Review Article

Lymphangiogenic growth factors, receptors and therapies

Marja Lohela, Anne Saaristo*, Tanja Veikkola*, Kari Alitalo

Molecular/Cancer Biology Laboratory and Ludwig Institute for Cancer Research, Haartman Institute and Helsinki University Central Hospital, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland

Summary

The lymphatic vasculature is essential for the maintenance of normal fluid balance and for the immune responses, but it is also involved in a variety of diseases. Hypoplasia or dysfuction of the lymphatic vessels can lead to lymphedema, whereas hyperplasia or abnormal growth of these vessels are associated with lymphangiomas and lymphangiosarcomas. Lymphatic vessels are also involved in lymph node and systemic metastasis of cancer cells. Recent novel findings on the molecular mechanisms involved in lymphatic vessel development and regulation allow the modulation of the lymphangiogenic process and specific targeting of the lymphatic endothelium.

Recent results show that the homeodomain transcription factor Prox-1 is an important lymphatic endothelial cell (LEC) fate-determining factor which can induce LEC-specific gene transcription even in blood vascular endothelial cells (BECs). This suggests that the distinct phenotypes of cells in the adult vascular endothelium are plastic and sensitive to transcriptional reprogramming, which might be useful for future therapeutic applications involving endothelial cells

Vascular endothelial growth factor-C (VEGF-C) and VEGF-D are peptide growth factors capable of inducing the growth of new lymphatic vessels *in vivo* in a process called lymphangiogenesis. They belong to the larger family which also includes VEGF, placenta growth factor (PlGF) and VEGF-B. VEGF-C and VEGF-D are ligands for the endothelial cell specific tyrosine kinase receptors VEGFR-2 and VEGFR-3. In adult human as well as mouse tissues VEGFR-3 is expressed predominantly in lymphatic endothelial cells which line the inner surface of lymphatic vessels. While VEGFR-2 is thought to be the main mediator of angiogenesis, VEGFR-3 signaling is crucial for the development of the lymphatic vessels. Heterozygous inactivation of the VEGFR-3 tyrosine kinase leads to primary lymphedema due to defective lymphatic drainage in the limbs. Other factors that seem to be involved in lymphangiogenesis include the Tie/angiopoietin system, neuropilin-2 and integrin $\alpha 9$.

VEGF-C induces lymphatic vessel growth, but high levels of VEGF-C also resulted in blood vessel leakiness and growth. The VEGFR-3-specific mutant form of VEGF-C called VEGF-C156S lacks blood vascular side effects but is sufficient for therapeutic lymphangiogenesis in a mouse model of lymphedema. As VEGF-C156S is a specific lymphatic endothelial growth factor in the skin, it provides an attractive molecule for pro-lymphangiogenic therapy.

Keywords

LEC, VEGF, AAV

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Development of the lymphatic vessels

During embryogenesis, the development of the blood vascular system occurs via two processes, vasculogenesis and angiogenesis. Vasculogenesis involves the *de novo* differentiation of endothelial cells (ECs) from mesoderm-derived precursor cells, called hemangioblasts (1). The EC precursors (angioblasts) and the hematopoietic cell precursors are thought to be derived from common precursor cells. The angioblasts cluster and reorganize to form capillary-like tubes. Circulating endothelial progenitor cells have been isolated from peripheral blood of adult tissue, and

Correspondence to:
Dr. Kari Alitalo
Molecular/Cancer Biology Laboratory and Ludwig Institute for Cancer Research
Haartman Institute and Helsinki University Central Hospital
Biomedicum Helsinki
University of Helsinki
00014 Helsinki, Finland
Tel.: 358-9-1912 5511, Fax: 358-9-1912 5510
E-mail: Kari.Alitalo@Helsinki.FI

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*Both authors contributed equally to this work.

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some data suggest that these cells can participate in postnatal formation of new blood vessels (postnatal vasculogenesis/angiogenesis) (2-4).Once the primary vascular plexus is formed, new capillaries form by sprouting or by splitting (intussusception) from pre-existing vessels in a process called angiogenesis (5). The newly formed vasculature is further remodeled into a more mature tree-like hierarchy of vessels containing vessels of different sizes. Excess branches are pruned, some vessels regress and others fuse to form larger ones. In the primary capillary plexus the ECs start to differentiate into arterial or venous type (6).

In humans, the first lymph sacs have been found in six to seven week old embryos (7). This is nearly one month after the development of the first blood vessels. There are two theories about the origin of the lymphatic vessels. A century ago Sabin proposed that the primitive lymph sacs originate by EC budding from the pre-existing embryonic veins (8). The lymphatic system would then spread from these primary lymphatic sacs by sprouting. An alternative model suggests that the initial lymph sacs arise in the mesenchyme from precursor cells, independent of veins, and that the connection to the venous system is formed later in development (9). In a chick-quail chimera model mesodermal lymphangioblasts, lymphatic precursor cells, were shown to participate in the development of the lymphatic system, supporting the theory that the peripheral lymphatic vessels may develop by multiple mechanisms at least in the avian species (10-13). It is possible that both of the proposed mechanisms contribute to the formation of the lymphatic system also in mammals.

Recently two lymphatic specific markers, VEGFR-3 and the transcription factor Prox-1, have been shown to be expressed in the endothelial cells forming the budding lymph sacs in mouse embryos, supporting Sabin's theory of lymphatic development (14-16). Prox-1 was shown to be essential for the development of the murine lymphatic system, as Prox-1 null mice are devoid of lyphatic endothelial cells (16). Detailed analysis of Prox-1 null mice has led to the formulation of a model for early lymphatic vascular development (17). After the initial formation of the blood vascular system, venous endothelial cells become competent to respond to a lymphatic-inducing signal. The first indication that lymphangiogenesis has begun, at around embryonic day 9.5 (E9.5), is the specific expression of Prox-1 in a restricted subpopulation of endothelial cells located on one side of the anterior cardinal vein. At this stage, the lymphatic marker LYVE-1 is uniformly expressed in the cardinal vein endothelial cells. Around E10.5, the Prox-1 and LYVE-1 double positive cells start budding and spreading in a polarized manner (18). These budding lymphatic endothelial cells eventually give rise to the primary lymph sacs, from which lymphatic vessels then spread to peripheral tissues of the embryo (16). All venular endothelial cells may originally be bipotent, but upon the asymmetric expression of at least Prox-1 in a restricted cell population these cells become committed to lymphatic differentiation (18). Prox-1 activity is not required for the initiation of endothelial cell budding from the cardinal vein, but rather for maintaining it (16). As the cells bud in a polarized manner, they start expressing additional lymphatic endothelial markers, such as secondary lymphoid organ chemokine (SLC) (18). Also VEGFR-3 expression is maintained at a high level in the budding lymphatic endothelial cells, while its expression becomes weaker in the blood vasculature. The simultaneous expression of LYVE-1, Prox-1, VEGFR-3 and SLC may indicate irreversible commitment to the lymphatic edothelial cell lineage (18). At least three other transcription factors seem to be involved in lymphatic development. Net, a member of the Ets-domain family, is expressed in lymphatic vessels, and Net-deficient mice die neonatally from insufficient lymph drainage and ensuing chylothorax (19, 20). Mutations in the gene for the forkhead factor FoxC2 have been linked to the human lymphedema-distichiasis syndrome, and haploinsufficient mice mimic the disease phenotype (21, 22). Recently *SOX18*, the human orthologue of the *ragged* gene, has been found to be mutated in both recessive and dominant forms of a rare hypotrichosis-lymphedema-telangiectasia syndrome (23).

Both angiogenesis and lymphangiogenesis are tightly regulated by intercellular signalling mechanisms, growth factors and cytokines. The angiogenic switch, in tumors for instance, is thought to be caused by a shift in the net balance of positively acting angiogenic mediators and negatively acting angiogenesis inhibitors. The mechanisms underlying this shift of balance are incompletely understood, but several factors including oncogenes, tumor suppressor genes and hypoxia are known to contribute to the regulation of this balance by causing up- or down-regulation of endogenous angiogenesis inhibitors and pro-angiogenic growth factors (24, 25). Regulation of lymphangiogenesis is, however, much less well understood. Table 1 summarizes the results of a range of knockout studies of various ligands and receptors involved in the development of the circulatory system.

