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REVIEW

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Genesis and pathogenesis of lymphatic vessels

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Abstract The lymphatic system is generally regarded as supplementary to the blood vascular system, in that it transports interstitial fluid, macromolecules, and immune cells back into the blood. However, in insects, the open hemolymphatic (or lymphohematic) system ensures the circulation of immune cells and interstitial fluid through the body. The *Drosophila* homolog of the mammalian vascular endothelial growth factor receptor (VEGFR) gene family is expressed in hemocytes, suggesting a close relationship to the endothelium that develops later in phylogeny. Lymph hearts are typical organs for the propulsion of lymph in lower vertebrates and are still transiently present in birds. The lymphatic endothelial marker VEGFR-3 is transiently expressed in embryonic blood vessels and is crucial for their development. We therefore regard the question of whether the blood vascular system or the lymphatic system is primary or secondary as open. Future molecular comparisons should be performed without any bias based on the current prevalence of the blood vascular system over the lymphatic system. Here, we give an overview of the structure, function, and development of the lymphatics, with special emphasis on the recently discovered lymphangiogenic growth factors.

Keywords Lymphatic endothelium · Drainage · Lymphedema · Lymphangiogenesis · VEGF · Vertebrates · Insects

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Introduction

Large multi-cellular organisms with a high metabolic demand use carrier molecules to distribute oxygen within their body. In vertebrates, a closed vascular system guides the transport of these molecules. Therefore, the vascular system has to be functional early in development. When the human embryo reaches the size of approximately 3 mm at embryonic day 22, its heart starts beating. Later, when the cardiovascular system is functioning, the lymphatic system develops, forming a second vascular system. Unlike the cardiovascular system, it is not a circulatory system. Lymphatic flow starts in blind-ended capillary networks that penetrate most of the body tissues (Figs. 1, 2). Collecting lymphatics drain the capillary networks, and after collecting fluid from many tributaries, the largest collecting lymphatic vessels (the thoracic duct and the right lymphatic duct) ultimately reach the veins.

Compared with our tremendous knowledge about the cardiovascular system, presumably our understanding of the lymphatic system is still rudimentary. The discovery and exploration of the lymphatic system have lagged behind those of the cardiovascular system, because of the immediately obvious importance of the cardiovascular system.

Retrospectively, three heydays have shaped our understanding of the lymphatic system. The first took place during the first years of the 20th century when researchers such as Sabin, Kampmeier, Huntington, and McClure were studying the ontogeny of the lymphatic system (for a review, see Wilting et al. 1999). The second boost of knowledge resulted from the use of the electron microscope during the 1960s to solve questions about the fine structure of the initial lymphatics and their function (for a review, see Leak 1970). At the moment, lymphatic research is experiencing an impressive comeback thanks to molecular biology, to genetics, and last but not least, to the discovery of markers specific for the lymphatic endothelium (for a review, see Oliver and Detmar 2002).

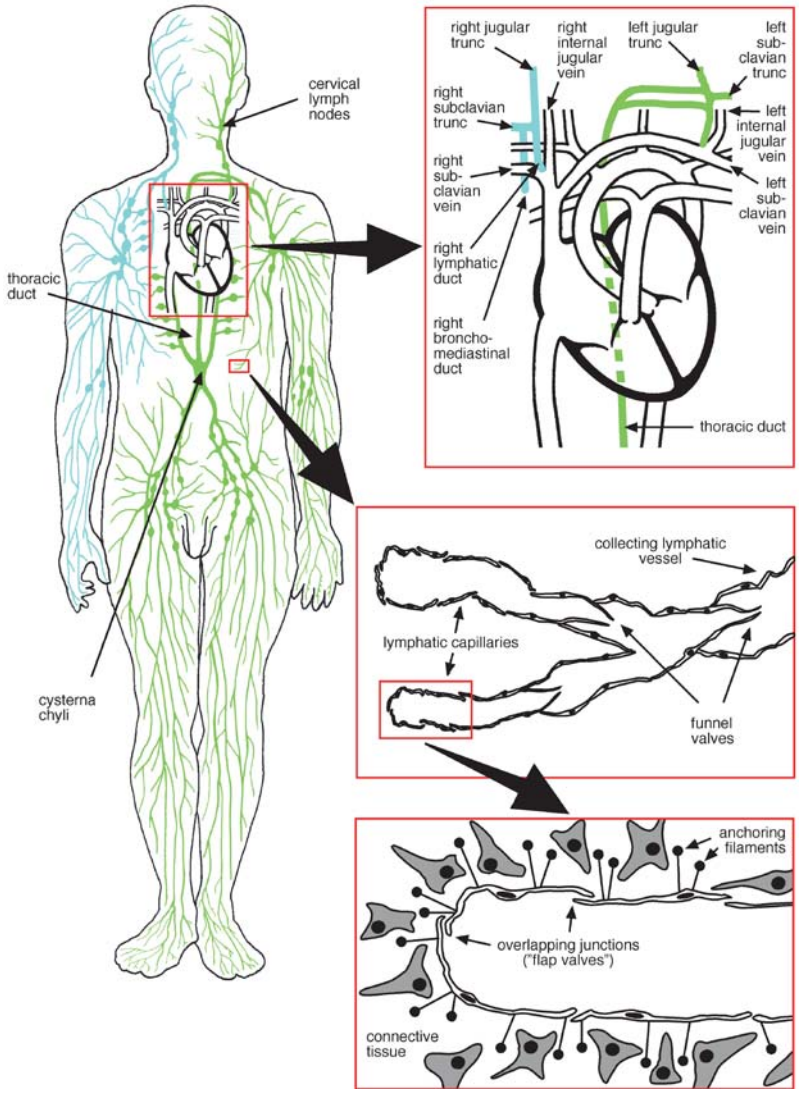

Fig. 1 Schematic view of the lymphatic system

Cardiovascular system

During early embryogenesis, most of the blood vessels form by vasculogenesis, the in situ formation of an immature network of endothelial channels by the differentiation of precursor cells (angioblasts). Vasculogenesis starts in extra-embryonic tissues where putative mesodermal precursor cells (hemangioblasts) aggregate into blood islands. Cells in the center of a blood island develop into hematopoietic cells, and those at the periphery differentiate into angioblasts. In the embryo, vasculogenesis gives rise to the heart endocardium, the paired dorsal aortas, and the primary vasculature of endoderm-derived organs (e.g. lung, liver, and pancreas; for a review, see Wilting and Christ 1996). The primary vascular network grows and is remodeled into a functional hierarchical system containing large caliber conduit vessels and small capillaries for diffusion. Various cellular mechanisms (splitting, fusion, sprouting, and intercalation) participate in this remodeling and expansion, a process collectively referred to as angiogenesis (for a review, see Risau 1998).

