VEGFR-3 in Angiogenesis and Lymphangiogenesis

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Contents

Abbreviations

aa	amino acid
Ang	angiopoietin
BEC	blood vascular endothelial cell
E.	embryonic day
EC	endothelial cell
ECM	extracellular matrix
Flk-1	fetal liver kinase 1 (mouse VEGFR-2)
Flt-1	*fms*-like tyrosine kinase-1 (VEGFR-1)
Flt-4	*fms*-like tyrosine kinase-4 (VEGFR-3)
Ig	immunoglobulin
kD	kilodalton
KDR	kinase insert domain containing receptor (human VEGFR-2)
KS	Kaposi's sarcoma
LEC	lymphatic endothelial cell
LYVE-1	lymphatic vessel endothelial hyaluronan receptor -1
MoAb	monoclonal antibody
mRNA	messenger ribonucleid acid
NRP	neuropilin
P.	postnatal day
PoAb	polyclonal antibody
PECAM-1	platelet endothelial cell adhesion molecule-1
PDGF	platelet-derived growth factor
PDGFR	PDGF receptor
PlGF	placenta growth factor
RTK	receptor tyrosine kinase
SMC	smooth muscle cell
Tek	tunica interna endothelial cell kinase (Tie-2)
Tie	tyrosine kinase with Ig and EGF homology domains (Tie-1)
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
VWF	von Willebrand factor

List of Original Publications

This thesis is based on following original articles, which are referred to in the text by their Roman numerals. Some unpublished data are also presented.

I Jussila, L., Valtola, R., Partanen, T.A., Salven, P., Heikkilä, P., Matikainen, M-T., Renkonen, R., Kaipainen, A., Detmar, M., Tschachler, E., Alitalo, R. and Alitalo K.: Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor-3. *Cancer Res.* 58:1599-1604, 1998.

II Dumont, D.∗, Jussila, L∗, Taipale, J.∗, Mustonen, T., Pajusola, K., Breitman, M. and Alitalo, K: Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science* 282: 946-949, 1998.

III Veikkola, T.∗, Jussila, L.∗, Jeltsch, M., Thurston, G., McDonald, D.M., Achen, M.G., Stacker, S.A., Alitalo, K.: Signalling via VEGFR-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J.* 20:1223-1231, 2001.

IV Mandriota, S.J., Jussila, L., Jeltsch, M., Compagni, A., Baetens, D., Prevo, R., Banerji, S., Huarte, J., Montesano, R., Jackson, D.G., Orci, L., Alitalo, K., Christofori, G., Pepper, M.S. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J.* 20:672-682, 2001.

∗ equal contribution

Abstract

Blood and lymphatic vessels develop in a parallel, but independent manner, and together form the circulatory system allowing the passage of fluid and delivering molecules within the body. Although the lymphatic vessels were discovered 300 years ago, at the same time as the blood circulation was described, the lymphatic system has remained relatively neglected until the present. This is in part due to the difficulties in recognizing these vessels in tissues because of a lack of specific markers. Over the past few years several molecules expressed specifically in the lymphatic endothelial cells have been characterized, and knowledge about the lymphatic system has started to accumulate again.

The VEGF (Vascular Endothelial Growth Factor) family of growth factors and receptors is involved in the development and growth of the vascular endothelial system. Two of its family members, VEGF-C and VEGF-D regulate lymphatic endothelial cells via their receptor VEGFR-3. These are the first molecules found to be involved in the biology of the lymphatic vessels, and their discovery has opened new lines of inquiry in the study of lymphatic endothelial cell regulation. The role of the lymphatic vessels in immune responses and certain pathological conditions can be studied in more detail as the blood and lymphatic vessels seem to be involved in many diseases in a coordinated manner. Discoveries made so far will be helpful in the diagnosis of certain vascular tumors, in the design of specific treatments for lymphedema, and in the efforts to prevent the metastatic tumor spread via the lymphatic system.

The present study was undertaken to characterize the biological role of growth factors VEGF-C and VEGF-D and their receptor VEGFR-3. VEGFR-3 is shown to have an important role in the embryonic development of the cardiovascular system, before the lymphatic vessels start to form. In adults the expression of VEGFR-3 is mainly restricted to lymphatic endothelial cells, where it serves as a molecular marker for these vessels. In experimental models, VEGF-C and VEGF-D are shown to induce the growth of new lymphatic vessels via VEGFR-3 and this process is also shown to be a critical step in the metastatic processes of the tumor cells.

Review of the literature

Blood vessel development

The oxygen and nutrients supplied by the vascular system are crucial for cell function and survival. The cardiovascular system is the first organ system to develop in embryos, as it supplies oxygen and nutrients to the growing tissues. During organogenesis, the proximity of growing cells to the circulation is ensured by the coordinated growth of blood vessels and organ parenchyma. Embryonic vascular development involves a complex series of events during which the endothelial cells differentiate, proliferate, migrate and undergo maturation into an organized network of vessels (158, 159). The first step in the development of the blood vessels is called vasculogenesis, in which endothelial cells are generated from their mesenchymal precursors and spontaneously assemble into tubules that fuse to form the primary vascular plexus of the embryo. Remodelling and expansion of these primary vessels into arteries, veins and capillaries of different sizes is called angiogenesis (Fig. 1). In the yolk sac blood islands, mesenchymal cells give rise to both endothelial and hematopoietic cells (30). These cells organize into clusters consisting of future endothelial cells in the outer layer surrounding the inner hematopoietic cells. The endothelial cells then coalesce with those of the neighboring blood islands to form a primitive honeycomb-like blood vessel network, and the hematopoietic cells differentiate into erythrocytes.

A complex orchestration of molecular regulators is needed for the blood vessels to grow. Sprouting of new vessels from pre-existing ones is the most frequent mechanism of angiogenesis in embryos, and it involves several sequential steps (199). The extracellular matrix components are degraded locally by proteases produced by the endothelial cells. This allows the chemotactic migration of endothelial cells towards angiogenic stimuli. The endothelial cells proliferate and form loops, which become perfused with circulating blood. The loops between the vessels can also form by another mechanism called intussusceptive growth, a form of angiogenesis involving the *in situ* remodelling of the vessels by protruding interstitial tissue columns. In this process a large sinusoidal capillary can be divided into smaller capillaries, which then grow separately (158).

While endothelial cells initiate angiogenesis, they cannot complete the process. Newly formed capillary sprouts are fragile and remain susceptible to remodelling as long as they lack appropriate perivascular structures. The maturation of new blood vessels into stable and functional vessels requires the accumulation of a basal lamina and recruitment of pericytes and smooth muscle cells to cover tightly the abluminal side of the vessel (80). The smooth muscle cells provide structural support to the larger vessels and are important regulators of blood flow and pressure by their contractile abilities.

The vascular system is a highly heterogeneous and non-uniform organ system (64, 195, 205). Endothelial cells differ considerably in the arterial, capillary and venous compartments and there is further heterogeneity between the different organs (39). Recent molecular probing of the endothelial cell surface by phage display library panning *in vivo* has revealed striking molecular specificity for the availability of molecular determinants in different vascular endothelia (164). Endothelial cells in different vessels also have distinct characteristics, such as fenestrations, cell junctions, enzymes and carrier systems. Differentiation of endothelial cells is dependent on interactions with local parenchymal cells in the target tissues. Although it is not always known which factors induce the organotypic differentiation of endothelial cells, the existence of such cell-cell interactions seems to be widely accepted.

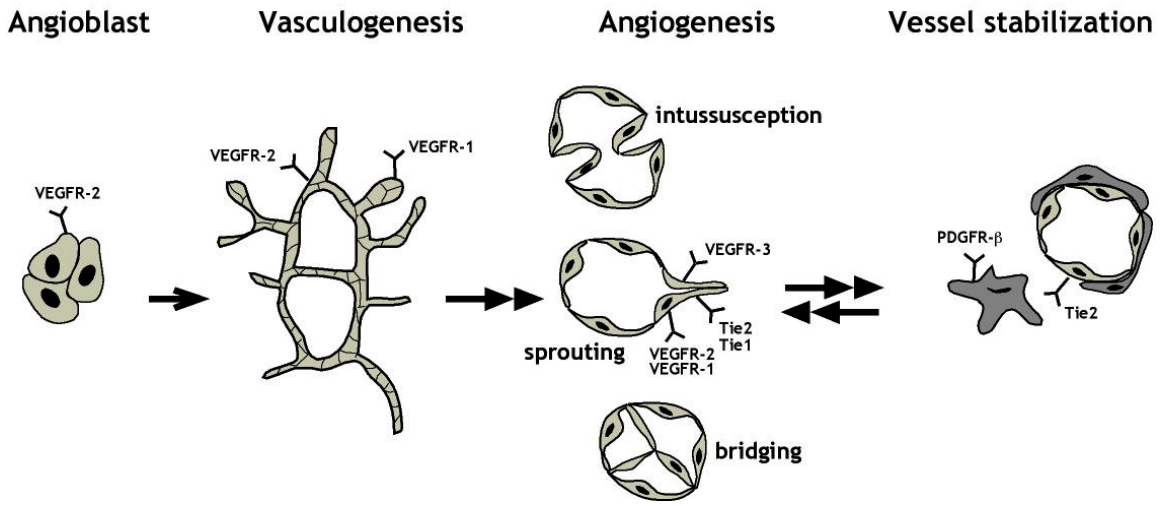

Figure 1. Embryonic blood vessel development. Endothelial precursors, angioblasts, assemble into a primitive network of vessels, which further expand and remodel to form circulatory system. Perivascular structures cover and stabilize the mature vessels. Adapted from Carmeliet (30).

Physiological and pathological angiogenesis

Angiogenesis is also required for the maintenance of the functional and structural integrity of tissues during post-natal life. Vasculogenesis is probably restricted to early development, while new vessels in adults appear to be formed by angiogenesis (30). However, adults are apparently able to mobilize bone marrow-derived endothelial precursor cells for angiogenesis (156). In healthy adults the endothelial cell turnover is usually very low and the vascular endothelia is maintained in quiescence by a balance of positive and negative regulators of angiogenesis. Angiogenesis is limited to sites where the metabolic demands of the tissue are such that new blood vessels are needed. Cells suffering from hypoxia start to release angiogenic factors in order to establish better contact with the circulating blood. In wound healing, fracture repair, inflammation, folliculogenesis and ovulation during the menstrual cycle, as well as in situations of ischemia, the positive regulators predominate, leading to the activation of angiogenic mechanisms (59).

In contrast to developmental angiogenesis, angiogenesis in adults originates mostly in mature blood vessels. In embryos endothelial cells are loosely connected and actively growing, whereas in adults they are quiescent and encapsulated by a thick mural coat. Therefore the blood vessels must first become destabilized to allow new growth. In contrast to angiogenesis in embryos, there is often inflammation associated with adult angiogenesis, attracting monocytes/macrophages, platelets, mast cells and other leukocytes. Angiogenesis results in a higher capillary density, but also the larger vessels are modified by the lack of an adequate oxygen supply. In the case of acute or chronic occlusion of a major artery (coronary, femoral artery), preexisting arteriolar connections can be recruited to bypass the site of occlusion. Arteriogenesis produces rapid growth of the preexisting collateral vessels, which are not perfused with blood under normal flow conditions. These vessels have the ability to dramatically

increase their lumen by proliferation of endothelial and smooth muscle cells (26).

One of the most extensively studied forms of pathological angiogenesis is tumor angiogenesis (58). Like normal cells, tumor cells need to be located at a close distance from the blood vessels serving the metabolic demands of the growing tumor. The stage in tumor development, when a solid tumor grows beyond a few millimeters in diameter and starts to generate its own microcirculation is called the angiogenic switch (60). It means the transition of an avascular tumor to a tumor with its own blood supply. At this stage the endothelial cells transit from a quiescent into an angiogenic state. The positive regulators are induced and negative regulators often decrease. Tumor blood vessels are leaky and immature, at least partly because the pericytes and smooth muscle cells are usually poorly recruited to the tumors (58). These vessels resemble angiogenic vessels in other settings, such as in wound healing, with the exception that tumor vessels do not mature properly and some of the endothelial cells in the tumor vessels are replaced by tumor cells (35). Angiogenesis also takes place in other pathological conditions such as proliferative retinopathy, rheumatoid arthritis, psoriasis and juvenile hemangioma (59).