The lymphatic endothelial cell phenotype

Recent studies have shown that isolated primary human lymphatic endothelial cells have a distinct gene expression profile, differing clearly from the blood vascular endothelial cells (26-29). The most striking differences between LECs and BECs were detected in the expression of pro-inflammatory cytokines and chemokines (interleukin-8, interleukin-6, monocyte chemotactic protein-1) and receptors (UFO/axl, CXCR4, IL-4R) as well as genes involved in cytoskeletal and cell-cell or cell-matrix interactions, such as integrins and proteins associated with cadherin junctions (26, 27). Integrin α5 (a subunit of the fibronectin receptor) was mainly expressed in BECs, while

Table I: Summary of the knockout studies on receptors and ligands regulating the development of the vascular system. For references, see text.

Gene	Null phenotype	Lethal at	Functions
VEGF	Lack of endothelial cells and blood vessels	E8-9 (-/-) E11-12 (+/-)	Induction of endothelial cells, vasculogenesis, angiogenesis
PlGF	Impaired pathological angiogenesis	survive	Recruitment of EPC?
VEGF-B	Heart defects	survive	?
VEGFR-1	Increased hemangioblast commitment	E8.5	Modulation of VEGFR-2 activity, pathological angiogenesis
VEGFR-2	Lack of endothelial and blood cells	E8.5-9.5	Hemangioblast migration, differentiation, proliferation
VEGFR-3	Defective vascular remodeling	E9.5-10.5	Blood vessel maturation, lymphatic vessel development
Ang-1	Defective vascular remodeling	E12.5	Blood vessel stabilization
Ang-2	Defective postnatal vascular remodeling, lymphatic abnormalities	<P14	Blood vessel destabilization, lymphatic vessel development and function
Tie-1	Compromised endothelial integrity	E13.5	EC-pericyte interactions
Tie-2	Defective vascular expansion and maintenance	E9.5-10.5	EC-pericyte interactions
NP-1	Neuronal and cardiovascular defects	E12.5-E13.5	Axon guidance, VEGF-C coreceptor?
NP-2	Neuronal and lymphatic defects	survive	Axon guidance, VEGF coreceptor
PDGF-B	Hemorrhaging, microaneurysms, loss of perivascular cells	P0	Pericyte recruitment
PDGF-Rβ	Hemorrhaging, microaneurysms, loss of perivascular cells	P0	Pericyte maturation, blood vessel stabilization
Ephrin-B2	Defective vascular remodeling	E11	Establishment of arterial EC identity
EphB4	Defective vascular remodeling	E10	Establishment of venous EC identity

integrin α9 (a subunit of the osteopontin and tenascin receptor) was LEC-specific: antibodies against integrin α9 stained lymphatic capillaries specifically, while blood vessel endothelia were negative (26). Interestingly, mice lacking integrin α9β1 were reported to develop respiratory failure due to the accumulation of a milky pleural (presumably lymphatic) effusion and they died within six to 12 days after birth (30). This phenotype may relate to a co-operation between α9β1 and VEGFR-3 signaling (31).

In line with the results from early embryos (18), overexpression of Prox-1 was capable of inducing LEC-specific gene transcription in BECs and suppressed the transcription of of BEC-specific genes (26, 29). Prox-1 upregulated the expression of VEGFR-3 in BECs, and also strongly induced another LEC-specific factor, p57kip2. Although no lymphatic phenotype has been reported in p57kip2-null mice, their lens phenotype is reminiscent of that of the Prox-1 deficient embryos (32, 33). The p57kip2 levels were strongly decreased in the *Prox-1*$^{-/-}$ lens fiber cells, suggesting that Prox-1 acts upstream of p57Kip2 and that both molecules are part of a common transcriptional program (34). In other cell types, Prox-1 acted as a proliferation inducer via the induction of cyclin E1 and E2 (26). These results, in line with the Prox-1 null phenotype (18), shows that Prox-1 is an important fate-determining factor for LECs. They also suggest that the distinct phenotypes of cells in the adult vascular endothelium are plastic and sensitive to transcriptional reprogramming, which might be useful for future therapeutic applications involving endothelial cells.

Characteristics of lymphatic vessels

Lymphatic vessels differ from blood vessels in several ways. Lymphatic capillaries are essentially thin-walled and blind-ended endothelial tubes that, unlike typical blood capillaries, lack pericytes and a continous basal lamina and contain large interendothelial valve-like openings (35-40). Lymphatic capillaries also contain anchoring filaments that connect the vessels to the extracellular matrix (ECM) (35). These filaments are thought to maintain the patency of the vessels during increased tissue pressure and inflammation. Due to their greater permeability, lymphatic capillaries are more effective than blood capillaries in removing protein-rich fluid from intercellular spaces. In the small intestine, lymphatic vessels take up large proteins and lipids that can not get across the fenestrae of the absorptive capillaries (41). Large collecting lymphatic vessels contain connective tissue and bundles of smooth muscle in their wall as well as valves, which prevent the backflow of lymph (41). Unlike in the blood vessel system there is no central pump in the

lymphatic vessel network in mammals. Lymph is driven in the tissues by the compression of the primary lymphatic vessels by adjacent skeletal muscles. Contractility of the collecting lymphatic vessel wall also contributes to lymphatic vessel function (13, 42). When a lymph vessel becomes streched with fluid, the wall of the vessel automatically contracts. Valves in the lymphatic vessels further aid the unidirectional flow in the vessel network. Some tissues, such as brain, retina, bone marrow and cartilage, totally lack the lymphatic vessels.

Lymphoid organs such as lymph nodes, tonsils, Peyer's patches, spleen and thymus, are a part of the lymphatic system serving immune functions. Besides fluid transport, the lymphatic system has an important role in immunological responses. As lymph circulates in the lymphatic vessels, it passes through lymph nodes, where it is exposed to cells of the immune system. In the lymph nodes foreign substances (antigens) are concentrated by the dendritic cells and presented to lymphocytes. This leads to a cascade of steps that results in immune responses. Lymphocytes circulate between the lymphatic and blood vasculature. Lymphocytes that enter lymphatic vessels in peripheral tissue enter the lymph nodes via the afferent lymphatic vessels. Lymphocytes may also enter the lymph node through the wall of special postcapillary venules that are called the high-endothelial venules. Lymphocytes are then recirculated to the blood circulation along with lymph via the efferent lymphatic vessels and the thoracic duct. Lymphocyte passage across the endothelium is guided by several adhesion molecules, including the integrins, selectins and their ligands (43-46). The trafficking of antigen presenting cells, dendritic cells and Langerhans cell, from peripheral tissues to the lymphatic vessels is also regulated by chemokine signalling. Activated dendritic cells upregulate the cytokine receptor CCR7 in order to become sensitive to SLC that is constitutively produced by the lymphatic endothelial cells in the skin (47, 48). Interestingly, it has recently been shown that certain human breast cancer cell lines also express the CCR7 receptor (49). In an experimental animal model expression of CCR7 enhanced lymphatic metastasis of the melanoma cells 10-fold as compared to control tumor cells, and neutralizing anti-SLC antibodies were capable of blocking this effect (50). Tumor cells may therefore use the same trafficking pathways, possibly involving also other chemokines and adhesion molecules, as the lymphocytes and antigen presenting cells use in order to gain access to the lymphatic vessels.

The VEGF family of growth factors is involved in both angiogenesis and lymphangiogenesis

The members of the vascular endothelial growth factor (VEGF) family play critical roles in the growth of blood vascular as well as lymphatic endothelial cells (Table I). The VEGF family currently includes five members: VEGF, PlGF, VEGF-B, VEGF-C and VEGF-D. In addition, various viral homologues have been detected, such as the orf virus VEGF (also called VEGF-E). The VEGFs are secreted dimeric glycoproteins, all of which contain the characteristic regularly spaced eight cysteine residues, the cysteine knot motif. The VEGF family members bind to three known VEGF receptors (VEGFR-1, VEGFR-2 and VEGFR-3) with differing specificities (Fig. 1).

VEGF is expressed as several isoforms of different amino acid chain lengths (in human, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$) that all bind to VEGFR-1 and VEGFR-2 but differ in their ability to bind heparin and neuropilin-1 (NRP-1) (51-55). As a mitogen, VEGF is highly specific for vascular ECs (56-63). In vertebrate embryos, VEGF is widely expressed especially in tissues immediatedly adjacent to areas of active vessel formation (64). Inactivation of a single VEGF allele in mice resulted in embryonic lethality due to defective angiogenesis (65, 66). VEGF expression is critical for the earliest stages of vasculogenesis, as blood islands, endothelial cells, and major vessel tubes fail to develop in VEGF knockout embryos (65, 66). VEGF is a survival factor for ECs, both *in vitro* and *in vivo*, and it induces the expression of anti-apoptotic factors (67-70). It has been proposed that pericyte coverage of the newly formed vessels is the critical event that determines when ECs no longer require VEGF for survival *in vivo* (71). VEGF is also known as vascular permeability factor, as it is a potent inducer of vascular leak (72-74).

