEGFR-2, expressed on LEC (Fig. 8.2). VEGF-A induces proliferation of LEC

and overexpression in vivo induces lymphangiogenesis in tissue repair and inflammation (Hong et al. 2004; Kunstfeld et al. 2004; Nagy et al. 2002). Even neutralizing anti-VEGF-A antibodies reduce both lymphatic vessel density (LVD) and lymph node metastasis in xenograft models (Whitehurst et al. 2007). Recently, it has been suggested that VEGF-A predominantly promotes lymphatic enlargement, but not the formation of lymphatic vessels (Wirzenius et al. 2007). However, whether the effect is mainly direct or indirect is still not well understood, because VEGF-A also might stimulate lymphangiogenesis indirectly by recruitment of VEGF-C/-D secreting mononuclear cells (Cursiefen et al. 2004b).

Podoplanin is a transmembrane sialomucoprotein expressed at high levels on lymphatic vessel endothelium (Breiteneder-Geleff et al. 1999). It appears to be important for their correct function and formation. In humans, podoplanin is also expressed in osteoblastic cells, kidney podocytes, and lung alveolar Type-I cells (Wetterwald et al. 1996). The precise function of podoplanin is unclear; however, mice with a targeted gene deletion were shown to have impaired lymphatic function and lymphedema (Schacht et al. 2003). Podoplanin knockout mice having defects in lymphatic, but not blood vessel patterning, show symptoms of lymphedema and die at birth due to respiratory failure (Schacht et al. 2003). Podoplanin is also able to aggregate platelets by interaction with the -C-type lectin-like receptor2 (CLEC-2), preventing leaks between the both vasculatures (Kato et al. 2003; Suzuki-Inoue et al. 2007). Interestingly, Wicki et al. (2006) recently published that podoplanin is upregulated in the invasive front of a number of human carcinomas and promotes tumor-cell invasion.

LYVE-1, the primary lymphatic endothelial receptor for hyaluronan has been shown to be a highly specific marker for lymphatic endothelium in a wide variety of different tissues, and to distinguish lymphatic from blood vascular endothelium in numerous human tumors (Banerji et al. 1999). The considerable structural similarity between LYVE-1 and the leukocyte inflammatory homing receptor CD44 suggests a potential role for LYVE-1 in lymphatic trafficking (Banerji et al. 1999). However, the precise function of LYVE-1 remains unknown, and LYVE-1 $^{-/-}$ mice display no obvious phenotype (Gale et al. 2007). Recently, Lyve-1 expression has also been reported to be absent in some tumor- and inflammation-associated lymphatic vessels (Rubbia-Brandt et al. 2004). It could be downregulated upon incubation of cultured LEC with tumor necrosis factor-alpha (Johnson et al. 2007).

Evidence is mounting concerning the role of *integrins* in lymphangiogenesis. Especially α9β1 seems to have a crucial role in lymphangiogenesis. Mice deficient in the integrin α9 subunit show edema, extra vascular lymphocytes surrounding lymphatic vessels, and die shortly after birth (Huang et al. 2000). Because integrin β1 can stimulate to some degree VEGFR-3, and VEGF-C and VEGF-D can bind α9β1, the integrin-complex might be involved in lymphatic vessel formation and stabilization (Wang et al. 2001). Integrin α9β1 has a role in growth factor induced lymphangiogenesis as Prox-1 upregulates the integrin and VEGFR-3 (Mishima et al. 2007). Antagonism of α9β1 suppressed VEGF-C induced motility. Additional studies revealed that α1β1 and α2β1 are expressed on LEC in healing wounds, and antagonists could block lymphangiogenesis (Hong et al. 2004). Antagonists of α4β1, which is expressed on tumor lymphatic endothelium, has been shown to block tumor metastasis as well as lymphangiogenesis (Garmy-Susini et al. 2007).

While Neuropilin-1 is mainly expressed on arterial endothelial cells, Neuropilin-2 is restricted to veins and lymphatics and is known to mediate axonal guidance during neuronal development. Neuropilin-2 is expressed by LEC and deficient mice develop a reduced small lymphatic endothelium (Yuan et al. 2002). It is also a receptor for VEGF-C and VEGF-D,

raising the possibility that VEGF-C signaling is enhanced by Neuropilin, similar to Neuropilin-1 promotion of VEGF-A binding to VEGFR-2 (Karkkainen et al. 2001).