Lymphangiogenesis

Lymphatic vessels are also part of the vascular circulatory system. The lymphatic system comprises of an extensive network of capillaries, collecting vessels and ducts that permeates most of the organs (165). Unlike the blood vasculature, which forms a continuous loop, the lymphatic system is an open ended, one-way transit system. It assists in maintaining the blood volume, carries cells, interstitial fluid components and metabolites that leak from the capillaries and returns them to the venous circulation via the thoracic duct.

The lymphatic vessels also form part of the immune system by continuously circulating the white blood cells within the lymphoid organs (spleen, tonsils, thymus, Peyer patches and lymph nodes) and bone marrow and transporting antigen-presenting cells. Mononuclear phagocytes and also lymphocytes patrolling the tissues enter the afferent lymph vessels and the lymph nodes to elicit primary immune responses before re-entering the vasculature. Endothelial receptors and binding proteins are involved in this trafficking of specific lymphatic cell populations.

Lymphatic vessels start to develop in embryos around midgestation, in parallel with the development of blood vessels and most of the organs. When the embryo grows, these vessels are needed for the regulation of the interstitial tissue pressure. The origin of the lymphatic vessels has long been controversial. Historically, the best accepted view of lymphatic development is the one proposed by Sabin (166, 167). Sabin proposed that early in fetal development, isolated primitive lymph sacs originate by endothelial cell budding from embryonic veins. Sabin's model proposes that the peripherial lymphatic system then spreads from these primary lymph sacs by endothelial sprouting into the surrounding tissues and organs where local lymphatic capillaries form. An alternative model has suggested that the initial lymph sacs arise in the mesenchyme from precursor cells independent of the veins and secondarily establish venous connections (85). Although recent reports about the development of the lymphatic vessels support Sabin's theory (44, 198), the existence of primitive lymphangioblasts, which can be recruited by the developing lymphatic vessels has been shown at least in avian species (171). One should thus note, that a combination of the two mechanisms is possible, whereby centrifugally sprouting lymphatic vessels anastomose with lymphatics developing from lymphangioblasts in tissues.

The lymphatic vessels differ in many ways from the blood vessels, but they also share many properties. Both vascular systems are lined by the endothelium and the larger vessels are supported by a smooth muscle framework, particularly around luminal valves, which are present in the veins and in the large lymphatics (203). The smooth muscle layer in blood vessels controls the contractile tone of the vessels in response to vasoactive substances. Blood vessels have a continuos or

fenestrated basement membrane and tight interendothelial junctions, which make the vessel wall selectively permeable to cells, fluids and molecules, whereas lymphatic vessels have a relatively free import for interstitial fluid. Lymphatic endothelial cells have complex overlapping intercellular junctions and specialized anchoring filaments, which hold the vessel open as tissue pressure rises (203). It has been suggested that these properties provide the lymphatics with a second valvular function (189). Liquid, macromolecules and migrating cells pass through the blood capillary endothelia, enter the tissues and are gradually absorbed into the lymphatic system. The fluid is transported via the lymphatic capillaries into the collecting vessels and through the lymph nodes, returning eventually to the circulation.

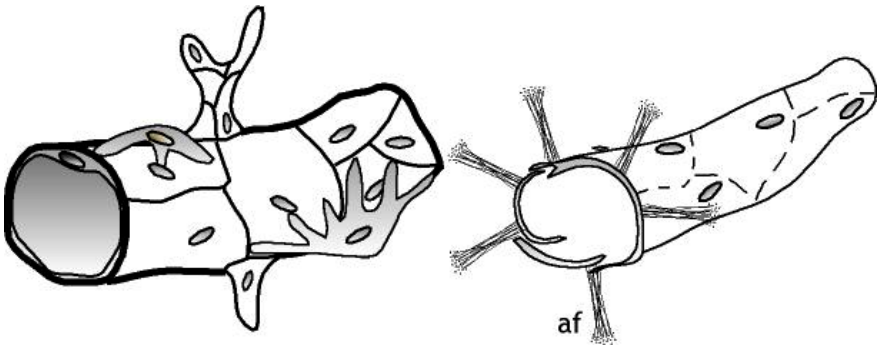

Figure 2. Structure of the blood (left) and lymphatic (right) capillaries. Lymphatic vessels resemble blood vessels but are thinner-walled and more irregular and allow relatively free import of interstitial fluid and macromolecules. Blood vessels are supported by the perivascular smooth muscle cells whereas typical for the lymphatic vessels are specific anchoring filaments (af), which attach the vessels to the surrounding tissue.

Molecular regulation of blood and lymphatic vessels

Intercellular signalling mechanisms which govern the formation of blood and lymphatic vessels have emerged relatively recently. The complexity of endothelial cells indicates that its regulation must involve many developmental and tissue-specific differentiation factors. Angiogenic signals are mediated by a number of growth factors and cytokines, and the balance between the positive and negative regulators maintains the adult vessels in a quiescent state (reviewed in (76)). Whenever the balance is disturbed the vessels react either by activating the angiogenic responses or regress by apoptosis when sufficient growth signals are not present. Interaction of angiogenic growth factors with their target cells triggers a cascade leading to the formation of blood vessels. Less is known about the regulation of the lymphatic vessels, although similar mechanisms seem to be involved.

Blood vessel development depends on members of the Vascular Endothelial Growth Factor (VEGF) family of proteins. This family consist of VEGF, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PlGF (placenta growth factor), which bind and activate cell surface receptors and regulate endothelial cell growth and differentiation (Fig.3) (96). The VEGFR family includes VEGFR-1 (also known as Flt-1), VEGFR-2 (Flk-1 / KDR) and VEGFR-3 (Flt-4) tyrosine kinase receptors. Neuropilins 1 and 2 (NRP-1/-2) are another class of high affinity non-tyrosine kinase receptors for VEGFs on endothelial and neuronal cell surfaces (138, 151). Recently, additional molecules similar to VEGF and capable of increasing capillary permeability were found in snake venom, suggesting that the family may be even larger (67, 105). The receptors have partly

overlapping but independent roles in the vascular development and maintenance. The expression levels of these genes modulate the abundance of different types of vessels in tissues. Other factors that are involved in the regulation of blood and lymphatic vessels, are the angiopoietins (Ang), ephrins and platelet-derived growth factors (PDGFs), which all act together in a co-ordinated manner during vessel formation (18, 30, 64). Interestingly, certain highly differentiated endothelia may have additional structurally unrelated regulators, such as EG-VEGF (112).

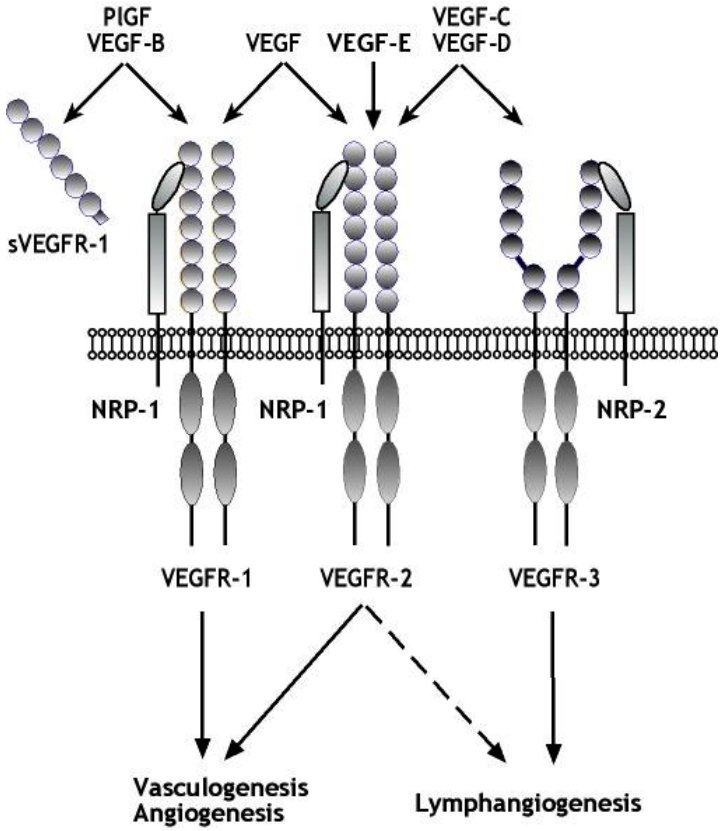

Figure 3. Receptor binding specificity of VEGFs. Growth factors activating VEGFR-1 and VEGFR-2 mediate the angiogenic signals, whereas lymphangiogenesis is mainly obtained via VEGFR-3.

VEGF

VEGF, discovered in 1989, is a major mediator of both vasculogenesis and angiogenesis (reviewed in (53)). In endothelial cells VEGF binds to VEGFR-1 and VEGFR-2 (52). VEGF is expressed as several isoforms consisting of polypeptides of different sizes (121, 145, 165, 183, 189 and 206 amino acid residues), which are all formed from the same gene by alternative splicing. They differ in their ability to interact with extracellular matrix components and with NRP-1 (41, 83, 145, 178, 186). These isoforms are thought to have distinct, but overlapping functions in angiogenesis. VEGF is also known as vascular permeability factor, as it promotes the extravasation of fluid and plasma proteins, including fibrin, from the blood vessels (45, 172). The increase in microvascular permeability and tissue deposition of fibrin is considered to enhance the migration of

endothelial cells in the extracellular matrix (46).

VEGF is essential for embryonic vasculogenesis and angiogenesis. Inactivation of only a single VEGF allele in mice resulted in embryonic lethality due to defective angiogenesis (31, 54). Also a reduced number of hematopoietic cells was observed. In mutant mice lacking the 164 and 188 amino acid isoforms of VEGF, half of the mice did not survive due to defects in for example postnatal angiogenesis in the myocardium, suggesting that the other forms of VEGF cannot completely replace the action of the others (34). Partial inhibition of VEGF by a soluble extracellular form of VEGFR-1 resulted in increased mortality and impaired organ development in the early postnatal period (69). It was shown that in addition to proliferation, VEGF is also required for the survival of endothelial cells. Consistent with this, other studies have also shown that VEGF supports the survival of endothelial cells by inducing the expression of anti-apoptotic proteins in endothelial cells (6, 14, 70).

VEGF is important in the etiology of several diseases characterized by pathological angiogenesis such as psoriasis, rheumatoid arthritis, and proliferative retinopathy (reviewed in (53)). Consistent with this, the expression of VEGF is potentiated in response to hypoxia and by activated oncogenes as well as by a variety of cytokines (74, 157, 174). Upregulated VEGF expression contributes to the development of solid tumors by promoting tumor angiogenesis (59). Tumor inhibition studies with neutralizing anti-VEGF antibodies suggested that other angiogenic factors may also be involved (102). However, the VEGF signalling pathway is currently considered as one of the most promising targets for the inhibition of tumor angiogenesis.

VEGF-B

VEGF-B is structurally closely related to VEGF and binds one of its receptors, VEGFR-1 (136). It has two splice variants, isoforms of 167 and 186 amino acids. They differ in binding to heparan sulphates in extracellular matrix and to NRP-1 (124). Both VEGF-B isoforms are able to form heterodimers with VEGF, and perhaps with other growth factors. This adds diversity to their biological roles by allowing a variety of combinations for cellular signal transduction.