VEGF-B has two splice isoforms, $VEGF\text{-}B_{167}$ and $VEGF\text{-}B_{186}$, that are differentially expressed with $VEGF\text{-}B_{167}$ being predominant (75, 76). VEGF-B is a ligand for VEGFR-1 and its isoforms differ in their binding to heparin and to NRP-1 (77). PlGF is another member of the VEGF family of growth factors that binds specifically to VEGFR-1 (78, 79). PlGF was originally discovered in the human placenta and in humans, it has two isoforms, PlGF-1 and PlGF-2 (78, 80-82). PlGF-2 is able to bind to NRP-1 and heparin. In culture, PlGF homodimers are chemotactic for monocytes and ECs (83), but PlGF alone is not capable of inducing EC proliferation or vascular permeability (79). PlGF deficient mice or even double knockout mice lacking both PlGF and VEGF-B do not have an obvious phenotype (84). However, loss of PlGF impairs angiogenesis, plasma extravasation and collateral growth during ischemia, inflammation, wound healing and cancer (84). Transplantation of wild type bone marrow rescued the impaired angiogenesis and collateral growth in PlGF deficient mice, indicating that PlGF might contribute to blood vessel growth by mobilizing bone-marrow derived EC precursor cells (84). Is seems that PlGF and possibly also VEGF-B play a role in pathological angiogenesis by increasing the recruitment of bone marrow derived myeloid and ECs precursor cells, inflammatory cells and pericytes and by enhancing the effects of VEGF (85-87).

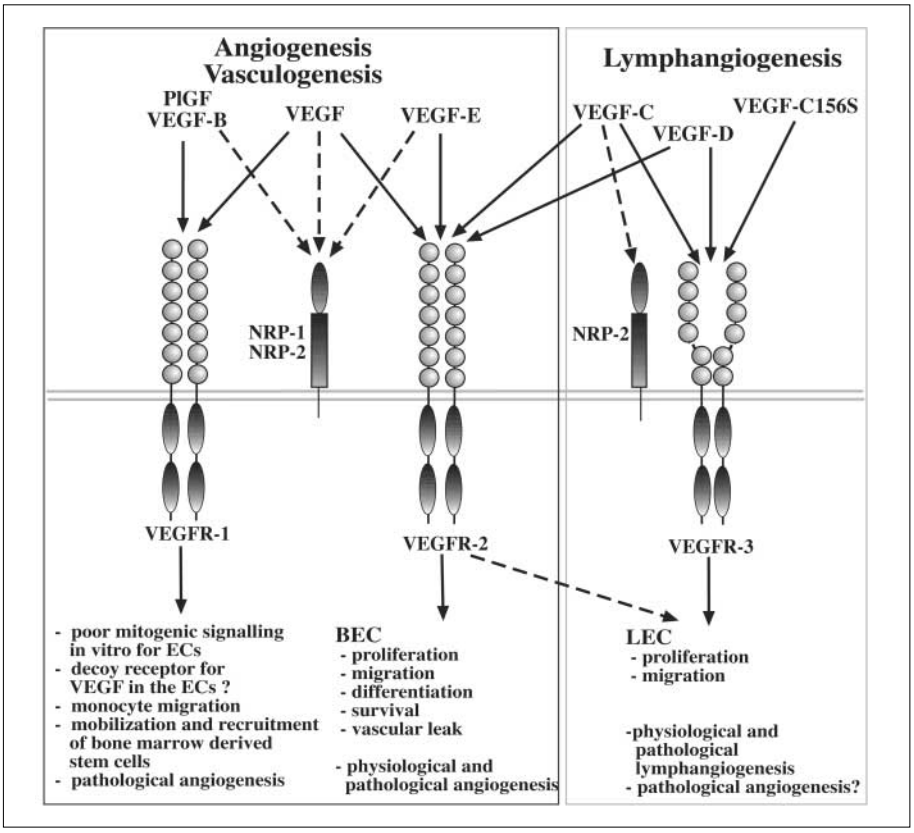

Figure 1: Receptor binding specificity of VEGF family members. VEGFR-2 is the main receptor for VEGF in ECs. VEGFR-1 signaling mediates monocyte migration and, according to recent data, recruitment of VEGFR-1 positive stem cells from the bone marrow. The role of VEGFR-1 signaling in ECs is poorly defined. VEGFR-3 signalling regulates lymphatic vessel growth. Neuropilins (NRPs) function as isoform specific accessory receptors for some VEGF family members.

VEGF-C and VEGF-D are the main regulators of lymphangiogenesis

VEGF-C was discovered as the first ligand for VEGFR-3 (88, 89), and VEGF-C receptor binding profile and affinity are regulated at the level of proteolytic processing (Fig. 2). VEGF-C is produced as a preproprotein with long N- and C terminal propeptides flanking the VEGF homology domain. Initial proteolytic cleavage of the precursor produces a form with intermediate affinity towards VEGFR-3, but a second proteolytic step is required to generate the fully processed form with a high affinity for both VEGFR-2 and VEGFR-3 (90). VEGF-D is also produced as a preproprotein, and undergoes proteolytic processing in a manner similar to VEGF-C: the fully processed human VEGF-D binds to both VEGFR-2 and VEGFR-3 (91-93). Interestingly, mouse VEGF-D differs from its human counterpart in at least two aspects. Mouse VEGF-D is a specific ligand for mouse VEGFR-3 (94, 95), and there are two isoforms of mouse VEGF-D generated by alternative RNA splicing, which differ in their C-termini (94, 95). This suggests that the biological functions of VEGF-D differ in different species or that VEGF-D signalling via VEGFR-2 is not significant for normal development and physiology.

VEGF-C and VEGF-D were the first characterized growth factors capable of inducing growth of new lymphatic vessels *in vivo* (88, 93). Overexpression of VEGF-C or VEGF-D in the epidermis of transgenic mice results in the development of a hyperplastic lymphatic vessel network (96, 97). Conversely, inhibition of lymphatic growth was obtained when VEGF-C/VEGF-D binding to their receptors was blocked by a soluble form of the extracellular domain of VEGFR-3 in a similar transgenic mouse model (98). When applied to mature chorioallantoic membrane (CAM), VEGF-C works as a highly specific lymphangiogenic factor (38). *In vitro*, VEGF-C stimulates migration and mitogenesis of cultured endothelial cells (88-90, 99). However, recombinant VEGF-C promotes angiogenesis in the early CAM where lymphatic vessels have not yet developed, in avascular mouse cornea, and in rabbit ischemic hindlimb (99, 100). The fully processed form of VEGF-C also induces vascular permeability via VEGFR-2 (90, 101). VEGF-D has been shown to be mitogenic for cultured microvascular endothelial cells (93) and angiogenic *in vivo* (93, 102).