Subsequent studies have also identified *additional lymphangiogenic factors*, including fibroblast growth factor-2 (bFGF), platelet derived growth factor (PDGF-BB), HGF, insulin-like growth factor (IGF), and angiopoietins (Ang-1/-2). bFGF promotes lymphangiogensis in a mouse cornea assay, but it is more likely that this is due to an indirect effect by inducing VEGF-C production (Chang et al. 2004; Kubo et al. 2002). Recently, HGF was described as a novel lymphangiogenic growth factor. HGF promoted lymphangiogenesis and promoted peritumoral lymphangiogenesis (Kajiya et al. 2005). Of interest, HGF-receptor, also known as MET/c-met has been reported to correlate with metastatic spread of cancer (Danilkovitch-Miagkova and Zbar 2002). Studies also revealed that the insulin-like growth factor 1 and 2 (IGF-1/-2) induce lymphangiogenesis, but the effect could not be blocked by antagonist of VEGFR-3 (Bjorndahl et al. 2005), although IGF-receptors promoted expression of VEGF-C and lymph node metastasis in a Lewis lung carcinoma model (Tang et al. 2003). Whether IGF-1/-2 has a direct or indirect effect has to be further analyzed. In addition to Prox-1, VEGF-C, VEGF-D, and VEGFR-3, several molecules are known to be especially important for *later stages of lymphatic development*.

While *angiopoietin-2 (Ang-2)* is not required for the formation of lymphatics, it plays a key role in their subsequent remodeling and maturation. Mice lacking Ang-2 develop subcutaneous oedema and chylous ascites and die shortly after birth, due to impaired lymphatic vessel formation (Gale et al. 2002). Ang-1 can rescue these effects, although the abnormal angiogenesis also observed in Ang-2 $^{-/-}$ mice is not corrected (Gale et al. 2002). Interestingly, VEGF-C induces Ang-2 expression in cultured LEC through VEGFR-2, indicating a possible connection between the VEGF and angiopoietin families during lymphangiogenesis (Veikkola et al. 2003). However, so far there are no data published about the angiopoietins enhancing tumor-lymphangiogenesis and lymphatic metastasis, although a majority of tumors show an increased expression (for review (Tait and Jones 2004).

The *PDGF*-family (Platelet derived growth factor) includes at least four structurally related members, PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD, that can form both homodimers and hetereodimers (Heldin and Westermark 1999). PDGF signaling is critical for proper embryonic development, whereas in the adult it plays a role in wound healing and in the control of interstitial fluid pressure. Besides stimulation of stromal cell recruitment, PDGF seems to be an important factor in regulating angiogenesis, pericyte recruitment, and tumor growth (Heldin and Westermark 1999; Ostman and Heldin 2007; Reinmuth et al. 2009). PDGF-BB plays a direct role in promoting lymphangiogenesis and metastasis. Expression of PDGF-BB in murine fibrosarcoma cells induce tumor lymphangiogenesis, leading to enhanced metastasis in lymph nodes (Cao et al. 2004). Cao et al. suggest that PDGF-BB acts as a survival factor for newly formed lymphatics through interaction with receptors PDGFR-alpha and -beta, both detected on isolated primary lymphatic endothelia cells. PDGFs may modulate the postnatal remodeling of lymphatic vessels, but not the development of rudimentary lymphatic vessels. This has to be validated in future.

