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VEGFR-2 AND VEGFR-3 SPECIFIC SIGNALING IN LYMPHANGIOGENESIS AND ANGIOGENESIS

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ACADEMIC DISSERTATION

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ABBREVIATIONS

AAV	Adeno associated virus
Ad	Adenovirus
Ang	Angiopoietin
ATP	Adenosine triphosphate
BEC	Blood vascular endothelial cell
BM	Basement membrane
cDNA	Complementary deoxyribonucleic acid
CAM	Chorioallantoic membrane
CUB	Protein domain: the name originates from the first three proteins the domain was found in (Complement subcomponents C1r/C1s, sea urhin EGF-like protein, bone morphogenic protein 1)
DMSO	dimethyl sulfoxide
E	Embryonic day
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FIGF	c-fos induced growth factor (VEGF-D)
Flk1	Fetal liver kinase 1 (mouse VEGFR-2)
Flt1	*Fms*-like tyrosine kinase 1 (VEGFR-1)
Flt4	*Fms*-like tyrosine kinase 4 (VEGFR-3)
HDMVEC	Human dermal microvascular endothelial cell
HIF	Hypoxia-inducible factor
HSPG	Heparan sulphate proteoglycan
IFP	Interstitional fluid pressure
Ig	Immunoglobulin
K14	Keratin-14
kb	Kilobase
kDa	Kilodalton
KDR	Kinase insert domain containing receptor (human VEGFR-2)
LEC	Lymphatic endothelial cell
LYVE-1	Lymphatic endothelial hyaluronan receptor 1
MAPK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid
NP	Neuropilin
NRTK	Nonreceptor tyrosine kinase
P	Postnatal day
PAE	Porcine aortic endothelial
PC	Pericyte
PDGF	Platlet derived growth factor
PDGFR	PDGF receptor
PECAM-1	Platelet endothelial adhesion molecule 1
PlGF	Placenta growth factor
PTK	Protein tyrosine kinase
Prox-1	*Prospero*-related homeobox protein 1
RTK	Receptor tyrosine kinase
SLC	Secondary lymphoid chemokine
SMA	Smooth muscle 〈-actin
SMC	Smooth muscle cell
TGF	Transforming growth factor
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
VPF	Vascular permeability factor (VEGF)
VRF	VEGF related factor (VEGF-B)
VRP	VEGF related protein (VEGF-C)

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original research articles, which are referred to in the text by their Roman numerals.

I Uutela M, **Wirzenius M***, Paavonen K*, Rajantie I, He Y, Karpanen T, Lohela M, Wiig H, Salven P, Pajusola K, Eriksson U and Alitalo K. 2004. PDGF-D induces macrophage recruitment, increased interstitial pressure and blood vessel maturation during angiogenesis. *Blood.* 104:3198-3204.

II **Wirzenius M,** Tammela T*, Uutela M*, He Y, Odorisio T, Zambruno G, Dvorak HF, Ylä-Herttuala S, Shibuya M and Alitalo K. 2007. Distinct vascular endothelial growth factor signals for lymphatic vessel enlargement and sprouting. *Journal of Experimental Medicine. In press.*

III Karpanen T, **Wirzenius M**, Mäkinen T, Veikkola T, Haisma HJ, Achen MG, Stacker SA, Pytowski B, Ylä-Herttuala S and Alitalo K. 2006. Lyphangiogenic growth factor responsiveness is modulated by postnatal lymphatic vessel maturation. *The American Journal of Pathology*. 169(2):708-718.

IV Heckman C, **Wirzenius M*,** Holopainen T*, Keskitalo S, Jeltsch M, Wedge SR, Jürgensmeier JM and Alitalo K. 2007. The tyrosine kinase inhibitor AZD2171 effectively prevents VEGFR-3 activity and lymphangiogenesis. *Submitted.*

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ABSTRACT

The circulatory system consists of the blood and lymphatic vessels. While blood vessels transport oxygen, cells, and nutrients to tissues, the lymphatic vessels collect fluid, cells, and plasma proteins from tissues to return back to the blood circulation. Angiogenesis, the growth of new blood vessels from pre-existing ones, is an important process involved in several physiological conditions such as inflammation, wound healing, and embryonic development. Furthermore, angiogenesis is found in many pathological conditions such as atherosclerosis and the growth and differentiation of solid tumors. Many tumor types spread via lymphatic vessels to form lymph node metastasis.

The elucidation of the molecular players coordinating development of the vascular system has provided an array of tools for further insight of the circulatory system. The discovery of the Vascular Endothelial Growth Factor (VEGF) family members and their tyrosine kinase receptors (VEGFRs) has facilitated the understanding of the vasculature in different physiological and pathological situations. The VEGFRs are expressed on endothelial cells and mediate the growth and maintenance of both the blood and lymphatic vasculatures.

This study was undertaken to address the role of VEGFR-2 specific signaling in maturation of blood vessels during neoangiogenesis and in lymphangiogenesis. We also wanted to differentiate between VEGFR-2 and VEGFR-3 specific signaling in lymphangiogenesis. We found that specific VEGFR-2 stimulation alone by gene therapeutic methods is not sufficient for production of mature blood vessels. However, VEGFR-2 stimulation in combination with expression of platelet-derived growth factor D (PDGF-D), a recently identified member of the PDGF growth factor family, was capable of stabilizing these newly formed vessels.

Signaling through VEGFR-3 is crucial during developmental lymphangiogenesis, but we showed that the lymphatic vasculature becomes independent of VEGFR-3 signaling after the postnatal period. We also found that VEGFR-2 specific stimulation cannot rescue the loss of lymphatic vessels when VEGFR-3 signaling is blocked and that VEGFR-2 specific signals promote lymphatic vessel enlargement, but are not involved in vessel sprouting to generate new lymphatic vessels *in vivo*, in contrast to the VEGFR-2 dependent sprouting observed in blood vessels. In addition, we compared the inhibitory effects of a small molecular tyrosine kinase inhibitor of VEGFR-2 vs. VEGFR-3 specific signaling *in vitro* and *in vivo.* Our results showed that the tyrosine kinase inhibitor could equally affect physiological and pathological processes dependent on VEGFR-2 and VEGFR-3 driven angiogenesis or lymphangiogenesis.

These results provide new insights into the VEGFR specific pathways required for pre- and postnatal angiogenesis as well as lymphangiogenesis, which could provide important targets and therapies for treatment of diseases characterized by abnormal angiogenesis or lymphangiogenesis.

REVIEW OF THE LITTERATURE

1. THE CARDIOVASCULAR SYSTEM

The blood vascular system

The cardiovascular system includes the heart and the blood vessels. Arteries deliver oxygenated blood to the capillaries where two-way exchange occurs between the blood and tissues. Veins collect deoxygenated blood from the microvasculature and carry it back to the heart.

Blood vessels consist of a monolayer of blood vascular endothelial cells (BECs) with molecular junctions tightly attaching the endothelial cells (ECs) to each other. These junctions form barriers, which regulate vessel permeability and the exchange of molecules between nearby ECs (reviewed in Dejana, 2004). The blood vessels rest on a basement membrane (BM), a thin sheet of extracellular matrix (ECM) composed of collagens, laminins, fibronectin, and heparan sulphate proteoglycans (HSPGs), which can vary depending on vessel type and tissue environment. Correct assembly of the BM is needed for vessel stability, as well as EC morphogenesis and vessel maturation (reviewed in Davis and Senger, 2005). In capillaries, the BM is shared by ECs and pericytes (PCs), which actively interact with each other and further support and stabilize the vessels. The larger vessels regulate blood pressure and transport blood to the capillaries. Layers of vascular smooth muscle cells (vSMCs), elastic fibers, and connective tissue surround the vessels making them resist high pressure.

Development of the blood vascular system

The blood vascular system is one of the first functional organ systems of the vertebrate embryo, and the creation of an accurately patterned network of blood vessels is essential for embryonic survival. Vasculogenesis initiates when the common precursor cells of both endothelial and blood cells, the hemangioblasts, form aggregates termed blood islands, in which the inner cells develop into hematopoietic precursors and the outer population into endothelial progenitor cells or angioblasts. Following commitment to the endothelial cell lineage, angioblasts may migrate extensively before assembling into a primitive vascular plexus of veins and arteries (reviewed in Carmeliet, 2000).

After formation of the primary vascular plexus, new capillaries are generated from the pre-existing vessels in a process termed angiogenesis. In developmental angiogenesis, new vessels are formed either by endothelial sprouting and elongation from pre-existing vessels, or by splitting or merging of the pre-existing vessels (reviewed in Lawson and Weinstein, 2002). After the onset of blood circulation, the emerging vascular plexus is rapidly remodelled to resemble a mature system with a hierarchy of large and small vessels. A large amount of different endogenous inducers and inhibitors of angiogenesis have been identified. These factors must be tightly controlled and in balance for physiological angiogenesis to occur properly. The maturation of blood vessels includes the deposition of the BM and recruitment and organization vascular mural cells (reviewed in Carmeliet, 2000).

Endothelial and vascular mural cells

Depending on the vessel type, ECs vary in their morphology, function, and gene-expression profiles. Morphologically, they differ in size, shape, thickness, number of microvilli, and the position of the nucleus. Blood and lymphatic vascular endothelial cells (BECs and LECs) represent two distinct cell populations (Hirakawa et al., 2003; Kriehuber et al., 2001; Makinen et al., 2001b; Podgrabinska et al., 2002). ECs in tumor vessels are abnormally shaped, overlap each other and are only loosely interconnected with openings between the cells (Hashizume, 2000). ECs express specific markers that are very helpful in identifying these cells *in vitro* and *in vivo*. Most of the markers are present on both ECs and hematopoietic precursors or mature blood cells, strengthening the idea of a common embryonic precursor (Risau, 1995). Some of the endothelial markers are constitutive and present in essentially all types of endothelium. Other molecules are expressed only after activation by inflammatory cytokines or growth factors (Reviewed in Garlanda and Dejana, 1997).

Vascular mural cells, pericytes (PC) and vascular smooth muscle cells (vSMCs), are a heterogenous group of cells, which stabilize the newly formed vessel wall together with BM deposition. vSMCs of large blood vessels are separated from the vascular BM by a layer of mesenchymal cells and extracellular matrix. In these vessels, the vSMCs make up a separate layer in the vascular wall. PCs are embedded in the endothelial BM and make close contacts with the EC. While the role of vSMCs is to mediate vascular contraction and strength, the PCs seem to facilitate and integrate cell-to-cell communication. A single PC is often in contact with several ECs and therefore suggested to be involved in neighboring EC stabilization, contacts, and signaling. The vascular mural cells can be distinguished from the ECs by specific marker proteins (reviewed in Armulik et al., 2005).

Vascular mural cells originate from multiple sources including mesenchymal cells, neural crest cells, and epicardial cells (Hungerford and Little, 1999). In embryonic mesodermal cells they have common progenitors with the ECs (Flk-1 positive embryonic stem cells), which can differentiate into ECs in the presence of vascular endothelial growth factor (VEGF) or into vSMCs in the presence of platelet–derived growth factor B (PDGF-B) (Yamashita et al., 2000).

Combinations of antiendothelial and antipericyte agents have been shown to have a synergistic effect in antiangiogenic therapy for diseases such as cancer and diabetic retinopathy (reviewed in Armulik et al., 2005). A combination of growth factors promoting both angiogenesis and PC recruitment has also shown to be synergistically effective in proangiogenic therapy.

Physiological angiogenesis

Usually ECs of adult, mature, quiescent vessels have a very low proliferating rate. However, new vessel growth occurs during some physiological events, such as in female reproductive organs (reviewed in Ferrara and Kerbel, 2005). In the adult, angiogenesis appears to be the primary mechanism of new vessel formation, although recent studies have suggested that bone marrow-derived endothelial precursor cells can be recruited and incorporated into sites of active neovascularization (Hristov et al., 2003).

Angiogenesis is initiated by an increase in vascular vasodilation and permeability in response to angiogenic growth factors. Periendothelial support cells detach and interendothelial connections loosen. Once ECs are activated, they start to produce proteases which decompose the BM and release factors from the ECM, such as basic fibroblast growth factor (bFGF), VEGF, and insulin-like growth factor-1 (reviewed in Carmeliet, 2000). Monocytes and macrophages are also recruited to the site of angiogenesis where they secrete growth factors (Conway et al., 2001). The growth factors futher stimulate ECs to migrate into the tissue, to proliferate, and differentiate to form new vessels. Finally, the ECs start to produce PDGF-B to attract PCs and vSMCs to stabilize the newly formed vessels (Lindahl et al., 1998).