Whereas most organs become vascularized by a combination of vasculogenesis and angiogenesis, avascular ectodermal tissues, such as the brain, initially become vascularized exclusively by angiogenic mechanisms (Plate 1999). The endothelial cell layer becomes invested with mesodermal cells: a covering of pericytes, muscular tissue, and connective tissue. Therefore, vessel formation also requires the recruitment and organization of non-endothelial cells (for a review, see Carmeliet 2000). Angiogenesis has been assumed to be the only means of neovascularization in adult organisms, but recently, a population of progenitor cells able to differentiate into endothelial cells has been isolated from the circulating blood of adults and identified as originating from the bone marrow (Asahara et al. 1997, 1999; Shi et al. 1998; Crosby et al. 2000; for a review, see Rafii and Lyden 2003). However, the relative contribution of circulating precursors to physiological or pathological angiogenesis needs to be determined. Only a few physiological processes in the adult involve endothelial cell proliferation, e.g., the female reproductive cycle (for a review, see

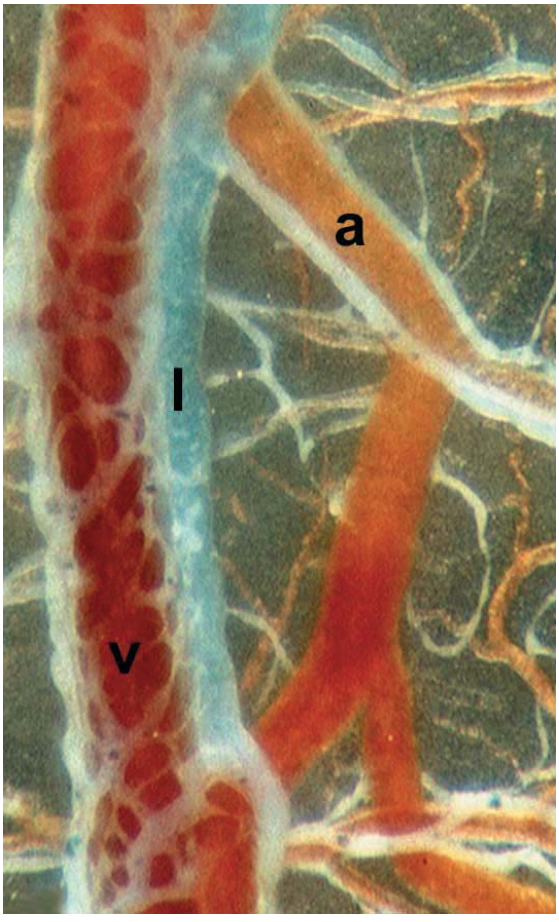

Fig. 2 Injection of Mercox-blue into the lymphatic system of the chorioallantoic membrane of a day-16 chick embryo. Note lymphatic capillaries and collecting lymphatics (*l*) accompanying an artery (*a*) and a vein (*v*). The vein is invested by a plexus of lymphatic capillaries

Reynolds et al. 1992) or exercise-induced muscular hyperplasia (for a review, see Tomanek and Torry 1994). In disease, angiogenesis frequently contributes to the pathological process (e.g., in tumorigenesis) and may, by itself, even be the main pathological process (for a review, see Carmeliet 2003).

Structure and function of the lymphatic system

The lymphatic system consists of a network of thin-walled vessels that drain fluid and particles from the interstitial spaces. Unlike blood vessels, the lymphatics do not form a circular system. The unidirectional flow of lymph returns fluid to the cardiovascular system. Lymphatic flow begins in the capillary networks (initial or terminal lymphatics). Lymphatic capillaries consist of a single layer of non-fenestrated endothelial cells resting on an incomplete basal lamina. Prenodal collectors drain the capillary networks and transport lymph to the regional lymph nodes. Lymphatic capillaries are not invested with mural cells, although collectors posses a smooth muscle cell layer (Schmid-Schonbein 1990b). Postnodal lymphatic trunks carry lymph to collecting lymph nodes or directly into lymphatic ducts. Lymph from the gastrointestinal and lumbar region drains into the cisterna chyli at the posterior end of the thoracic duct. The thoracic duct ascends upward behind the aorta to the left jugulo-subclavian junction and receives lymph from the body wall, except from its upper right quadrant from which the lymph is collected into the right lymphatic duct and returned to the veins at the right jugulo-subclavian junction (see Fig. 1; Foster 1996; Swartz 2001).

Drainage of interstitial fluid

The heart is a powerful pump, and when blood enters the capillary bed, it is still subject to a pressure of 4–5 kPa. Because of this pressure, 20–30 l plasma leak each day from the capillaries into the interstitium (Landis and Pappenheimer 1963). Osmotic forces cause the reabsorption of about 90% of the extravasated fluid at the venous end of the capillaries and post-capillary venules (Starling 1895–1896). Lymphatic vessels drain the remaining 10%. Approximately 2 l thoracic duct lymph are formed in humans each day (Bierman 1953; Linder and Blomstrand 1958). Lymph contains most of the components of plasma, although the concentration of high molecular weight components is lower because of capillary filtration. The total protein concentration is about one half that of serum (Bergstrom and Werner 1966; Werner 1966). Because of this leakiness, blood vessels are usually accompanied by lymphatics, a notable exception being the central nervous system. However, there is little doubt about the quasi-lymphatic function of the perivascular spaces in the brain. These spaces connect to the cervical lymph nodes (Casley-Smith et al. 1976), this connection being important for both drainage and the immune response to brain infections (for reviews, see Esiri and Gay 1990; Weller et al. 1996; Johnston and Papaiconomou 2002).

Immune defence

The lymphatics play many important roles in immune defence mechanisms. If pathogens invade the body, some are taken up together with the interstitial fluid by the lymphatics. The lymph passes through one or several lymph nodes before it enters the venous system. In collaboration with the antigen-presenting cells of the lymph nodes, T and B cells recognize non-self epitopes and mount an immune response. The activated immune cells proliferate in the lymph nodes and produce antibodies. Both cells and antibodies are delivered into the general circulation via the lymphatics. Not only the interstitial spaces, but also the blood is screened by the lymph nodes, since about 50% of the total amount of plasma protein passes through the lymphatics each day (Klein 1990).