VEGF-B is produced in large quantities by the developing myocardium and by muscle, bone, pancreas, adrenal gland, and the smooth muscle cell layer of several large vessels, but not by endothelial cells (1). VEGF-B is likely to act in a paracrine fashion as its receptor is almost exclusively located on endothelial cells. VEGF-B is a very weak endothelial cell mitogen when produced in mammalian cells (136), but otherwise its biological role is still unclear. Mice lacking a functional VEGF-B gene are healthy and fertile, but depending on the genetic background may have a conduction defect or reduced heart size (2, 13). The knockout mice display a striking vascular dysfunction after coronary occlusion and they show impaired recovery from experimentally induced myocardial ischemia (13). Considering such results, it is interesting to note that while VEGFR-1 and VEGFR-2 were expressed rather uniformly in the developing vasculature, only VEGFR-1 was prominently expressed in the human fetal coronary endothelium (148). These results suggest a role for VEGF-B in the coronary vasculature and potential clinical use in therapeutic angiogenesis.

PlGF

PlGF was discovered in the human placenta and it is about 50% homologous to VEGF (121). PlGF binds to VEGFR-1 and its heparin binding isoform, PlGF-2, also binds to NRP-1 (28, 129, 144). Binding of PlGF to VEGFR-1 is considered to increase the proportion of VEGF available to activate VEGFR-2 thereby potentiating the angiogenic properties of VEGF (144). A lack of PlGF has no major effect on embryonic development, even in combination with a loss of VEGF-B (33). However, loss of PlGF impairs angiogenesis associated with tumors, ischemia, myocardial infarcts and experimental retinopathy, and leads to prolonged healing of incisional skin wounds (33). During collateral growth after ligation of the femoral artery, PlGF was found to be essential for plasma extravasation,

monocyte recruitment and for the growth of endothelial and smooth muscle cells. These results indicate that PlGF activates membrane bound VEGFR-1 and specifically potentiates the angiogenic response to VEGF. In contrast to the essential role of VEGF in physiological and pathological angiogenesis, the role of PlGF is restricted to pathological vessel formation.

VEGF-C and VEGF-D

VEGF-C was cloned from human prostate carcinoma cells and its mature form consisting of the VEGF homology domain is 30% identical to $VEGF_{165}$ (90). VEGF-C is synthesized as a preproprotein from which a stepwise proteolytic processing generates several forms, with sequentially increasing binding and activity for its receptors, VEGFR-2 and VEGFR-3 (Fig. 4) (91). Like VEGF, VEGF-C stimulates the migration of endothelial cells, and increases vascular permeability and endothelial cell proliferation but at higher concentrations than VEGF. These signals for endothelial cells are probably mediated through VEGFR-2 in blood vascular endothelial cells and generally via VEGFR-3 in the lymphatic endothelial cells (91, 93). Unlike VEGF, the expression of VEGF-C does not appear to be regulated by hypoxia (49), but is increased in response to proinflammatory cytokines suggesting a role in inflammatory responses (160). The pattern of VEGF-C expression in embryos suggests that it plays a role in the development of the lymphatic vessels, since a paracrine expression pattern is seen between VEGF-C and VEGFR-3 at sites where the first lymphatic sprouts occur (108). Conversely, VEGF-C is already expressed before the emergence of the lymphatics, which also suggests the involvement in vasculogenesis/angiogenesis during early development.

VEGF-C regulates physiological and pathological blood vessel growth *in vivo*. It is able to stimulate angiogenesis in the mouse cornea and in limb ischemia (29, 204). On the other hand, VEGF-C has been shown to regulate the growth of lymphatic vessels in various experimental models. Overexpression of VEGF-C in skin keratinocytes leads to dermal lymphatic vessel hyperplasia (88). Signalling via VEGFR-3 alone was shown to be sufficient for the lymphangiogenesis, since transgenic mice overexpressing a mutant form of VEGF-C, which has lost its capacity to bind VEGFR-2 and only binds and activates VEGFR-3 (VEGF-C156S (89)), was able to induce a similar phenotype (194). VEGF-C was also studied in the mature, differentiated chorioallantoic membrane (CAM), that contains lymphatic vessels mainly around arterioles and veins (134). In this assay, VEGF-C acts as a highly specific lymphangiogenic factor. However, when VEGF-C was applied to the early CAM, where the lymphatics have not yet developed, it promoted angiogenesis. The angiogenic vs. lymphangiogenic responses to VEGF-C may depend on the degree of proteolytic processing of its precursor, and on the expression of it receptors in the blood vs. lymphatic endothelial cells of the target tissue. VEGF-C also has synergistic effects with VEGF, during the induction of angiogenesis, and this effect is more prominent in cells expressing both of its receptors (150). In addition, VEGF-C can compete with VEGF in binding to VEGFR-2.

VEGF-D (also known as c-fos-induced growth factor or FIGF) is the most recently discovered member of the mammalian VEGF family (3). It shares 61% sequence identity with VEGF-C and these two growth factors bind to the same receptors on human endothelial cells. VEGF-D is proteolytically processed similarly to VEGF-C and the proteolytic processing also appears to regulate VEGF-D biological activity and receptor specificity (181). Interestingly, in mice VEGF-D binds only to VEGFR-3, suggesting that VEGF-D may have a somewhat different function in mouse and man (9). This is uncommon within the VEGF family as these homologous and evolutionary conserved growth factors are assumed to exhibit similar receptor binding characteristics in different species. VEGF-D has been shown to be able to stimulate the proliferation of endothelial cells, and shows angiogenic properties *in vitro* and *in vivo* (127). Like VEGF-C, it was also shown to be lymphangiogenic when overexpressed in skin keratinocytes (194). Little is known about the expression of VEGF-D in physiological conditions, but its mRNA has

been observed in the developing melanocytes and fibroblasts, lung mesenchyme and in the adult vascular wall (4).

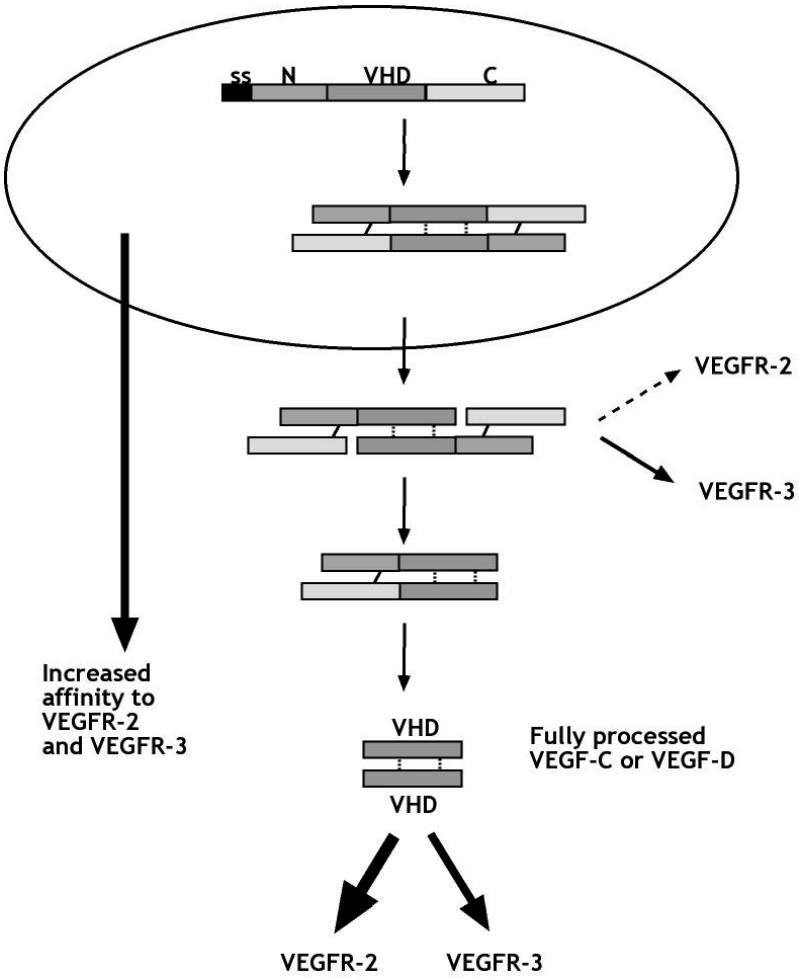

Figure 4. Proteolytic processing of the VEGF-C and VEGF-D protein. The growth factors are synthesized as prepropeptides containing N- and C- terminal propeptides (N and C) and a VEGF-homology domain (VHD). Proteolytic processing generates several forms with increased binding towards VEGFR-2 and VEGFR-3. This may regulate the angiogenic and lymphangiogenic properties of the VEGF-C and VEGF-D.

VEGF-E

A VEGF homologue, VEGF-E, was recently discovered in the genome of the parapoxvirus, Orf virus, that infects sheep, goats, and occasionally humans (120). Infection by this virus causes proliferative skin lesions in which extensive capillary proliferation and dilation are prominent histological features. Several strains of the virus encode different VEGF-E variants, which bind specifically to VEGFR-2 and NRP-1 and are able to stimulate endothelial cell mitogenesis and vascular permeability (133, 201). VEGF-E is not essential for viral replication but rather plays an important role in modulating the host environment during infection.

VEGFR-1 & VEGFR-2

VEGFR-1 and VEGFR-2 are important in blood vascular endothelial cell proliferation, migration and survival. Mice carrying a homozygous disruption in either of the two VEGF receptors die during early development due to defects in both vasculogenesis and angiogenesis. Embryos lacking functional VEGFR-2 are lacking mature endothelial and hematopoietic cells (173). In contrast, VEGFR-1 deficient mice have normal hematopoietic progenitor cells and endothelial cells that migrate and proliferate but do not assemble into tubes and functional vessels (61). More recent studies have shown that an excessive proliferation of endothelial progenitors is the main factor leading to this disorganization (62). This supports the view that VEGFR-1 is a negative regulator of VEGF-induced vasculogenesis in embryos.

VEGFR-1 alone has been shown to induce weak mitogenic signals *in vitro* (110), but it is thought that VEGFR-2 is the major receptor transducing the effects of VEGF in endothelial cells. For example, VEGF-E and site-directed mutants of VEGF, which bind only to VEGFR-2, stimulate endothelial cells similarly to VEGF (70, 101, 128, 201). VEGF also provides survival signals for endothelial cells via VEGFR-2 (70). Outside of the vascular system, VEGFR-1 is expressed in monocytes and macrophages, placental throphoblasts and renal mesangial cells, and VEGFR-2 in hematopoietic stem cells, megakaryocytes, platelets, retinal progenitor cells and in circulating endothelial precursor cells (11, 36, 38, 98, 207, 208). Despite the importance of these receptors during embryonic blood vessel development, VEGFR-1 and VEGFR-2 appear to be downregulated in the quiescent adult endothelium.

VEGFR-3

VEGFR-3 was cloned from a human leukemia cell line and from human placenta (65, 142). Two isoforms of VEGFR-3 have been described, designated VEGFR-3s (short) and VEGFR-3l (long), which differ as a result of alternative splicing. The long form is the predominant form in most tissues. An endogenous retroviral genome appears responsible for the short isoform in humans, but this form is missing from mice (84). VEGFR-3 is initially expressed in all embryonic vasculature, but during development its expression in blood vessels decreases and becomes restricted to the developing lymphatic vessels (93). VEGFR-3 deficient embryos die as a result of a defect in the remodelling of the primary vascular network and cardiovascular failure at midgestation, before the lymphatic vessels start to develop (44). In adults the expression of VEGFR-3 is mainly restricted to lymphatic endothelial cells, where it serves as a molecular marker for these vessels (92, 93). These results suggest that VEGFR-3 plays a dual role, in embryos in cardiovascular development and in adults in the regulation of the lymphatic vessels.