In the developing mouse embryo, VEGF-C mRNA is prominent in mesenchymal cells in regions where lymphatic vessels develop by sprouting from embryonic veins (103). High expression of VEGF-C is also detected in the murine mesenterium, lung, heart and kidney (103). Interestingly, at least two reports also document VEGF-C expression in blood vascular endothelial cells; during avian embryonic development and in Kaposi's sarcoma (104, 105). Both VEGF-D isoforms are expressed in several structures and organs in the developing mouse embryo,

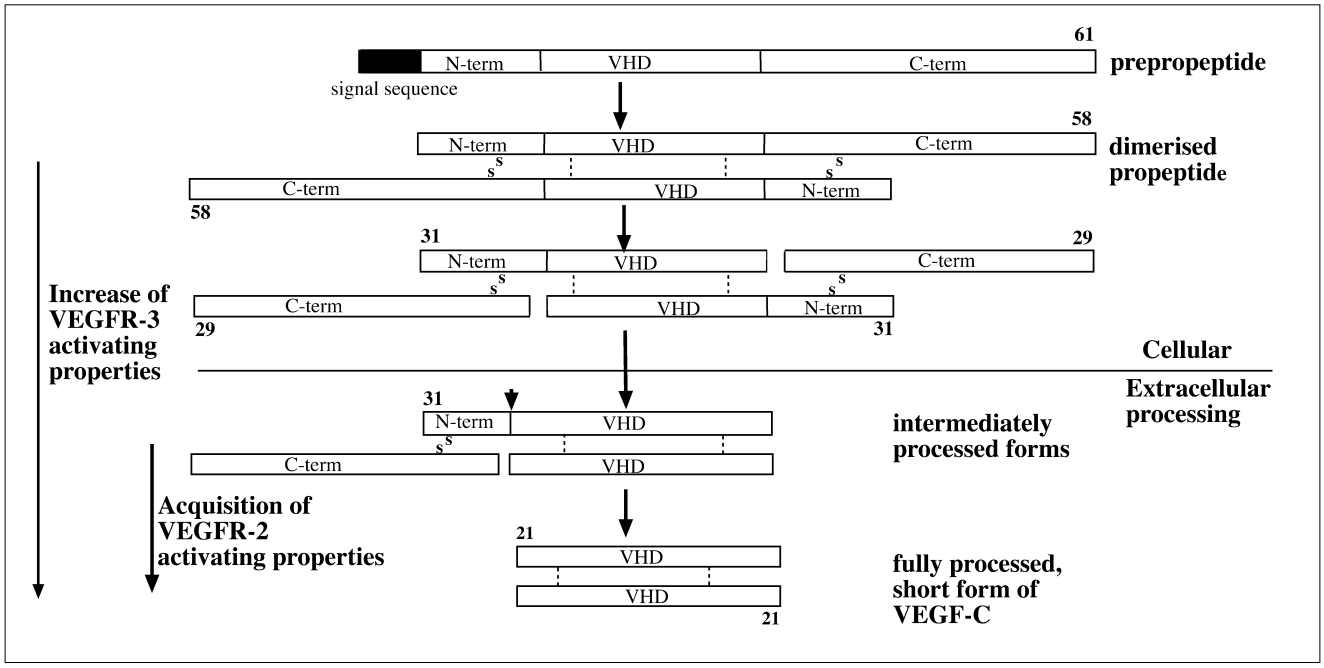

Figure 2: Proteolytic processing of VEGF-C (and VEGF-D). These growth factors are synthesized as prepropolypeptides containing signal sequence, N- and C- terminal propeptides and the VEGF-homology domain (VHD). Proteolytic processing increases the binding affinity to VEGFR-3 and only the fully processed 21 kDa form of VEGF-C is able to bind to VEGFR-2. The numbers indicate the approximate molecular masses (kDa) of the corresponding polypeptides under reducing conditions. Modified from Joukov, 1997

such as limb buds, heart, lung and skin (94, 95, 106-108). VEGF-C and VEGF-D were also found to be expressed in vascular smooth muscle cells, and intense cytoplasmic staining for VEGF-C was detected in neuroendocrine cells, such as the β-cells of the islets of Langerhans, prolactin secreting cells in the anterior pituitary, adrenal medullary cells and neuroendocrine cells of the gastrointestinal tract (108). The adjacent expression patterns of VEGFR-3 and VEGF-C suggest that VEGF-C acts in a paracrine manner during the formation of the venous and lymphatic vascular systems (15, 103). The proinflammatory cytokines interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) upregulate VEGF-C mRNA, whereas both dexamethasone and an IL-1 receptor antagonist inhibited this effect (109). *In vitro*, cultured blood capillary ECs express VEGF-C, but lymphatic ECs do not; LECs require the addition of VEGF-C in the medium for growth if cultured alone (110, 111).

VEGF receptor expression pattern and binding specificity are crucial for lymph/angiogenic signals

The VEGFs mediate their signals via high-affinity receptor tyrosine kinases (RTKs), the VEGFRs. The VEGFRs are structurally and functionally similar to the members of the platelet derived growth factor (PDGF) receptor family, and form a subfamily within that receptor class (112, 113). All VEGFRs have seven immunoglobulin (Ig) homology domains in their extracellular ligand binding part and an intracellular tyrosine kinase signaling domain split by a kinase insert. Like other RTKs, the VEGFRs are thought to dimerize and to undergo transphosphorylation upon ligand binding. Phosphorylated tyrosine residues may serve to control the kinase activity of the receptor, and to create docking sites for cytoplasmic signaling molecules, which provide substrates for the kinase.

VEGFR-1 (or Flt1) can exist either as a transmembrane glycoprotein or a shorter soluble form consisting only of the six first extracellular Ig homology domains (114-116). In the adult, VEGFR-1 is expressed mainly in blood vascular endothelium, vascular smooth muscle cells and monocytes (117). Targeted inactivation of the VEGFR-1 gene results in increased hemangioblast commitment, leading to overgrowth of endothelial-like cells and disorganization of blood vessels and to embryonic lethality at E8.5 (118, 119). Monocyte function is defective in mice deleted of the VEGFR-1 tyrosine kinase domain, indicating that VEGFR-1 signaling is needed to mediate monocyte migration in response to VEGF (120). Very recent results have shown that VEGFR-1 has important roles in activation and recruitment of bone marrow-derived hematopoetic stem cells that play a major role in diseases like inflammation, atherosclerosis, and both pathological and therapeutic angiogenesis and arteriogenesis (85-87). However, this receptor has not been implicated in lymphangiogenesis.

Both VEGFR-2 and VEGFR-3 are expressed in LECs *in vivo* and in culture. VEGFR-2 is first expressed at E7.0 in hemangiogenic lateral plate mesoderm, but later becomes restricted to the blood islands (121). Targeted disruption of the VEGFR-2 gene results in the absence of both blood vessels and blood cells, and results in lethality at E8.5-9.5 (121, 122). VEGFR-2 continues to be expressed in endothelial cells and apparently also in some hematopoietic cells (123). In mouse skin, VEGFR-2 is expressed in the veins and in the collecting lymphatic vessels, whereas arteries are negative for VEGFR-2 (124). There is some evidence that VEGFR-2 has a role also in lymphangiogenesis, as discussed in the chapter dealing with therapeutic lymphangiogenesis below (97, 125).

In vitro, several signal transduction molecules including phosphatidylinositol 3'-kinase (PI3K), phospholipase Cγ, Src family tyrosine kinases, focal adhesion kinase, Akt/protein kinase B, protein kinase C, extracellular signal-regulated kinase, and p38 mitogen-activated protein kinase (MAPK), are activated or modified by VEGFR-2 in primary endothelial cells (126). *In vivo*, activation of VEGFR-2 leads to the expression of proteases required for the breakdown of the basement membrane, the upregulation of specific integrins expressed on angiogenic endothelium, and to the initiation of endothelial cell proliferation and migration (112). VEGFR-2 also mediates vascular permeability, probably via Src and Yes tyrosine kinases (127, 128).

Two splice variants of human VEGFR-3 (or Flt4) mRNA have been characterized, a 4.5 kb transcript and a more prevalent 5.8 kb transcript (129). The smaller one, which does not exist in the mouse, is generated by alternative splicing into the long terminal repeat of a retrovirus integrated between the last two exons of the VEGFR-3 gene (130). The longer transcript encodes 65 additional amino acid residues and is the major form detected in tissues. After biosynthesis glycosylated VEGFR-3 is proteolytically cleaved in the fifth Ig homology domain, but the resulting polypeptide chains remain linked by a disulfide bond (89, 131).

During murine embryogenesis, VEGFR-3 is initially expressed in angioblasts of head mesenchyme, dorsal aorta, cardinal vein and allantois (14, 15). At E12.5 VEGFR-3 is expressed both in the developing venous and presumptive lymphatic endothelia, but later during development VEGFR-3 expression becomes largely restricted to the lymphatic endothelium (15, 132-134) However, in adult human tissues VEGFR-3 was shown to be expressed in certain fenestrated and discontinuous blood vessel endothelia, found in tissues with highly active transport/exchange of molecules accross the vessel wall (108). In most human tissues that have continuous capillary endothelium, VEGFR-3 expression was restricted to the lymphatic endothelium (108).