Intestinal absorption

Another significant function of the lymphatics is the intestinal absorption and transport of long-chain dietary triglycerides and lipophilic compounds, such as fat-soluble vitamins, or chlorinated organic compounds. After passing the gastro-intestinal epithelium, the majority of compounds will be absorbed into the portal blood. However, high molecular weight molecules and colloids are preferentially taken up by the lymphatics (chylous vessels or lacteals). For example, chylomicrons with a diameter of 200–800 nm are released by the enterocytes and gain access almost exclusively to chylous vessels. When pharmacological substances are absorbed by intestinal blood capillaries and transported via the portal blood to the liver, a large proportion becomes inactivated (first-pass metabolic effect). Intestinal lymphatic absorption bypasses this metabolic effect (Porter 1997).

Lymph propulsion

Lymphatic capillaries have no intrinsic contractility and depend entirely on extrinsic forces for lymph propulsion. The notable exceptions are the initial lymphatics in the bat wing, which possess contractile smooth muscle (Hogan and Unthank 1986). Alternating compression and dilation of the lymphatics by respiratory movement, contraction of skeletal and intestinal muscles, and pulsatile pressure of adjacent arteries propels lymph forward (Schmid-Schonbein 1990a). The directed flow of lymph is maintained by funnel-shaped valves. The unit between two valves in the collecting lymphatics is termed the "lymphangion". In contrast to initial lymphatics, collecting lymphatics are contractile. Lymph flow from one lymphangion to the next is maintained against a pressure gradient (Kinmonth and Taylor 1956; Olszewski and Engeset 1980). The median pressure in initial lymphatics is close to atmospheric values (Zweifach and Prather 1975). The pressure rises in the collecting lymphatics, and in the thoracic duct diastolic pressure ranges between –0.7 and 2.3 kPa, systolic pressure between 0.3 and 3 kPa (Kinnaert 1973).

Development of the lymphatic system

Our knowledge about the development of the lymphatic system is based on studies performed at the beginning of the 20th century in diverse species, such as pig, frog and chicken, which may explain some of the controversial findings. Despite the mouse being a model organism in developmental biology, it is still one of the less-characterized species concerning embryonic lymphatic development, although the situation is presently subject to change. In contrast to lower vertebrates, the mouse has a large number of lymph nodes. Nevertheless, it remains to be tested whether the mouse can serve as a good model for all aspects of lymphatic research. Limitations may reside in its small size (creating only a minute amount of hydrostatic pressure), differences regarding lymphatico-venous anastomoses, and differences from the human at the molecular level.

In the mouse, development of the lymphatic system starts, based on morphological criteria, at embryonic day (E) 10.5, which corresponds to week 6.5–7 of human embryonic development and E 4.5 in the chick. By that time the cardiovascular system is functional (Clark and Clark 1920; van der Putte 1975a, 1975b). A distinct population of endothelial cells expressing the lymphatic-specific transcription factor Prox1 can be identified as early as E 9.5. These cells are located in the endothelial lining of the anterior cardinal vein and seem to give rise to the lymphatic primordia that form the huge jugular lymph sac, which lies in the angle between the anterior and posterior cardinal veins (Wigle and Oliver 1999; Wigle et al. 2002). There is considerable inter-species variability in the number and location of the lymphatic primordia, although the jugular region consistently seems to be the main site of lymphatic induction. In mammalian embryos, eight lymph sacs have been described: the paired jugular, subclavian, and posterior lymph sacs, the unpaired retroperitoneal lymph sac, and the cisterna chyli (Sabin 1909; van der Putte 1975a). Numerous theories of the development of lymphatics have been proposed.

Centrifugal sprouting

According to the "centrifugal" theory, lymph sacs develop from neighboring veins, and the peripheral lymphatic system then develops exclusively from the lymph sacs by the sprouting of endothelial cells into the surrounding tissues and organs (Sabin 1902; Clark 1912). Recent data favor this theory, including results from studies on the Prox1 knock-out (k.o.) mouse (Wigle et al. 2002) and from expression studies of the highly lymphatic-specific marker vascular endothelial growth factor receptor-3 (VEGFR-3, flt-4; Kaipainen et al. 1995; Kukk et al. 1996). In Prox1 k.o. mice, development of the lymphatic system is arrested on day 11.5, whereas the blood vessels are not affected. This shows not only that Prox1 has an essential role for the development of lymphatics, but also that early lymphatic structures can develop in a Prox1-independent manner. In early embryos, VEGFR-3 is expressed in blood vascular endothelial cells (BECs) but successively becomes restricted to lymphatic endothelial cells (LECs). This indicates that LECs are derived from BECs, or in contrast, that early BECs fulfil lymphatic functions, especially as early embryonic vessels are highly permeable. However, there is no agreement among the advocates of the centrifugal sprouting theory regarding the relationship of the embryonic lymph sacs to adult lymphatico-venous communications. According to Huntington and McClure (1910), all lymph sacs lose their connections with the veins and re-establish permanent connections only at the jugulo-subclavian junction. Alternatively, the latter connection is a persisting embry-

onic structure, and studies in the mouse argue for this theory (van der Putte 1975b).

Centripetal development

A vasculogenic mechanism has been proposed for the establishment of the lymphatic system, by analogy with the development of the blood vascular system. In the mesenchyme, precursors of LECs might arise close to the veins, but independently from their endothelial lining, and fuse into a lymphatic network, which subsequently might connect to the centripetally located veins (Huntington and McClure 1910; Kampmeier 1912). In serial histological sections, the continuum of the lymphatic primordia to the veins is often not visible (van der Putte 1975a), which may argue for this theory, but this does not rule out the venous origin of LECs, as individual cells might migrate to form the lymphatic primordia.

A model that integrates both theories, viz., sprouting from veins and the in situ differentiation of mesenchymal precursors, has been proposed by van der Jagt (van der Jagt 1932). Support for this model has been obtained recently. The lymphatics of the avian chorioallantoic membrane (CAM) and probably also the lymphatics of the wing are not exclusively derived from sprouts of the lymph sacs but also arise by the in situ differentiation of the local mesenchyme. Homotopic grafting of Prox1-negative day-3 quail allantoic buds into chick hosts results in CAM lymphatics composed of both donor and host endothelial cells (Schneider et al. 1999; Papoutsi et al. 2001). In addition to having a mesenchymal origin, some lymphatics may be derived from the veins, e.g., the part that develops in Prox1 k.o.-mice. The parts of the lymphatics that form lymphatico-venous anastomoses may be more closely related to the veins than those parts of the lymphatics that remain strictly separated from the blood vessels. The differential behavior may reflect differential origin. Moreover, in vitro, LECs and BECs represent distinct cell types that do not show any spontaneous inter-conversion (Kriehuber et al. 2001; Makinen et al. 2001). In our eyes, the question of whether LECs are generally derived from BECs or whether a significant part of LECs differentiate from mesenchymal precursor cells without taking the BEC detour is still a matter of controversy and requires further study.