Pathological blood vessel growth

A wide range of diseases, including chronic inflammatory diseases, diabetic retinopathy, rheumatoid arthritis, preeclampsia, and cancer are characterized by abnormal angiogenesis. In contrast to physiological angiogenesis, pathological angiogenesis is often induced by inflammation. Monocytes and macrophages, platelets, mast cells and other leukocytes are attracted to sites of inflammation, in part by angiogenic factors such as VEGF (reviewed in Takahashi and Shibuya, 2005).

Tumor angiogenesis

Mammalian cells need oxygen and nutrients for survival and therefore have to be located within the diffusion limit of oxygen from blood vessels. In order to grow to a size beyond a few cubic millimeters, solid tumors must promote new blood vessel growth. Tumor vessels develop by sprouting or intussusception from pre-existing vessels. Also, circulating endothelial precursor cells from the bone marrow may contribute to tumor angiogenesis (Lyden et al., 2001).

Tumor vessels are not always formed only by ECs; rather they can be composed of only cancer cells or consist of a mosaic of cancer cells and ECs (Jain, 1988). Tumor vessels are structurally and functionally abnormal. They are dilated and tortuous, have an uneven diameter, and have excessive branching points, probably due to an imbalance of the angiogenic factors (reviewed in Carmeliet and Jain, 2000). Tumor vessel blood flow is variable and the vessels are very leaky (Dvorak et al., 1999; Hashizume, 2000). Also, tumor vessels may either lack PCs or be covered by abnormal PCs (Morikawa et al., 2002). Tumor vessels have also been proposed to express surface proteins that are absent or scarcely detectable in normal mature vessels (Huang et al., 1997).

Therapeutic angiogenesis

Therapeutic angiogenesis (promoting new vessel growth to treat ischaemic disorders) has been shown to be effective in a variety of animal models of coronary or limb ischemia. Clinical trials using the angiogenic factors VEGF or FGF have unfortunately not yielded in convincingly positive results (reviewed in Simons, 2005). Recently, studies have shown that therapy using hypoxia inducible factor-1 (HIF-1) which promotes expression of growth factors both for vessel growth and maturation would be more potent than using single angiogenic factors (Heinl-Green et al., 2005; Pajusola et al., 2005).

Already in 1971, it was proposed that tumor growth and metastasis are angiogenesis-

dependent, and hence, blocking angiogenesis could be a strategy to arrest tumor growth (reviewed in Folkman et al., 2000). Several promising strategies to inhibit tumor angiogenesis have been designed and tested *in vitro* and in animal models. These include inhibitors that interfere with angiogenic ligands, their receptors or downstream signaling, endogenous inhibitors of angiogenesis, or directly targeting of molecules that are specifically expressed on the tumor vasculature (Arap et al., 2002). However, only a few of these have had any effect in the clinics, despite extensive trials (reviewed Jain et al., 2006). So far, three drugs which interfere with VEGF have been approved by Federal Drug Administration (FDA) in the USA: Bevacizumab (Avastin^{TM}) is a neutralizing antibody for VEGF which was shown to improve the efficacy of chemotherapy in colorectal cancer (Hurwitz, 2004; Hurwitz et al., 2004); Pegaptanib (Macugen^{TM}), an oligonucleotide that binds VEGF; and Ranibizumab (Lucentis^{TM}), an antibody that binds VEGF. The latter two are both used for treatment of age-related macular degeneration (AMD) (Gaudreault et al., 2005; Gragoudas et al., 2004).

2. THE LYMPHATIC SYSTEM

The main function of the lymphatic system is to maintain tissue fluid balance by returning interstitial fluid to the venous circulation via the larger lymphatic collecting vessels and the thoracic duct. The lymphatic system also has an important role in the immune system and in absorption and transportation of digested fats from the intestine (reviewed in Karpanen and Makinen, 2006).

The lymphatic system is composed of a vascular network of thin-walled, blind-ended capillaries that drain protein-rich lymph from the extracellular spaces within most organs. The lymphatic capillaries are valveless endothelial single-cell layer tubes, which have an irregular basement membrane, overlapping EC junctions, and they lack PCs and SMCs, making them highly permeable to large macromolecules. Collecting lymphatic vessels have sparse SMC coverage, which helps moving lymph forward, and valves, which prevent backflow (reviewed in Karpanen and Makinen, 2006). Anchoring filaments attach the lymphatic vessels to the surrounding tissue. In addition to the lympahtic vessels, the lympahtic system includes lymphoid organs such as the lymph nodes, tonsils, Peyer's patches, spleen, and thymus, all of which play an important role in immune responses. Lymphatic vessels are normally not present in avascular structures such as epidermis, cartilage and cornea, or in some vascularized organs such as brain, retina and bone marrow (reviewed in Oliver and Detmar, 2002).

Development of the lymphatic vessels

During embryogenesis, the development of lymphatic vessels begins after the establishment of the blood vasculature when a subset of venous ECs becomes committed to the lymphatic endothelial cell lineage. These cells sprout from the major veins in the jugular and perimesonephric areas to form primitive lymphatic sacs, from which lymphatic vessels then spread by endothelial sprouting to the periphery of the embryo (reviewed in Wigle and Oliver, 1999). The homeodomain transcription factor Prox-1 (*prospero*-related homeobox protein 1) has been identified as a critical regulator of lymphatic endothelial cell (LEC) differentiation (reviewed in Oliver and Detmar, 2002). Induction of polarized expression of Prox-1 in the cardinal vein leads to upregulation of lymphatic-specific genes. The

simultaneous expression of the lymphatic-specific genes LYVE-1, Prox-1, VEGFR-3, and SLC may lead to irreversible specification of the lymphatic pathway (Wigle et al., 2002).

Physiological and pathological lymphangiogenesis

In the adult, the growth of lymphatic vessels follows the growth of blood vessels, similar to the situation occurring in embryonic development. During wound healing, the growth of lymphatic vessels lags behind that of blood vessels, and shortly after their formation, lymphatic vessels tend to regress (Paavonen et al., 2000). Lymphangiogenesis is not detected in chronic wounds, suggesting disturbed VEGFR-3 signaling in an abnormal wound healing process, which might be further impaired by the lack of lymphatic vessels. Lymphangiogenesis is thus probably an essential feature of tissue repair and inflammatory reactions in most organs.

Tumor lymphangiogenesis and lymphatic metastasis

In most human cancers, the lymphatic system serves as the primary route for the metastatic spread of tumor cells, and metastasis to the regional lymph nodes is one of the most important indicators of tumor aggressiveness (reviewed in Stacker et al., 2002a). VEGF-C and -D promote tumor lymphangiogenesis and the metastatic spread of tumor cells (reviewed in Saharinen et al., 2004).

Intratumoral lymphatic vessels and metastasis to lymph nodes and lungs have been documented in tumor xenografts expressing VEGF-C or VEGF-D (Skobe et al., 2001a; Skobe et al., 2001b; Stacker et al., 2001), as well as in VEGF-C or -D transgenic mouse tumors (Mandriota et al., 2001; Von Marschall et al., 2005). VEGF-C-induced tumor growth, lymphangiogenesis and lymphatic metastasis were inhibited by adenoviral expression of a soluble VEGFR-3-Ig fusion protein, which “traps” available VEGF-C and VEGF-D (He et al., 2002; Karpanen et al., 2001). Tumor lymphatic vessels have distinct molecular expression patterns from normal LECs (Laakkonen et al., 2002). Therefore, drugs specifically targeted to peritumoral lymphatic vessels might inhibit lymphatic metastasis. However, destruction of these vessels would further elevate the high interstitial pressure inside the tumor, which is known to interfere with the delivery of anti-cancer drugs (Jain and Padera, 2002).

Lymphedema

Lymphedema is caused by insufficient lymph transport by lymphatic vessels, which results in build-up of protein rich fluid in tissues causing chronic swelling of the extremities (Witte et al., 2001). Lymphedema is generally divided into two main categories. Primary lymphedema is a rare developmental disorder, characterized by hypoplasia or hyperplasia of the cutaneous lymphatic vessels. Kinase inactivating mutations in the VEGFR-3 gene were identified in several cases of hereditary, early-onset lymphedema (Milroy disease) (Ferrell et al., 1998; Irrthum et al., 2000; Karkkainen et al., 2000). Mutations in the transcription factors FoxC2 and Sox18 were also linked to lymphedema-distichiasis and hypotrichosis-lymphedema-telangiectasia, respectively (Fang et al., 2000; Finegold et al., 2001; Irrthum et al., 2003). Secondary lymphedema is caused by damage of the lymphatic vessels due to infection, surgery, or trauma. Secondary lymphedema can develop as the result of inflammatory obstruction of the draining lymphatic vessels or, for example, after breast cancer surgery (reviewed in Rockson, 2000). The most common cause of secondary

lymphedema is filariasis caused by the parasitic Wucheriya bancroftii or Brugia malayi with a prevalence of over 120 million cases worldwide.

Inflammation and wound healing

Edema occurs in inflammatory diseases when the speed of plasma leakage from blood vessels exceeds the drainage through lymphatic vessels. Inflammatory cells express VEGFRs and are therefore attracted by angiogenic and lymphangiogenic signals. Macrophages secrete VEGF, VEGF-C and VEGF-D and can therefore stimulate lymphangiogenesis (Cursiefen et al., 2004; Mimura et al., 2001; Schoppmann et al., 2002; Skobe et al., 2001a). These cells can also transdifferentiate into LECs, which can incorporate into the lymphatic endothelium (Kerjaschki, 2005; Maruyama et al., 2005). Chronic airway inlammation by *Mycoplasma pulmonis* resulted in massive lymphangiogenesis induced by VEGF-C and VEGF-D producing inflammatory cells. Whereas the newly formed blood vessels regressed after antibiotic treatment, the lymphatic vessels stayed intact much longer (Baluk et al., 2005). Proliferation of lymphatic vessels has also been reported in rejected kidney transplants and in cornea models of inflammatory neovascularization (Chen et al., 2004; Kerjaschki et al., 2006).

Therapeutic lymphangiogenesis

Currently, therapeutic options for the management of lymphedema are limited, consisting primarily of physiotherapy, massage, and external compression (Witte et al., 2001). The discovery of specific genes involved in the regulation of lympahtic vessels and in the pathology of lymphedema has lead to experimental approaches for treating lymphedema. Adenoviral VEGF-C gene transfer into the skin was shown to result in a strong lymphangiogenic response (Enholm et al., 2001), and viral VEGF-C gene therapy induced the growth of functional lymphatic vessels in the skin of a mouse model for hereditary lymphedema (Karkkainen et al., 2001). However, high levels of VEGF-C also led to blood vascular effects such as increased vessel leakiness, presumably through the interaction of VEGF-C with VEGFR-2 expressed on the blood vascular endothelium (Saaristo et al., 2002a).

Adenoassociated virus (AAV)-mediated VEGF-C156S and VEGF-C gene transfer in the skin of lymphedema mice induced the formation of functional lymphatic vessels that persisted for at least eight months (Saaristo et al., 2002b). VEGF-C lymphangiogenic gene therapy has been shown to accelerate diabetic wound healing by inducing persistent angiogenesis and lymphangiogenesis (Saaristo et al., 2006). VEGF-C and VEGF-C156S lymphangiogenic gene therapy was used to reconstruct the lymphatic vessel network severed by the incision wound in free flap operations in an animal model (Saaristo et al., 2004).

