Regulation of lymphangiogenesis—From cell fate determination to vessel remodeling

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Abstract

Lymphatic vessels are important for the maintenance of normal tissue fluid balance, immune surveillance and adsorption of digested fats. During the past decade, the identification of lymphatic-specific markers and growth factors has enabled detailed studies of the lymphatic system, and gain- and loss-of-function experiments have greatly increased our understanding of the mechanisms of normal lymphatic development. Understanding the basic biology has provided novel insights into the pathologic conditions of the lymphatic system that contribute to lymphedema, inflammation or lymphatic metastasis, and opened possibilities for the development of better therapeutic strategies. Here we review the current knowledge about the molecular mechanisms regulating the development of the lymphatic vasculature; of the differentiation of lymphatic endothelial cells, of the regulation of the growth of lymphatic vessels, and of remodeling of the vasculature into a network consisting of lymphatic capillaries and collecting lymphatic vessels. Furthermore, we will discuss the molecular mechanisms involved in the pathological conditions of the lymphatic vessels.

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Lymphatic vascular system

The main function of the lymphatic vasculature is to maintain normal tissue fluid balance by restoring interstitial fluid to the cardiovascular system. In addition, the lymphatic system is an important part of immune surveillance and involved in absorption and transportation of digested fats from the intestine [1]. Lymphatic vessels usually extend through a tissue accompanying larger blood vessels, sometimes ensheathing the veins as a plexiform net. They arise in the peripheral connective tissue as blind-ended capillaries that collect excess of extravasated tissue fluid, originating as capillary infiltration from the blood serum, and drain into larger lymphatic vessels. These vessels converge and unite, pass to the lymph nodes, and return the fluid to the venous circulation via the final collecting trunk, the thoracic duct. Different types of lymphatic vessels can be distinguished morphologically; the lymphatic capillaries are valveless endothelial tubes which have discontinuous basement membrane, overlapping endothelial cell junctions and lack pericytes and smooth muscle cells (SMCs), making them highly permeable to large macromolecules. In contrast, collecting lymphatic vessels have sparse SMC coverage, which helps in propelling lymph forward, and numerous, irregularly located valves, which prevent backflow. The luminal valves are, however, rare in the thoracic duct. Its two most prominent valves are situated at its termination in the subclavian vein where the free borders of the valves are directed towards the venous lumen in order to oppose influx of venous blood.

Establishment of lymphatic endothelial cell identity

During embryogenesis, the development of lymphatic vessels starts after the establishment of the blood vasculature when a subset of venous endothelial cells becomes committed to lymphatic endothelial lineage and sprouts from the major veins in jugular and perimesonephric area to form primitive lymphatic sacs (Fig. 1). The homeodomain transcription factor Prox-1 has been identified as a critical regulator of lymphatic endothelial cell (LEC) differentiation [2]. Targeted disruption of Prox-1 in mouse leads to arrest in the budding of the presumptive LECs and failure in lymphatic vessel development, while the development of blood vasculature is not affected [3]. Induction of polarized expression of Prox-1 in cardinal vein leads to upregulation of lymphatic-specific genes, such as vascular endothelial growth factor receptor 3 (VEGFR-3) and LYVE-1 [4]. In Prox-1-deficient embryos the lymphatic-specific gene expression was not induced but the mutant cells continued expressing blood vascular markers, suggesting that the cells were not committed to the lymphatic lineage [4]. In contrast, ectopic expression of Prox-1 in blood vascular endothelial cells (BECs) induced expression of lymphatic-specific genes.
genes and resulted in the downregulation of blood vascular genes [5,6]. Together, these studies suggest a role for Prox-1 as a fate-determining factor for the LECs. However, the signal that induces Prox-1 expression in a subset of venous endothelial cells remains to be elucidated. Interleukin 3 (IL3) and IL7 have been shown to induce Prox-1 expression in cultured BECs, but whether they provide signals for lymphatic differentiation in vivo has not yet been addressed [7,8].