Several other molecules were additionally found to be required for the development. The tyrosine kinase Syk and the adaptor protein SLP-76 were found to be involved in the separation of blood and lymph vessels (Abtahian et al. 2003). Deficiency resulted in arteriovenous shunting and connections between blood vessels and blood-filled lymph vessels. A similar role has been reported for Spred-1/Spred-2. In knockout mice, blood-filled lymphatic vessels have been reported indicating a possible role in vascular separation (Taniguchi et al. 2007) and angiopoietin-like

protein-4 might be required for sustained separation of the two vasculatures (Backhed et al. 2007). Recently, two membrane proteins have been described specifically expressed in activated tumor-associated LEC. Applying double-staining techniques with established LEC markers, Fiedler et al. (2006) have screened endothelial cell differentiation antigens for their expression in LECs. Their experiments identified the sialomucin CD34 as being exclusively expressed by LECs in human tumors but not in corresponding normal tissues. LyP-1, a molecular marker of tumor lymphatics in the MDA-MB-435 breast carcinoma cell line, which was grown in nude mice, was identified by combining ex vivo screening of phage-displayed peptides and in vivo screening for tumor homing. LyP-1 does not appear in normal lymphatics, and it remains to be determined whether it is expressed in other tumor types (Laakkonen et al. 2002). Since LEC's can be successfully isolated by tissue micropreparation from lymphatic channels, embryonic stem cells, even when established in primary culture, provide a valuable opportunity to further explore molecular mechanisms of lymphangiogenesis and the biology of lymphatic metastasis (Hirakawa et al. 2003; Kono et al. 2006; Petrova et al. 2002; Podgrabinska et al. 2002; Wick et al. 2007). This may lead to the identification of endothelial lineage specific signatures.

8.4 Pathology of the Lymphatic Vasculature

Lymphatic vessels have multiple functions and play an important role in various diseases. Impaired function of lymphatic vessels results in lymphedema. Based on the cause, lymphedema occurs as a hereditary (primary) edema or acquired (secondary) edema, but share common features—the dysfunctional lymphatic vessel showing fibrosis and susceptibility to inflammation and infection. The secondary lymphedema is a frequent clinical finding in industrialized countries due to cancer treatment including surgery, radiotherapy, and chemotherapy.

8.4.1 Secondary Lymphedema

In the setting of *inflammation*, lymphatic vessels have multiple functions. In acute inflammation, edema is one typical sign and a significant feature. It results when the amount of inflamed tissue fluid exceeds the capacity of lymphatic vessel for drainage. Lymphatic vessels have the passive role to transport the interstitial fluid and cytokines to the sentinel lymph nodes. In addition, the lymphatic vessels actively participate in the inflammatory process and are responsible for the afferent immune response by enhancing the migration of dendritic cells, which could be induced in two different ways. One is the increasing level of markers such as the secondary lymphoid chemokine (CCL21) or by increased lymphangiogenesis, triggered by infiltrating immune cells. In the case of an inflammatory response, the infiltrating immune cells are a major source of growth factors and even stromal fibroblasts secrete chemokines and other cytokines such as VEGF-A, VEGF-C, and monocyte-colony stimulating factor (M-CSF). They are chemotactic for further monocytes and macrophages (Barleon et al. 1996; Melder et al. 1996). Macrophages, in particular, secrete many angiogenic and lymphangiogenic factors, including VEGF-C and VEGF-D (Schoppmann et al. 2002), and therefore trigger lymphangiogenesis. It has even been reported that macrophages contribute to lymphangiogenesis by incorporation into newly formed lymphatic vessels in the inflamed cornea (Kerjaschki et al. 2006). Thus, VEGFR-3 might have crucial roles in amplification of pathological lymphangiogenesis. Cornea inflammation increased the expression of VEGFR-3 and induced VEGF-C in dendritic cells, possibly by the secretion of proinflammatory cytokines (Hamrah et al. 2003).

It further induces pronounced recruitment of dendritic cells to lymph nodes and triggers graft-rejection. VEGF-C producing macrophages were also found to participate in lymphangiogenesis in human renal transplant rejection (Kerjaschki et al. 2004). Therefore, antilymphangiogenic strategies may improve transplant survival in the setting of transplantation (Cursiefen et al. 2004a, 2003).