Lymphatics in various vertebrates

Until recently, there have been misconceptions about the existence of lymphatics in some vertebrate classes. Additionally, sparse data rule out any attempts to draw conclusions about the phylogeny of the lymphatic system, and almost all of the research on comparative anatomy was carried out during the 19th and early 20th century with the limitations of that period (for a review, see Kampmeier 1969).

Fish

Mayer (1919) tried to explain the contradictory findings regarding piscine lymphatics by the existence of a secondary vascular system. The existence of such a secondary vascular system was confirmed by Vogel and Claviez (1981). It constitutes a separate parallel circulatory system and includes vessels earlier assumed to be lymphatics (Hoyer 1934). It starts from the systemic arteries, forms its own capillary networks, which supply mainly the oral mucous membranes and the skin, and returns to the systemic venous system. It functions presumably in skin respiration, osmoregulation, and immune defence. Based on its anatomical and functional characteristics, the secondary circulation has been hypothesized as being an evolutionary predecessor of the lymphatic system (Steffensen and Lomholt 1992). There is evidence for a "true" lymphatic system in lungfish (Laurent et al. 1978), and it is thus reasonable to speculate that the separation between blood vascular and lymphatic system was associated with the transition from aquatic to terrestrial life.

Ligands and receptors of the VEGF family that regulate lymphatic growth and development in higher vertebrates are also present in fish, but their relevance for the secondary vascular system has not been analyzed (Stainier et al. 1995). Molecules of the VEGF/platelet-derived growth factor (PDGF) family have also been identified in invertebrates, which lack endothelial cells altogether. In *Drosophila*, VEGFR directs embryonic immune cell migration. These molecules have probably only recently, on the evolutionary scale, assumed their roles in blood vessel and lymphatic development, and it is certainly conceivable that endothelial cells evolved from immune cells (Duchek et al. 2001; Heino et al. 2001; Cho et al. 2002). In ontogenesis, however, the inverse has been observed (Ciau-Uitz et al. 2000; de Bruijn et al. 2000).

Amphibians and reptiles

All amphibian orders seem to possess a lymphatic system that is comparable to the mammalian system, except in frogs and toads, where the superficial initial lymphatics fuse during metamorphosis to form dermal lymph sacs of the adult (Hoyer 1934; Kotani 1990). A characteristic structure found in amphibians are the lymph hearts, which are numerous and located at the lymphatico-venous anastomoses. Such entry points of the lymph into the blood circulation can generally be found in vertebrates in three different areas: jugular, lumbar, and caudal. The amphibian lymphatic system mostly communicates with the venous system in all of these areas, and lymph hearts range in number from four or six in frogs to more than 200 in caecilians. The important function of lymph hearts is to maintain the directed lymph flow and entry of lymph into the circulation. In reptiles, lymphatico-venous anastomoses exist in the caudal and the jugular regions, but

lymph hearts are found only in the caudal region (Hoyer 1934).

Birds and mammals

Mammals and most birds do not possess lymph hearts, but in birds, these are still found as transient embryonic organs. However, unlike other vertebrates, mammals and aquatic birds possess lymph nodes. Vertebrates differ significantly from each other in the number of their lymph nodes: most, approximately 400–500, are found in humans, whereas ducks have only four (Weidenreich et al. 1934). In most mammals, several lymphatico-venous junctions are formed during development. Usually, only the paired junction in the jugular region persists into adulthood, but additional lymphatico-venous anastomoses at both central and peripheral locations have been observed (Wolfel 1965; Threefoot and Kossover 1966; Pressman et al. 1967; Aboul-Enein et al. 1984). Several mammalian species maintain lumbar junctions into the inferior vena cava and the renal vein, draining the lymphatics of the lower extremities and the mesentery (Silvester 1912; Job 1918).

Pathology

Lymphedema

Insufficiency of lymphatic transport causes lymphedema. This may either be hereditary or of unknown etiology (primary lymphedema), or a consequence of a previous disease or trauma (secondary or acquired lymphedema). Iatrogenic lymphedema, and especially postmastectomy edema, represents probably the most common lymphatic condition in developed countries (Petrek and Heelan 1998). Surgical damage to the lymphatics has been regarded to be the main cause of post-surgical edema, but its etiology appears not to be straightforward, as the disease often develops more than 10 years after the surgical insult (for a review, see Pain and Purushotham 2000). Worldwide, the most common cause of lymphedema is, however, filariasis, which is mostly caused by infection by *Wuchereria bancrofti* or *Brugia malayi*. These parasitic nematodes are transmitted by mosquito bites. The parasite lives and reproduces in the lymphatic system causing a massive lymphatic dilation at the early stages of the disease. In advanced disease, lymphatic transport is blocked leading to an extreme enlargement of the limbs or other areas of the body termed elephantiasis (Rao et al. 1996; Dreyer et al. 2000).

In hereditary lymphedema, lymphatic vessels can either be hypoplastic or hyperplastic. In addition to some broad-spectrum syndromes, such as those of Ulrich-Turner and Noonan, a large number of distinct lymphedema syndromes have been described (Kääriäinen 1984; Ferrell et al. 1998; Finegold et al. 2001).

Hereditary lymphedema types I and II

Type I hereditary lymphedema (Milroy disease, OMIM 153100) is an early onset form of hereditary lymphedema. In these patients, the initial superficial lymphatics of edematous areas cannot be demonstrated by fluorescence micro-lymphangiography and seem to be absent or highly hypoplastic. However, superficial lymphatics are present in non-edematous areas (Bollinger et al. 1983). In some families, inheritance is strongly linked to dominant missense mutations in the *VEGFR-3* gene on chromosome 5 (Karkkainen et al. 2000). However, penetrance is incomplete or variable in these families. Other families show additional linkage to multiple loci on chromosomes 3, 11, and 18, unrelated to any known target genes. Thus, an oligogenic pattern of inheritance, modifier genes, and environmental factors might also be involved.

Type II hereditary lymphedema (Meige disease, OMIM 153200) differs from type I by a later disease onset (around puberty), and its etiology appears to be even more complex, since only 10% of the families show dominant patterns of inheritance and a penetrance of 40% (Holberg et al. 2001).

Lymphedema-distichiasis syndrome

Dominant mutations in the transcription factor *FOXC2* gene have been identified as the cause of lymphedema-distichiasis syndrome (OMIM 153400; Fang et al. 2000; Finegold et al. 2001). In addition to lymphedema distichiasis, three other lymphedema syndromes co-segregated with *FOXC2* mutations: type II hereditary lymphedema, lymphedema/ptosis (OMIM 153000), and lymphedema/yellow-nail syndrome (OMIM 153300). All identified *FOXC2* mutations result in a truncated protein, and the observed phenotype is probably a result of haploinsufficiency.