In adults, VEGFR-3 is expressed in a subset of capillary and venous endothelium, although it is absent in endothelia of all large blood vessels (147). VEGFR-3 is re-activated in the blood vessel endothelium in certain pathological conditions and upregulation of VEGF-C/VEGF-D ligands may accompany this (4, 168, 193). Similarly, VEGFR-2 can be expressed by both blood vascular and lymphatic endothelia (148). During wound healing, acute inflammation is followed by the deposition of fibrin and connective tissue and the growth of blood vessels into the granulation tissue. Most blood vessels then regress as the wound is remodelled into scar tissue. VEGFR-3 positive lymphatic vessels have been observed to sprout from pre-existing lymphatics and grow into the granulation tissue in healing skin wounds (141). These lymphatic vessels persisted in the wound for some time but regressed as the healing proceeded. This suggests that transient lymphangiogenesis is needed during wound healing, in parallel with angiogenesis. On the other hand, no lymphatic vessels were seen in chronic human wounds (141). The absence of lymphatic vessels may contribute to the impaired healing in these conditions. The angiogenic vessels in wound healing remained negative for VEGFR-3, suggesting that there are differences in the regulation of

angiogenesis in various pathological conditions.

Little is known about the characteristic features of lymphatic endothelial cells, mainly because isolated lymphatic endothelial cells have not been available for molecular studies. Recently, primary cultures of human dermal endothelial cells were shown to consist of distinct lines of blood vascular and lymphatic endothelial cells (106, 125). Cells of the lymphatic lineage could be isolated by antibodies against VEGFR-3 or podoplanin. Signalling via VEGFR-3 was shown to be critical for growth, migration, and survival of the isolated lymphatic endothelial cells (125). Also VEGFR-2 was detected in the lymphatic endothelial cells, suggesting that activation of both VEGF-C receptors may be required for their maximal survival (125, 148).

Neuropilins

NRP-1 and NRP-2 are transmembrane receptor proteins that are required for axon guidance and, according to recent discoveries, also for the regulation of angiogenesis (72, 177, 178). Both neuropilins bind certain isoforms of VEGF, VEGF-B, VEGF-E and PlGF-2 (124, 129, 178, 201). NRP-1 is expressed in the tips of actively growing axons of particular classes of neurons (63), but also in the blood vascular endothelial cells and in certain tumor cells (177, 178). NRP-1 enhances $VEGF_{165}$ binding to VEGFR-2 and VEGF-mediated chemotaxis. Embryos lacking functional NRP-1 die due to defects in VEGF mediated angiogenesis and subsequent cardiovascular failure (99) and ectopic overexpression of NRP-1 leads to an excess of dilated blood vessels and hemorrhage, apparently due to inappropriate VEGF activity (104). Mice having targeted deletion of NRP-2 are viable, but have defects in the development of central and peripheral nervous systems (37, 71). Recently, NRP-2 has been shown to bind VEGF-C and to be expressed together with VEGFR-3 in the endothelial cells of a sub-population of the lymphatic vessels (94).

Angiopoietins and Tie-Receptors

Tie-1 and Tie-2 (Tek) are expressed in endothelial cells throughout embryonic development as well as in hematopoietic progenitor cells (42). Gene targeting experiments indicate that Tie-1 and Tie-2 are essential to the angiogenic expansion of the vasculature during development. In mouse embryos lacking the Tie-2 receptor endothelial cells are present in slightly reduced numbers and are assembled into tubes, but the vessels remain immature, lacking branching networks and proper organization into a hierarchy of large and small vessels (43, 169). The vessels lack intimate encapsulation by periendothelial support cells, and the endocardium is only loosely attached to the myocardium. Thus Tie-2 appears to control the capability of endothelial cells to recruit stromal cells, which stabilize the vessel structure and modulate the function of blood vessels (76). Tie-1 is required cell autonomously for endothelial cell survival and extension of the vascular network during the later part of embryogenesis (155, 169). Vasculogenesis proceeds normally in embryos lacking both Tie-1 and Tie-2, since the angioblasts differentiate normally (154). It appears that one of the earliest critical functions of these receptors concerns endocardial development, but that rescue of the embryos is possible if one bypasses the critical period using transgenic expression of Tie-2 (Dr. D. Dumont, personal communication).

Ang-1 and Ang-2 bind to Tie-2 with similar affinities, but only Ang-1 can activate the receptor directly. Ang-2 is capable of inhibiting the effects of Ang-1 in endothelial cells in short-term experiments. However, if endothelial cells of human umbilical vein are stimulated with Ang-2 for longer periods, activation of the Tie-2 receptor is obtained (185). Ang-2 is also capable of stimulating Tie-2 in transfected nonendothelial cells. Thus, Ang-2 has both agonistic and antagonistic properties, which may relate to its ability to dimerize or oligomerize less efficiantly than Ang-1, or to binding to an inhibitor that needs to be downregulated.

Ang-1 is widely expressed in both embryonic and adult tissues (184). Ang-2 is also expressed in embryos around large vessels, but in adults the expression pattern is restricted to sites of physiological angiogenesis, where vascular remodelling occurs (122). Transgenic overexpression of Ang-2 under the Tie-2 promoter in the embryonic endothelium indicates that Ang-2 inhibits the recruitment of supporting perivascular cells, resulting in a phenotype similar to that of the Ang-1 knockout embryos (184). In adults, Ang-2 allows vascular remodelling, which otherwise is restricted by encapsulation by the basement membrane and periendothelial support cells. When the expression of Ang-1 overcomes that of Ang-2, such remodelling ceases and vessels stabilize (reviewed in (64)).

Several lines of evidence indicate that there is significant collaboration between VEGF, Ang-2 and Ang-1 in angiogenic processes. Vascular regression is associated with very high levels of Ang-2 in the absence of activating survival signals from VEGF. In the skin of transgenic mice VEGF increases the number of capillaries, whereas Ang-1 causes a massive enlargement of postcapillary venules (188). Interestingly, Ang-1 was able to rescue the permeability effects of VEGF (187). Co-expression of both factors was required to obtain an increased number of large vessels in transgenic mice. Identification of the ligand(s) for the Tie-1 receptor should provide further insights into the mechanistic basis for this asymmetric regulation of vascular development. Ang-2 may also be involved in the regulation of lymphatic vessels, since knock out mice lacking functional Ang-2 have chylous ascites with a disorganized and leaky lymphatic vasculature (personal communication with Dr. G. Thurston and Dr. G. Yancopoulos).

Table 1.
Summary of the biological roles of VEGFs, VEGFRs, TIEs and Angiopoietins

Gene	*Phenotype in gene deficient mice*	*Lethal*	*Biological role*
VEGF	defective blood vessel development and blood islands (31, 54)	-/- E8-9 +/- E11-12	vasculogenesis, developmental and pathological angiogenesis
VEGF-B	1) normal heart development (2) or 2) defective heart development (13)	Viable	modulates biological activity of VEGF (?)
PlGF	normal vascular development (33)	Viable	pathological angiogenesis, amplifies responsiveness to VEGF
VEGFR-1	disorganization of vascular system, increased number of endothelial precursors (61, 62)	E 8.5	negative regulator of VEGF-mediated angiogenesis ? monocyte recruitment
VEGFR-2	no mature endothelial or hematopoietic cells (173)	E 8.5-9.5	hemangioblast differentiation, proliferation and migration, vascular permeability
VEGFR-3	pericardial effusion, defective large blood vessels (44)	E 9.5-10.5	vascular remodelling, migration and survival of lymphatic ECs
NRP-1	anomalies in cardiovascular system and efferent nerve fibers (99, 104)	E12.5	axon quidance in PNS, cardiovascular development, enhances effect of $VEGF_{165}$
NRP-2	defects in the development of central and peripheral nerves (37, 71)	Viable	axon quidance
Ang-1	fewer branches, homogeneously sized vessels, defects in endocardium and myocardium (184)	E12.5	recruitment of perivascular cells, vessel stabilization
Tie-1	defective angiogenic expansion and integrity of vessels (155, 169)	E10.5	vascular network formation, survival of EC
Tie-2	defects in maturation and organization of blood vessels (169)	E10.5	recruitment of perivascular cells, vessel stabilization

EC; endothelial cell, PNS; periferal nervous system

PDGFs

The platelet-derived growth factor (PDGF) family consist of homodimers or heterodimers made from the pairwise assembly of the related PDGF polypeptide chains (reviewed in (79)). These effects of the PDGFs depend on the target cell type, in particular on the cell's

repertoire of PDGF receptors, α and β. The α receptor can bind PDGF-A, PDGF-B and PDGF-C chains, whereas the β-receptor is selective for the PDGF-B and PDGF-D chains (16, 18, 115). Based on the gene deficient studies, both receptors are essential for embryonic development. PDGF-A and PDGFR-α are prominently expressed at sites of epithelium-mesenchyme interaction, whereas PDGF-B takes part in blood vessel development (81, 116, 140). PDGF-D is the first known PDGFR-β-specific ligand, and its unique receptor specificity indicates that it may be important in the development and pathophysiology of several organs. The expression of PDGF-C and PDGF-D in the arterial wall and cultured vascular cells suggests that they can transduce proliferation/migration signals to pericytes and smooth muscle cells (192).

During blood vessel development, PDGF-B is expressed in endothelial cells, while pericytes and smooth muscle cells covering the blood vessels express PDGFR-β, indicating paracrine signalling between these two cell types (81, 116). Targeted gene disruption studies of PDGF-B or PDGFR-β gave similar phenotypes. In both mouse strains, blood vessel development was deficient because of the inability of blood vessels to attract pericytes (80, 81, 116). Also the development of the renal and hematopoietic systems were affected (114, 179). Lack of pericytes in embryonic angiogenesis leads to hyperplasia of endothelial cells, supporting the notion that pericytes inhibit endothelial cell proliferation (80). Smooth muscle cell proliferation in response to the release of growth factors from neighboring cells is one mechanism postulated to account for the development of atherosclerotic lesions. PDGFs may be involved in initiation and progression of atherosclerotic changes in arterial intima by promoting proliferation of smooth muscle cells of the vascular wall (17, 130). These molecules may also have an important role in tumor biology, since expression of the mRNA of the receptors and ligands has been observed in wide variety of human tumors (79).

Ephrins

Before the heart starts to beat and circulate blood, the vascular hierarchy must be organized and arteries and veins must be ready to properly transport blood. In studies of the ephrin family of molecules it has become obvious that the fate of endothelial cell is already marked in early embryonic development when the whole endothelium is still rather uniform in nature (reviewed in (205)). Unlike ligands for other receptor tyrosine kinases, the ephrins cannot act as soluble mediators, but rather must be membrane-bound in order to activate their receptors. Interestingly, bidirectional signalling was observed between the ligand and receptor. Ephrin-B2 was shown to mark future arteries while its receptor Eph-B4 reciprocally marks the venous endothelium (195). Furthermore, embryos lacking Ephrin-B2 displayed severe defects in vascular remodelling in both arterial and venous domains. These findings provide some of the earliest known markers distinguishing the arterial and venous endothelia. This data for the first time shows the existence of bidirectional signalling between these vessel types. This suggests that the molecular differences are at least partly programmed genetically in arterial vs. venous endothelia and that these differences may be critical to normal development of the vasculature.

Markers for the lymphatic vessels

A major advance in the field of lymphangiogenesis has come from the discovery of lymphatic endothelium-specific markers (Table 2). In addition to VEGFR-3, other molecules have now been found to express relatively specifically in lymphatic endothelial cells. Podoplanin is a glomerular podocyte membrane mucoprotein, which occurs together with VEGFR-3 in the lymphatic endothelium and in benign vascular tumors and angiosarcomas, but is also expressed in certain non-endothelial cells (22, 23). Prox-1, a homeobox transcription factor, is involved in the sprouting of lymphatic vessels from

embryonic veins during development (198). Prox-1 is also expressed in nonendothelial cells. The third marker, LYVE-1 (Lymphatic vessel endothelial hyaluronan-receptor-1), is a receptor for extracellular matrix/lymphatic fluid glycosaminoglycan in lymphatic endothelial cells (10). This molecule, which is related to the CD44 receptor for hyaluronan, is distributed equally among the luminal and abluminal surfaces of lymphatic vessels and is involved in the uptake of hyaluronan by lymphatic endothelial cells and its transport from the tissues to the lymph (153).