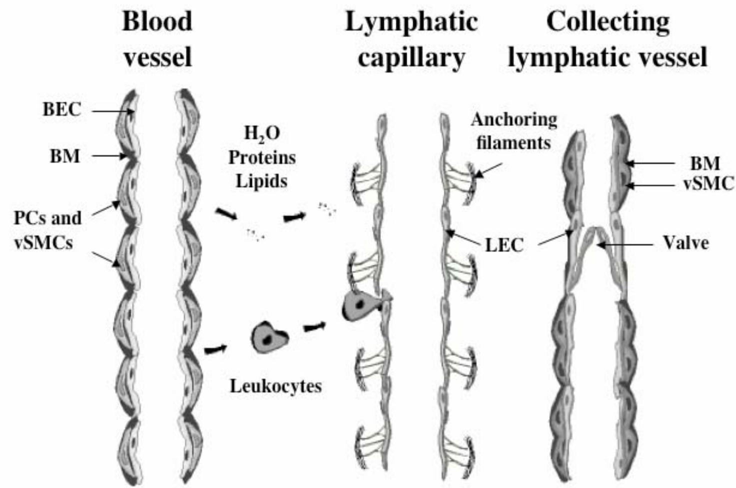

Figure 1. Schematic structure of blood and lymphatic vessels. Blood vessels consist of a monolayer of blood vascular endothelial cells (BECs), which form tight and adherent juntions. Vascular mural cells, pericytes (PCs), and vascular smooth muscle cells (vSMCs) surround the BECs. The BECs and PCs share a basement membrane (BM), which is needed for vessel stability. The lymphatic capillaries are valveless, single endothelial cell layer tubes with overlapping lymphatic endothelial cell (LEC) junctions and lacking PCs, making them highly permeable to large macromolecules. Anchoring filaments attach the lymphatic capillaries to the surrounding tissue. Collecting lymphatic vessels have sparse vSMC coverage, which helps move lymph forward, and valves, which prevent backflow.

3. CELL-TO-CELL SIGNALING AND PROTEIN PHOSPHORYLATION

The growth and survival of multicellular organisms is a complex process, which therefore must be strictly regulated. The synchronized differentiation of cells into different organs is largely dependent on the capability of the cells to react to extracellular stimulus and the ability to communicate with other cells. Signaling between cells occurs either locally as a result of direct contact between cells, or as a result of secreted signal mediators. These signal mediators can either be locally acting secreted growth factors or cytokines, or systematically circulating endocrine hormones, which mediate signals at their target tissue. Secreted growth factors bind to their receptors on the cell surface, which leads to an activation of intracellular signaling cascades. The final purpose for this activation is often regulation of different transcription factors and therefore reprogramming of the gene expression of the cell.

The key mechanism for intracellular signal transduction is covalent modification of proteins through reversible phosphorylation by protein kinases. Growth factor receptors have been characterized to be essential regulators of cellular functions. The receptors transmit the extracellular signals into the cell and regulate the important cellular processes such as cell migration and survival. Many intracellular signaling molecules contribute to a number of different signaling cascades. How the cell reacts to a specific stimulation is dependent on the length and strength of the signal, the cell type specific expression of receptors and modifying

proteins, and the availability of ligands (reviewed in Lowes et al., 2002). Many important cellular signals for growth, differentiation, adhesion, migration and apoptosis are transmitted by tyrosine phosphorylation of signaling modulators.

Protein tyrosine kinases

Protein tyrosine phosphorylation is the most common mechanism for signaling through membranes, mediated principally by all types of transmembrane receptors (reviewed in Hunter, 2000). The phosphorylation is carried out by protein tyrosine kinases (PTKs), which are divided into receptor tyrosine kinases (RTKs) and nonreceptor tyrosine kinases (NRTKs, also called cytoplasmic or intracellular tyrosine kinases). Over 90 PTKs have so far been identified in the human genome. These enzymes catalyze the transfer of the gamma phosphate of adenosine triphosphate (ATP) to tyrosine residues on protein substrates (reviewed in Blume-Jensen and Hunter, 2001; Hubbard and Till, 2000).

The kinase domain in protein tyrosine kinases consists of about 300 amino acids. The three dimensional structure of the kinase domain is very well conserved and composed of two lobes. The N-terminal lobe is comprised of a five-stranded β-sheet and one α-helix, αC, which functions in the regulation of the kinase. The C-terminal lobe is primarily α-helical and responsible for binding of the substrate. The linker region between the N- and C-terminal lobes forms a cleft at the ATP binding site (reviewed in Hubbard and Till, 2000).

Phosphorylation of tyrosine residues in the activation loop (A-loop), located in the C-terminal lobe of the kinase domain, typically leads to conformational changes and an increase in enzymatic activity. This regulation occurs either via *trans*-autophosphorylation or via phosphorylation by different NRTKs (reviewed in Huse and Kuriyan, 2002).

Ligand binding to RTKs induces receptor dimerization and activation, apparently by causing a conformational change of the receptor chains. As a result, the receptor becomes transphosphorylated on regulatory tyrosines in the activation loop, and promotes phosphorylation of target protein tyrosines (reviewed in Jiang and Hunter, 1999). The phosphorylated tyrosine residues on the activated receptor serve as docking sites for proteins containing phosphotyrosine recognition domains such as PTKs, PTPases, adaptors, scaffolds, small GTPase signaling proteins, phospholipid signaling proteins, cytoskeletal regulation proteins, transcription factors, etc. These proteins convert extracellular stimuli to a biological response through intracellular signaling pathways leading to changes in cellular functions.

4. THE VASCULAR ENDOTHELIAL GROWTH FACTORS AND THEIR RECEPTORS

The growth and differentiation of blood vascular as well as lymphatic endothelial cells are highly complicated and coordinated processes that involve many different growth factors and their receptor systems, such as members of the angiopoietin (Ang), ephrin, PDGF, and transforming growth factor-β (TGF-β) families (Carmeliet, 2003; Jain, 2003; Yancopoulos et al., 2000). However, signaling mediated through the vascular endothelial growth factors (VEGFs) and associated receptor systems often represents the major rate-limiting step in developmental and physiological angiogenesis. The human VEGF family currently includes five members: VEGF, placenta growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D. Recently, two non-human growth factors, orf virus VEGF (also called VEGF-E) and snake

venom VEGF (also called VEGF-F) have been added to the family. The VEGFs are secreted, dimeric glycoproteins, which all contain characteristic, regularly spaced eight cysteine residues, which compose the so-called cystine knot motif. Disulfide bonds covalently link the subunits together.

The VEGFs mediate their signals via three receptor tyrosine kinases, VEGFR-1, VEGFR-2, and VEGFR-3. The VEGFRs demonstrate structural and functional similarities to the platelet derived growth factor (PDGF) receptor family, and form a subfamily within that receptor class. All VEGFRs have seven immunoglobulin (Ig) homology domains in the extracellular ligand binding part, a transmembrane part, and an intracellular tyrosine kinase domain. Like other RTKs, the VEGFRs dimerize and undergo trans-autophosphorylation upon ligand binding.

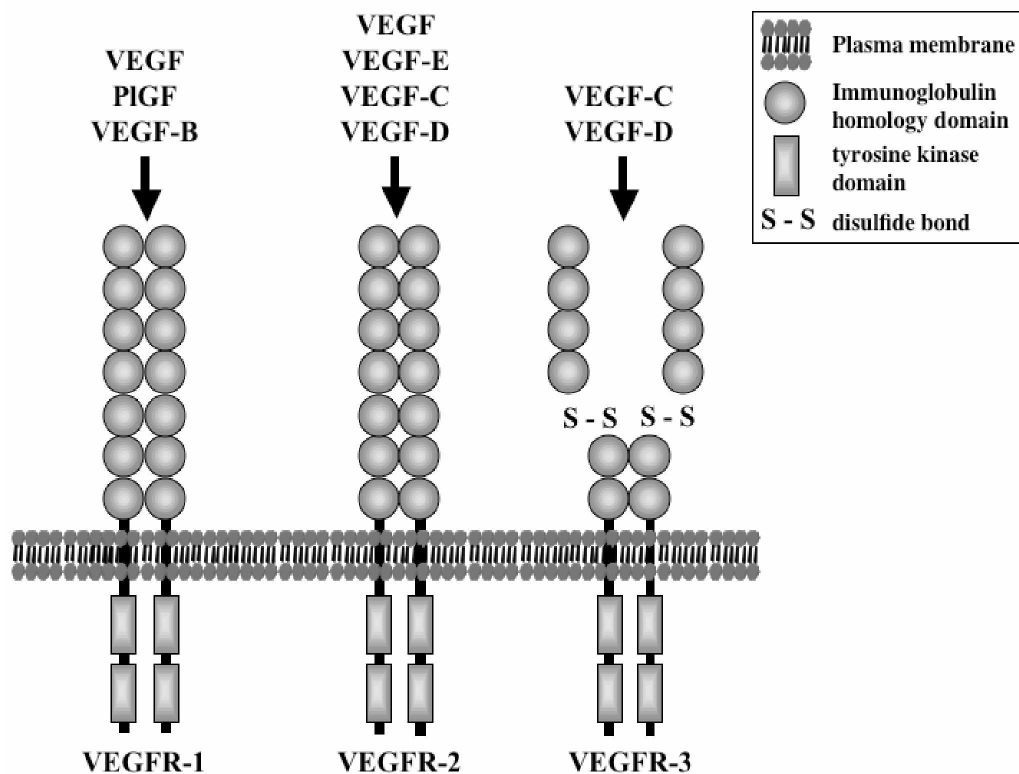

Figure 2. Receptor binding of VEGF family members to the VEGFRs.

VEGF

VEGF, also referred to as vascular permeability factor (VPF) is a major regulator of both physiological and pathological angiogenesis, as well as vascular permeability (reviewed in Ferrara, 1999b). Human VEGF exist in eight different protein isoforms, having subunit polypeptides of 121, 145, 148, 162, 165, 183, 189, and 206 amino acid residues (reviewed in Takahashi and Shibuya, 2005). The mouse isoforms are one amino acid residue shorter. VEGF is a ligand for the VEGF receptors (VEGFR)-1 and -2. In addition, $VEGF_{165}$ also binds neuropilin-1 (NP-1). The different isoforms differ in their bioavailability: whereas $VEGF_{121}$ is freely soluble and does not bind heparin, the larger isoforms contain increasingly

basic and heparin binding C-terminal domains. The major portion of secreted $VEGF_{165}$ remains bound to the cell surface and ECM, and $VEGF_{189}$ and $VEGF_{206}$ are almost completely bound in the ECM (Lee et al., 2005; Park et al., 1993). The longer isoforms can be released by proteolytic cleavage, thus increasing local VEGF concentrations during tissue growth and remodeling.

VEGF has a wide expression pattern and is produced by a variety of cell types (Ferrara, 1999a). VEGF expression is critical for the earliest stages of vasculogenesis, as blood islands, ECs, and major vessel tubes fail to develop in VEGF knockout embryos. Even loss of a single VEGF allele leads to embryonic lethality, indicating a strict dose-dependence for VEGF during development (Carmeliet et al., 1996; Ferrara et al., 1996).

VEGF induces angiogenesis by stimulating EC proliferation, migration and survival (Alon et al., 1995; Benjamin et al., 1999). Gene expression of VEGF is regulated by a variety of stimuli such as growth factors and cytokines as well as by hypoxia, transformation, p53 mutation and nitric oxide (NO) (reviewed in Takahashi and Shibuya, 2005).

PlGF

Human PlGF has been described in four isoforms PlGF-1, PlGF-2, PlGF-3, and PlGF-4 (Hauser and Weich, 1993; Maglione et al., 1991; Maglione et al., 1993). PlGF-2 and PlGF-4 bind heparin via a basic peptide insert near its C-terminus whereas PlGF-1 and PlGF-3 are non-heparin binding. PlGF homodimers bind only to VEGFR-1 (Park et al., 1994), but heterodimers of PlGF-1 and $VEGF_{165}$ have been characterized, which also can bind to soluble VEGFR-2 (Cao et al., 1996; DiSalvo et al., 1995; Park et al., 1994). PlGF-2 also binds NP-1 and -2 (reviewed in Takahashi and Shibuya, 2005). Overexpression of PlGF has been reported to induce increased vascularization and vessel permeability *in vivo* and shown to be mitogenic and chemotactic for ECs *in vitro* (Odorisio et al., 2002; Ziche et al., 1997). Loss of PlGF did not affect vasculogenesis or developmental angiogenesis, but resulted in impaired pathological angiogenesis during ischemia, inflammation, wound healing, and cancer (Carmeliet et al., 2001).