Genetic disruption of VEGFR-3 results in defective remodeling of the primary vascular plexus, disturbed hematopoiesis, cardiovascular failure and embryonic death by E9.5 (14). However, differentiation of endothelial cells, formation of primitive vascular networks and vascular sprouting are normal in VEGFR-3 null embryos, indicating that VEGFR-3 function is not necessary for these early stages of vascular development (14). VEGFR-3 signaling is required for the formation of the lymphatic vasculature, as lymphangiogenesis can be inhibited with a soluble VEGFR-3 that competes for ligand binding with the endogenous receptors (135). Missense mutations in the VEGFR-3 gene resulting in inactivation of the tyrosine kinase have been linked to primary lymphedema both in humans and in the mouse (94, 136). Interestingly, NRP-2, a receptor for various VEGFs on venous endothelia and semaphorins on neural cells, may act as a co-receptor for VEGF-C in some lymphatic vessels (137, 138).

In vitro experiments with a VEGFR-3-specific mutant of VEGF-C, called VEGF-C156S, have shown that VEGFR-3-stimulation alone is sufficient to protect lymphatic ECs from apoptosis and to stimulate their migration (101, 110). Consistent with this, VEGFR-3 phosphorylation has been shown to lead to PI3K-dependent Akt activation and protein kinase C-dependent activation of the p42/p44 MAPK (110). Only VEGF and VEGF-C, both ligands for VEGFR-2, were able to mediate the survival and migration of VEGFR-3-negative blood vessel ECs (110).

Tie-receptors and angiopoietins in lymphatic development

In addition to VEGFs, angiopoietins and the Tie-receptors have also been shown to play a role in the formation of the vascular system (for reviews, see (139-141)). There are two members of the Tie class of RTKs, Tie-1 and Tie-2 (or Tek). Both have extracellular domains consisting of two Ig homology domains, three epidermal growth factor homology peptides and three fibronectin type III homology domains. The cytoplasmic region of the Tie receptors contains a tyrosine kinase domain interrupted by a kinase insert. Tie-1 and Tie-2 are predominantly expressed by vascular endothelial cells. To date there are four known angiopoietins, which all bind to the Tie-2 receptor, mediating vessel stabilization signals, whereas the Tie-1 receptor has no known ligand (142-145). Ang-1 and Ang-4 activate Tie-2, while Ang-2 and Ang-3 appear to function as specific antagonists and inhibit Tie-2 signaling at least in some settings.

The phenotypes of Tie-2 and Ang1 deficient mice suggest a role for this ligand-receptor system in maintaining the communication between ECs and the surrounding mesenchyme. Deletion of Tie-2 results in embryonic death between E9.5 and E10.5 as a consequence of insufficient expansion and maintenance of the primary capillary plexus (146, 147). In embryos lacking Tie-1, endothelial cell integrity is compromised, leading to edema, hemorrhages and death between E13.5 and birth (147,

148). Deletion of Ang-1 results in angiogenic defects very similar to those seen in mice lacking Tie-2, including the absence of perivascular cells (149). Adenovirally mediated Ang-1 administration protected adult vasculature against plasma leakage, but it essentially lacked the effects on blood vessel morphology seen in the K14-Ang-1 transgenic model (150).

Ang-2 is highly expressed at sites of active vessel remodeling, such as the ovary and the placenta, and also some tumors (143, 151). These expression patterns and the phenotypes of Tie-2 and Ang-1 knockout mice have led to a model for angiogenic vessel remodeling, in which Ang-2 is required to block a constitutive stablilizing action of Ang-1, leading to the detachment of perivascular cells that inhibit endothelial cell proliferation, and allowing the blood vessels to revert to a more plastic state for initiation of angiogenesis (143). The study of the Ang-2 null mouse has revealed that the angiopoietins are also likely to play roles in the lymphatic development (152). Mice lacking Ang-2 have lymphatic defects, but the expression of Ang-1 in the Ang-2 locus is sufficient to rescue the lymphatic phenotype, suggesting that both Ang1 and Ang2 may play role in lymphangiogenesis as agonists. Tie-2 expression pattern in blood and lymphatic vessels is poorly characterized. Tie-1 has been shown to be expressed by lymphatic endothelium during embryogenesis since E13.5 and in adults, but its function in LECs is unknown (153). As in angiogenesis, angiopoietins may contribute to the remodeling and stabilization of the newly formed lymphatic vessels. Further studies are clearly needed to characterize the role of angiopoietins in lymphatic vessel development.

Neuropilin-2 is implicated in lymphatic development

NRP-1 and NRP-2 are widely expressed cell surface glycoproteins with short cytoplasmic tails. They bind class three semaphorins, secreted molecules mediating repulsive signals during axon guidance, as well as several VEGF family members (126). NRPs are believed to be non-signaling and therefore to require the presence of a signal-transducing receptor in order to mediate their effects. By binding to various VEGFs, neuropilins may act as coreceptors for the VEGFRs, and seem to increase the efficiency of their tyrosine kinase signals (154). Interestingly, in developing chick embryos endothelial NRP-1 expression is mostly restricted to arteries, whereas NRP-2 preferentially marks veins, indicating that NRPs may play a role in determining endothelial arterial and venous identity (155). Mice deleted of the NRP-2 gene show absence or severe reduction of small lymphatic vessels and capillaries during development, indicating a selective requirement of NRP-2 in lymphatic development (156). NRP-2 has been shown to bind VEGF-C, suggesting that it may be involved in VEGFR-3 signaling in lymphatics (138).

Lymphangiogenesis in disease and prospects for therapy

In adult tissues lymphangiogenesis appears to follow but lag behind angiogenesis. The requirement for lymphatic growth in the setting of angiogenesis can be understood by the need to drain fluid leaking out of the immature blood vessels. For example, during wound healing VEGFR-3 positive lymphatic vessels sprout from pre-existing lymphatics into the granulation tissue (157, 158). Therefore, some degree of lymphangiogenesis is probably an integral feature of tissue repair and inflammatory reactions in most organs.

Impairment of lymphatic function is involved in various diseases characterized by inadequate transport of interstitial fluid, edema, impaired immunity and fibrosis (159). On the other hand, abnormal proliferation of lymphatic endothelial cells takes place in lymphangiomas, lymphangiosarcomas and possibly in Kaposi's sarcoma (160). Lymphatic vessels also serve as an important route for tumor metastasis. Very little was known about the molecular mechanisms behind lymphatic diseases until very recently. The first gene mutations causing human lymphedema have now been found and several mouse models have facilitated the development of new therapeutic applications for lymphedema. Animal tumor models have also been used to analyse mechanisms of tumor metastasis and to test strategies for the inhibition of metastasis.

Lymphedema

Lymphatic vessels play a key role in the immune response to various antigens and in maintaining fluid homeostasis in the body. Blockage of lymphatic drainage or an abnormal development of the superficial lymphatic vessels leads to lymphedema, which is characterized by disfiguring and disabling swelling of the extremities (160). Lymphedemas can be etiologically divided into two main categories. Primary lymphedemas are rare developmental disorders whereas the more common secondary lymphedema syndromes are caused by infections, surgery or trauma. Secondary lymphedema can develop as the result of inflammatory or neoplastic obstruction of the draining lymphatic vessels or for example after breast cancer surgery. Approximately 35% of primary lymphedema patients have a family history of the disease and it has been estimated that 1:6000 of newborns develop primary lymphedema, with a sex ratio of one male to three females (161). Secondary lymphedema is a relatively common disorder and it has been estimated that there are three to five million patients with secondary lymphedema in the United States. Lymphatic filariasis is the second leading cause of permanent and long-term disability globally. Lymphatic filariasis is caused by a parasitic infection of the lymphatic vessels and may lead to massive edema and deformation of the limbs or genitals (160). According to the World

Health Organization, over 120 million people suffer from filarial lymphedema worldwide. In all its forms, lymphedema is a chronic disease in which persistent dysfunction of the lymphatic vessels gradually results in dermal fibrosis, thickening of the skin and accumulation of adipose tissue.

Genetic alterations in lymphedema

The molecular pathogenesis of various lymphedema phenotypes has been unclear, but recent reports indicate several chromosomal regions and genes which are involved in the development of lymphedema. Congenital hereditary lymphedema (Milroy's disease) was linked to the *VEGFR3* region in the distal chromosome 5q and missense mutations that inactivate VEGFR-3 were found to be involved in the disease (113, 136, 162-164). While mutations which inhibit the biological activity of VEGFR-3 are one cause of primary lymphedema, there are several families with Milroy's disease and other lymphedema syndromes, which involve other genetic loci. It is estimated that 5% of patients with primary lymphedema carry a mutation in the *VEGFR3* gene (D. Finegold, R. Ferrel, personal communication). For example, the more common *FOXC2* gene mutations result in the hereditary lymphedema-distichiasis syndrome (21). Characterization of other genes involved in the development of lymphedema syndromes will give us more insight into the molecular mechanisms of lymphedema.