In one setting of *lymphatic dysfunction*, the clinical finding of lymphedema is associated with a blockade of the lymphatic fluid uptake. Filiariasis, a parasitic worm infection (*Brugia malayi* or *Wuchereria bancrofti*), often causes massive fibrosis of the lymph nodes and lymph channels in the inguinal region. The resulting edema of the external genitalia and the lower limbs is so extreme that it is called elephantiasis. In Europe, one often finds edema resulting from trauma, surgery, tissue grafting, and congenital edema (Daroczy 1995; Gerber 1998; Mortimer 1998; Witte et al. 1998). Treatment of cancer by removal or irradiation of lymph nodes induces posttreatment lymphedema. Impaired lymphatic drainage produces swelling, scarring, and immundysregulatory disorders. Lymphedema can be a result of an induced imbalance between lymph formation and absorption. The induced fluid accumulation causes pain, chronic and disabling swelling, tissue fibrosis, adipose degeneration, poor immune function, and susceptibility to infections, as well as impaired wound healing (Rockson 2001). Recent studies of experimental lymphedema revealed that VEGF-C protein injection into the wounded area and virus-mediated VEGF-C gene therapy induce the growth of functional lymphatics (Karkkainen et al. 2001; Szuba et al. 2002). Furthermore, it has been shown that adenoviral delivery regenerated lymphatic vessels in mice (Tammela et al. 2007). Postsurgical lymphedemas might be a future indication for VEGF-C-based therapies; however, in the case of cancer treatment related lymphedema, future studies are warranted. VEGF-C might increase the risk of distant organ metastasis if not all tumor cells have been removed.

8.4.2 Primary Lymphedema

Primary lymphedemas are rare genetic developmental disorders which can manifest at birth (Milroy's disease) or at the onset of puberty (Meige's disease) (Witte et al. 1998). Milroy's disease is a congenital form of disease. It has been mapped to the telomeric part of chromosome 5q, in the region 5q34-q35 and Irrthum et al. (2000) have shown that this region includes a VEGFR-3 intragenic polymorphism. Several heterozygous VEGFR-3 missense mutations have been found in Milroy's disease, resulting in the expression of an inactive tyrosine kinase (Irrthum et al. 2003; Karkkainen et al. 2000). The effect of these mutations was the inhibition of autophosphorylation of the receptor causing this congenital hereditary lymphedema (Irrthum et al. 2000). In Milroy's disease, the superficial or subcutaneous lymphatic vessels are usually aplastic or hypoplastic, whereas in other lymphedema syndromes, such as in lymphedema distichiasis (LD), the microlymphatic network is normal or larger than in healthy controls (Bollinger et al. 1983). The inactivating mutation of the forkhead transcription factor FOXC2 in autosomal dominant LD syndrome relates to pubertal onset of lymphedema and double row of eyelashes (distichiasis) (Fang et al. 2000). FOXC2 is a member of the forkhead/winged helix family of transcription factors involved in developmental pathways. FOXC2 knockout mice display aortic arch and ventricular septal defects and also defective lymphatic valve formation and abnormal pericyte recruitment (Petrova et al. 2004). FOXC2 is necessary in lymphatic maturation and is expressed in the developing lymphatic vessels and lymphatic valves of adults (Dagenais et al. 2004; Petrova et al. 2004). Dysfunction of SOX18, a transcription factor of the SOX family, has been identified as a cause for hypotrichosis ly mphedema-teleangiectasia syndrome in humans (Irrthum et al. 2003), and is interestingly regulated by VEGFR-3 activation (Cermenati et al. 2008).

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However, the detailed function is unclear. Reelin mutations, a gene coding for a protein guiding neuronal-cell migration, is accompanied with congenital lymphedema and chylous ascites (Hong et al. 2000).

In many lymphedema patients none of the aforementioned genetic defects are visible, indicating more relevant genes in human lymphatic development. Different familial lymphedema syndromes emphasize even bigger phenotypic and genotypic heterogeneity in inherited lymphedema angiodysplasia syndromes, where the mutated genes have not been characterized yet (Northup et al. 2003).

8.5 Role of Lymphangiogenesis in Cancer

The metastatic spread of tumor cells is responsible for the majority of cancer deaths, and with few exceptions, all cancers can metastasize. The lymphatic system is the primary pathway of metastasis for most human cancers. For migrating tumor cells, the lymphatic system has many advantages over the blood circulation. Even the smallest lymphatic vessels are larger than blood capillaries; flow velocities are lower and there is less interference with serum factors. High shear stress and mechanic deformation in the blood vascular system often kills metastatic cells (Liotta et al. 1991; Weiss 1992). Lymphatic vessels have no or a discontinuous basal membrane, intercellular gaps, and lymphatic capillaries are not surrounded by pericytes.