VEGF-B

VEGF-B, also referred as VEGF related factor (VRF), exists as two isoforms, $VEGF\text{-}B_{167}$ and $VEGF\text{-}B_{186}$, and it can form heterodimers with VEGF (Olofsson et al., 1996a; Olofsson et al., 1996b). VEGF-B is a ligand for VEGFR-1 and NP-1 (Makinen et al., 1999; Olofsson et al., 1998). Both forms of VEGF-B are broadly expressed, being most abundant in heart and skeletal muscle (Aase et al., 1999; Olofsson et al., 1996a). Deletion of the VEGF-B gene did not have a vascular effect, but resulted in abnormal cardiac development and function (Aase et al., 2001; Bellomo et al., 2000).

VEGF-C and VEGF-D

VEGF-C, also referred to as VEGF-related protein (VRP), and VEGF-D, also referred to as c-Fos-induced growth factor (FIGF), are the primarly lymphangiogenic factors (Achen et al., 1998; Joukov et al., 1996; Lee et al., 1996; Veikkola et al., 2001). No splicing variants have been reported in humans, while two isoforms of VEGF-D, $VEGF\text{-}D_{358}$ and $VEGF\text{-}D_{326}$, have been identified in mouse (Baldwin et al., 2001). VEGF-C and VEGF-D have precursor proteins with both N- and C-terminal propeptides flanking the VEGF homology region. They

are converted into mature forms by specific cleavage enzymes via two proteolytic steps (Joukov et al., 1997; McColl et al., 2003; Siegfried et al., 2003; Stacker et al., 1999). Upon proteolytic cleavage the affinity of VEGF-C and VEGF-D towards VEGFR-3 increases and the fully processed, mature forms also binds to and activates VEGFR-2. Interestingly, the mouse VEGF-D is a specific ligand of VEGFR-3, unable to bind VEGFR-2 (Baldwin et al., 2001). VEGF-C and VEGF-D are also able to bind NP-1 and NP-2 (Karpanen et al., 2006). Deletion of the VEGF-C gene in mice shows that VEGF-C is required for the initial steps in lymphatic development (Karkkainen et al., 2004), whereas VEGF-D-deficient mice are healthy and display no pathologic changes consistent with a defect in lymphatic function (Baldwin et al., 2005). Even though both VEGF-C and VEGF-D induce EC proliferation and migration *in vitro* (Achen et al., 1998; Joukov et al., 1996; Lee et al., 1996; Makinen et al., 2001b), only VEGF-C induces vascular permeability (Joukov et al., 1997; Stacker et al., 1999).

VEGF-C is most abundant in the heart and lung and is expressed mainly by mesenchymal cells and vascular SMCs (Karkkainen et al., 2004; Kukk et al., 1996). VEGF-D has a broader expression pattern, in the lung, heart, skeletal muscle, colon, and small intestine (Achen et al., 1998). VEGF-C acts as a highly specific lymphangiogenic factor in the mature chorioallantoic membrane (CAM) and in the skin of transgenic mice (Jeltsch et al., 1997; Oh et al., 1997). However, recombinant VEGF-C also promotes angiogenesis when applied to early CAM where lymphatic vessels have not yet developed, to avascular mouse cornea, to mouse cornea or to rabbit ischemic hindlimb (Cao et al., 1998; Rissanen et al., 2003; Witzenbichler et al., 1998). In both zebrafish and in Xenopus tadpoles, VEGF-C is required for angiogenesis (Ny et al., 2005; Ober et al., 2004). VEGF-D has been reported to promote angiogenesis in rabbit cornea and muscle, but in other systems, such as mouse skin, VEGF-D induces exclusively lymphangiogenesis (Marconcini et al., 1999; Rissanen et al., 2003; Veikkola et al., 2001).

Orf virus VEGFs and snake venom VEGF

Genes with sequence homology to VEGF have been discovered in Orf viruses and in pseudocowpox viruses. These virus-encoded VEGFs, commonly named VEGF-E, cause highly vascularized and pustular dermatitis in sheep, goats, and occasionally in humans (Lyttle et al., 1994; Meyer et al., 1999; Ogawa et al., 1998). The virus-encoded VEGFs can be separated into two groups, with VEGF-E_{D1701} and VEGF-E_{NZ2} most closely related to VEGF and PlGF, while VEGF-E_{NZ7} is similar to VEGF-C and VEGF-D (Holmes and Zachary, 2005). The virus-encoded VEGFs bind to VEGFR-2 and induce its autophosphorylation to almost the same extent as VEGF, but do not bind to VEGFR-1 (Meyer et al., 1999; Ogawa et al., 1998; Wise et al., 1999). Although VEGF-E does not play a role in vascular physiology, it has been used as a VEGFR-2 specific agonist in experimental models of angiogenesis *in vitro* and *in vivo*. Such studies have indicated that VEGF-E expression in the skin of transgenic mice results in an angiogenic phenotype (Kiba et al., 2003). Chimeric molecules composed of VEGF-E_{NZ7} and human PlGF-1 (VEGF-E_{NZ7}/PlGF) have been proposed to be potent therapeutic angiogenic factors by stimulating angiogenesis in response to tissue ischemia and by accelerating skin wound healing (Inoue et al., 2007; Zheng et al., 2006; Zheng et al., 2007)

VEGF-like proteins have also been identified from snake (viper) venom. Different snake venom VEGFs bind VEGFR-1 and/or VEGFR-2, and vary in their ability to induce

angiogenesis and vessel permeability (Gasmi et al., 2000; Gasmi et al., 2002; Junqueira de Azevedo et al., 2001; Komori et al., 1999; Takahashi et al., 2004).

Table 1. The molecular features of members of the human VEGF gene family. The asterisks indicate heparin binding. References appear in the text.

	VEGF	**PlGF**	**VEGF-B**	**VEGF-C**	**VEGF-D**
Gene (Geneatlas database)	15.9 kb	13.9 kb	3.71 kb	109.2 kb	38.86 kb
Chromosome location	6p12	14q24.3	11q13	4q34	Xp22.31
Protein splice isoforms	$VEGF_{121}$, $VEGF_{145}$*, $VEGF_{148}$*, $VEGF_{162}$*, $VEGF_{165}$*, $VEGF_{183}$*, $VEGF_{189}$*, $VEGF_{206}$ *	PlGF-1 ($PlGF_{131}$), PlGF-2* ($PlGF_{152}$), PlGF-3 ($PlGF_{203}$), PlGF-4* ($PlGF_{276}$)	VEGF-B_{167}*, VEGF-B_{186}	Precursor VEGF-C_{388}*	Precursors VEGF-D_{358}*, VEGF-D_{326}*
Sequence homology		42% sequence identity to VEGF	45% sequence identity to VEGF	30% sequence identity to VEGF	61% sequence identity to VEGF-C; 31% identical to VEGF
Dimerization	Homodimers; heterodimer with VEGF-B and PlGF	Homodimers, heterodimers with VEGF	Homodimers; heterodimers with VEGF	Homodimers; heterodimers with VEGF-D	Homodimers; heterodimers with VEGF-C

VEGFR-1

VEGFR-1, also known as Flt1 (Fms-like tyrosine kinase 1), is a 180 kDa transmembrane glycoprotein, but its mRNA can also be spliced to produce a shorter soluble protein consisting only of the first six extracellular Ig homology domains (De Vries et al., 1992; Kendall and Thomas, 1993; Shibuya et al., 1990). In the adult, VEGFR-1 is expressed mostly on the blood vascular endothelium, vSMCs, monocytes/macrophages, dendritic cells, hematopoietic stem cells, trophoblasts, and osteoclasts (reviewed in Shibuya, 2001). The VEGF binding domains have been mapped to the 2nd and 3rd extracellular Ig homology domains (Keyt et al., 1996).

Several autophosphorylation sites of VEGFR-1 have been identified but the associated downstream signaling pathways are poorly known mainly due to its weak tyrosine kinase activity (reviewed in Shibuya and Claesson-Welsh, 2006). Interestingly, the pattern of VEGFR-1 autophosphorylation induced by VEGF and PlGF were not identical, suggesting that the two ligands induce different conformational changes of the intracellular part of the receptor, or different association patterns with modifying molecules, such as heparin sulfated glycans or neuropilins (Autiero et al., 2003). Targeted inactivation of the VEGFR-1 gene

results in embryonic death at E8.5-9.0 due to overgrowth and disorganization of blood vessels (Fong et al., 1995; Fong et al., 1999). Deletion of only the intracellular domain of VEGFR-1 is compatible with normal vascular development, but impairs pathological angiogenesis such as tumor angiogenesis (Hiratsuka et al., 2001; Hiratsuka et al., 1998). VEGFR-1 signaling is also involved in monocyte/macrophage migration in response to VEGF (Hiratsuka et al., 1998) and in the reconstitution of hematopoiesis by recruiting hematopoietic stem cells (Hattori et al., 2002). These results indicate that the ligand-binding domain together with the transmembrane domain of VEGFR-1 are adequate to act as a negative effector of angiogenesis, most likely by functioning as a "decoy" receptor for secreted VEGF (Fong et al., 1999; Hiratsuka et al., 1998). VEGFR-1 activation is also reported to result in inhibition of VEGFR-2 (Gille et al., 2000; Zeng et al., 2001). Conversely, VEGFR-1 has been suggested to positively adjust VEGFR-2, possibly through heterodimerization of the receptors (Autiero et al., 2003; Carmeliet et al., 2001; Huang et al., 2001). VEGFR-1 stimulation has been shown to be involved in progression of diseases such as rheumatoid arthritis and cancer metastasis (reviewed in Shibuya and Claesson-Welsh, 2006)

VEGFR-2

VEGFR-2, denoted as KDR (kinase insert domain containing receptor) in human and Flk-1 (Fetal liver kinase 1) in mouse, is a 220 kDa protein with no reported splice variants. VEGFR-2 is the main VEGF induced signal transducer in both physiological and pathological angiogenesis, mediating proliferation, migration, and survival signals in ECs (reviewed in Shibuya and Claesson-Welsh, 2006). VEGFR-2 expression by tip cells confers VEGF sensitivity and guided migration of angiogenic sprouts during vascularization (Gerhardt et al., 2003). VEGFR-2 is essential for the differentiation of VEGFR-2 positive endothelial precursor cells into vascular ECs and VEGFR-2 gene knockout mice die at E8.5-9.0 due to lack of vasculogenesis (Shalaby et al., 1995). In addition to ECs, VEGFR-2 is expressed in hematopoietic stem cells and megakaryocytes (Katoh et al., 1995; Ziegler et al., 1999). The ligand-binding domains have been mapped to the 2nd and 3rd extracellular Ig homology domains (Fuh et al., 1998).

VEGFR-2 stimulation by VEGF leads to autophosphorylation of tyrosine residues (Y) at the carboxyterminal region of the receptor. Y1175 appears to be one of the most essential since it is a binding site for the SH2 domain of phospholipase-Cγ (PLCγ), which is then tyrosine phosphorylated and activates the downstream PKC-c-Raf-MEK-MAP-kinase pathway (Takahashi et al., 2001). Knock-in mutant mice with a Tyrosine-to-Phenylalanine substitution at position 1173 (equivalent to 1175 in humans) die at E9.0 due to lack of vasculogenesis/angiogenesis (Sakurai et al., 2005), suggesting this pathway is important *in vivo*. VEGFR-2 has also been shown to interact with the adapter molecule Shb, TSAd (T-cell specific adapter), Akt, VE-cadherin, and integrins such as $\alpha v\beta 3$ (Gerber et al., 1998; Holmqvist et al., 2004; Shay-Salit et al., 2002; Zeng et al., 2001). VEGFR-2 also mediates vascular permeability, probably via Src and Yes (Eliceiri et al., 1999). Recent studies have also suggested a role for VEGFR-2 in lymphangiogenesis (Hirakawa et al., 2005; Nagy et al., 2002).

Lately, a naturally occurring soluble form of VEGFR-2 has been detected in mouse and human plasma (Ebos et al., 2004). Similar to soluble VEGFR-1, soluble VEGFR-2 may have a regulatory effect in VEGF-mediated angiogenesis.

VEGFR-3

VEGFR-3, also denoted as Flt4 (Fms-like tyrosine kinase 4), is a 195 kDa protein, which is, unlike VEGFR-1 and VEGFR-2, proteolytically cleaved within the fifth extracellular Ig loop into a 120 kDa and a 75 kDa form during synthesis, with the two forms linked by a disulfide bond (Lee et al., 1996; Pajusola et al., 1993). The presence of a smaller transcript in humans, but not in the mouse, was shown to be due to the insertion of a human retrovirus between the last two exons of VEGFR-3 (Hughes, 2001; Pajusola et al., 1993).