Grafting experiments indicate that allantoic mesoderm as well as paraxial/somitic mesoderm of the avian wing bud have potential to differentiate into lymphatic endothelium, suggesting that differentiation can also occur from mesodermal precursor cells, lymphangioblasts [9,10]. In addition, Prox-1-positive lymphangioblasts, which share a common origin with vascular progenitor cells, contribute to lymphatic vessel formation in Xenopus tadpoles [11]. However, the existence of a putative lymphangioblast has not yet been reported in mammals. In addition, the inductive signal for the in situ differentiation of LECs remains unclear. In mouse embryonic stem cell cultures VEGF-C promoted the differentiation of VEGF-R-3-positive vascular progenitor cells into endothelial cells that expressed LYVE-1 [12]. However, VEGF-C is unlikely to provide the signal for the initiation of the lymphatic differentiation program in vivo because Prox-1-positive endothelial cells, thus representing differentiated LECs, are found in VEGF-C-deficient embryos [13].

**Regulation of the growth and maintenance of lymphatic vessels**

**Vascular endothelial growth factors (VEGFs) and VEGF receptor tyrosine kinases**

VEGF-R-3, one of the genes upregulated by Prox-1 in lymphatic endothelium, is a major regulator of lymphangiogenesis. During early development VEGF-R-3 is expressed in all endothelia and it is implicated in blood vascular remodeling [14,15]. However, after the development of lymphatic vessels starts, VEGF-R-3 expression becomes restricted to lymphatic endothelium [15]. The two known ligands for VEGF-R-3, VEGF-C and VEGF-D, can induce lymphangiogenesis in vivo and stimulate proliferation, migration, and survival of LECs in vitro [16–18] (see Fig. 2). After proteolytic processing VEGF-C and VEGF-D are also ligands for the major regulator of blood vascular endothelia, VEGFR-2 [19,20], and the fully processed mature forms are also capable of inducing angiogenesis in certain conditions [21,22]. Genetic ablation of VEGF-C leads to failure in the migration and proliferation of the LECs, and as a result the embryos do not develop lymphatic vessels [13]. Furthermore, VEGF-C haploinsufficiency or insufficient VEGF-R-3 signaling due to inactivating mutation in the kinase domain results in hypoplastic lymphatic vascular networks indicating that normal levels of this growth factor/receptor signaling are essential for proper lymphatic development [13,23]. In contrast, VEGF-D appears to be dispensable for lymphatic vascular development in vivo [24].

Continuous signaling via VEGF-R-3 is required not only for the growth but also for the survival of lymphatic endothelial cells and for the maintenance of the vessels. Inhibition of VEGF-R-3 signaling using a soluble VEGF-R-3 protein leads to apoptosis of LECs and regression of lymphatic vessels during embryogenesis [25] and during the first postnatal weeks (Karpanen et al., submitted for publication). However, in adult mice VEGF-R-3 inhibition blocks specifically the growth of new lymphatic vessels while the survival or function of preexisting vessels is not affected [26; Karpanen et al., submitted for publication]. These results suggest that there is a transient developmental period for the requirement of VEGF-C/VEGF-R-3 signaling for the survival of newly formed vessels. The maturing lymphatic vessels may receive additional survival signals from the extracellular matrix, which can promote LEC survival in vitro by modulating integrin signaling [18,27]. Integrin α5β1 can associate with VEGF-R-3 and thereby regulate VEGF-R-3 phosphorylation and function upon stimulation with VEGF-R-3 ligands or extracellular matrix proteins [27,28]. In addition, integrins may have more direct functions in lymphangiogenesis. Integrin α9β1, which is required for normal lymphatic development in vivo [29], was shown to directly bind VEGF-C and VEGF-D and thereby promote endothelial cell adhesion and migration [30].