8.5.1 Lymphvascular Invasion

Lymphatic vessels in comparison to blood vessels are easier to invade and provide ideal conduits. In addition, LEC's secret chemotactic agents attract malignant tumor cells toward areas of high LVD (Shields et al. 2007). Tumor cells protrude and migrate between LEC, known as lymphovascular invasion (LVI), an important parameter in the prognosis of cancer patients associated with relapse-free and overall survival in various cancers (Lee et al. 2006, 2007; Lotan et al. 2005; May et al. 2007). Once tumor cells gain access to lymphatic vessels, they embolize as single cells or in clusters to the sentinel lymph node (SLN) (Yancopoulos et al. 2000). When tumor cells infiltrate, the SLN further metastasis to distant lymph nodes or distant organs occurs. Through lymphaticovenous connections cancer cells metastasize via blood vessels, although hematogenous metastasis could also occur without SLN metastasis (Fisher and Fisher 1966).

8.5.2 Tumor-Lymphangiogenesis

Lymphangiogenesis has been found in the tissue of many malignancies. Studies revealed that tumors can actively induce the formation of tumor lymphangiogenesis and promote metastasis (Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001). VEGF-C and VEGF-D induced lymphatic vessel proliferation intratumorally and peritumorally. Size of the peritumoral lymphatic vessel was observed to be a most reliable and significant predictor for cutaneous melanoma metastasis and survival (Dadras et al. 2003). There is still an ongoing controversy regarding the significance and functionality of intratumoral lymphatics or intratumoral lymphangiogenesis. Although there have been studies demonstrating that intratumoral lymphatics are nonfunctional for fluid drainage (Padera et al. 2002), others could describe the prognostic influence of intratumoral lymphatics in immunhistochemical analysis (Dadras et al. 2003). Tumor lymphangiogenesis predicts the presence of melanoma metastasis in sentinel lymph nodes at time of surgery (Dadras et al. 2003). In addition, several clinical studies have correlated intratumoral LVD with metastasis (reviewed by (Achen et al. 2005; Stacker et al. 2002), but nevertheless

the importance of intratumoral lymphangiogenesis in regard to metastasis is debatable and may depend on the organ and/or experimental model used. This leads to the general problem that current methodology of lymphangiogenesis quantification is still characterized by high intra- and inter-observer variability. For using the amount of lymphatic vessels in a tumor as a clinically useful parameter, a reliable quantification technique needs to be developed.

8.5.3 Lymphatic Endothelial Cell Activation

Aside from peritumoral lymphangiogenesis, activation of lymphatic vessels has also been proposed as a way to enhance tumor cell infiltration and sentinel lymph node metastasis. He et al. (2005) recently noted that peritumoral LEC proximal to subcutaneous LNM35 lung tumors often displayed an activated phenotype – characterized by increased vessel sprouting, dilation, and permeability. VEGF-C may also activate lymphatics to promote tumor cell chemotaxis, lymphatic intravasation, blood vessel leakage with enhanced lymphatic vessel dilatation and hence tumor cell dissemination (Hoshida et al. 2006). Others have speculated that activated lymphatics might upregulate secretion of chemokines that could attract tumor cells (Alitalo et al. 2004). This activated phenotype can apparently be reversed by adenoviral delivery of soluble Flt-4 (He et al. 2005). Experimental evidence has been obtained suggesting that LEC's could attract tumor cells by secreting chemokines, and therefore actively promote lymphatic metastasis. One of the chemokines, named secondary lymphoid chemokine (SLC/CCL21), is highly expressed in lymph nodes, specifically in endothelial cells of high endothelial venules and T cell-rich areas, and also in the lymphatic endothelium of multiple organs (Gunn et al. 1998). CCL19 and CCL21, chemokines produced by LEC (Saeki et al. 1999), induce a biochemical change when bound to CCR7. Inactivation of CCR7 or CCL21 blocked dendritic cells to migrate from peripheral tissues to draining lymph nodes (Gunn et al. 1999). Recent reports have also shown that human (Takeuchi et al. 2004) and murine (Wiley et al. 2001) melanomas express CCR7, the receptor for CCL21 and CCL19 and that in gastric carcinoma, head/neck squamous cell carcinoma, nonsmall cell lung cancer, and breast cancer, these two factors are associated with lymph node metastasis (Mashino et al. 2002; Muller et al. 2001; Takanami 2003; Wang et al. 2004; Yan et al. 2004). It has been reported earlier that CCR7 and CXCR4, receptors for SLC/CCL21 and CXCL12, respectively, are significantly expressed in human breast cancer cells. Their ligands exhibit high levels of expression in regional lymph nodes, bone marrow, lung, and liver, which represent the first destinations of breast cancer metastasis (Muller et al. 2001). Inhibiting the interaction between this receptor–ligand pair in vivo reduced the ability of MDA-MB-231 breast cancer cells to metastasize to both lung and lymph nodes. These data suggest active interactions between tumor cells and endothelial cells. Furthermore, overexpression of CCR7 by B16 murine melanoma cells enhanced the incidence of lymph node but not lung metastasis when the tumor cells were implanted into the footpads of mice (Wiley et al. 2001). CCR7-mediated enhancement of lymphatic metastasis could be completely suppressed by treatment with neutralizing anti-SLC antibodies (Wiley et al. 2001). These data indicate that chemokines and their receptors play a critical role in determining the metastatic destination of tumor cells.