Recently, a β-chemokine receptor D6 was shown to be present in a subset of lymphatic vessels in the skin, intestine and lymphoid tissues (131). Interestingly, lymphatic vessels in most of the organs remained negative for its expression. The existence of this receptor on only a subset of lymphatics suggests a functional heterogeneity within the lymphatic vasculature. Consistent with this, recent findings revealed the co-expression of NRP-2 and VEGFR-3 in lymphatic vessel endothelia of the intestine, whereas dermal lymphatic vessels did not show NRP-2 expression (94). The mannose receptor of macrophages is also expressed by lymphatic endothelia in addition to macrophages and other non-endothelial cells (117). The biological role of this receptor in lymphatic vessels is not known, but it may play a role in inflammation and immunity.

5'-nucleotidase and desmoplakin have also been used to distinguish the lymphatic from the blood vascular endothelium (170, 191) and since lymphatic capillaries lack a continuous basement membrane, immunohistochemistry for extracellular matrix components type IV collagen and laminin have also been used to distinguish them from blood capillaries (12). VEGFR-2 is occasionally expressed in lymphatic endothelia (148) and Tie-1 and Tie-2 may also have a role in lymphatic vessel regulation, as they also appear in lymphatic endothelia (92, 148) and mice deficient for the Ang-2 have a lymphatic phenotype (unpublished data of G. Thurston and G. Yancopoulos).

Table 2. Markers for the lymphatic vessels

Molecule	*Protein class*	*Biological effect*
VEGFR-3	receptor tyrosine kinase on endothelial cell (92, 93)	lymphangiogenesis, survival of LEC
LYVE-1	receptor for extracellular matrix glycosaminoglycan (10)	transport of HA from tissues to lymph nodes
PROX1	transcription factor (198)	developmental lymphangiogenesis
Podoplanin	integral membrane protein (23)	unknown
β-chemokine receptor D6	chemokine receptor in afferent lymphatics (131)	leukocyte recirculation
Macrophage mannose receptor	receptor in macrophages (117)	phagocytosis of microbes, viral endocytosis
Desmoplakin	component of intercellular adhering junction (170)	adhesion of LECs

LEC; lymphatic endothelial cell, HA; hyaluronan

Diseases of the lymphatic vessels

Pathological processes similar to those that affect blood vessels, such as thrombosis, inflammation, vessel wall hypertrophy, and sclerosis may also occur to some extent in lymphatic vessels. However, the slow flow of the lymphatic fluid makes lymphatic disorders more chronic in character (203). Lymphatic vessel defects are associated with intense

fibrosis and overgrowth rather than the dramatic occlusive events such as occur in blood vessels when the blood flow is interrupted.

Lymphedema

An important function of the lymphatic vessels is to regulate the pressure of interstitial fluid in tissues by transporting excess fluid back into the circulation. Clinical situations in which the lymphatic system is involved include lymphedema due to impaired lymphatic drainage. This can be caused by inflammatory or neoplastic obstruction of the lymphatic vessels including accumulation of ascites fluid due to lymphatic obstruction in peritoneal carcinomatosis or edema of the arm following surgery or radiotherapy for breast cancer. Lymphatic filariasis is one of the leading causes of permanent and long-term disability globally. It is a parasitic infection in the lymphatic vessels, which leads to abnormal transport function, massive edema and deformation of the limbs (203).

Primary lymphedema is a rare developmental disorder, in which the transport failure of the cutaneus lymphatic vessels results in interstitial lymph fluid accumulation. Chronic lymphatic dysfunction gradually results in thickening of the skin, accumulation of adipose tissue and dermal fibrosis of the affected area (113). Recently, several groups have reported linkage of congenital lymphedema (Milroy's disease) to the VEGFR-3 gene (50, 57, 202) with autosomal dominant inheritance. This mutation was shown to lead to reduced VEGFR-3 tyrosine kinase activity, and subsequent failure in transducing sufficient physiological VEGF-C/VEGF-D signals to the lymphatic endothelial cells (95). In all lymphedema families studied, the affected individuals had only one mutant allele (86, 95), compatible with the results that inactivation of both VEGFR-3 alleles in mice is embryonic lethal (44). The mutation affecting the biological activity of VEGFR-3 is probably one cause of primary lymphedema, but some other lymphedema genes also exist, for example FOXC2 (51).

Kaposi's sarcoma

Kaposi's sarcoma is a multicentric neoplasm consisting of multiple vascular nodules appearing in the skin, mucous membranes, and viscera. Individuals with specific conditions of immunodysregulation, especially AIDS patients, develop these tumors. Molecular and epidemiological studies indicate that development of Kaposi's sarcoma is associated with infection by the human herpesvirus-8 (HHV-8) (183). The nodules are characterized by clusters of spindle-shaped tumor cells and by prominent vasculature consisting of small, irregular, endothelial-lined spaces. It is thought not to be a neoplastic transformation of cells in the classic sense, but rather a manifestation of excessive proliferation of the spindle cells. A central question in the pathogenesis of Kaposi's sarcoma has long been, which cell type in early lesions gives rise to the uniform tumor cells of late nodules (66). The spindle cells are most likely endothelial in origin, but there has been controversy as to whether they are of lymphatic or blood vascular derivation.

Vascular tumors

Vascular tumors can be divided into benign tumors (hemangioma) and malignant vascular tumors (angiosarcoma) and to the tumors of lymphatic vessels (lymphangioma) and perivascular cells (glomus tumors and hemangiopericytoma). The molecular characteristics of these tumors are so far mostly unknown but VEGF and its receptors have been shown to be expressed in the endothelial cells of these tumors (162). Although normal mesenchymal tissues show VEGFR-3 in the lymphatic endothelial cells, benign and malignant vascular tumors show widespread VEGFR-3 expression, suggesting that VEGFR-3 is upregulated in the proliferating blood vascular endothelial cells. This is consistent with the VEGFR-3 expression in the embryonic developing vessels, as well as reactivation in adult angiogenic blood vessels (44, 193). Although strong expression of VEGFR-3 would be consistent with lymphatic differentiation, the extensive erythrocyte content in the vascular lumina of

these lesions supports the idea that VEGFR-3 expression in these tumors reflects a proliferative vascular phenotype rather than a lymphatic phenotype. The expression of VEGFR-3 among vascular proliferations demonstrates that blood vessel endothelia can acquire VEGFR-3 expression independently of lymphatic vascular differentiation.

Classification of angiosarcomas is mostly based on morphological criteria. It has been suggested that some of the angiosarcomas contain components of a lymphatic lineage, but there was not proof of this (135). The reactivity for VEGFR-3 was seen in poorly differentiated angiosarcomas, indicating that this receptor is conserved on malignant transformation (146). Some cell populations in angiosarcomas are positive for podoplanin, which retains its lymphatic endothelial cell specificity in vascular tumors, supporting the idea of mixed expression of both blood and lymphatic phenotypes in angiosarcomas (23). Since expression of VEGFR-3 is seen in the majority of benign and malignant vascular tumors, but expression is less consistent in malignant epithelioid vascular tumors and absent in malignancies of nonendothelial origin, VEGFR-3 could be used as a lineage marker to identify endothelial cell differentiation in the tumors. However, further studies are needed to evaluate the sensitivity and specificity of these markers.

Lymphangiomas result from abnormal development of lymphatic vessels which prevents lymph fluid draining from the affected area. Lymphangiomas can originate in most organs, although they are most often found in the soft tissues of the head and neck (cystic hygroma) and axilla. They consist of a benign multicystic mass of dilated networks of lymphatic channels. Both VEGFR-3 and podoplanin specifically stain the endothelia of lymphangiomas and could be used for diagnostic purposes (23, 92, 146).

Tumorigenesis and metastasis

Lots of evidence indicates that tumorigenesis is a multistep process, and these steps reflect the genetic alterations that drive the progressive transformation (reviewed in (77)). Whereas normal cells require mitogenic signals before they can move from a quiescent state into an active proliferative stage, malignant cells are self-sufficient for the growth signals, and insensitive to the growth-inhibitory signals. Tumor cells generate many of their own growth signals leading to autocrine stimulation, thereby reducing their dependence on stimulation from the normal tissue micro-environment. It has long been thought that tumors are independent from the surrounding cells and their action, but now it seems more likely that cancer development depends upon interactions between tumor cells and their benign neighbors. Tumors and metastases tend to harbor complex mixtures of several cell types that collaborate to create a malignant growth, including fibroblasts, immune cells and endothelial cells (58, 77).

The cells within aberrant proliferative lesions initially lack angiogenic ability, preventing their expansion. The ability to induce and sustain angiogenesis seems to be acquired in discrete steps during tumor development via an angiogenic switch from vascular quiescence to proliferation (77). In studies of transgenic mouse tumorigenesis, angiogenesis was found to be activated in midstage lesions prior to the appearance of full-blown tumors (60). These observations indicate that neovascularization is necessary for the rapid clonal expansion associated with the formation of macroscopic tumors. Tumors appear to activate the angiogenic switch by changing the balance of angiogenic inducers and inhibitors (15, 58). Another regulatory mechanism is the function of proteases, which can control the availability of angiogenic activators and inhibitors stored in the extracellular matrix (40).

Tissue hypoxia is a fundamental angiogenic stimulus characteristic also of malignant tumors. The VEGF gene has been shown to contain specific hypoxia-responsive elements and to be upregulated in response to low oxygen tension (55). Ang-2 levels are also increased by hypoxia, suggesting a collaboration of VEGF and Ang-2 in the regulation of neovascularization of ischemic tissue (reviewed in (111)). Tumor-derived signals such as VEGF may specifically induce

Ang-2 expression in tumor endothelia, and this may be one important component in angiogenic switch and in the formation of an endogenous tumor microcirculation.

Vasculature and growth factors in tumors

Microvascular density has been used as a measure of tumor angiogenesis and its correlations to tumor growth, metastasis and prognosis has been studied (197). Levels of VEGF are upregulated in a large number of human tumors (reviewed in (55)) and inhibition of VEGF activity results in the suppression of growth of a wide variety of tumor cell lines in murine models (56). It was long thought that lymphatic vessels may be lost, collapsed or could not penetrate in the expanding primary tumors because they cannot survive in the high interstitial pressure inside the tumors (reviewed in (149)). However, recently some intra-tumoral lymphatic vessels have been observed (180).

Few data are available on the influence of lymphatic microvessel density on survival in cancer. In ovarian cancer, the lymphatic vessel density had no influence on the progression of the disease, and in cervical cancer an increased amount of lymphatic vessels may even be associated with a favorable prognosis (19, 20). It is likely that human tumors demonstrate heterogeneity with regard to the presence or absence of intra-tumoral lymphatics. The nature of the marker may also influence the determination of the lymphatic vessel density. VEGFR-3 has been seen in the endothelial cells of the proliferating vasculature in certain solid tumors and vascular tumor cells of endothelial origin (146, 193) and therefore cannot be used alone to confirm the intratumoral lymphatic vessel density. Other markers for the lymphatic vessels, podoplanin and LYVE-1, would better suit for this purpose and help in elucidating any correlation between lymphatic vessel density and tumor growth, metastases and prognosis.

All VEGFRs are present in tumor neovasculature and tumor cells have been reported to be able to secrete VEGF, VEGF-B, VEGF-C and VEGF-D (4, 55, 168). However, the angiogenic switch is thought to be carefully regulated and at least some specific genetic events in tumor progression correlate with lymphatic metastasis, suggesting that a "lymphangiogenic switch" mechanism is also a formal possibility.