Lymphedema mouse models

Several experimental models of secondary lymphedema have been described including lymphedema in the mouse tail (165), rat hindlimb (166, 167), or rabbit ear (168, 169). In addition, two existing mouse strains show phenotypes of primary lymphedema. In the Chy mouse model, an inactivating VEGFR-3 mutation results in persistent hypoplasia of the superficial lymphatic vessels. The subserosal lymphatic vessels are enlarged in these mice, and this leads to the formation of chylous ascites shortly after birth (138). In another mouse model, overexpression of the soluble extracellular domain of VEGFR-3 in transgenic mouse skin competes for VEGF-C/VEGF-D binding with the endogenous receptor, leading to the regression of developing lymphatic vessels in several organs and resulting in lymphedema (98). However, in this model the lymphatic vessels regenerate during later postnatal development in most organs, although not in the skin. Like the human lymphedema patients, both of these mouse models show swelling of the limbs due to hypoplastic/aplastic cutaneous lymphatic vessel network. Thus, these animal models provide us with tools to develop and test new therapies for lymphatic dysfunction.

Prolymphangiogenic gene therapy

Strategies for local and controlled induction of lymphangiogenesis could benefit the development of treatment for both primary and secondary lymphedema. The discovery of specific genes and signalling cascades involved in the regulation of lymphatic vessel growth and in pathogenesis of lymphatic dysfunction have established a basis for the development of new targeted treatments

Previously, proangiogenic gene therapy in humans has shown promise in the treatment of cardiovascular ischemic diseases (170-172). Angiogenesis has been stimulated by the overexpression of VEGF or various fibroblast growth factors, using several different gene transfer vectors (173). However, while VEGF is a potent inducer of angiogenesis, the vessels it helps to create are immature, tortuous and leaky, often lacking in perivascular support structures (174, 175). In addition, only a fraction of the blood vessels induced in response to VEGF in the dermis and in subcutaneous fat tissue are stable and functional (176, 177), and intramuscular vessels develop into an angioma-like proliferation or regress (4, 176). Furthermore, edema induced by VEGF overexpression complicates VEGF-mediated neovascularization, although recent evidence suggests that it can be avoided by providing Ang-1 for vessel stabilization (150, 178).

VEGF-C and VEGF-D induce lymph/angiogenesis and angiopoietin blocks vascular leak

VEGF-C, VEGF-C156S and VEGF-D have been shown to induce hyperplasia of cutaneous lymphatic vessels, while causing very little or no angiogenesis, when expressed as transgenes in the skin (96, 97). Adenovirally administered VEGF-C (AdVEGF-C) has also been reported to induce mainly lymphangiogenesis in mouse ears (179). However, recent results show that in addition to lymphangiogenesis, viral VEGF-C also induced enlargement, tortuosity and leakiness of veins and venules both in the skin and in nasal mucosa in a dose-dependent manner (124). Actual angiogenic sprouting of new blood vessels was not observed in response to AdVEGF-C or adeno-associated virus-encoded VEGF-C (AAV-VEGF-C) (124). Smaller concentrations or longer periods (14-21 days) of adenoviral VEGF-C expression resulted in a weaker blood vascular response while the lymphangiogenic response was maintained (124).

VEGF-C has been shown to stimulate the release of endothelial nitric oxide, which is a potent mediator of vasodilation and which could also contribute to enhanced vascular permeability (180). The enlargement of blood vessels in response to both AdVEGF-C and AdVEGF in the skin may in part result from receptor-mediated vasodilation as the veins and venules of the skin were shown to express VEGFR-2, but EC proliferation

may also play a role as some ECs of the blood vessels were stained for the cell proliferation marker PCNA one week after infection with the highest concentrations of AdVEGF-C (124). Although VEGF was unable to induce the sprouting of new lymphatic vessels, it induced their enlargement and the proliferation of LECs, at least some of which might be indirectly induced via increased permeability and tissue edema resulting in lymphatic vessel volume overload (124, 125). However, enhanced expression of VEGF by epidermal keratinocytes in a transgenic (K14) mouse model was not reported to cause changes in the skin lymphatic vasculature, despite substantial edema (178, 181).

Expression of Ang-1 blocked the increased leakiness of blood vessels induced by VEGF-C, while vessel enlargement and lymphangiogenesis were not affected (124). Previously both transgenic and adenovirally administred Ang-1 have been reported to protect adult blood vasculature against the plasma leakage induced by VEGF or inflammatory mediators (150, 178). In the transgenic model Ang-1 had effects on blood vessel morphology, but adenovirally mediated expression of Ang-1 did not change the morphology of the blood vessels in the skin (124). Data from several mouse models suggests that Ang-1 signaling via Tie-2 is needed for the stabilization of blood vessels and for the maintenance of the interactions between the ECs and the surrounding ECM and mesenchyme (149, 182, 183). Ang-1 and Ang-2 might play a similar stabilizing role in the development of the larger lymphatic collector vessels, which normally have tightly associated SMCs but display a very disorganized SMC coverage in the absence of Ang-2 (152).

VEGF-D has been reported to be angiogenic in the rabbit avascular cornea (102), but no blood vascular effects were seen in the K14 skin model (97). In the the rat cremaster muscle, adenoviral delivery of the short form of human VEGF-D induced a predominantly angiogenic response whereas both lymphangiogenesis and angiogenesis were induced in rat skin (184). In the rabbit hindlimb ischemia model, adenoviral human VEGF-D was a very potent inducer of blood vessel permeability and angiogenesis, whereas human VEGF-C had only marginal effects on the blood vessels (J. Markkanen, T. Rissanen, S. Ylä-Herttuala, personal communication). In another study VEGF-C was reported to induce angiogenesis in the rabbit hindlimb ischemia model (180). Thus, the *in vivo* effects of VEGF-C and VEGF-D seem to differ in different animal models and tissues. We do not know to which rabbit VEGF receptors human VEGF-C and VEGF-D bind to as such receptors have not been cloned. Differences in the proteolytic processing, availability of the receptors and vessel types may also modulate the biological effects of VEGF-C and VEGF-D. Furthermore, VEGF is know to be upregulated normally during ischemia whereas VEGF-C is not (185, 186). Therefore in the ischemic tissues VEGF-C may act as an adjunctive pro-angiogenic growth factor by enhancing VEGF mediated angiogenesis.

VEGF-C156S as a lymphangiogenic agent

In order to develop a lymphatic specific gene therapy approach without the unwanted blood vascular side effects, Saaristo and coworkers (187) studied the potential of the VEGFR-3-specific mutant, VEGF-C156S, (101) as a therapeutic agent in lymphedema. The effects of native VEGF-C and VEGF-C156S were compared using two approaches: transgenic mice that overexpress these growth factors under the K14-promoter in the skin and viral administration of VEGF-C and VEGF-C156S in the skin. VEGF-C156S was shown to potently induce lymphangiogenesis both during embryonic development and in the adult skin.

The mechanisms of lymphangiogenesis appear to be very similar to those of angiogenesis (5) in that lymphangiogenesis seems to proceed via vessel enlargement, sprouting and splitting (Fig. 3B) (187, 188). Native VEGF-C and VEGF-C156S differ in their lymphangiogenic mechanisms: in the transgenic skin model, VEGF-C156S induced lymphatic vessel hyperplasia, whereas VEGF-C induced a strong increase in the number of lymphatic vessels (187). This suggests that the activation of VEGFR-2 is required for the induction of lymphatic sprouting during embryonic development. In adult skin, however, recombinant VEGF-C156S viruses were capable of inducing the growth of lymphatic vessels and the formation of new lymphatic sprouts, although this was less pronounced than with native VEGF-C (187). In one study, mice treated with AdVEGF developed abnormal, large lymphatic channels that persisted, suggesting that also VEGFR-2 may participate in lymphangiogenesis (125). For efficient formation of vessel sprouts, signalling via both VEGFR-2 and VEGFR-3, or possibly even receptor heterodimers, may be required. On the other hand, it is also possible that VEGF-C is more effective than VEGF-C156S in recruiting other effector cells and factors needed for lymphangiogenic sprouting.