VEGFR-3 stimulation leads to autophosphorylation of intracellular tyrosine residues (Dixelius et al., 2003). The adaptor molecule Shc has been shown to bind to VEGFR-3 as well as Pyk2, which leads to activation of ERK and Jun NH2-terminal kinase (Jnk) and tyrosine phosphorylation of the focal adhesion protein paxillin (Wang et al., 1997). VEGFR-3 also induces PKC dependent activation of the p42/p45 MAPK signaling cascade and PI3K dependent Akt phosphorylation to mediate growth and survival of cultured LECs (Makinen et al., 2001b). The tyrosine residue Y1068 is necessary for the activation of the VEGFR-3 kinase domain, whereas Y1063 was shown to be the site for survival signaling via the CRK/MKK4/JNK pathway and Y1230/1231 activate the classic PI3K/Akt and ERK pathways upon GRB2 reqruitment to the receptor (Salameh et al., 2005). VEGFR-3 has also been shown to heterodimerize with VEGFR-2, which leads to differential phosphorylation of the receptor and potential activation of different signal transduction pathways in comparison to the VEGFR-3 homodimeric configuration (Dixelius et al., 2003).

Loss of the VEGFR-3 gene leads to defective remodeling of the primary vascular plexus and cardiovascular failure resulting in embryonic death by E9.5 (Dumont et al., 1998). Therefore, the early embryonic function of VEGFR-3 involves blood vascular development. Later, VEGFR-3 expression becomes quite specific for LECs (reviewed in Oliver and Detmar, 2002). VEGFR-3 signaling is required for the formation of the lymphatic vasculature, as lymphangiogenesis can be inhibited with a soluble VEGFR-3 (Makinen et al., 2001a). In adults, VEGFR-3 is also expressed in fenestrated blood vessels, in monocytes/macrophages, in neural progenitor cells, and in osteoblasts (Le Bras et al., 2006; Orlandini et al., 2006; Partanen et al., 2000; Schoppmann et al., 2002; Skobe et al., 2001a). VEGFR-3 is also upregulated in tumor blood vessels (Laakkonen et al., 2007; Partanen et al., 1999; Valtola et al., 1999). Missense mutations in the VEGFR-3 gene that result in inactivation of the tyrosine kinase have been linked to primary lymphedema both in humans and in the mouse (Karkkainen et al., 2000).

Other VEGF receptors

Neuropilins

Neuropilins (NP-1 and NP-2) are widely expressed cell surface glycoproteins (Kolodkin et al., 1997). They function as receptors for the class 3 semaphorins (Sema3s), which are secreted molecules involved in axon guidance, as well as coreceptors for several VEGF family members (reviewed in Neufeld et al., 2002). NP1 binds $VEGF_{165}$, $VEGF\text{-}B_{167}$, $VEGF\text{-}B_{186}$, PlGF-2, VEGF-C, and VEGF-D (Karpanen et al., 2006; Makinen et al., 1999; Migdal et al., 1998; Ober et al., 2004; Soker et al., 1998), whereas NP-2 binds $VEGF_{165}$, $VEGF_{145}$, PlGF-2, VEGF-C, and VEGF-D (Gluzman-Poltorak et al., 2000; Karpanen et al., 2006). Due to their short cytoplasmic tails, the NPs probably have no independent signaling function, but may act as coreceptors for the VEGFRs. VEGFR-1 interacts with both NP-1

and NP-2 (Fuh et al., 2000; Gluzman-Poltorak et al., 2001), while VEGFR-2 interacts only with NP-1 (Soker et al., 2002) but not NP-2 (Karpanen et al., 2006). VEGFR-3 has been shown to interact with both NP-2 and NP-1 and NP-1 is suggested to modulate the angiogenic function of the VEGFR-2 and VEGFR-3 ligands VEGF-C and VEGF-D (Karpanen et al., 2006).

Sema3A and $VEGF_{165}$ compete with each other for NP-1 binding, and Sema3A binding to NP-1 in the presence of VEGFR-2 can inhibit VEGF-dependent angiogenesis (Miao et al., 1999). Both NPs are expressed in yolk-sac ECs during vasculogenesis (Herzog et al., 2001). Later, NP-1 is preferentially expressed in arterial ECs, while NP-2 is expressed in venous and lymphatic ECs (Herzog et al., 2001; Yuan et al., 2002). Overexpression of NP-1 in transgenic mice resulted in excess capillary formation, blood vessel dilation, and extensive hemorrhage (Kitsukawa et al., 1995). Mice defective of NP-1 die at around E13 with both severe neuronal defects and disturbed blood vascular development (Kawasaki et al., 1999; Kitsukawa et al., 1997). On the other hand, NP-2 deficint mice are viable and only have mild neuronal defects and lack small lymphatic capillaries, while blood vessels and larger lymphatic vessels are intact (Chen et al., 2000; Giger et al., 2000; Yuan et al., 2002). Double knockout of both NP-1 and -2 leads to defects in vasculogenesis and a failure to assemble the primary vascular plexus (Takashima et al., 2002).

Heparan sulphate proteoglycans

Heparan sulphate proteoglycans (HSPGs) are a constituent of the ECM and plasma membrane. One function of HSPGs is to anchor heparin binding growth factors, thus forming protein gradients and thereby altering ligand availability to their receptors. The longer splice isoforms of the VEGFs, as well as PlGF-2 and $VEGF\text{-}B_{167}$, bind heparin and heparan sulphate proteoglycans (HSPG) on the cell surface and in the ECM via a distinct heparin binding domain (Gitay-Goren et al., 1992; Hauser and Weich, 1993; Olofsson et al., 1996b; Persico et al., 1999). Heparin binding sites have also been detected in extracellular domains of both VEGFR-1 and VEGFR-2, indicating that these receptors may also associate with HSPGs on the cell surface (Cohen et al., 1995; Dougher et al., 1997).

Interaction of VEGF with HSPGs anchor VEGF to the cell surface and ECM and creates a growth factor gradient which is necessary for correct vessel guidance (Ruhrberg et al., 2002). Cell surface HSPGs might induce conformational changes of the receptor or ligand, which modifies their interaction. HSPGs might also take part in the formation of signaling complexes (Yayon et al., 1991) or in enhancing signaling properties (Jakobsson et al., 2006) or protect proteins from proteolytic degradation (Rosengart et al., 1988).

Integrins

Integrins are one of the major families of cell adhesion receptors and are essential for embryonic development, proliferation, survival, adhesion, differentiation, and migration of cells (Brakebusch and Fassler, 2003).
Integrins are heterodimeric transmembrane glycoproteins, composed of non-covalently linked α and β subunits. 18α and 8β subunits form 24 known $\alpha\beta$ heterodimers (Hynes, 2002). Most integrin receptors can bind a wide variety of ligands. On the other hand, many extracellular matrix and cell suface adhesion proteins can bind to multiple integrin receptors. Many different integrins have been shown to have important roles in VEGF-induced angiogenesis (Friedlander et al., 1995; Kim et al., 2000; Senger et al., 1997). Integrin $\alpha v\beta 3$ directly

interacts with VEGFR-2, enhancing kinase activity and mitogenic signaling (Borges et al., 2000; Soldi et al., 1999). Mice lacking integrin α9β1 die 6-12 days after birth due to chylothorax, suggesting a role for α9β1 in lymphatic development or function (Huang et al., 2000). VEGF-C and VEGF-D induced migration and adhesion of ECs is shown to be dependent on integrin α5β1 (Vlahakis et al., 2005). The ECM components collagen and fibronectin can, through their binding to β1 integrin, stimulate the tyrosine phosphorylation of VEGFR-3 receptor and induce the formation of a β1 integrin-VEGFR-3 receptor complex. Furthermore, EC migration requires signals from both the ligand-activated VEGFR-3 and the ECM-activated β1 integrin (Wang et al., 2001).

VE-cadherin

Vascular endothelial (VE)-cadherin is the transmembrane component of the adherens junctions specifically in ECs. The vascularization process is arrested in VE-cadherin-deficient embryos at a very primitive stage, indicating that VE-cadherin is required in developmental angiogenesis (Gory-Faure et al., 1999). VE-cadherin associates with VEGFR-2 upon VEGF induction or angiogenic stimulation. This association inhibits VEGFR-2 phosphorylation by phosphatases at cell-cell contacts (Grazia Lampugnani et al., 2003), leading to decreased EC growth and proliferation (Carmeliet et al., 1999; Lambeng et al., 2005). VEGFR-2-VE-cadherin association is needed for VEGF transduced survival signals of ECs via the PI3K/Akt pathway (Carmeliet et al., 1999). VEGF stimulation of VEGFR-2 triggers association with the VE-cadherin-Src complex which leads to Src phosphorylation and then VE-cadherin phosphorylation which again triggers cell-cell disruption (Lambeng et al., 2005).

5. PLATELET-DERIVED GROWTH FACTORS AND THEIR RECEPTORS

Platelet-derived growth factors (PDGF) constitute a family of disulfide-bonded dimeric growth factors: PDGF-AA, -AB, -BB, -CC, and -DD. These factors exert their cellular effects through two tyrosine kinase receptors; the structurally related PDGF α- and β-receptors. The PDGFs contain regularly spaced eight cysteine residues, the cystine knot motif, which is involved in inter- and intramolecular bonds. PDGFs are major mitogens for several cell types of mesenchymal origin and have important functions during development, including regulation of vessel formation and control of the development of organs, e.g. the lung and kidney (reviewed in Betsholtz, 2004).

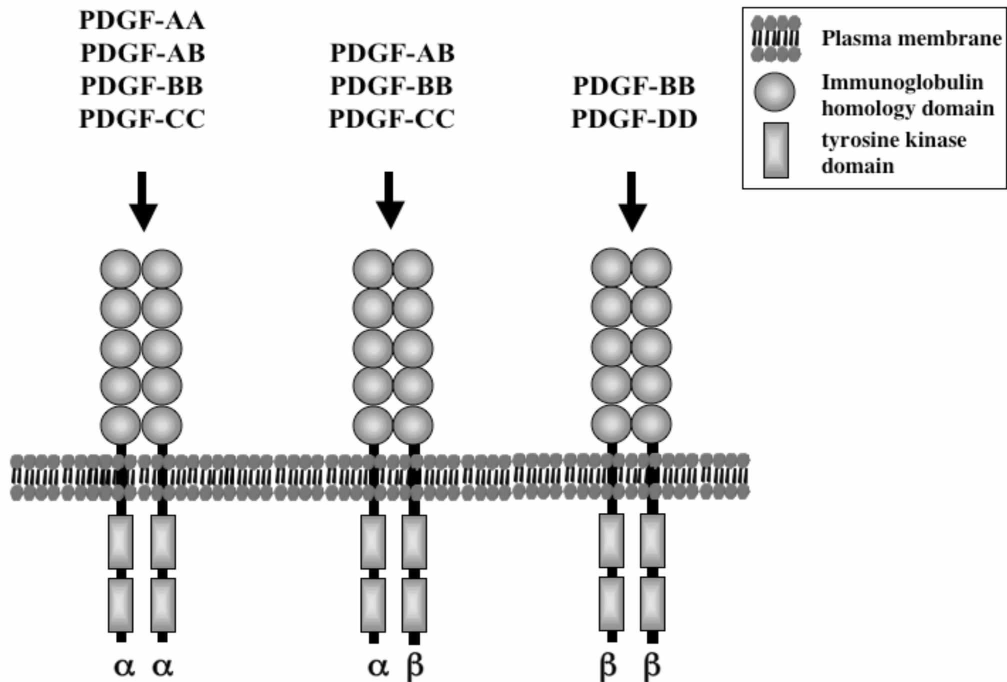

Figure 2. Receptor binding of PDGF family members to the PDGFRs.