Lymphangiectasia

Lymphangiectasia is a lymphatic disorder characterized by dilated dysfunctional lymphatics. The condition can be limited to a specific organ. The lungs are affected in hereditary pulmonary cystic lymphangiectasia (OMIM 265300), and the intestine in Hennekam lymphangiectasia-lymphedema syndrome (OMIM 235510; Hennekam et al. 1989; Gilewski et al. 1996). The causes of lymphangiectasia and lymphedema are thought to be similar, and both conditions occur jointly in syndromes such as Noonan type I (OMIM 163950), Hennekam, or hereditary intestinal lymphangiectasia (OMIM 152800). Moreover, acquired forms similar to lymphedema are known (Celis et al. 1999).

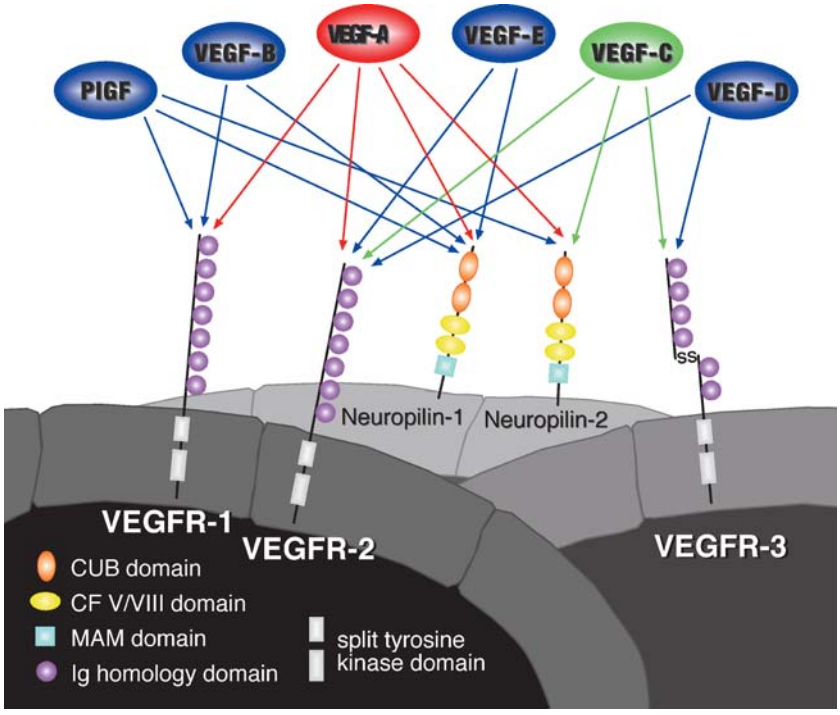

Fig. 3 VEGF family of ligands and their receptors. VEGFRs have an extracellular domain composed of immunoglobulin homology domains, a transmembrane domain and intracellular tyrosine kinase domain split by a kinase insert sequence. The neuropilins contain an extracellular part of two complement-binding (CUB) domains, two coagulation factor (*CF*) V/VIII homology domains, a MAM (meprin, A5, μ) domain, which is important for homo- and heterodimerization, a transmembrane segment, and a short cytoplasmic domain of about 40 amino acids. Note that human VEGF-D binds both VEGFR-2 and VEGFR-3, whereas mouse VEGF-D binds only VEGFR-3 (*PlGF* placenta growth factor)

Lymphatic neoplasms

In rare cases, neoplasms can develop from LECs. Lymphangiomas constitute approximately 5% of all benign lesions of infancy and childhood (Zadvinskis et al. 1992). Since lymphangiomas can be present either as a localized mass or as a diffuse tumor, it is questionable whether all lymphangiomas represent true neoplasms (Scalzetti et al. 1991). Unlike lymphangioma, lymphangiosarcoma is a true malignant lesion, mostly occurring as a complication of post-mastectomy edema (Stewart Treves syndrome; Janse et al. 1995).

Tumor lymphangiogenesis

The lymphatic system serves as the primary pathway for the metastatic spread of tumor cells to regional lymph nodes, and possibly also to distant organs. The prognostic value of lymph node metastasis was recognized long before the concept of lymphangiogenesis both within and adjacent to tumors became widely accepted (Fisher et al. 1969; Carter et al. 1989). Tumor lymphangiogenesis occurs in experimental models in the chick and the mouse with important implications for metastasis (Papoutsi et al. 2000; Karpanen et al. 2001; Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001). Nevertheless, it remains unclear as to what extent lymphangiogenesis occurs in human cancers, and what the consequences are with respect to cell dissemination (Leu et al. 2000; Birner et al. 2001; Jackson et al. 2001; Schoppmann et al. 2001). According to a recently published study, tumor lymphangiogenesis provides a prognostic indicator for metastasis and survival in human cutaneous melanoma (Dadras et al. 2003).

Therapeutic lymphangiogenesis

The high regenerative capacity of lymphatic vessels is well known (Clark and Clark 1932). However, expertise in therapeutic lymphangiogenesis is just emerging (Karkkainen et al. 2001). Animal models are being developed for aquired lymphedema (Witte et al. 2001), and a mouse model of hereditary lymphedema also exists in the form of the *Chy* mouse. These mice carry a mutant VEGFR-3 allele coding for a receptor with a kinase domain that is incompetent in signaling. In this model, VEGF-C has been used to compensate for insufficient VEGFR-3 signaling. This results in the growth of new functional lymphatics (Karkkainen et al. 2001). On the other hand, hyperplasia of the lymphatic vessels and impaired lymphatic transport occur in the keratin-14 VEGF-C transgenic mouse (Jeltsch et al. 1997). From the visceral lymphatics in *Chy* mice, only the lacteals are aplastic, although the VEGFR-3 mutation is assumed to be dominant-negative in all LECs. Local differences may reside in the differential expression of additional growth factor (co)receptors on LECs: neuropilins (NRPs) are a small family of transmembrane proteins (for a review, see Neufeld et al. 2002). In the human, two related genes exist, viz., NRP-1 and -2, which are expressed in various splice forms including soluble forms. NRP-2 binds VEGF-C and might modulate its activity (Fig. 3). NRP-2 null mutants are viable until adulthood and show a grossly normal cardiovascular system but display selec-