VEGF is known for its functions in regulating angiogenesis. However, in addition to its strong angiogenic effect, VEGF overexpression in mouse skin induced enlargement of lymphatic vessels and promoted lymphangiogenesis during wound healing and in tumors [31–33]. The lymphangiogenic effect is caused, at least partly, due to VEGF induced recruitment of monocytes and macrophages, which can produce lymphangiogenic growth factors [34,35]. However, VEGF-R-2, the receptor for VEGF, is expressed in at least some lymphatic vessels [36], and VEGF can promote proliferation and migration of cultured LECs [18,33], suggesting that VEGF/VEGF-R-2 signaling may also directly stimulate lymphatic endothelium. VEGF-R-2 may also regulate lymphangiogenesis via formation of heterodimeric receptor complexes with VEGF-R-3 [37]. The VEGF-R-3 tyrosine phosphorylation sites are differentially used in homo- versus heterodimeric receptor complexes, which may lead to activation of distinct downstream signaling pathways and thereby to a different biological response [37]. Interestingly, overexpression of VEGF-C or a VEGF-R-3-specific mutant form of VEGF-C appears to result in distinct effects on lymphangiogenesis; whereas simultaneous activation of VEGF-R-2 and VEGF-R-3 by VEGF-C induced extensive lymphatic vessel sprouting, activation of VEGF-R-3 alone
induced enlargement of preexisting lymphatic vessels in mouse embryos [36].

Angiopoietins

Three members of the angiopoietin family (Ang1, Ang2, and Ang3/4) are ligands for the Tie1 and/or Tie2 receptor tyrosine kinases, which are expressed both in blood vascular and lymphatic endothelia [38,39] (Fig. 2). Ang1 can activate both Tie1 and Tie2 that appear as performed heteromeric complexes between the two receptor molecules [40]. In contrast, Ang2 can mediate either agonistic or antagonistic function in Tie2 signaling [41,42]. During the formation and stabilization of the blood vasculature Ang2 seems to act as a Tie2 antagonist [41], while agonistic function of Ang2 is required for proper patterning of the lymphatic vasculature [42]. Overexpression of Ang1 stimulated lymphatic endothelial cell proliferation and promoted vessel enlargement and generation of new sprouts [39,43]. Ang1 upregulated the expression of VEGFR-3, and a soluble form of VEGFR-3 inhibited the observed lymphatic sprouting, suggesting a crosstalk between the VEGF and angiopoietin signaling during lymphatic development [43].

Other lymphangiogenic growth factors

Other growth factors with lymphangiogenic potential include fibroblast growth factor-2 (FGF-2), platelet-derived growth factors (PDGFs), and hepatocyte growth factor (HGF) [44–46]. While the effect of FGF-2 appears to be indirect due to upregulated expression of VEGF-C and VEGF-D by blood endothelial and periendothelial cells [44,47], PDGFs and HGF may act directly on lymphatic endothelium [45,46]. Based on the observation that their receptors were upregulated in newly formed vessels and in activated lymphatic endothelia during wound healing, in inflamed skin and in tumor-associated lymphatic vessels, both PDGFs and HGF were suggested to have a role in pathological lymphangiogenesis in adults [45,46]. However, their functions during the
development of embryonic lymphatic vasculature have not been studied.

**Separation of blood and lymphatic vascular domains**

After the lymphatic vasculature is formed, it needs to be strictly separated from the blood vessel network, and the only sites where these vessels retain direct contacts with each other are at the junction where the thoracic and right lymphatic ducts empty their contents into subclavian veins. Loss of hematopoietic intracellular signaling molecules Syk or SLP-76 leads to arteriovenous shunting and mixing of blood and lymphatic endothelial cells, which resulted in hemorrhaging and perinatal death [48]. This study suggested a non-cell-autonomous mechanism where signals from circulating hematopoietic cells regulate the separation of blood and lymphatic vascular networks. However, it remains to be elucidated what the signals originating from hematopoietic cells are. In addition, the mechanisms, which must exist at the endothelial cell surface level to keep the emerging lymphatic vessels separate from preexisting blood vessels, remain unknown.

**Remodeling and maturation of the lymphatic vasculature**

Remodeling of the blood vasculature is known to play a critical role in the development of a functional blood vessel network. Although lymphatic vasculature also undergoes significant remodeling after its initial establishment, the molecular mechanisms involved in these processes are largely unknown. Eph–ephrin signaling has recently been implicated in the postnatal lymphatic maturation processes. EphrinBs are transmembrane ligands for Eph receptor tyrosine kinases, and these molecules regulate a variety of morphogenetic processes, including remodeling of the arterial–venous plexus during cardiovascular development [49]. EphrinB ligands have intrinsic signaling capacities; their cytoplasmic domains can be phosphorylated on tyrosine residues and they have a carboxylterminal motif for binding of PDZ domain containing proteins [50–52]. Mouse mutants deficient in the PDZ binding motif of ephrinB2 develop normal blood vasculature but display chylothorax and failure in the remodeling of the lymphatic vasculature into a hierarchically organized vessel network consisting of lymphatic capillaries and collecting lymphatic vessels [53]. In addition, the formation of luminal valves in the collecting vessels is defective in ephrinB2 mutant mice. The functional failure of the lymphatic system in ephrinB2 mutant mice demonstrates that postnatal remodeling is a critical process during the establishment of a normal lymphatic vascular network.