Previously VEGFR-3 signalling has been shown to be important for lymphatic endothelial cell survival *in vitro* (98, 189). A majority of the lymphatic vessels formed in response to adenoviral VEGF-C expression were seen to regress when the adenovirus was no longer active. In contrast, the AAV-mediated gene transfer resulted in long-lasting transgene expression and therefore the VEGF-C induced lymphatic vessels were sustained (187). The study by Saaristo and coworkers showed that VEGF-C156S had minimal effects on blood vessels, in contrast to VEGF-C; making VEGF-C156S an attractive candidate for lymphangiogenic therapy. AAVs encoding native VEGF-C and VEGF-C156S were also used in this study to treat the Chy lymphedema mice which have hypoplasia of cutaneous lymphatic vessels. Both factors induced sprouting of new lymphatic vessels from the sac-like lymphatic remnants in the skin of Chy mice (Fig. 3 C, D) three to five weeks after the AAV infection

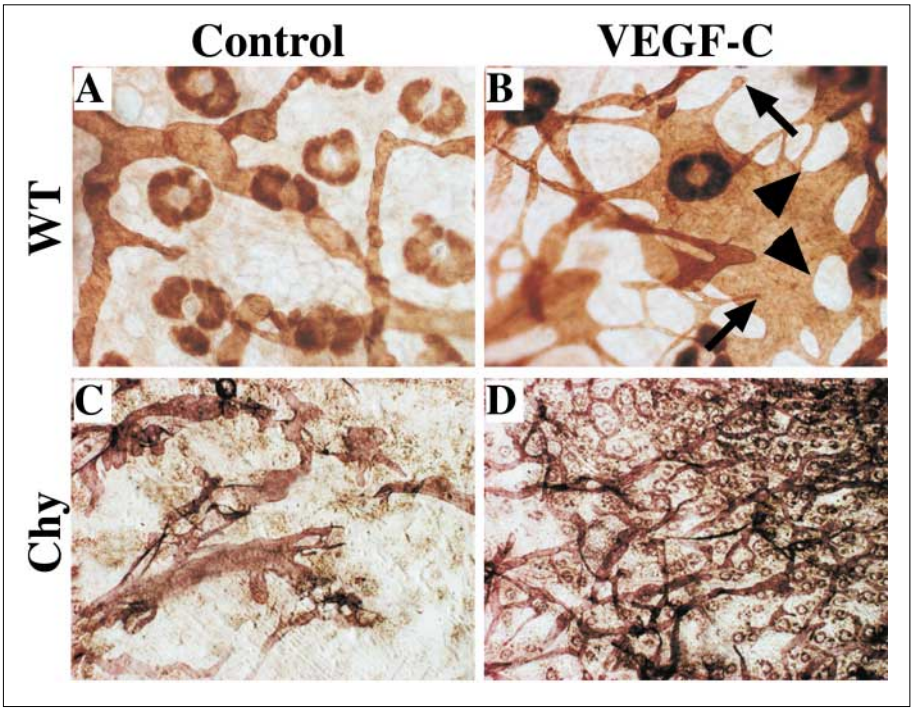

Figure 3: VEGFR-3 whole mount staining of the lymphatic vessels in the skin. A, B) Ongoing lymphangiogenesis in AAV-VEGF-C infected wild-type mouse skin (B) as compared to AAV-EGFP – infected control (A). Enlargement of the lymphatic vessels, sprouting (arrow) and splitting (arrowhead) of the vessels. C,D) In the Chy lymphedema mice AAV-mediated VEGF-C gene transfer is sufficient for inducing the formation of a functional lymphatic vessel network in the skin. Only few abnormal lymphatic remnant structures can be seen in the untreated Chy mouse skin (C), whereas in the VEGF-C treated Chy mouse lymphatic vessels form a hierarchic vessel network (D).

(187). After two months of VEGF-C156S or VEGF-C gene therapy, the formation of a branched network of functional, normal-looking, but slightly dilated lymphatic vessels was detected in the skin, and these vessels have persisted for at least eight months so far (187).

In addition to primary lymphedema, VEGF-C therapy could probably also be used in secondary lymphedema. In fact in regional lymphedema the treatment would be easier because local gene or protein transfer could be used. - Angiogenic gene therapy has raised concerns regarding the potential stimulation of dormant tumor growth due to increased tumor angiogenesis in response to elevated systemic VEGF levels. As tumor induced lymphangiogenesis has been associated with enhanced metastasis to the lymph nodes (190-195), the risk of enhanced growth and spread of dormant metastases during VEGF-C/D therapy needs to be carefully evaluated. Secondary lymphedema is common after lymphadenectomy for the treatment of cancer. One possibility to improve the safety of the VEGF-C/D therapy in these patients could be to combine the growth factor therapy with microsurgical treatment. Surgeons have performed lymphatic-venous anastomoses in order to reconstruct lymphatic drainage in patients with obstructive lymphedema (196). By combining this treatment with VEGF-C gene therapy one might be able to reduce the concentration of the growth factor used for therapy and thus increase the safety of the treatment. The half-life of VEGF-C in the blood circulation is short (97) and local VEGF-C therapy is therefore likely to function without systemic effects.

Tumor lymphangiogenesis

Tumorigenesis and tumor metastasis are multistep processes, and the accumulation of several genetic mutations is required for both processes. For a tumor cell to metastasize it must pass through several barriers and finally survive and grow in the target tissue. In metastasis via the vascular system, tumor cells must first enter the vessels of the primary tumor. VEGF and bFGF, secreted by the tumor cells, induce the expression of plasminogen activators and matrix metalloproteinases, contributing to the degradation of basement membranes (197). Because lymphatic vessels start out as thin-walled, blind-ended sacs in the extracellular tissue and because they lack a continuos basal lamina and have valve-like structures between the endothelial cells (depending on the degree of lymphatic distension) (198), they can generally be penetrated by tumor cells more easily than the blood vessels. However, tumor blood vessels in contrast to normal blood vessels also have fragmented and leaky basement membranes, and according to recent reports a considerable percentage of tumor blood flow is in direct contact with the tumor cells (199). After overcoming the first vascular barrier, the tumor cells have to survive in blood or lymph and attach to the new target tissue via the endothelium (lymph node or other target organ). Furthermore, in order to grow to a macroscopic size, the micrometastatic cells must also be able to induce angiogenesis in the target tissue. Therefore many factors, including proteolytic and migratory activity, expression of adhesion molecules, and deposition of the ECM surrounding stromal and tumor cells, contribute to tumor growth and metastasis.

Most human tumor types have a characteristic way of metastasizing via lymphatic or blood vessels to specific target tissues. Metastasis to the regional lymph nodes is one of the most important indicators of tumor aggressiveness (for references, see (200, 201)). Mechanisms determining the metastatic patterns in different tumor types remain poorly understood. In addition, certain tumor types rarely metastasize and some may remain dormant due to failure to initiate angiogenesis (202, 203).

While angiogenesis is required for tumor growth (204), it is not yet clear to what extent active lymphangiogenesis occurs in human tumors as the identification of lymphatic vessels has been difficult in the absence of molecular markers. Of the newly discovered lymphatic specific markers, at least VEGFR-3 has been shown to be expressed also in some tumor blood vessels (205-207). According to some analyses, functional lymphatic vessels are absent from the interior of solid tumors, possibly at least partly due to collapse of the vessels caused by the interstitial pressure induced by growing cancer cells (208-210). Enlarged, but functional lymphatics are often detected in the tumor periphery, and they frequently contain clusters of tumor cells (208). However, also intratumoral lymphatic vessels have been documented in tumor xenografts overexpressing VEGF-C or VEGF-D (191, 192, 211), and in human head and neck squamous cell carcinomas (212). On the other hand, no intra-tumoral lymphatic vessels were detected in invasive breast cancer (213) or in mice overexpressing VEGF-C as a transgene in pancreatic tumor cells (190). This discrepancy may at least in part be explained by the trapping of pre-existing lymphatic vessels between the rapidly growing tumor foci in the xenografts. Lymphangiography by intravital microscopy in experimental tumors has indicated that while lymphatic vessels in the tumor periphery are perfused and often enlarged, those inside the tumor often appear compressed and non-functional (208, 209). In principle, tumor cells can either invade the pre-existing lymphatic vessels directly, or enter new lymphatic vessels formed at the tumor periphery by tumor-induced lymphangiogenesis. As discussed in the previous chapter, tumor cells may also use the same trafficking pathways, chemokines and adhesion molecules as lymphocytes and antigen presenting cells in order to gain access to the lymphatic vessels (49, 50).