PDGF-A and PDGF-B

PDGF was purified from platelets in the 1970s and was later found to consist of a PDGF-A and -B chain, which can act as homodimers or heterodimers. PDGF-AA activates only the PDGFR-αα receptor while PDGF-BB and the heterodimer PDGF-AB activate all three PDGFR variants, PDGFR-αα, -αβ and -ββ (reviewed in Betsholtz, 2004). The aminoterminal prodomains of these proteins are cleaved intracellularly and are secreted in an active form (reviewed in Heldin and Westermark, 1999). Both the A- and B- chains involve a basic sequence, which mediates interactions with ECM components and may act as a retention signal (Kelly et al., 1993; LaRochelle et al., 1992). PDGF is synthesized by many different cell types, such as fibroblasts, vSMCs, ECs, and macrophages (reviewed in Heldin and Westermark, 1999). Most cell types expressing PDGF make both A- and B-chains, but the expression of the two chains are independently regulated at the transcriptional as well as posttranscriptional levels (reviewed in Dirks and Bloemers, 1995).

Deletion of PDGF-A in mice leads to defective development of alveoli of the lung (Lindahl et al., 1997b). Loss of PDGF-A also results in a reduced number of intestinal villi. Secretion of PDGF-A from intestinal epitelium induces proliferation of PDGFR-α expressing mesenchymal cells (Karlsson et al., 2000). PBGF-B is expressed mainly in the developing vasculature and has a critical role in the recruitment of PCs to new blood vessels. ECs of arteries and angiogenic vessel sprouts secrete PDGF-B, which induces proliferation and migration of PDGFR-ββ expressing vascular SMCs and PCs (reviewed in von Tell et al., 2006). The deletion of PDGF-B in mice leads to embryonic death due to vascular and renal dysfunction (Lindahl et al., 1997a).

PDGF-C and PDGF-D

PDGF-C and PDGF-D are the most recently discovered growth factors in the PDGF family (Bergsten et al., 2001; LaRochelle et al., 2001; Li et al., 2000; Tsai et al., 2000). While the classical PDGF-A and PDGF-B primarily encode the growth factor domain, PDGF-C and PDGF-D encode a unique two-domain structure with N-terminal complement subcomponents C1r/C1s, urchin endothelial growth factor (EGF)-like protein and bone morphogenic protein 1 (CUB) domain (Bork and Beckmann, 1993), in addition to the C-teminal growth factor domain. PDGF-C and PDGF-D proteins form only homodimers as proteins (Bergsten et al., 2001; Li et al., 2000). PDGF-CC activates the PDGFR-αα and -αβ, while PDGF-DD is regarded as PDGFR-ββ specific (Bergsten et al., 2001; Gilbertson et al., 2001; LaRochelle et al., 2001; Li et al., 2000). Knock-out studies of PDGF-C have shown that PDGF-C has a specific role in palatogenesis and in morphogenesis of the skin (Ding et al., 2004). A PDGF-D knockout has not been reported. PDGF-C and PDGF-D have been shown to have broad expression patterns and to have potentially important roles during normal embryogenesis and maintenance of adult tissues, as well as in various pathological conditions such as renal disease, fibrosis, and cancer (reviewed in Reigstad et al., 2005).

PDGFR-α and PDGFR-β

PDGFR-α and -β have a molecular size of 170 kDa and 180 kDa, respectively, and consist of five extracellular immunoglobulin-like domains and an intracellular tyrosine kinase domain (Claesson-Welsh, 1989; Matsui et al., 1989). The receptors dimerize upon growth factor binding and can form both homo- and heterodimeric receptor complexes (Kanakaraj et al., 1991). PDGFRs are present on connective tissue cells such as fibroblasts and vSMCs, but they have also been detected on other cell types (Heldin and Westermark, 1999). PDGF receptor signaling is associated with various pathological conditions, including different fibrotic diseases and various vasculoproliferative diseases (reviewed in Bonner, 2004; Raines, 2004).

Deletion of either PDGFR leads to embryonic lethality. PDGFR-α appears to be needed for promoting the proliferation of precursor populations in cell types including lung alveolar SMCs, oligodendrocytes, intestinal villi, and dermal papillae (Lindahl, 1998). PDGFR-β null animals have severe defects in the vSMC and PC populations, similar to PDGF-B deficient mice. The most severely affected of this cell type are vSMCs and PCs in different organs such as the brain, heart, kidney, skin, and eye (Lindahl et al., 1997a; Lindahl, 1998; Soriano, 1994).

AIMS OF THE STUDY

This study was undertaken to address the role of VEGFR specific signaling in angiogenesis and lymphangiogenesis. The specific aims for the study were:

I To study VEGFR-2 induced vessel formation and establishment by PDGF-D

II To clarify the role of VEGFR-2 in lymphangiogenesis and to study angiogenesis mediated by specific VEGFR-1 or VEGFR-2 stimulation

III To study inhibition of VEGFR-3 postnatally under normal conditions and VEGFR-3 ligand dependency on postnatal lymphatic vessel maturation

IV To investigate the effects of a small-molecular tyrosine kinase inhibitor, AZD2171, on lymphangiogenesis

MATERIALS AND METHODS

The materials and methods are described in detail in the original publications, which are here referred to using Roman numerals.

Production and use of adeno-associated viruses (I)

Full-length VEGF-E, full-length and short-form of PDGF-D, full-length PDGF-B and human serum albumin cDNAs were cloned into the AAV vector. Recombinant AAVs were produced as described previously and tested *in vitro* using metabolically labeled cell lysates with analysis by immunoprecipitation and SDS-PAGE.

Fifty microliters purified AAV (5×10^{11} genomic particles/ml) was injected into the subcutis of the ear or the gastrocnemius muscle of FVB/n or NMRI nu/nu mice. Four weeks later, the mice were sacrificed and the tissues were analyzed.

The blood vessels in whole mount preparations of the AAV-transduced mouse skin were visualized using antibodies against BECs (PECAM-1) and vSMCs (SMA). The results of the whole mount analysis were confirmed by staining frozen or paraffin embedded tissue sections with antibodies against BECs (PECAM-1), vSMCs (SMA), and macrophages (F4/80). Blood vessel permeability was studied by comparing vascular leakage in ear skin after intravenous injection of a fluorescent dye.

Generation and analysis of K14-PDGF-D transgenic mice (I)

Human PDGF-D cDNA was cloned into the K14 promoter expression vector. A 5 ng/ml solution of the DNA was injected into fertilized eggs of the FVB/n-strain of mice, and the resultant transgenic mice were maintained in this strain. For the analysis of transgene expression, we examined PDGF-D mRNA levels by northern blot and protein expression by immunohistochemistry using anti-PDGF-D antibodies.

The skin was analyzed by immunohistochemistry using antibodies against BECs (PECAM-1), smooth muscle cells (SMA), lymphatic vessels (VEGFR-3 and LYVE-1), hematopoietic cells (CD45), T-lymphocytes (CD3), B-lymphocytes (B-220), granulocytes (Ly-6G), and macrophages (F4/80). Interstitial fluid pressure was measured from the skin by using the modified Wick technique. The function of skin lymphatic vessels was analyzed by fluorescent microlymphangiography.

To investigate the possible role for PDGF-D in wound healing, two circular wounds were made on both sides of the back of the mice using a 5 mm punch-biopsy tool. The wounds were allowed to heal for up to 10 days, after which the mice were sacrifized and the wounds were collected. Sections of the wounds were stained with haematoxylin and eosin. To evaluate the amount of connective tissue in skin and wound, the samples were stained with Van Gieson and Masson trichrome stains.

Analysis of blood and lymphatic vessels (II- IV)

The blood and lymphatic vessels in whole mount preparations of mouse tissue were visualized using antibodies against BECs (PECAM-1 and VEGFR-2), SMCs (SMA), or LECs (LYVE-1 and VEGFR-3).

Staged embryos of the different transgenic mice mated in the VEGFR-2+/LacZ or VEGFR-3+/LacZ background were stained for ß-galactosidase activity at 37°C.

The results from the whole mount analysis were confirmed by staining frozen or paraffin embedded tissue sections with antibodies against BECs, smooth muscle cells, inflammatory cells (CD11b), LECs, or the proliferation marker BrdU.

The effects of VEGFs on blood vessel permeability were studied by comparing vascular leakage in transgenic mouse ear skin after an intravenous injection of Evans blue dye or FITC-dextran. Microcirculation was measured from the base of the ear by a laser-doppler flowmeter. The function of skin lymphatic vessels was analyzed by fluorescent microlymphangiography.

Cell stimulation and western blot analysis (II)

Porcine aortic endothelial (PAE) cells stably overexpressing VEGFR-2 or VEGFR-3 were starved overnight in serum-free medium and incubated for 20 min with soluble VEGFR-2-Ig or VEGFR-3-Ig fusion proteins or rat monoclonal antibodies against human VEGFR-2 at a concentration of 1 µg/ml. The cells were then stimulated for 10 min with recombinant VEGF-E or VEGF-C, lysed, and immunoprecipitated with antibodies against VEGFR-3 or VEGFR-2. Phosphotyrosine-specific antibodies and antibodies against VEGFR-2 or VEGFR-3 were used for detection of proteins in western blot.

Generation and use of recombinant adenoviruses (II, III, IV)

Full length VEGF-E cDNA was cloned into the pAdapt vector. The adenoviruses encoding VEGF-E, VEGF164, VEGF165, VEGF-C, VEGFR-2-Ig, VEGFR-3-Ig, and nuclear-targeted LacZ were produced as previously described. Analysis of protein expression was performed *in vitro* using metabolically labeled cell lysates and immunoprecipitation with SDS-PAGE.

Lymphatic sprouting inhibition studies with adenoviruses (II)

2.5×10^8 pfu of recombinant adenoviruses encoding VEGF-C, VEGF-E, VEGF164, VEGF165, or LacZ were injected intradermally into female NMRI nu/nu mice. The skin was analyzed 4 days later. For inhibition experiments, 1×10^9 pfu of VEGFR-3-Ig or LacZ adenoviruses were injected intravenously 3 days prior to the ligand encoding adenoviruses.

For sprouting inhibition experiments, the mice were injected intraperitoneally with 600 µg of mF4-31C1, a rat monoclonal antibody against mouse VEGFR-3, DC101, a rat monoclonal antibody against mouse VEGFR-2, or rat IgG every second day in a volume of 200 µl. One day later, 2.5×10^8 pfu of recombinant adenoviruses encoding VEGF-C, VEGF-C156S, VEGF-E, or LacZ were injected intradermally into the ears of the same mice. The lymphatic and blood vessels in the skin were analyzed by whole mount stainings and immunohistochemistry as described above.

Matings and analysis of transgenic mice (II)

The single transgenic mice used in this study were all previously published. The aim was to study the angiogenic and lymphangiogenic effects of overexpression of different VEGFR-

specific ligands alone and in combinations. The growth factors were expressed under the K14-promoter, which has been shown to target expression of transgenes appropriately to cells of squamous epithelia. The heterozygous VEGFR-2+/LacZ and VEGFR-3+/LacZ mice were used for visualization of VEGFR-2 and VEGFR-3 expression in blood and lymphatic vessels. All the mouse strains used are listed in Table 2. The analysis of blood and lymphatic vessels in the mice were analyzed as described above.

Table 2. Transgenic mice lines used in study II.

Mouse line	Reference
K14-VEGF-E	(Kiba et al., 2003)
K14-PlGF	(Odorisio et al., 2002)
K14-VEGF-C156S	(Veikkola et al., 2001)
K14-VEGFR-3-Ig	(Makinen et al., 2001a)
K14-VEGF-D	(Veikkola et al., 2001)
VEGFR-2+/LacZ	(Shalaby et al., 1995)
VEGFR-3+/LacZ	(Dumont et al., 1998)
K14-VEGF165	(Zheng et al., 2006)
K14-VEGF-C	(Jeltsch et al., 1997)
K14-VEGF-E × K14-PlGF	II
K14-VEGF-E × K14-VEGF-C156S	II
K14-VEGF-E × K14-VEGFR-3-Ig	II
K14-VEGF-E × VEGFR-2+/LacZ	II
K14-VEGF-E × VEGFR-3+/LacZ	II

Treatment of mice with adenovirus-encoded ligand traps, blocking antibodies, or recombinant proteins (III)

The transgenic mice used in the study were VEGFR-3+/LacZ, K14-VEGF-C, K14-VEGF-C156S, and K14-VEGF-D mice and they have all been described previously. NMRI nu/nu and nu/+ mice were from obtained from Harlan.