Pericytes and SMCs have important roles during developmental angiogenesis, for example, in the establishment and maintenance of vessel stability. Similar to the blood vascular development, proper endothelial–SMC interactions appear to have a critical role in supporting normal development of lymphatic vessels. Impaired recruitment of SMCs onto collecting lymphatic vessels in Angiopoietin-2-deficient mice results in disorganization and hyperplasia of the vessels [42]. In contrast, excessive coverage of lymphatic vessels by SMCs in FOXC2 null mice leads to abnormal patterning and failure in luminal valve formation and results in retrograde lymphatic flow [54]. These studies suggest that the SMC interaction can regulate the behavior of the endothelial cells and indicate the importance of SMCs in the poorly understood valve morphogenesis. Ectopic SMC coverage, together with disturbed valve formation, is also observed in the lymphatic vessels of the mice lacking ephrinB2 PDZ binding motif [53]. However, the appearance of the lymphatic defects prior to the acquisition of SMC coverage suggests a cell-autonomous function of ephrinB2 in the lymphatic endothelium.

**Lymphatic endothelial heterogeneity**

The antigenic profiles of blood vascular and lymphatic endothelia reflect the specialization of blood and lymphatic vessels for their distinct functions. Many of the lymphatic-specific molecules, including VEGFR-3, Prox-1, podoplanin, and beta-chemokine receptor D6, have important functions in the regulation of normal lymphatic development and/or function [3,23,55,56]. The roles of others, such as LYVE-1, remain unclear. Isolation of primary LECs [18,57–59] has recently allowed extensive gene expression profiling and identification of novel lymphatic-specific markers, and functional studies may reveal important roles for these molecules in the development of lymphatic vessels. In contrast, the knowledge of the heterogeneity within lymphatic endothelium, in different types of vessels and in different organs, is limited.

VEGFR-2 and ephrinB2 appear to be expressed specifically in collecting lymphatic vessels [36,53], while VEGFR-3 and neuropilin-2 are predominantly expressed in lymphatic capillaries [60,61]. Consistent with the high VEGFR-3 expression in lymphatic capillaries, overexpression of VEGFR-3 ligands, VEGF-C and VEGF-D, in transgenic mice leads to hyperproliferation of capillaries but does not affect collecting lymphatic vessels [17,36]. On the other hand, inhibition of VEGFR-3 signaling by a soluble receptor or by a kinase inactivating mutation leads to hypoplasia of lymphatic capillaries, while the collecting lymphatic vessels remain largely unaffected ([23,25]; Karpanen et al., submitted for publication). Further support for the heterogeneity of lymphatic endothelia comes from the observation that the hyaluronan receptor LYVE-1, which during early development is expressed in all lymphatic endothelia, becomes downregulated in the collecting lymphatic vessels in adult tissues while high expression is maintained in lymphatic capillaries [53] (see Fig. 3). In
addition, the chemokine receptor D6 is expressed only in a subset of lymphatic endothelia, including places involved in leukocyte trafficking [62]. The apparent heterogeneity suggests the existence of molecularly distinct differentiated cell types. Further studies are needed to reveal the basis for such heterogeneity and to uncover the genetic programs coordinating capillary versus collecting lymphatic vessel differentiation. Contact with SMCs resulted in downregulation of VEGFR-3 expression in BECs in an in vitro coculture system [60], suggesting that SMC interaction is involved in regulating the gene expression and possibly the differentiation of endothelial cells in collecting lymphatic vessels.