Mechanisms of blood vascular and lymphatic metastasis

The capacity to spread enables cancer cells to escape the primary tumor mass and colonize new areas in the body, where nutrients and space are not growth limiting. Tumor cell dissemination is mediated by mechanisms including invasion, spread via blood or lymphatic vessels or direct seeding of body cavities or surfaces (39). Although the biochemical mechanisms are not completely understood it is thought that the metastatic spread of a tumor is not a random process. Distinct patterns of metastasis can be discerned which vary from tumor type to tumor type. A common metastatic pattern for carcinomas is that regional lymph nodes are often the first organs to develop metastases either draining via pre-existing afferent lymphatic vessels and/or via newly formed lymphatic capillaries. This pattern of metastasis is central to the utility of the sentinel lymphnodectomy as a surgical technique. However, not all tumors and tumor types metastasize to the regional lymph nodes first (161). The mechanisms determining whether regional lymph nodes or other sites first develop metastases remain poorly understood. In fact, most disseminated tumor cells have a limited life span and only a few develop into clinically detectable micrometastases, but identification of those occult tumors cells, and prevention of their growth and spread would be of great clinical significance.

VEGF-C, VEGF-D and tumor metastases

VEGFR-3 may play an important role in the formation of tumor-induced neo-vascularization, since it is expressed in capillary vessels during tumor angiogenesis

(146, 193). Inactivation of VEGFR-3 by neutralizing antibodies suppressed tumor growth by destabilizing large vessels in tumor xenografts in mice. Micro-haemorrhages were seen in these vessels, suggesting that VEGFR-3 could be involved in maintaining the integrity of the endothelial cell lining in the neovasculature (107). Frequent administration of VEGFR-3 antibody was required for the suppression of tumor growth but the architecture of the non-angiogenic blood and lymphatic vessels remained unaffected. It has also been shown that even a prolonged suppression of VEGF activity in adult mice has no effect on the maintenance of the vascular system, though it suppressed angiogenesis severely in embryos (69). The fully established blood and lymphatic vessels seem to be resistant to treatment with these kinds of anti-angiogenic agents.

Recent work using experimental models has highlighted the role of VEGF-C and VEGF-D in tumor biology. Transgenic mice overexpressing VEGF-C in ß-cells of the endocrine pancreas developed extensive lymphangiogenesis around the endocrine islets of Langerhans (126). Furthermore, when tumors were induced in these VEGF-C overexpressing islets, metastatic tumor cell aggregates of ß-cell origin were observed in the surrounding lymphatic vessels. These mice also frequently developed metastases in the lymph nodes, which drain the pancreas, whereas tumors in mice lacking the VEGF-C transgene never metastasized nor were tumor cells observed inside the lymphatic vessels (126). Similarly, human breast cancer cells expressing ectopic VEGF-C were shown to induce lymphangiogenesis in and around the orthotopically implanted tumors (97, 176). However, VEGF-C did not have a significant effect on angiogenesis although it increased tumor growth. Increased spreading of the cells to the regional lymph nodes was observed and the degree of tumor lymphangiogenesis correlated with lymph node metastases (Mattila et al., submitted for publication and (176)). VEGF-C induced tumor growth, lymphangiogenesis and intra-lymphatic tumor growth was inhibited by adenoviral expression of the soluble VEGFR-3 receptor (97).

VEGF-D was also shown to promote the metastatic spread of tumor cells via the lymphatics (180). In addition to lymphangiogenesis and increased metastases, the tumors secreting VEGF-D also had an increased growth rate and tumor angiogenesis. The growth of the tumor, angiogenesis and formation of metastases were inhibited by neutralizing antibodies against VEGF-D. The differences between the tumor angiogenic properties of VEGF-C and VEGF-D may be due to differences in their proteolytic processing in different tumors. Some of the heterogeneity in the effects of these growth factors may also result from variable expression of their receptors, VEGFR-2 and VEGFR-3, on the blood vascular and lymphatic endothelia. In particular, in the above case, enhanced tumor angiogenesis was probably obtained for VEGF-D because of its increased proteolytic processing, which resulted in an increased affinity to VEGFR-2 (180), when compared to the VEGF-C models. Also, intra-tumoral lymphatic vessels were observed in tumor xenografts (97, 176, 180), but not in the transgenic tumors (126), which may be at least partially explained by the trapping of vessels in between the rapidly growing tumor foci in the xenografts. On the basis of these observations, tumor vessel formation can be dissected into pathways that preferentially activate angiogenesis via VEGFR-2 and pathways that activate lymphangiogenesis driven by VEGFR-3, although there is evidence that the receptors occasionally share overlapping expression patterns (148).

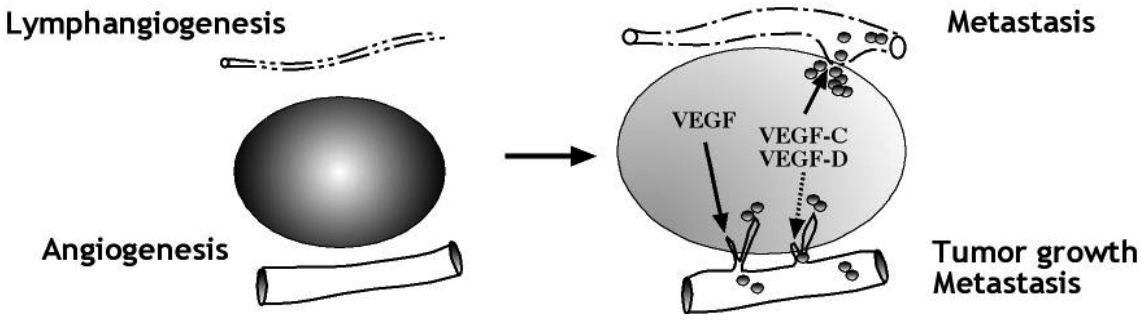

Figure 5. Angiogenesis and lymphangiogenesis in tumors. At certain stage tumor starts to secrete growth factors that induce the angiogenic and/or lymphangiogenic responses. This allows the spread of metastatic tumor cells via blood or lymphatic vessels.

It is still unknown whether VEGF-C or VEGF-D expression also promotes lymphangiogenesis in human tumors, and if so, does this increase the rate of metastasis to the lymph nodes. VEGF-C expression has been detected in about half of human cancers analyzed (168). In breast cancer VEGF-C expression seems to correlate with lymph node positive tumors, whereas VEGF-D may be expressed predominantly in inflammatory breast carcinomas, suggesting that these growth factors have distinct roles in various tumors despite their biochemical similarities (109). A number of reports have described a correlation between VEGF-C expression in human tumors and the formation of metastases in regional lymph nodes. So far, VEGF-C levels in primary tumors have been shown to correlate significantly with lymph node metastases in thyroid, prostate, gastric, colorectal, lung and eosophageal carcinomas (5, 25, 103, 132, 190, 206). Less is known about the presence of VEGF-D in human tumors, but VEGF-D was shown to be upregulated in human melanomas when compared to melanocytes (4). In melanomas VEGF-D was detected in the tumor cells and in vessels adjacent to immunopositive tumor cells, but not in vessels distant from the tumors. This suggests that VEGF-D binds to the endothelial cells of nearby vessels and contributes in a paracrine manner to the regulation of tumor angiogenesis.

It is not known to what extent tumor cell secreted factors are directly responsible for the large lymphatic vessels occasionally detected around human tumors. Inflammatory cells for example could contribute to the lymphangiogenesis, as VEGF-C is chemotactic for macrophages and induced by proinflammatory cytokines (49, 160). It is not clear whether the newly formed lymphatic vessels mature in a way similar to the blood vessels, or whether they are more prone to tumor cell invasion for example because of differences in the expression of adhesion receptors. VEGF is known to be able to upregulate the expression of adhesion molecules in the vasculature, but such a role for VEGF-C and VEGF-D is not known.

Therapeutic approaches

Anti-angiogenic and anti-metastatic therapy

Despite advances in surgery, radiotherapy and chemotherapy, the prognosis of many cancers remains poor. One of the goals of gene therapy in cancer treatment is to target the therapeutic gene to all tumor cells, as each untreated tumor cell has the potential to progress and to metastasize. The purpose of combining conventional cancer therapy with anti-angiogenic agents is that the anti-

vascular effects of the chemotherapy and radiotherapy are selectively enhanced in the cells of newly formed vessels, for example when survival signals mediated by VEGF are blocked (reviewed in (53, 100)). However, one needs also to consider the unwanted toxic effects of the cancer therapy on the vasculature, some of which could be alleviated by provision of vascular survival factors (143). Therapy resistance in tumor cells depends on tumor cell heterogeneity, genetic instability and a high mutation rate. Compared to conventional cytostatics, there may well be less of a risk of resistance to anti-angiogenic agents, since the endothelial cells are assumed to be genetically more stable and have a lower mutation rate than the tumor cells (21, 53). However, the immature nature of tumor blood vessels should provide a therapeutic window where the tumor vascular endothelium can be targeted leaving the rest of the vasculature intact.

Several anti-angiogenic agents, alone or in combination with conventional therapies, have advanced to clinical trials. Many of them target angiogenic growth factors, their receptors or downstream signalling. For example, neutralizing antibodies against VEGF or VEGFR-2 have been used in the treatment of various solid tumors with and without combination with traditional cancer therapy (32). Although pre-clinical results are promising it is not yet clear how anti-angiogenic therapies will perform clinically.

Mechanisms of angiogenesis differ in various tissues. Therefore therapeutic inhibition of angiogenesis needs to be modified for each target tissue (56). There is evidence indicating that different types of tumor have distinct molecular mechanisms to activate the angiogenic switch. Whether a single anti-angiogenic molecule will suffice to treat all tumor types, or whether an ensemble of such molecules needs to be developed, remains to be seen. The differences between the surface molecules of blood vascular and lymphatic endothelia can be taken into account when targeting therapeutic agents selectively to tumor lymphatic vessels. This would increase the potency of the drug in the target tissue and limit the possibility of side effects (8, 163). Methods such as cDNA microarray analysis and phage display screening have been used to identify such markers. Toxic or vaso-occlusive therapy has already been used to target directly tumor vasculature (7, 47, 73). The targeting of lymphatic vessels in human tumors would help in imaging these vessels and facilitate studies into the role of lymphatic vessels in the metastatic processes. Anti-cancer drugs specifically targeted to peritumoral lymphatic vessels could be used to inhibit lymphatic metastasis. However, the destruction of these vessels would further elevate the high interstitial pressure inside the tumors impairing the delivery of other drugs. As VEGF-D expression has been shown to become upregulated by direct cell-cell contacts, the increased intratumoral pressure could increase close contacts between the tumor cells and lead to a compensatory increase of the lymphangiogenic growth factor levels (139). Increased intratumoral pressure could also enhance the likelihood of hematogenous metastasis (32, 182).

Gene and recombinant protein therapy of myocardial and peripheral ischemia

Ischemic heart disease stems from poor oxygenation of the heart muscle as a consequence of coronary vessel obstruction (39). Promoting angiogenesis in this situation, or in ischemia of the lower limb, may have a positive impact by increasing collateral vessel formation. Various angiogenic approaches to treating ischemic diseases are already in clinical trials (56, 87). Many of them involve the delivery of VEGF to ischemic tissue in order to stimulate the growth of new vessels. One outstanding question is whether a single angiogenic factor can promote functional and sustainable angiogenesis, or if a combination of angiogenic molecules is required. For example, vessels induced by VEGF are leaky and tortuous, so it may be possible to control leakiness by combining VEGF with Ang-1, as was done in a mouse model (187).

Recombinant VEGF-C may also be used as a therapeutic angiogenic growth factor in the treatment of tissue ischemia, possibly even in combination with VEGF (82). The angiogenic

activity of VEGF-C in ischemic conditions may relate to the increased expression of VEGFR-2 and the presence of relatively high endogenous VEGF levels in such conditions. On the other hand, lymphangiogenesis has never been studied in ischemia, but no evidence exists at present concerning the possible role of hypoxia in the regulation of the lymphatic vessels. The findings that VEGF has an important role in bone angiogenesis and endochondral bone formation suggest that these factors could also be used to enhance revascularization in orthopedic conditions such as nonhealing fractures (68).