Role of VEGF-C and VEGF-D in tumors

Double transgenic experiments where mice overexpressing VEGF-C under the rat insulin promoter (Rip) were mated with Rip1Tag2 mice that spontaneously develop pancreatic beta cell tumors have provided insight into the possible role of VEGF-C in tumor metastasis (190). The tumors of Rip1Tag2 mice are capable of local invasion, but do not induce lymphangiogenesis nor do they form metastases (214). In the double transgenic animals VEGF-C induced excessive lymphangiogenesis around the pancreatic beta-cell tumors and this resulted in metastatic spread of tumor cells to pancreatic and regional lymph nodes (190). These data suggest that the VEGF-C-induced increase in peritumoral lymphatic vessels makes the lymphatics more accessible to tumor cells. Similarly, human breast cancers expressing ectopic VEGF-C were shown to induce lymphangiogenesis but no angiogenesis in and around the implanted tumor cells in immunocompromised mice (191, 193), resulting in enhanced tumor metastasis to the regional lymph nodes, and to the lung (191). In a human malignant melanoma xenoplant model, overexpression of VEGF-C resulted in both tumor lymphangiogenesis and angiogenesis (211). Although tumors overexpressing VEGF-C exhibit an increase in lymphatic metastasis, this may not be accompanied by an increase in the (hematogenous) lung metastasis (194, 208). Also VEGF-D has been shown to promote the metastatic spread of tumor cells via the lymphatic vessels (192). In addition, tumors secreting VEGF-D had an increased growth rate and tumor angiogenesis. The differences between tumor angiogenic properties of VEGF-C and VEGF-D in different studies may be related to differences in the proteolytic processing of these growth factors in different tumor types. *In vitro*, VEGF-C overexpression did not significantly alter the growth or migration of tumor cells, nor could it convert human lung carcinoma cells to an aggressive metastatic phenotype (194, 208). - As tumor growth and metastasis are multistep processes, other factors in addition to VEGF-C seem to be required for the highly metastatic phenotype.

VEGF-C induced tumor lymphangiogenesis and lymphatic metastasis can be inhibited by adenoviral expression of soluble VEGFR-3, which "traps" the available VEGF-C and VEGF-D before they can interact with their receptors (193, 194). In a human lung cancer xenoplant model, blocking of the VEGFR-3 signalling by a soluble form of the receptor suppressed tumor lymphangiogenesis and lymph node metastasis, but it did not have an effect on lung metastasis, suggesting that the mechanisms of lymphatic and lung metastasis differ at least in some tumor types (194). Inhibition of VEGFR-3 with neutralizing antibodies also suppressed the growth of experimental tumors by destabilizing the tumor-associated angiogenic vessels without affecting the established blood and lymphatic vasculature (215). In addition, the lymphatic spread of tumors induced by VEGF-D was blocked with an antibody specific to VEGF-D (216). Therefore, drugs specifically targeted to peritumoral lymphatic vessels might be used to inhibit lymphatic metastasis. However, destruction of these vessels could further elevate interstitial pressure inside the tumor, which is known to interfere with the delivery of anti-cancer agents (210) and which might also facilitate the entry of tumor cells into the blood vessels.

Several recent reports have suggested a correlation between VEGF-C expression and lymphatic metastasis in human tumors, (217-224), but less is known about VEGF-D in human tumors

(216, 224). It is still unclear if VEGF-C and VEGF-D expression promotes lymphangiogenesis in human tumors and if this increase could then translate into a higher rate of metastasis. Activation of the endothelium of lymphatic vessels in the tumor periphery by growth factors or cytokines secreted by the tumor cells may be sufficient to promote the interaction of tumor cells with the lymphatic vessels and thus facilitate tumor metastasis. Furthermore, both VEGF-C and VEGF-D increase vascular permeability and the resulting increased interstitial pressure could facilitate tumor cell entry to both blood and lymphatic vessels. - Thus, although the preliminary results from animal models and patient studies have suggested that VEGF-C and VEGF-D are involved in tumor metastasis, further studies are required to establish their importance in human cancers.

Concluding remarks

The first lymphangiogenic growth factors, VEGF-C and VEGF-D, isolated in 1996, have opened lymphangiogenesis for molecular analysis. Additional molecules that have been reported to be necessary for normal lymphatic development include the transcription factors Prox-1, FoxC2 and Sox18; the α9 integrin and Ang-2 (16, 21-23, 30, 152). As Prox-1 has now been established as a crucial fate-determining factor for lymphatic endothelial cells (16, 18, 26, 29), the processes regulating its expression in the developing vessel endothelium need to be examined. An interesting question is whether VEGF-C and VEGF-D are involved already in the triggering of the lymphatic endothelial phenotype – the expression pattern of VEGFR-3 indicates that this is possible.

Although the basic functions and effects of the lymphangiogenic factors are now better known, molecular control of the formation of a patterned hierarchy of lymphatic vessels is still unclear. Current data suggests that lymphangiogenesis in adults occurs by the outgrowth of new lymphatic vessels from the pre-existing vessels (157). However, the potential role of lymphangioblasts in adult lymphagiogenesis requires further studies. Also, little is known of the signaling pathways and networks in lymphatic endothelial cells and their interacting pericellular matrix and perivascular cells. The exact roles of the different receptors, for example VEGFR-2 and VEGFR-3 or Tie-1 and Tie-2, remain to be clarified in the lymphatic endothelium, and there may be other important yet undiscovered factors participating in lymphangiogenesis.

Lymphatic capillaries and collecting vessels are both needed for lymphatic drainage, and the characteristics of these distinct types of lymphatic vessels are likely to differ significantly. The collecting vessels express higher levels of VEGFR-2, and they are surrounded by a smooth muscle cell layer. Lymphatic vessels in different locations interact with different sets of surrounding cells. Cell-cell and cell-matrix interactions must contribute to the patterning and function of the lymphatic vessel network, but the specific signaling cascades mediating these interactions are not yet known. The Tie/angiopoietin system seems to be involved in the interaction of blood vessel endothelial cells and their support cells, cooperating with the VEGF signaling system (reviewed in (139)). Recent data suggests that this is also the case in lymphatic endothelial cells (152).

The emerging understanding of the molecular mechanisms of different forms of lymphedemas and the discovery of lymphangiogenic growth factors and markers now allow us to develop targeted therapies for diseases involving lymphatic vessel dysfunction. VEGF-C gene therapy may be a powerful tool in the treatment of various forms of human lymphedema, once the problems with gene delivery can be solved. Administration of the VEGF-C protein may also prove to be effective in the treatment of this disease (225). Treatment of late stage lymphedema may be difficult because long-term lymphedema results in the accumulation of secondary changes in the tissues, such as dermal fibrosis and fat deposition. However, advanced diagnostic methods, such as lymphoscintigraphy, allow the diagnosis of lymphedema at an early stage. Patients with local secondary lymphedema, without malignancies, may be the most suitable group for the initial testing of pro-lymphangiogenic therapy. Although the half-life of VEGF-C is short in the systemic blood circulation (97), the possibility that VEGF-C or VEGF-D can enhance the growth or lymphatic metastasis of dormant tumors in these patients needs to be carefully considered. The blood vessels formed in response to VEGF treatment are immature, tortuous and leaky and they often lack perivascular support structures (174). This may also apply to the newly formed collecting lymphatic vessels. To achieve safe and successful therapeutic lymphangiogenesis, the characteristics and function of the new lymphatic vessels formed in response to VEGF-C should be studied in detail. Growth factor combinations could be utilized to create a better-functioning hierarchic lymphatic network.

In the future, narrowing the target-cell range of vector mediated gene expression would be advantageous for all gene therapy approaches. Therapeutic applications can include targeting of either gene expression or viral entry to the desired endothelial cells of the specific tissue (226). Gene therapy vectors have been modified with homing peptides or antibodies. Importantly, now that we have lymphatic and blood endothelial cell as well as pericyte specific markers we could use these molecules for the targeted homing. Alternatively, endothelial cell specific promoters could be used to achieve endothelial expression of the therapeutic genes. The lymphatic endothelial cell specific promoters, such as the VEGFR-3 gene regulatory region, could also be used to target the lymphatic endothelium. Combining growth factor or gene therapy with lymphatic microsurgery may also be beneficial. Future studies will show the therapeutic potential and relevance of the pro-lymphangiogenic and anti-lymphangiogenic therapies in human diseases.

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