**Pathologic conditions of lymphatic vessels**

**Lymphedema**

Malfunctions of the lymphatic system rarely result in life-threatening diseases; however, failure of lymph transport can lead to lymphedema, a disfiguring disorder for which no effective treatment is currently available. Lymphedema may be an inherited disease (primary) or it may be caused when the lymphatic vessels or tissues are obstructed or damaged due to infection, radiation therapy, or surgery (secondary or acquired). Unlike edema, caused by heart failure or chronic venous insufficiency, primary lymphedema develops due to hypoplasia or hyperplasia of the lymphatic vessels, leading to insufficient lymph drainage. Primary lymphedemas are a group of disorders that can be associated with additional malformations in other organs. The genetic basis for some of these diseases has been determined during the recent years. A congenital, autosomal dominant form of lymphedema (Milroy disease) is caused due to kinase inactivating mutations in the VEGFR-3 gene [63–65]. On the other hand, mutations identified in genes encoding for the transcription factors FOXC2 and Sox18 underlie the genetic causations of lymphedema-distichiasis and hypotrichosis-lymphedema-telangiectasia, respectively [66–68]. FOXC2 appears to act downstream of and cooperate with VEGFR-3 in lymphatic development [54], but the target genes for FOXC2 and Sox18 in lymphatic endothelium remain to be elucidated. The mouse mutants for all these lymphedema-associated genes also show lymphatic abnormalities, allowing their use for further studies as models for lymphedema and for testing of new therapeutic strategies [23,69,70]. Encouraging results have already been obtained by using VEGF-C therapy in animal models for lymphedema [23,36,71].

**Inflammation**

Recent new discoveries reveal the active involvement of the lymphatic system in inflammatory diseases. In chronic airway inflammation, lymphangiogenesis prevented mucosal edema but interestingly, whereas the newly formed blood vessels regressed after antibiotic treatment the lymphatic vessels persisted [72]. Rejected kidney transplants were found to contain a massive increase in the amount of lymphatic vessels as compared to normal kidneys, and these lymphatics produced the secondary lymphoid chemokine (SLC/CCL21), which further attracted CCR7+ lymphocytes and dendritic cells [74]. Macrophages are suggested to have a dual role in inflammation induced lymphangiogenesis by secreting lymphangiogenic growth factors VEGF-C and -D, which stimulate the growth of existing lymphatic endothelial cells and by trans-differentiating to lymphatic endothelial cells, which incorporate into the lymphatic endothelium [35,74,75].

**Tumor lymphangiogenesis and lymphatic metastasis**

Malignant tumors can activate lymphangiogenesis and metastasize through the lymphatic system. Clinical studies have shown that expression of VEGF-C and VEGF-D by the tumor cells correlates with high lymphatic vessel density in the vicinity of and/or inside the tumor and provides a prognostic indicator of the metastatic potential (reviewed in [76]). In transgenic and xenotransplanted mice, overexpression of VEGF-C or VEGF-D induces tumor lymphangiogenesis, intralymphatic tumor growth, and formation of...
lymph node metastases [77–80]. Administration of soluble VEGFR-3 protein via adenovirus or neutralizing antibodies against VEGF-D inhibited lymphatic metastasis in the experimental mouse models, suggesting that inhibition of VEGFR-3 signaling may provide an important strategy for blocking tumor metastasis also in human patients [77,78,81]. However, recent studies suggest that additional factors besides VEGF-C are also involved in regulating lymph node metastasis [45,82], and that targeting of both factors besides VEGF-C are also involved in regulating lymphangiogenesis and tumor cell invasion may be required in order to accomplish a complete blockade of lymphatic metastasis [82]. Nevertheless, the evidence from experimental and clinical studies highlights the importance of lymphangiogenic signaling in tumor biology and demonstrates the critical role of VEGFR-3 signaling pathway in this context, making it a promising target for anti-cancer therapeutics aimed to limit metastatic spreading.

Conclusive remarks

Recent discoveries have demonstrated the important role of lymphatic vessels in numerous pathological conditions, including tumor metastasis, lymphedema, and inflammatory diseases. The challenge for future studies is to identify new molecular players and increase our understanding of the basic biology of lymphatic development, which may open possibilities for novel therapeutic strategies for different lymphatic disorders.

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References