8.5.4 Lymph Node Lymphangiogenesis

Paget (1889) concluded that metastasis occurred only when certain favored tumor cells (the seed) had a special affinity for the growth milieu provided by certain specific organs (the soil). The

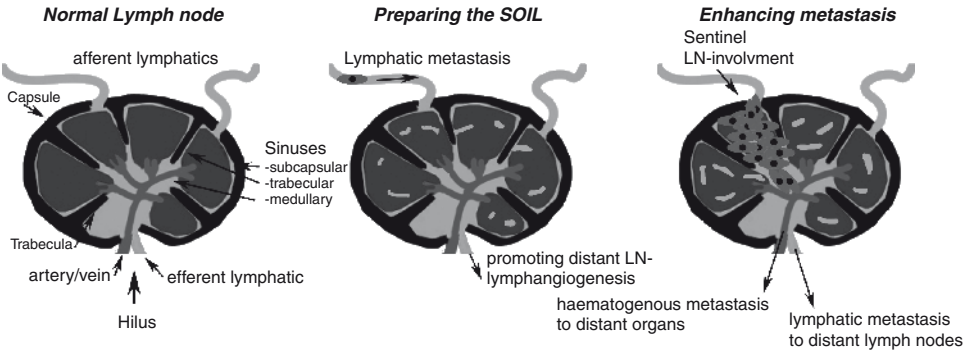

Fig. 8.3 Lymph node lymphangiogenesis. Cancer promotes tumor-associated lymphangiogenesis leading to enhanced metastasis to sentinel lymph nodes (SLN). Lymphangiogenic factors (VEGF-C or VEGF-A) are drained to the SLN where they induce expansion of the lymphatic network (lymph node lymphangiogenesis) preparing the lymph node for the later arrival of the metastatic cells. Metastatic cells then further stimulate sentinel lymph node lymphangiogenesis and distant lymph node lymphangiogenesis, enhancing cancer spread to distant lymph nodes and organs

concept of the *"Seed and soil hypothesis"* (Fig. 8.3) for tumor lymphangiogenesis has been recently described by Hirakawa et al. (2007, 2005). They describe an increased lymphangiogenesis in the sentinel lymph node, even prior to, and after metastatic colonization (Hirakawa et al. 2007, 2005) (Fig. 8.3). Similar observations have been also made in malignant melanoma experiments (Harrell et al. 2007) and in hematological malignancies such as lymphomas (Ruddell et al. 2003). Interestingly, these investigators also observed a 20-30-fold increase in lymph flow. Equivalent changes of lymph node lymphangiogenesis have been recently described in uninvolved axillary lymph nodes of human breast cancer patients (Qian et al. 2006), and lymph node lymphangiogenesis was even associated with nonsentinel lymph node metastasis (Van den Eynden et al. 2006, 2007). That lymph nodes respond to inflammation or neoplasia is a long-known fact. Activated lymph nodes can increase many-fold in size and weight (Cahill et al. 1976; Hall and Morris 1965; Hay and Hobbs 1977). This can be a morphological change known as reactive lymphadenopathy also observed during inflammatory processes. Although the exact mechanism underlying cancer-associated lymph node lymphangiogenesis remains unclear, it could be proposed as a possible way for tumors to disseminate faster throughout the lymphatic system and, subsequently, to distant sites.