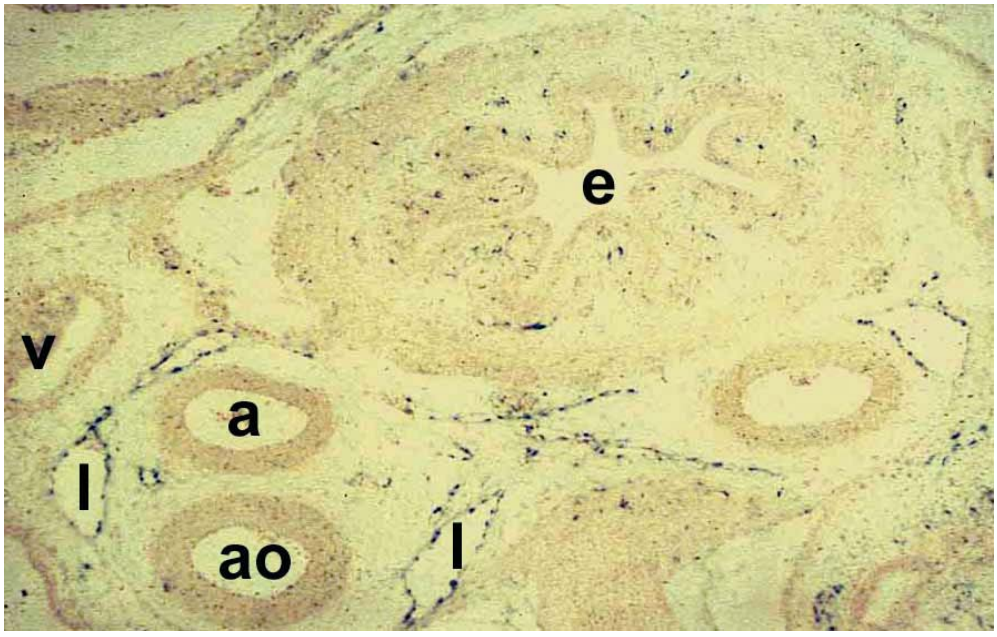

Fig. 4 In situ hybridization with the VEGFR-3 antisense probe showing the chick lymphatic vessels (*l*) accompanying the mesenteric artery (*a*) and vein (*v*) of a day-10 chick embryo (*ao* aorta, *e* esophagus)

tive malformations of the lymphatic vascular system (Yuan et al. 2002).

VEGF family

The last 20 years of angiogenesis research have been dominated by molecular biology. The identification of the molecular players started with the discovery of VEGF-A in 1989 (Keck et al. 1989; Leung et al. 1989; Plouët et al. 1989) and the discovery of VEGF-C in 1996 (Joukov et al. 1996; Lee et al. 1996). Several large-scale sequencing projects have contributed to the identification of genes involved in angiogenesis, and the focus of today's research is to understand their functions. Polypeptide growth factors and their receptors are major components of the regulatory machinery governing angiogenesis. Central positions in this machinery are occupied by the VEGFR tyrosine kinases. VEGFRs are largely specific for endothelial cells. In mammals, three VEGFRs interact with five different VEGFs, each of which has a characteristic receptor-binding pattern (Fig. 3). However, other growth factor and receptor families provide major contributions to vessel differentiation, notably the Tie receptors with their angiopoietin ligands (for a review, see Jones et al. 2001), the ephrins and their receptors (for a review, see Cheng et al. 2002), the neuropilins, which can act as co-receptors for several VEGF family members (for a review, see Neufeld et al. 2002), and the PDGFs, which do not act directly on endothelial cells but, instead, recruit pericytes and smooth muscle cells to coat the endothelial tube, which is indispensable for vessel stabilization (for a review, see Betsholtz et al. 2001). The molecules and their functions are discussed elsewhere in this issue, and we will therefore focus on the lymphangiogenic pathways.

VEGF-A is a master effector of hemangiogenesis

The key molecule "par excellence" of vascular development is VEGF, which is also called VEGF-A to distinguish it from the other VEGF family members. The function of VEGF-A in vasculogenesis and angiogenesis has been extensively analyzed (for reviews, see Neufeld et al. 1999; Ferrara et al. 2003). VEGF-A binds to VEGFR-1 and VEGFR-2. VEGFR-2 seems to mediate most, if not all, of the biological effects of VEGF, whereas VEGFR-1 probably has a modifying function, mostly by acting as a decoy receptor. In VEGFR-2 k.o. mice both hematopoiesis and vasculogenesis are blocked (Shalaby et al. 1995), whereas in VEGFR-1 k.o. mice, the remodeling of the primary vascular plexus fails because of the increased hemangioblast commitment of mesenchymal precursors (Fong et al. 1995, 1999). A lymphangiogenic function for VEGF-A is generally not accepted (Wilting and Christ 1996), despite one controversial recent report (Nagy et al. 2002). The C-terminal domain of VEGF-A_{165} (also called $VEGF_{55}$) constitutes a heparin-binding domain similar to that of plasminogen, but unrelated to the fibroblast-growth-factor-type heparin-binding domain (Fairbrother et al. 1998).

Lymphangiogenic growth factors VEGF-C and VEGF-D

VEGFR-3 is a paralog of VEGFR-1 and VEGFR-2. Its expression becomes restricted to LECs during embryogenesis (Fig. 4; Kaipainen et al. 1995; Kukk et al. 1996; Wilting et al. 1997). The search for its ligand has led to the identification of two lymphangiogenic growth factors: VEGF-C and VEGF-D (Joukov et al. 1996; for a review, see Jussila and Alitalo 2002), which specifically induce the proliferation of lymphatic endothelial cells in trans-