An important question concerning the pro-angiogenic therapies is how the therapeutic molecules should be administered. Is it possible to deliver systemically a potent molecule like VEGF in therapeutic quantities without causing toxic side effects, like hypotension or edema and could these be prevented by local therapy? Suitable methods and routes of therapy would also avoid the infiltration of inflammatory cells, such as macrophages, which express VEGFR-1. It is not clear for how long these factors should be administered, whether the therapy leads to a functional vasculature and whether the vessels will regress upon the completion of therapy. At least some of the vessels generated in response to VEGF gene therapy eventually stabilize and acquire periendothelial structures (152). Such stabilization of vessels may depend on the level of intraluminal blood flow. However, concern about potential side effects, such as inappropriate blood vessel growth in patients with diabetic retinopathy or solid tumors, has decreased the enthusiasm for the use of these powerful agents (196).

Therapeutic lymphangiogenesis

The discovery of specific genes involved in the regulation of lymphatic vessels, and in the pathology of lymphedema should make the design of more targeted treatments for this disease possible. As transgenic VEGF-C/D overexpression is able to induce the postnatatal growth of new lymphatic vessels in the skin (88, 194), treatment using these molecules may also be useful in lymphedema patients. Subcutaneous viral gene transfer of VEGF-C in mice has already been shown to induce lymphangiogenesis within two weeks of treatment (48, 94). The effect of VEGF-C was explored by both gene therapy and transgene approaches in the Chy lymphedema mouse model (94). These mice, like human patients, have a heterozygous mutation in the VEGFR-3 gene, resulting in partial loss of VEGFR-3 activity, and features typical for lymphedema (94, 119, 118). This impairs the development of the cutaneous lymphatic vasculature and leads to hypoplastic, non-functional vessels. When VEGF-C was overexpressed in the skin of Chy mice, growth of functional cutaneus lymphatic vessels was induced, suggesting that VEGF-C/D gene therapy may be applicable to human lymphedema. Such therapy could also be used in non-hereditary, regional forms of lymphedema resulting from trauma, surgery or lymphatic vessel destruction after filariasis. As VEGFR-3 signalling plays a role in lymphatic endothelial cell survival (123, 125), long term growth factor expression may be needed to obtain lymphangiogenesis, and maintain these vessels in chronic lymphedema. The functional characteristics of the newly formed lymphatic vessels, for example their connections to draining lymphatic vessels, still require additional studies.

Aims of the study

Previous studies showed that VEGFR-3 is expressed in lymphatic endothelial cells and that the growth factors VEGF-C and VEGF-D bind and activate it. This study was done to obtain more information about the biological role of this receptor/ligand system in the regulation of lymphatic vessels.

I. Production of antibodies for lymphatic endothelial cells

Relatively little is known about the role of lymphatic vessels in human pathological conditions. One explanation for this has been the lack of specific markers for lymphatic endothelia leading to difficulties in the recognition of these vessels. We produced monoclonal antibodies against human VEGFR-3 in order to stain the lymphatic endothelial cells in human tissue samples and to study the lymphatic vessels in physiological and pathological conditions in more detail.

II. Biological function of VEGFR-3

Gene deletion studies for two other receptors in the VEGF family of growth factors, VEGFR-1 and VEGFR-2, showed embryonic lethal phenotype due to defective blood vessel development. Previous descriptive studies had shown that VEGFR-3 is expressed in the developing blood vessels in embryos before lymphatic vessels are formed, but its role in early blood vessel development and adult lymphatic vessels was not known. We created mice lacking a functional gene encoding VEGFR-3 in order to find out its biological role.

III. Overexpression of ligands of VEGFR-3 in transgenic mice

It was known that VEGF-C is able to stimulate both angiogenesis and lymphangiogenesis, but since this growth factor binds both VEGFR-2 and VEGFR-3 it was not clear which receptor mediates the lymphangiogenic signals. VEGF-D was shown to induce angiogenesis, but its lymphangiogenic properties were not known. To answer these questions, we overexpressed mutant form of VEGF-C, which only binds to VEGFR-3 (VEGF-C156S) and VEGF-D in the skin of transgenic mice.

IV. VEGF-C in tumor development

Increased expression of VEGF-C in primary tumors had been shown to correlate with dissemination of tumor cells to regional lymph nodes. However, the direct role for VEGF-C in tumor lymphangiogenesis and metastasis was not known. In order to answer this question, we studied the role of VEGF-C in tumorigenesis in transgenic mice, which had overexpression of VEGF-C during pancreatic β-cell tumor development.

Materials and Methods

Production of monoclonal antibodies (I)

The extracellular part of the VEGFR-3 protein was produced in a baculovirus expression system and recombinant protein was purified from the culture medium of the infected cells. This protein was used for the immunization of mice by intraperitoneal injection. After sacrificing the mice the splenic lymphoid cells were fused with plasmocytoma cells for the production of antibodies. The clones were tested by flow cytometry and purified from hybridoma ascites fluid. One clone, designated 9D9 was found to stably secrete a monoclonal antibody. The specificity of this antibody for VEGFR-3 was confirmed by FACS (fluorescent activated cell sorting), immunoprecipitation and Western blotting analyses. The antibodies were used for immunohistochemistry of human lymph node, lymphoma, breast cancer and Kaposi's sarcoma samples.

VEGFR-3 gene deletion in mice (II)

Mice lacking a functional VEGFR-3 gene (VEGFR-3-LacZ) were generated by homologous recombination. In the gene deletion construct the bacterial β-galactosidase gene (LacZ) replaces the first coding exon of the VEGFR-3 gene, leaving the LacZ-marker gene under the transcriptional regulatory sequences of VEGFR-3. Gene targeting was confirmed by Southern blot analysis. The product of this marker gene, which mimics the expression of endogenous VEGFR-3, can be visualized by the β-galactosidase staining allowing the spatial and temporal monitoring of the VEGFR-3 gene expression in these mice.

Overexpression of VEGF-C and VEGF-D in mice (III, IV)

Two different promoters were used to overexpress VEGF-C or VEGF-D in transgenic mice. Human keratin-14 (K14) promoter was used to direct the expression of full-length human VEGF-C156S (89), VEGF-C or VEGF-D to the basal cells of the epidermis in the skin of transgenic mice. Rat insulin promoter (Rip) was used to target the human VEGF-C expression to pancreatic β-cells. Transgenic mice were generated by microinjection of transgenic DNA constructs into the pronucleus of fertilized oocytes. The resulting mouse lines were analysed for the expression of the transgene by Northern and Southern blotting. The biological consequences of the overexpression of these growth factors were studied in skin or pancreatic tissue samples. Rip-VEGF-C transgenic mice were further crossed with the Rip1Tag2 (SV40 Tag oncogene) transgenic line, which develop pancreatic β-cell tumors, in order to study the role of VEGF-C in tumorigenesis.

Visualization of blood and lymphatic vessels (I-IV)

Several methods were used to visualize the lymphatic vessels in human and mouse tissues. Antibodies against VEGFR-3 (mouse-anti-human MoAb clone 9D9 or rat-anti-mouse MoAb from Dr. Hajime Kubo) or LYVE-1 (rabbit-anti-mouse PoAb from Dr. D. Jackson) were used for the immunohistochemical staining of lymphatic vessels. Blood vessels were stained for antibodies against vWF (mouse-anti-human PoAb, Dako), CD31 (mouse-anti human MoAb, Dako and rat-anti-mouse PECAM1, Pharmingen), Pal-e (mouse anti-human MoAb, Pharmingen) and MECA-32 (from Dr. Hallman). Also antibodies against Tie2 (mouse-anti-human, from Dr. Toshio Suda), for high endothelial venules (Heca 452 from Dr. Sirpa Jalkanen and Slex CD15s from Pharmingen), VEGFR-2 (from Dr. Hajime Kubo), VEGF-C (rabbit-anti-human 882 (91)), and VEGF-D (biotinylated goat-anti-mouse, R&D Systems) was used.

Heterozygous VEGFR-3-lacZ mice were used for the visualization of lymphatic vessels in whole mounts of tissue. After β-galactoside staining VEGFR-3 expression sites can be seen in blue. These mutant mice were further crossed with K14-VEGF-C156S and K14-VEGF-D

mice to allow the comparison of the lymphatic vessels after transgenic overexpression of these growth factors. Fluorescent dextran (Sigma) and ferritin (Sigma) macromolecules as well as Evans blue dye (Sigma) were used for the intradermal injections in order to visualize the intake and transport capacities of the lymphatic vessels. Intravenous injections of *L. esculentum* lectin (Vector) following the intracardial perfusion of the fixative were used to visualize the blood vessels in whole mounts of mouse tissue.

Results and Discussion

I. Antibodies against VEGFR-3 stain lymphatic vessels

Monoclonal antibodies produced against the extracellular part of the VEGFR-3 protein stained specifically the endothelial cells of lymphatic vessels. By using these antibodies I could visualize the lymphatic endothelial cells for the first time by single immunohistochemical staining and a comparison with the blood vessels was possible. The lymphatic vessels were stained in both physiological (lymph node and tonsil) and pathological (lymphoma, breast cancer) human tissue samples. Interestingly, the spindle cells of the Kaposi's sarcoma stained positive for VEGFR-3, providing further evidence for the origin of these cells from lymphatic endothelial cells rather than from the blood vascular endothelial cells. This was recently confirmed by similar results obtained by other group with antibodies against podoplanin, another marker for lymphatic endothelial cells (23, 92). The spindle cells of Kaposi's sarcoma have also been shown to express VEGFR-2, Ang-2, Tie-1, and Tie-2 (24, 146, 175). The anti-VEGFR-3 antibodies introduced in this study provide a useful tool for the studies of lymphatic vessels in disease and inflammatory processes.

In further studies by using these antibodies, new information about VEGFR-3 expression has been gained. In addition to lymphatic endothelial cells, VEGFR-3 is seen in a subset of capillary cells and the venous endothelium in adults (147). The fenestrated capillary endothelia of the endocrine organs express VEGFR-3, implying that it may have a role in the endothelial transport functions. VEGFR-3 is also seen in blood vascular endothelia at sites of hematopoiesis or blood cell trafficking, such as in the sinusoids of the liver, spleen and bone marrow. Also some nonendothelial expression of VEGFR-3 has been observed in embryonic notochordal cells and in the throphoblasts of the placenta (147, 200).

Upregulation of VEGFR-3 expression was seen in the angiogenic blood vessels associated with tumor growth (193). Conversely, no upregulation of VEGFR-3 was seen in the angiogenic vessels of a healing wound, whereas transient invasion of VEGFR-3 positive lymphatic vessels was seen to follow the growth of blood vessels into the granulation tissue (141). Suprisingly, VEGFR-3 was widely expressed in the endothelial cells of benign and malignant vascular tumors. VEGFR-3 may thus have more complicated role in the regulation of blood and lymphatic vessels that my previous studies suggested. VEGFR-3 is expressed in the embryonic arteries and veins but in adults it is predominantly seen in lymphatic endothelia. However, it seems to become upregulated in immature blood vascular endothelial cells, such as in proliferating vascular tumors or in tumor angiogenic vessels. This is consistent with the finding that VEGFR-3 is seen in the developing blood vessels of embryos (44, 93). In addition to visualization of lymphatic vessels in adult physiological conditions the anti-VEGFR-3 antibodies can thus be used for the diagnostic of malformations and tumors of both blood and lymphatic vessels. The knowledge about the regulation of lymphatic vessels has just began to accumulate and further studies will show whether the lymphangiogenesis is differently regulated in various physiological and pathological situations.

Recently, lymphatic endothelial cells were isolated from cell cultures by these monoclonal antibodies against VEGFR-3 (125). The availability of lymphatic endothelial cells now allows a more specific study of the genetic and functional properties of these cells and will help to uncover their contribution to molecular pathogenesis where the lymphatic vessels are involved. Freshly isolated lymphatic endothelial cells from malignant and normal tissues would be useful for example in understanding the interactions of stromal lymphatic endothelial cells and tumor cells in for example breast carcinomas.

Isolated lymphatic endothelial cells along with their growth factors could also prove useful in the reconstitution of a functional lymphatic network in the axillar region after radical surgery for advanced breast carcinoma.