8.6 Targeting Lymphangiogenesis

Dissemination of tumor cells is an early and common event and is associated with poorer prognosis for human cancer patients. Targeting lymphangiogenesis could prevent lymphatic metastasis and further dissemination to distant lymph nodes or even distant organs. In the setting of adjuvant tumor therapy, antilymphangiogenic treatment may be an interesting approach after the primary tumor has been surgically removed. Preventing the dissemination of micrometastasis and keeping the metastasis in a localized stage might increase the therapeutic opportunities and improve prognosis. Thus far, therapeutic agents include antibodies, soluble receptors, and tyrosine kinase inhibitors.

8.6.1 Antibodies

The most extensively targeted molecular system is the VEGFR-3/VEGF-C and VEGFR-3/VEGF-D system. Inhibition by neutralizing antibodies reduced lymphangiogenesis and prevented lymphatic metastasis in various animal models (He et al. 2002, 2005; Hoshida et al. 2006; Roberts et al. 2006; Stacker et al. 2001). Interestingly, neutralizing VEGFR-3 antibody blocked the formation of new lymphatics, while the preexisting lymphatics have not been affected (Pytowski et al. 2005). Of further importance is the expression of VEGFR-2 and the stimulation of LEC by VEGF-A and by proteolytically processed VEGF-C. Clinical studies inhibiting the activation of VEGFR-2 by the neutralizing VEGF-A antibody (bevacizumab) showed to be beneficial in human tumors (for review (Ferrara et al. 2004). An antilymphangiogenic effect of this antibody has never been evaluated systematically so far. Treatment of breast carcinoma in animal models with an anti-VEGF-A antibody revealed a reduced LVD and lymph node metastasis (Whitehurst et al. 2007). The effect might be more pronounced by a combined blockade of both VEGF-C and VEGF-A, leading to dual blocking of angiogenesis and lymphangiogenesis. Double blockade by an anti-receptor targeting may lead to enhanced antiangiogenic and antitumor effect (Tammela et al. 2008).

8.6.2 Soluble Receptors

Soluble receptors compete with membrane-bound receptors. They comprise their extracellular portions and retain the ability to bind their ligand. Even due to the binding of multiple soluble factors they might be very effective. Inhibition of VEGFR-3 signaling with a soluble receptor, VEGFR-3-Ig, suppressed tumor lymphangiogenesis and lymphatic metastasis in a breast and lung carcinoma model (He et al. 2002; Karpanen et al. 2001).

8.6.3 Small Molecule Inhibitor

A similar approach, but interacting intracellularly with the signal transduction are the receptor tyrosine kinase inhibitors such as sorafenib and sunitinib. Both interact with the VEGFR-2 and VEGFR-3 phosphorylation pockets and inhibit consecutive signaling pathways, but no studies have been published so far on their specific antilymphangiogenic effect. Cedarinib and vandetanib, which block VEGFR-2 and VEGFR-3 signaling, yielded no inhibition of lymphatic metastasis in animal models, suggesting that kinase inhibition of both receptors may not be enough (Padera et al. 2008). But further studies are warranted to determine the role of tyrosine kinase inhibitors in antilymphangiogenic treatment.

8.7 Conclusions

Lymphangiogenesis is currently receiving increasing scientific and clinical interest. The identification of novel mediators of lymphangiogenesis will likely lead to new advances in our understanding of the mechanisms underlying tumor metastasis. Comprehensive research strategies have revealed a number of novel targets supporting biologically based therapeutic studies. Novel lymphangiogenic targets for the treatment of cancers and inflammation support the future development of individualized therapies, possibly avoiding adverse side effects.

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