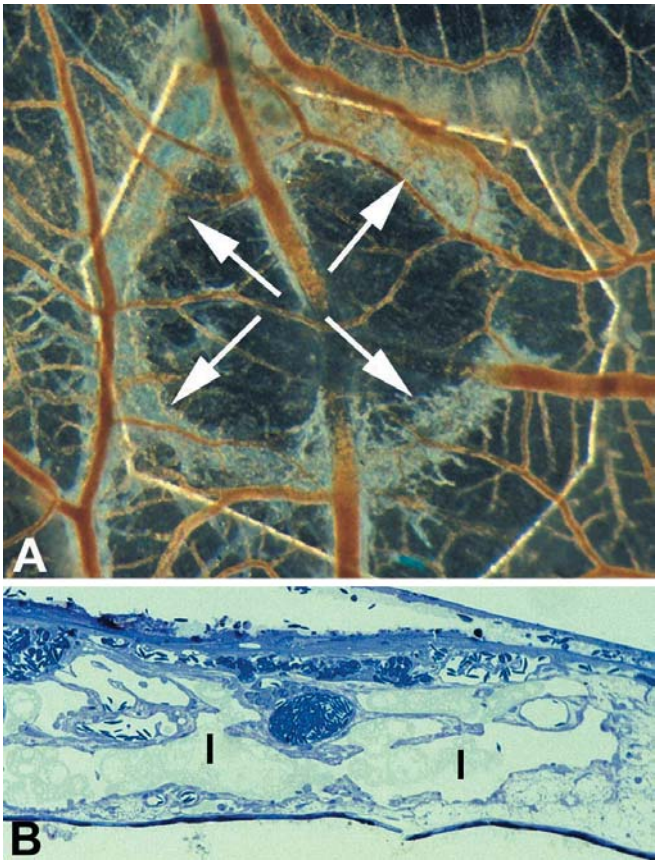

Fig. 5A, B Lymphangiogenic effect of VEGF-D on the chick chorioallantoic membrane (CAM). **A** Note numerous Mercox-filled lymphatic capillaries that have developed below the rim (*arrows*) of the filter disk containing the highest growth factor concentrations. **B** Semi-thin section through the CAM shows the delicate structure of the wide lymphatic channels (*l*). Blood vessels were weakly affected (own unpublished data)

genic mice and in the CAM (Fig. 5; Jeltsch et al. 1997; Oh et al. 1997; Veikkola et al. 2001). In the same assays, VEGF-A is specific for blood vessels and does not induce any changes in the lymphatic vasculature (Oh et al. 1997; Detmar et al. 1998). However, several reports confirm that both VEGF-C and VEGF-D can exercise significant angiogenic potency both in vitro and in vivo (Lee et al. 1996; Cao et al. 1998; Witzenbichler et al. 1998; Marconcini et al. 1999; Byzova et al. 2002). Discrepancies concerning their angiogenic versus lymphangiogenic potential might be a result of differences in the extracellular processing of the proteins (Joukov et al. 1997; Stacker et al. 1999).

Mice deficient in VEGFR-3 die from cardiovascular failure at E 9.5 (Dumont et al. 1998) because of impaired remodeling and maturation of large vessels. VEGFR-3 thus appears to a have an essential role before the formation of the lymphatic system, when it is still ubiquitously expressed on all endothelial cells. In VEGF-A k.o. mice, endothelial differentiation is not completely blocked, unlike in the VEGFR-2 k.o. mice, suggesting that VEGF-C does indeed activate VEGFR-2 in early embryonic development (Carmeliet et al. 1996; Ferrara et al. 1996; Shalaby et al. 1997). At least in vitro, VEGF-C can take over VEGF-A functions in a differentiation assay with avian hemangioblasts (Eichmann et al. 1997). Further insights into the role of VEGFR-3 in lymphatic development could be obtained by a conditional knock-out strategy. Contrary to the mouse, an endogenous retrovirus disrupts the splice pattern in humans, giving rise to a C-terminally shortened receptor isoform, whose signaling properties differ from the long form (Pajusola et al. 1993; Borg et al. 1995; Hughes 2001).

Molecular characteristics of VEGF-C and VEGF-D

VEGF-C and VEGF-D differ from other VEGF family members by the presence of long N- and C-terminal extensions flanking the VEGF-homology domain (Joukov et al. 1996; Lee et al. 1996; Orlandini et al. 1996; Yamada et al. 1997; Achen et al. 1998). The N-terminal propeptide does not contain any known motifs, whereas the C-terminal domain contains a repetitive cysteine pattern (Cys-X_{10}-Cys-X-Cys-X-Cys), homologous to a motif first identified in a secreted silk-like Balbiani ring 3 protein produced by larval salivary glands of the midge *Chironomus tentans.* The VEGF homology domain (VHD) of VEGF-C exhibits 35% identity to VEGF-A. Similar to the other VEGF family members, the VHD of VEGF-C is encoded by exons 3 and 4 (Chilov et al. 1997; Tischer et al. 1991; Maglione et al. 1993; Olofsson et al. 1996). The VEGF homology domains of VEGF-C and VEGF-D are 60% identical. VEGF-C is synthesized as a precursor protein, which undergoes subsequent proteolytic processing (Joukov et al. 1996, 1997; Siegfried et al. 2003). The C-terminal domain is cleaved off by furin or related protein convertases upon secretion but remains bound to the N-terminal domain by disulfide bonds, giving rise to a disulfide-linked tetramer composed of 29-kDa and 31-kDa polypeptides. Proteolytic processing of the N-terminal propeptide releases the mature form, which consists of two 21-kDa polypeptide chains, corresponding largely to the VEGF homology domain (Joukov et al. 1997). The 29-kDa and 31-kDa forms seem to be the most prevalent in various biological systems (Lee et al. 1996; Hu et al. 1997; Joukov et al. 1997; Eichmann et al. 1998; Hiltunen et al. 2000). Incomplete and additional proteolytic processing leads to two minor fragments that migrate on reducing SDS-polyacrylamide gel electrophoresis with a molecular weight of approximately 15 kDa and 43 kDa. The 15-kDa fragment represents the N-terminal propeptide, whereas the 43-kDa form presumably represents the VHD and the C-terminal domain. VEGF-D is processed in a similar way (Stacker et al. 1999).

Both the full-length and the mature forms of human VEGF-C bind VEGFR-3 with high affinity (Joukov et al. 1997), whereas high affinity binding to VEGFR-2 is dependent on proteolytic processing. The receptor-binding profile of VEGF-C seems to be conserved among

different species. In contrast, mouse VEGF-D does not bind mouse VEGFR-2 (Baldwin et al. 2001). Similar to VEGF-A, glycosylation of VEGF-C is not a prerequisite for receptor binding but is necessary for efficient secretion.

Structure of VEGFR-3 and its ligands

Neither the structure of VEGFR-3 nor of its ligands has been solved. The high homology of VEGF-C and VEGF-A in the VHD allows for molecular modeling of this domain and, together with mutational data, offers some limited insights into the nature of the receptor interaction of VEGF-C. The primary sequences of VEGF-C and VEGF-A show major differences in three regions that code for the N-terminal part, for the segment between β-strands 1 and 2, and for the flexible loop connecting β-strands 5 and 6. The flexibility of this loop might be of functional importance in the promiscuous receptor-binding properties of VEGF-A (Muller et al. 1997a). The N-terminal part of the VHD forms an α-helix in both VEGF-A and placenta growth factor (Muller et al. 1997b; Iyer et al. 2001) but is disordered in PDGF-BB (Oefner et al. 1992), which might also be the case for VEGF-C. Mutation analyses and X-ray structures show that these three regions are involved in the determination of receptor specificity of VEGFs and PDGFs. VEGF-C loses its ability to bind and activate VEGFR-2 when Cys-156 is mutated into serine (Joukov et al. 1998). In other members of the PDGF/VEGF family, cysteine residues homologous to Cys-156 are involved in inter-chain disulfide binding, whereas VEGF-C and VEGF-D are non-covalent dimers (Joukov et al. 1997; Stacker et al. 1999) indicating a possible role for the bottom groove between the two monomers in receptor interaction. Such a mechanism has also been proposed for VEGF-A and VEGFR-1 (Wiesmann et al. 1997).