One-, four-, or seven-day-old mouse pups were injected intraperitoneally with 5×10^8 pfu and mice 14 days or older with 1×10^9 pfu of AdVEGFR-3-Ig, AdVEGFR-2-Ig, AdLacZ, or PBS. Mice were injected intraperitoneally once a day or every second day with 30 mg/kg or 60 mg/kg of mF4-31C1, a rat monoclonal antibody against mouse VEGFR-3 that blocks ligand binding; 25 mg/kg of recombinant VEGFR-3-Ig fusion protein; 20 mg/kg AFL4, a rat monoclonal antibody against mouse VEGFR-3 that blocks ligand binding; or control antibodies in a volume of 20 to 100 µl. Mice were sacrificed at different time points and analysis of lymphatic and blood vessels was done using whole mount stainings, immunohistochemistry and lymphangiography as described above.

To test VEGFR-3-Ig binding properties, sera obtained from AdVEGFR-3-Ig-transduced mice were used to precipitate metabolically labeled VEGF-C from the conditioned medium of VEGF-C-transfected 293T cells.

Systemic half-life measurement of mF4-31C1 and VEGFR-3-Ig (III)

Female nu/nu mice were injected intraperitoneally with 20 mg/kg of mF4-31C1 or intravenously with 10 μg of recombinant VEGFR-3-Ig protein and blood samples were collected at the indicated time points. Plasma concentration of mF4-31C1 or VEGFR-3-Ig was determined using an enzyme-linked immunosorbent assay. The change in optical density was measured using a microplate reader. Pharmacokinetic parameters of mF4-31C1 were calculated by noncompartmental analysis using the WinNonlin program.

Cell stimulation, co-precipitation, and proliferation assays (IV)

For the receptor phosphorylation studies, HDMVECs were plated on 15-cm dishes pretreated with human fibronectin and grown to confluency. The cells were serum starved overnight at 37°C. AZD2171 or DMSO as a control were added to the cells and incubation carried out for 15 min at 37°C. VEGFC-156S, VEGF-E or BSA (control protein) was added to the cells at a final concentration of 500 ng/ml and the cells incubated at 37°C for 10 min. Following stimulation the cells were lysed and immunoprecipitated with anti-VEGFR-2 or anti-VEGFR-3 antibodies and analyzed by western blot. The phosphorylation of the receptors was detected using the anti-phosphotyrosine antibody.

For detection of downstream signaling mediators of VEGFR-2 and VEGFR-3 activity, HDMVECs were grown to confluency on fibronectin coated 10 cm dishes. The cells were serum-starved overnight and DMSO or AZD2171 was added and cells incubated as above before stimulation with VEGF-C156S or VEGF-E. Detection was carried out from cell lysates by western blot using anti-phospho-p44/42 MAPK, anti-phospho-Akt, and anti-phospho-CREB and anti-α-Actin antibodies.

For EC proliferation assay, the HDMVECs were seeded on fibronectin coated glass coverslips and grown to near-confluency. The medium was replaced with serum-free medium and the cells treated with the AZD2171 compound or DMSO for 15 min followed by the addition of VEGF-E or VEGF-C156S at 500 ng/ml. The incubation was continued for 20 hours. The last hour of incubation was carried out with 50 μM BrdU supplemented to the media. The cells were fixed and stained. Replicating nuclei were detected using the anti-BrdU antibody and LECs distinguished from BECs using the LYVE-1 antibody.

Inhibition of adenovirally or tumor cell-induced lymphangiogenesis and angiogenesis by the tyrosine kinase inhibitor AZD2171 (IV)

5 x 10^8 pfu of recombinant adenoviruses encoding VEGF-C, VEGF-C156S, VEGF-E, nuclear targeted *LacZ*, or GFP-tagged LNM35 (human non-small cell lung adenocarcinoma) cells suspended in Matrigel at a concentration of 2×10^5 cells/10μl were injected intradermally into 6-8 weeks old male NMRI nu/nu mice. The tyrosine inhibitor AZD2171 or 1% polysorbate (control vehicle) were given orally to the same mice once a day. The skin was analyzed 6 days later for the adenovirus treated ears and 7 days later for the tumor implanted ears. The ears were prepared for whole mount staining to visualize lymphatic and blood vessels as described above.

RESULTS AND DISCUSSION

The main results of the studies are summarized here. The results are presented and discussed in more detail in the accompanying original publications, which are here referred to using Roman numerals.

PDGF-D in blood vessel maturation and macrophage accumulation (I)

Despite its promise for treating ischemic diseases, VEGF can induce the growth of aberrant blood vessels and this could limit its therapeutic utility. In addition, some animal experiments have indicated that instead of stimulating angiogenesis, constant delivery of high levels of VEGF can result in the development of vascular malformations or hemangiomas (Dor et al., 2002; Lee et al., 2000; Pettersson et al., 2000).

Genes with sequence homology to VEGF have been discovered in Orf viruses and in pseudocowpox viruses. These virus-encoded VEGFs, commonly named VEGF-E, specifically bind VEGFR-2 but not VEGFR-1, and although VEGF-E does not play a role in vascular physiology, it can be used in experimental models to study VEGFR-2 specific responses *in vitro* and *in vivo*. VEGF-E has a strong angiogenic effect in transgenic mice (Kiba et al., 2003). We wanted to determine if we could obtain the same effect in a more therapeutially applicable model using recombinant AAVs.

AAVs encoding VEGF-E, PDGF-B, or PDGF-D were generated and shown to produce active transgene-encoded proteins in infected cell cultures. AAV-VEGF-E indeed induced a strong angiogenic effect when injected into mouse ear or into gastrocnemius muscle. On the other hand, neither PDGF-B nor PDGF-D had any angiogenic effect. Functional analysis of blood vessel permeability showed that VEGF-E induced blood vessels were leaky and the smooth muscle cell layer that surrounds the larger arteries and veins was irregular, presumably the cause for the vascular leakage. Interestingly, when we used combinations of AAV-VEGF-E and either AAV-PDGF-B or AAV-PDGF-D, the vascular leakage was significantly reduced and the smooth muscle cell layer appeared normal. This indicates that PDGF-D and PDGF-B are capable of stabilizing newly formed vessels through their effects on the SMCs.

The PDGF-D induced stabilization of VEGF-E induced new blood vessels was also confirmed in transgenic mice. When K14-VEGF-E mice (Kiba et al., 2003) were mated with the K14-PDGF-D mice the leakiness was reduced similar to the results obtained with the AAVs (M. Wirzenius and M. Uutela, unpublished data)

To study the biologic functions mediated by PDGF-D, we also created transgenic mice where full-length PDGF-D was expressed under the control of the K14 promoter in the basal epidermal keratinocytes. Our results show that overexpression of PDGF-D in skin increased macrophage recruitment into the unperturbed skin and that this effect was enhanced during the wound healing process. Previous studies have shown that PDGF-B induces macrophage migration through PDGFR-β (Siegbahn et al., 1990) although PDGF-D appeared to be more potent in stimulation of these cells.

The K14-PDGF-D mice also displayed increased interstitial fluid pressure (IFP), consistent with the fact that PDGFR-β is essential for the maintenance of steady state IFP (Pietras et al.,

2001). Tumors often have increased IFP, making it more difficult to deliver cancer chemotherapeutics. Using PDGFR-β inhibitors to lower the tumor IFP, and which would simultaneously decrease the SMC coverage of the tumor blood vessels, could have clinical benefits for cancer patients.

Since its discovery six years ago, PDGF-D has been linked to important functions both in embryogenesis and in adult tissues (reviewed in Reigstad et al., 2005). Most information on PDGF-D biology has been obtained from studies using the kidney as a model organ. PDGF-D is a likely candidate for controling the progression of metastatic renal cell carcinoma since its overexpression enhanced tumor progression and metastasis in mice. As a summary, our results indicate that PDGF-D has significant ability to regulate macrophage recruitment and participates in the control of tissue IFP. Both PDGF-D and PDGF-B improve the SMC coating of blood vessels during neoangiogenesis and decrease their permeability, thus being attractive candidates for use in local angiogenic gene therapy.

VEGFR-2 signaling in lymphangiogenesis (II)

Even though VEGF is one of the most important regulators of both physiological and pathological angiogenesis, recent studies have suggested that VEGF also can induce lymphangiogenesis (Hirakawa et al., 2005; Nagy et al., 2002). However, the signaling mechanisms mediating this response have been unclear.

We analyzed the roles of different VEGF receptors in lymphangiogenesis by using adenoviral and transgenic overexpression of VEGF family members in mouse skin, combined with the use of antibodies blocking specific VEGF receptors. In particular, overexpression of PlGF and VEGF-E allowed specific stimulation of VEGFR-1 and VEGFR-2, respectively. Our data showed that stimulation of VEGFR-2 induces hyperplasia of the lymphatic vessels but very few vessel sprouts in postnatal mice. On the other hand, we could not detect any lymphatic phenotype when only VEGFR-1 was activated by overexpression of PlGF. This indicates that the lymphatic vascular effects induced by overexpression of VEGF, as seen by us and others, are indeed mediated primarily via VEGFR-2.

VEGFR-2 expression has been shown to be high in the endothelial tip cells of blood vessels, which through their filopodia sense the VEGF gradient to guide blood vascular sprouting (Gerhardt et al., 2003). We showed that the sprouting mechanism of lymphatic vessels is not dependent on VEGFR-2 in adults. Overexpression of VEGF-E did not lead to sprouting of lymphatic vessels but rather to circumferential growth. In addition, the sprouting of lymphatic vessels induced by adenoviral VEGF-C could not be blocked with neutralizing antibodies against VEGFR-2. These results suggest that the sprouting mechanisms of lymphatic vessels in adults do not rely on VEGFR-2, and thus differ from those of blood vessels.

Surprisingly, VEGF-E did not induce changes in the lymphatic vasculature during embryonic development. Although VEGF-E stimulated lymphatic vessel enlargement in postnatal mice, we did not detect any increase in the number of lymphatic vessels in the K14-VEGF-E mice at any age of analysis. This indicates that signaling through VEGFR-2 does not induce lymphangiogenesis during embryonic development, but its effects become apparent during the postnatal period upon maturation of blood and lymphatic vessels. One possibility was

that at least some of the hyperplasia was a secondary effect resulting from the vascular leakage promoted by VEGF-E overexpression. The lymphatic hyperplasia started after the effects of VEGF-E in the blood vessels became apparent and both the vascular leakage and the lymphatic hyperplasia could be reduced by PlGF overexpression that apparently reduced blood flow via increased coating of small vessels by SMCs. However, from additional experiments we learned that vascular leakage alone was not sufficient for the hyperplasia, as the lymphatic vessels of the K14-PlGF mice appeared to be normal although their blood vessels were even leakier than those in the K14-VEGF-E mice. We cannot currently explain the late onset of the effects of the K14-VEGF-E transgene despite its high-level expression starting during the embryonic period. It may be speculated that *in vivo* particular pericellular matrix proteins and integrins are required for specific VEGFR-2 activation by VEGF-E and that they are not present until later in development.

We also found that VEGF-E overexpression was not able to rescue the lymphatic regression induced by blocking VEGF-C and VEGF-D. This result indicates that VEGFR-2 cannot substitute for VEGFR-3 signals during the onset of lymphangiogenesis. VEGFR-3 was expressed strongly in lymphatic capillaries, and it was internalized into the lymphatic ECs after adenoviral VEGF-C stimulation, but not after VEGF-E stimulation, suggesting that VEGF-E does not lead to signaling via this receptor. VEGFR-2 was expressed in both lymphatic capillaries and collecting lymphatic vessels in wild-type mice. However, VEGFR-2 was internalized in both types of vessels upon AdVEGF-E or AdVEGF-C transduction, indicating that the receptor was functional.