II. VEGFR-3 in embryonic development studied by gene disruption strategy

I studied the biological role of VEGFR-3 in relation to the vascular development in VEGFR-3 gene deficient mice. Mice heterozygous for VEGFR-3 were healthy and fertile and the development of the blood and lymphatic vessels appeared normal. However, no mice lacking the both alleles of VEGFR-3 were born. The homozygous mutant embryos failed to thrive and died in utero around midgestation. Analysis of the homozygote embryos revealed cardiovascular failure with deficient blood vessel formation. VEGFR-3 was thus shown to have an important role in embryonic blood vessel development before the lymphatic vessels start to develop. In embryos deficient for VEGFR-3, yolk sac lacked major blood vessels and the large vessels of the embryo, anterior cardinal vein and dorsal aorta, were rudimentary. In further studies, VEGFR-3 deficient embryos were found to suffer from anemia (75), probably due to defects in the vitelline vessels of the yolk sac impairing the yolk sac hematopoiesis. In the head region the vascular plexus failed to undergo remodelling. On the other hand, no major defects occurred in the differentiation of the endothelial cells or in the formation of the primary vascular networks by vasculogenesis or sprouting angiogenesis. As the lack of both alleles of VEGFR-3 was lethal at midgestation, the effects of VEGFR-3 in the development of the lymphatic vasculature remain to be seen. However, the normally developing, healthy heterozygote embryos and mice can be used to visualize the lymphatic vessels during development and in physiological and pathological processes.

The majority of blood vessel endothelial cell populations around midgestation are positive for both VEGFR-2 and VEGFR-3, suggesting that these receptors play essential roles in angiogenesis (75). VEGFR-1 is also seen in the developing vasculature at this stage (61). Endothelial cells are apparently activated by both VEGF and VEGF-C at this stage but it is not known whether these receptors transduce similar signals. The VEGF/VEGFR-2 system appears to be responsible for most of the growth signals for vascular endothelial cells, but it has also been proposed that VEGF-C induces proliferation and differentiation of embryonic vascular endothelial cells through VEGFR-2. VEGF-C signalling through VEGFR-2 and VEGFR-3 may thus have distinct roles in embryogenic vasculogenesis (75). In addition, in VEGF deficient mice some endothelial cells survive and this may be due to VEGF-C.

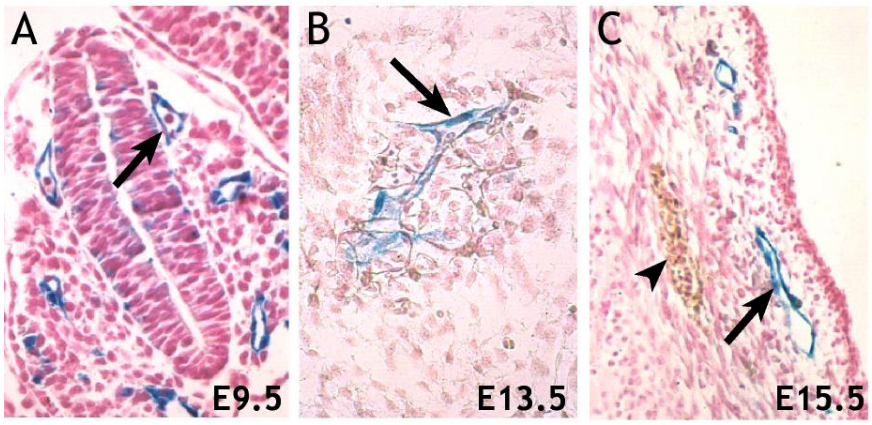

Figure 6. VEGFR-3 expression during embryonic development. β-galactosidase activity reveals VEGFR-3 expression in mice embryos deficient for VEGFR-3 (arrows in A-C). VEGFR-3 is essential for the development of blood vessels at midgestation (A), but at later stages its expression is seen in the developing lymphatic vessels (B). In older embryos (C) as well as in adults the VEGFR-3 expression is mainly seen in the lymphatic vessels and the expression in the blood vessels is downregulated (arrowhead in C).

III. Lymphatic hyperplasia in the skin of transgenic mice overexpressing VEGF-C and VEGF-D

In addition to VEGFR-3, VEGF-C also binds VEGFR-2, which is expressed in both blood and lymphatic endothelia. Therefore it was not clear which receptors mediates the lymphangiogenic effects of VEGF-C. VEGF-D, which binds to the same receptors, was reported to induce angiogenesis, but its role in lymphangiogenic processes was not known. In order to study the lymphangiogenic signalling pathway more specifically, we created transgenic mice overexpressing a VEGFR-3 specific mutant of VEGF-C (VEGF-C156S (89)) or VEGF-D in epidermal keratinocytes of the skin. Both transgenes were seen to be able to induce hyperplasia of lymphatic vessels by both proliferation of the endothelial cells and also by the growth of new lymphatic sprouts. On the other hand, the blood vessel architecture was not affected in these mice, demonstrating that stimulation of the VEGFR-3 is sufficient specifically to induce lymphangiogenesis *in vivo*. These transgenic models for lymphatic hyperplasia now allow studies of the biology of lymphatic vessels in pathological conditions such as wound healing, tumor progression and metastasis.

Recently, mice expressing the soluble ligand-binding part of the VEGFR-3 in the skin keratinocytes were generated (123). This soluble receptor protein inhibits the binding of endogenous VEGF-C and VEGF-D to the membrane bound receptor and thus neutralizes the activity of these growth factors. Interestingly, the mice were devoid of dermal lymphatics due to the inhibition of skin lymphangiogenesis and subsequent regression of existing lymphatic vessels during later embryonic development. This indicates that continuous signalling by VEGFR-3 is needed for the maintenance of the lymphatic vasculature and that soluble VEGFR-3 could be used as a specific inhibitor of lymphangiogenesis. The effect was specific for the lymphatic vessels, as the blood vasculature remained normal. However, the lymphatic vessels began to regrow after a few weeks postnatally even thought transgene encoded protein could be detected in the serum. It is thus possible that lymphatic vessels undergo maturation or otherwise become resistant to loss of VEGFR-3 ligands after early postnatal development.

The exact roles of VEGF-C and VEGF-D during embryonic vascular development are still unknown due to a lack of gene deletion studies. Their receptors VEGFR-2 and VEGFR-3 can be expressed variably in both blood and lymphatic endothelia and proteolytic processing regulates their binding affinity to the receptors. Therefore, VEGF-C and VEGF-D may regulate the responses of lymphatic vessels in adults, but they may also have important roles in physiological and pathological angiogenesis in various conditions. VEGF-C and VEGF-D may also affect the fluid dynamics of lymphatic vessels, and be involved in the formation of valves and recruitment of smooth muscle cells to the developing lymphatic collecting vessels. Unpublished data indicates that VEGF-C and VEGF-D can heterodimerize (Michael Jeltsch personal communication), as has been reported for PlGF and VEGF as well as VEGF-B and VEGF (27, 137). This may make their biological properties even more diverse.

IV. A genetic model of tumor metastasis in transgenic mice

VEGF-C overexpression was targeted to the β-cells of the pancreatic Langerhans islets in order to study the biological role of the VEGF-C in another organ system. An extensive network of lymphangiogenic vessels was seen around the pancreatic islets in the transgenic mice, whereas no such vessels were seen in the control mice. The transgenic mice where further crossed with Rip1Tag2 mice, which develop pancreatic β-cell tumors, in order to study VEGF-C during carcinogenesis. These tumors display morphological features typical of human insulinomas and they are capable of local invasion, but do not metastasize. Double transgenic tumors retained VEGF-C expression during the tumor progression. Interestingly, the tumors were surrounded by wide lymphatic channels, which contained aggregates of tumor cells. In 37% of the doubletransgenic mice, metastatic tumor cells were observed in the regional mesenteric lymph nodes, whereas no tumor cells were observed in the lymphatic vessels or lymph nodes of the control mice. These findings identify VEGF-C-induced lymphangiogenesis as a critical mediator of tumor metastasis. However, no increase in angiogenesis by VEGF-C was observed in these mice.

Similarly, human breast cancer cells expressing ectopic VEGF-C was reported to induce lymphangiogenesis in orthotopically implanted tumors (97, 176). Increased spreading of the cells to the regional lymph nodes was observed and the degree of tumor lymphangiogenesis was highly correlated with the distant metastases. Also, VEGF-C induced lymphangiogenesis was inhibited by adenoviral treatment with soluble VEGFR-3 receptor (97) but no differences in tumor growth or angiogenesis was observed.

Although it seems evident that both VEGF-C and VEGF-D can induce the growth of new lymphatic vessels, several questions remain unanswered regarding tumor lymphangiogenesis and metastasis. For example, it is not known whether it is sufficient for preexisting lymphatic vessels to expand by circumferential growth, or whether new vessels are required for the enhancement of the metastatic process. On the other hand, lymphatic vessels may either actively penetrate into existing tumors or become trapped in between expanding tumor foci. The intratumoral lymphatic vessels observed are usually collapsed due to the high interstitial pressure in solid tumors, impairing their transport capacity. Also, as in angiogenesis, lymphangiogenesis may occur by several mechanisms and different regulatory factors may be involved.

Activation of lymphatic endothelial cells by tumor cell secreted factors may promote the interaction of tumor cells with lymphatic endothelial cells, and thereby facilitate tumor cell entry into the lymphatics. In spite of the increased metastatic tendency of VEGF-C overexpressing tumor cells, metastases were only seen in about one third of the tumor bearing mice. Overall, on the basis of studies in which VEGF-C or VEGF-D has been overexpressed in tumors, one could suggest that there are additional, rate-limiting steps in the metastatic process. The simpliest explanation for the metastasis-enhancing effects of VEGF-C and VEGF-D is that they eliminate one rate-limiting step by increasing

the surface area between invading tumor cells, which are in contact with the hyperplastic lymphatic endothelium. However, they could also facilitate metastasis by increasing vascular permeability, by changing the adhesive properties, or cytokine or chemokine expression patterns of the lymphatic endothelium. VEGF-C and VEGF-D secreted by the tumor cells could also have an important effect on the tumor interstitial pressure. Both can increase vascular leakage, but not as efficiently as VEGF, and a parallel increase in lymphangiogenesis could alleviate this effect. The increased interstitial pressure could be a major determinant of tumor cell seeding into the blood vascular and lymphatic circulation, especially as recent studies have shown that a proportion of the lumen of tumor blood vessels themselves consists of tumor cells (35, 78).

Conclusions

The development and regulation of endothelial cells requires many growth factors orchestrating in a carefully co-ordinated manner. Blood and lymphatic vessels are formed in parallel during embryonic development but both vessel types are maintained in a rather quiescent stage in adults, being activated only in sites of new growth of the tissue. The role of lymphatic vessels in various diseases has not been studied much, but the recent discoveries of specific molecules involved in the biology of lymphatic vessels now allows more extensive studies of these vessels. Similarities between the regulation of blood and lymphatic vessels have been observed and these two vessel systems seem to work in a tightly regulated manner. Lymphangiogenesis may occur at sites of angiogenesis, either following the growth of blood vessels, like in wound healing, or acting independently as has been seen in experimental tumor models. The findings made so far will be helpful in diagnosis of certain vascular tumors, designing specific treatments for lymphedema and attempts to regulate the metastatic spread of tumor cells via lymphatic vessels. In addition, the isolation and culturing of lymphatic endothelial cells offers additional tools for the study of the molecular characteristics of these cells.

The growth factors VEGF-C and VEGF-D and their receptor VEGFR-3 are the first molecules found to regulate the lymphatic endothelial cells. This study presents further data on the biological role of VEGFR-3 during embryonic development as well as in adult physiological and pathological conditions. The experimental studies presented here show the potential of the growth factors VEGF-C and VEGF-D to induce the growth of lymphatic vessels. The challenge for future studies will be the use of these molecules in therapeutic purposes, or for the inhibition of VEGFR-3 signalling to prevent the metastatic spread of tumors via lymphatic vessels.

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