VEGF-C expression and its regulation

In the mouse, VEGF-C mRNA expression starts around E 8.5 in the head mesenchyme and the developing vertebrae. At E 12.5, VEGF-C expression is strong in the mesenchyme of the metanephric and jugular region, in which the embryonic lymph sacs develop near the large veins (Kukk et al. 1996). This pattern is conserved between species. In quail and chick embryos, VEGF-C has been observed in areas that become rich in lymphatic endothelium (Eichmann et al. 1993, 1998). In adult mice, the expression of VEGF-C decreases, but its mRNA can still be found in the lung, heart, liver, and kidney (Kukk et al. 1996; Fitz et al. 1997; Lymboussaki et al. 1999).

Although VEGF-C expression overlaps with VEGF-D, e.g., in the heart, important differences exist both during development and in adult life. VEGF-D is highly expressed in the lung (Avantaggiato et al. 1998; Farnebo et al. 1999). The regulation of VEGF-C and VEGF-D expression is less well understood than that of VEGF-A (Shweiki et al. 1992; Shima et al. 1996; Stein et al. 1998; Oosthuyse et al. 2001). Unlike the VEGF-A promoter, the VEGF-C promoter lacks hypoxia-response elements (Chilov et al. 1997), and thus VEGF-C mRNA levels are not regulated by hypoxia (Enholm et al. 1997). However, various growth factors and inflammatory cytokines upregulate the expression of VEGF-C (Enholm et al. 1997; Ristimaki et al. 1998), whereas steroid hormones act as antagonists (Laitinen et al. 1997; Ruohola et al. 1999). The VEGF-D promoter has also not been characterized in detail, but both hypoxia and cell-cell contacts have been implicated in its regulation (Orlandini and Oliviero 2001; Teng and Johns 2002).

VEGF-C signaling

The signal transduction pathways of VEGF receptors are still under investigation. Endothelial cells typically express more than one VEGF receptor, making it difficult to assign a specific effect to a specific receptor. On the other hand, non-endothelial cells transfected with a single receptor might lack the endothelial-cell-specific signal transduction machinery. The use of receptor-specific mutants to activate receptors on isolated primary cultures of endothelial cells has improved the situation (Gerber et al. 1998b; Joukov et al. 1998; Makinen et al. 2001).

In endothelial cells, the major VEGF-induced mitogenic signal is thought to be routed independently from Ras via PLCγ-PKC to the MAP kinase cascade (Waltenberger et al. 1994; Seetharam et al. 1995; Cunningham et al. 1997; Takahashi and Shibuya 1997; Takahashi et al. 1999). Akt phosphorylation by PI3 kinase seems to be important for survival signaling (Xia et al. 1996; Gerber et al. 1998b; Jiang et al. 2000). This process is dependent on the association of VEGFR-2 with vascular endothelial cadherin (Carmeliet et al. 1999). Akt is also one of the pathways by which VEGF-A activates the vasorelaxant endothelial nitric oxide synthase (Parenti et al. 1998; Dimmeler and Zeiher 1999; Fulton et al. 1999). Additionally, VEGF-A up-regulates the endothelium-specific $\alpha_V\beta_3$ integrin (Senger et al. 1997), which in turn associates with VEGFR-2 to potentiate VEGF-A-induced signaling (Soldi et al. 1999). Other target genes of VEGF-A that have been implicated in various aspects of angiogenesis include proteases, such as plasmin and matrix metalloproteinases (Pepper et al. 1991; Unemori et al. 1992), cytoskeletal components, such as focal adhesion kinase (Abedi and Zachary 1997), anti-apoptotic proteins, such as Bcl-2 (Gerber et al. 1998a), and transcription factors of the STAT family (Korpelainen et al. 1999).

Compared with VEGFR-2, little is known about VEGFR-3-initiated signal transduction. From the two human splice variants, the short isoform is compromised in its signaling capabilities, presumably because of the absence of the phosphorylation sites at the C-terminus (Pajusola et al. 1993; Fournier et al. 1995). When

transfected into PAE cells, VEGFR-3-initiated signal transduction appears to be similar to that of VEGFR-2 (Kroll and Waltenberger 1997). Thereby, Shc becomes tyrosine phosphorylated, and cell proliferation increases (Pajusola et al. 1994; Fournier et al. 1996; Wang et al. 1997). Like VEGFR-2, VEGFR-3 associates with Grb2 and PLCγ (Pajusola et al. 1994; Fournier et al. 1995, 1996). Moreover, the biological response is similar in PAE cells, as migration, actin reorganization, and proliferation have all been reported to occur (Joukov et al. 1996; Cao et al. 1998). Recently, VEGFR-3-expressing isolated LECs have been used to study signal transduction. These studies show that there are distinct differences between VEGFR-2 and VEGFR-3 mediated signaling (Gerber et al. 1998b; Taipale et al. 1999; Makinen et al. 2001).

Summary

During embryonic vascular development, the differentiation of ECs (vasculogenesis) and angiogenesis are of central importance. Later, angiogenesis is essential for growth, the female reproductive cycle, and the remodeling and regeneration of tissues during wound healing. Neovascularization also plays a major role in severe pathological conditions, such as tissue ischemia, tumor growth, and metastasis. The lymphatic vasculature is essential for the maintenance of fluid balance in the body, for immune defence, and for the uptake of dietary fat. Absent or damaged lymphatic vessels can lead to lymphedema, a chronic and disfiguring swelling of the extremities, sometimes necessitating amputation of the affected limb. In addition, lymphatic vessels promote the metastatic spread of cancer cells to distant organs, which is a leading cause of death in patients with cancer and a major obstacle in the design of effective therapies. The limited progress in lymphangiogenesis research is partly attributable to the difficulties in recognizing these vessels in tissues, because of the lack of specific markers. However, the recent discovery of key lymphangiogenic factors (including VEGF-C and VEGF-D and their receptor VEGFR-3) has allowed us to obtain novel insights into how lymphatic vessels and blood vessels coordinately grow and affect human disease. Additional effort is required for resolving the complex cascades behind these phenomena at the molecular level.

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