It is still not clear to what extent the VEGFR-2 stimulated lymphatic effect is primary or secondary. The K14 expression cassette has been shown to target expression of transgenes appropriately to epithelial cells, such as epithelial basal cells of the skin, the cornea, eyelids, ears, tongue, esophagus and forestomach (Nelson and Sun, 1983). We analyzed the blood and lymphatic vasculature also in these organs and found increased angiogenesis, but interestingly no hyperplasia of lymphatic vessels (unpublished data by M. Wirzenius). These results indicate that at least to some extent the lymphatic effects through VEGFR-2 are of secondary character. We have also observed that K14-VEGF-E+PlGF double transgenic mice developed highly vascularized hemangiomas around their mouth. These lesions also contained more and larger lymphatic vessels than the wild type skin taken from the mouth area, presumably secondary to the edema in the snout skin. The K14-VEGF-E+PlGF double transgenic mice were considerably smaller in size than the K14-VEGF-E and K14-PlGF or wild type littermate mice. We also wanted to investigate if the small size of the double K14-VEGF-E+PlGF mice affected the relative weight of the hearts of these mice. The hearts of the double K14-VEGF-E+PlGF mice were significantly bigger in relation to the size of the mice compared to their wild type littermates. The heart sizes of both K14-VEGF-E and K14-PlGF mice were normal. We did not observe any hypertrophy of the hearts of the K14-VEGF-E+PlGF mice and no differences of the blood or lymphatic vasculature between the hearts of the K14-VEGF-E+PlGF mice and their wild type littermates.

In conclusion, we showed here that overexpression of a VEGFR-2 specific ligand induces circumferential hyperplasia of the lymphatic vessels in adult, but not in embryonic skin. However, VEGFR-2 activation is not sufficient for the generation of new lymphatic vessels and it was not able to rescue the lymphatic regression induced by blocking the VEGFR-3 ligands VEGF-C and VEGF-D. These results specify the contribution of the different VEGF receptor pathways to lymphangiogenesis and reveal previously unknown postnatal changes that occur in the sensitivity of both blood and lymphatic vessels towards VEGF family

ligands. Taken together, the VEGF/VEGFR system needs to be strictly controlled during development but also in adult life.

Lymphatic vessels are independent of VEGFR-3 signaling in adults but not during postnatal lymphatic vessel maturation (III)

The spread of cancer cells through lymphatic vessels into regional lymph nodes is commonly the first step in the dissemination of human cancer. A correlation between VEGF-C expression by the primary tumor and metastasis to the sentinel lymph nodes has been reported for several types of human cancers (reviewed in Stacker et al., 2002b). Overexpression of VEGF-C and VEGF-D in experimental tumors has been shown to induce tumor lymphangiogenesis and to promote tumor metastasis (reviwed in Saharinen et al., 2004). Furthermore, VEGF-C/D-induced tumor lymphangiogenesis and lymphatic metastasis can be inhibited by blocking the interaction of VEGFR-3 with its ligands (He et al., 2002; Karpanen et al., 2001).

In this study we treated mice of different ages with adenoviral overexpression of the soluble VEGFR-3-Ig fusion protein, blocking antibodies against VEGFR-3, or recombinant VEGFR-3-Ig fusion proteins. We showed that inhibition of VEGFR-3 signaling results in regression of lymphatic capillaries and medium-sized lymphatic vessels during the first 2 weeks after birth. However, the lymphatic vasculature starts to regenerate by the age of 4 weeks.

Mice heterozygous for *VEGF-C* deficiency (Karkkainen et al., 2004), the Chy mice, which have an inactivating mutation in the *VEGFR-3* gene (Karkkainen et al., 2001), as well as mice expressing VEGFR-3-Ig under a keratin promoter in the skin (Makinen et al., 2001a) are born essentially without lymphatic capillaries, whereas their large collecting lymphatic vessels appear normal. However, in these mice, lymphatic vascular regeneration occurs in the internal organs but not in the skin starting at 2 weeks of age. Postnatal regeneration of lymphatic capillaries in most tissues, including the skin, has also been observed in mice deficient of NP-2 (Yuan et al., 2002), which acts as a co-receptor for VEGF-C. It is not yet known where lymphatic regeneration starts from in these various mice. The large lymphatic vessels in the NP-2-deficient mice and the lymphatic remnants in the AdVEGFR-3-Ig-treated mice could serve as origins of lymphatic sprouting. In contrast, the lymphatic vessels are almost completely absent from the skin of the heterozygous *VEGF-C* gene targeted mice, Chy mice, and K14-VEGFR-3-Ig mice.

Although the VEGF-C/VEGFR-3 pathway is not needed in adult mice for the growth of lymphatic vessels, it is apparently functional, because lymphangiogenesis can be stimulated by VEGF-C, when delivered for example via an adenovirus (Enholm et al., 2001) (Saaristo et al., 2002a). Possible postnatal factors responsible for the VEGF-C/VEGF-D/VEGFR-3-independent pathway could be VEGF (Hirakawa et al., 2005; Nagy et al., 2002), HGF (Kajiya et al., 2005), and the angiopoietins (Tammela et al., 2005b), which have all been shown to stimulate lymphangiogenesis. Another possibility is that the extracellular matrix provides important stabilizing growth-promoting signals for lymphatic vessels in adults, possibly mediated by integrins. It has previously been shown that integrin β1 can directly interact with VEGFR-3 and modulate its phosphorylation, thus affecting lymphatic EC migration (Wang et al., 2001). Integrin α9β1-deficient mice die 6 to 12 days after birth because of chylothorax caused by a failure of lymphatic vessels, which indicates a crucial

function for integrin $\alpha9\beta1$ in lymphatic development (Huang et al., 2000). Indeed, integrin $\alpha9\beta1$ was shown to directly bind VEGF-C and VEGF-D and to promote EC adhesion and migration (Vlahakis et al., 2005).

We also used adenoviral VEGFR-2-Ig similar to VEGFR-3-Ig in an attempt to postnatally inhibit the growth of lymphatic or blood vessels. We did not notice any effects on the lymphatic vessels, which could suggest that the VEGFR-2 signaling system in lymphatic vessels is not as active and important in postnatal life, even though signaling through VEGFR-2 has been shown to be involved in postnatal lymphatic vessel growth (Hirakawa et al., 2005; Nagy et al., 2002). Interestingly, we did not notice any effect on the blood vessels either, which is surprising considering that *VEGF* gene deletion or daily administration of VEGFR-1-Ig in postnatal mice has been shown to result in EC apoptosis, leading to severely reduced numbers of blood vessels and increased postnatal lethality (Gerber et al., 1999).

In summary, our study suggests that the blocking of VEGF-C and VEGF-D should be a safe method to inhibit tumor metastasis, because normal lymphatic vessels are not affected by such treatment in adults.

The small-molecular tyrosine kinase inhibitor AZD2171 potently inhibits lymphangiogenesis (IV)

Anti-angiogenic therapy by antibodies against the VEGFR-2 signaling system has been shown to be beneficial for treatment of some solid tumors. In addition, the formation of lymph node metastasis can be inhibited with a soluble form of VEGFR-3 or a monoclonal antibody targeting the receptor (He et al., 2002; Roberts et al., 2006). VEGFR-3 may also have a role in pathological angiogenesis, such as tumor blood vessel formation, and the use of a VEGFR-3 specific antibody has been shown to reduce primary tumor growth (Laakkonen et al., 2007). Inhibition of VEGFR-3 activity will therefore likely be therapeutically beneficial. The disadvantage of using therapies against specific molecules is the fact that tumors sometimes become resistant to treatment. In addition, the processes mediating cancer growth and progression are complex and involve a number of molecules. Inhibitors targeting multiple molecules or pathways could make it more difficult for the tumors to become resistant and therefore these drugs could be more efficacious.

AZD2171 is an indole-ether quinazoline, which competes for ATP binding on the catalytic domain of VEGFR-2, and has been shown to be an effective inhibitor of VEGF-induced signaling and angiogenesis, with additional activity against VEGFR-1 and VEGFR-3 (Wedge et al., 2005). In this study we used the specific VEGFR-2 ligand VEGF-E and the specific VEGFR-3 ligand VEGF-C156S to compare the ability to AZD2171 to block signaling from the main angiogenic and lymphangiogenic receptors. Activation of VEGFR-2 in primary human ECs by VEGF-E results in autophosphorylation of the receptor as well as increased phosphorylation of downstream signaling molecules and increased cellular functions such as proliferation, survival, migration, and tube formation. Specific activation of VEGFR-3 by VEGF-C156S results in a similar cascade of signaling events all of which could be blocked with the same dose of AZD2171 required for inhibition of VEGFR-2 activity. These results demonstrate that AZD2171 has comparable inhibitory activity against VEGFR-2 and VEGFR-3-induced cell phenotypes, and cells dependent on signaling from either receptor would be inhibited by similar doses of AZD2171.

In vivo, stimulation of VEGFR-2 by VEGF-E results in increased angiogenesis as well as lymphatic vessel enlargement. Similar to the effects of VEGF-C, VEGF-C156S induces lymphangiogenesis *in vivo*. Once daily oral treatment with AZD2171 was sufficient to block VEGF-E induced neovascularization as well as VEGF-C and VEGF-C156S induced lymphagiogenesis *in vivo*. These results suggest that physiological and pathological processes dependent on VEGFR-2 or VEGFR-3 driven angiogenesis or lymphangiogenesis would be equally affected by AZD2171 treatment. To investigate tumor cell induced lymphangiogenesis, we injected the human lung carcinoma cell line LNM35 subcutneously into the mouse ear. We observed the formation of lymphatic vessels around the VEGF-C expressing tumor cells, but lymphatic vessel formation was inhibited with AZD2171 treatment.

Tumor progression involves a number of processes each of which may be mediated by a number of different molecules. Tumor-induced neovascularization is a complex event and may require inhibition of more than one molecule. Small molecular tyrosine kinase inhibitors such as AZD2171 are therefore attractive for their ability to block activity of multiple targets. These results indicate that AZD2171 may prove beneficial in the clinics, not only by inhibiting new tumor blood vessel growth, but also by preventing the spread of cancer cells through simultaneous inhibition of tumor-associated lymphangiogenesis.

CONCLUDING REMARKS

Both blood and lymphatic vessels have been identified to play an important role in several physiological and pathological conditions and the capability to control their growth is of great importance. While anti-angiogenic therapy could be useful for patients suffering from increased angiogenesis, as in cancer or some forms of diabetes, stimulation of angiogenesis could be useful for patients suffering from heart ischemia or diabetes-related ischemia in the extremities. Similarly, while anti-lymphangiogenic therapy may be beneficial in preventing further cancer cell spread via lymphatic vessels, stimulation of lymphangiogenesis might help patients with hereditary or surgery-related lymphedema.

In the present study we have further characterized the VEGFR specific effects on angiogenesis as well as lymphangiogenesis by overexpressing different VEGFR specific ligands. We showed that while stimulation of blood vessels by the VEGFR-2 specific ligand VEGF-E lead to new and extremely permeable blood vessels, a combination of VEGF-E and either PDGF-B or PDGF-D leads to mature functional vessels. In addition, we showed that overexpression of VEGF-E induces circumferential hyperplasia of lymphatic vessels in the adult, while the VEGFR-1 specific ligand PlGF did not have any effect on lymphatic vessels. However, VEGFR-2 activation alone was not sufficient for the generation of new lymphatic vessels and it was not able to rescue the lymphatic regression induced by blocking the VEGFR-3 ligands VEGF-C and VEGF-D. Our results suggest that the sprouting mechanisms of lymphatic vessels in adults do not rely on VEGFR-2, and thus differ from those of blood vessels. These results specify the contribution of the different VEGF receptor pathways to lymphangiogenesis and reveal previously unknown postnatal changes that occur in the sensitivity of both blood and lymphatic vessels towards the VEGF family of ligands.

A challenge for future research is to find out why some members of the VEGF growth factor family are activated early during development while others are activated only later in postnatal life. Why does overexpression of VEGF-D not induce lymphangiogenesis during embryogenic development while VEGF-C does, even though their functions are both primarily mediated by VEGFR-3? Why does VEGFR-2 stimulation by VEGF-E not induce angiogenesis prenatally while VEGFR-1 stimulation by PlGF does? Can the growth factors and their receptors utilize alternative signaling systems and if so, what are these other systems? This could shed light on why for example some tumors become resistant to anti-angiogenic therapy. How these different growth factors and receptors work during pathological conditions is only starting to be elucidated.

The members of the VEGF family and their receptors appear to provide promising and versatile targets for the therapeutic manipulation of angiogenesis and lymphangiogenesis and the pathological situations associated with them. Therefore it’s extremely important to acquire a deeper understanding of the basic mechanisms connected with the different VEGF growth factors and their receptors and how they are involved in angiogenesis and lymphangiogenesis. Only this can result in the discovery of new therapies.

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