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Dissecting VEGFR-2 and VEGFR-3 function: VEGFR-3 mediates lymphangiogenic signals

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Academic dissertation

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Contents

ABBREVIATIONS **4**

LIST OF ORIGINAL PUBLICATIONS **5**

ABSTRACT **6**

REVIEW OF THE LITERATURE **7**

1. THE BLOOD VASCULAR SYSTEM 7
   Development of the blood vessels *7*
2. THE LYMPHATIC SYSTEM 8
   Lymphatic markers *8*
   Development of the lymphatic vessels *10*
3. MOLECULAR REGULATORS OF ANGIOGENESIS AND LYMPHANGIOGENESIS 11
   The VEGF family *11*
   The VEGF receptors *14*
   Other VEGF binding molecules *17*
   Molecules interacting with VEGF receptors *17*
   Molecules regulating blood vessel remodeling and stabilization *18*
4. BLOOD VESSEL GROWTH IN THE ADULT 22
   Physiological angiogenesis *22*
   Pathological angiogenesis *22*
   Arteriogenesis *23*
   Angioproliferative disease associated with virus infection *24*
   Therapeutic angiogenesis *24*
5. LYMPHATIC VESSEL GROWTH IN THE ADULT 25
   Physiological lymphangiogenesis *25*
   Pathological lymphangiogenesis *25*
   Therapeutic lymphangiogenesis *27*

AIMS OF THE STUDY **28**

MATERIALS AND METHODS **29**

RESULTS AND DISCUSSION **31**

CONCLUDING REMARKS **35**

ACKNOWLEDGEMENTS **36**

REFERENCES **37**

Abbreviations

AAV	adeno associated virus
Ad	adenovirus
Ang	angiopoietin
bFGF	basic fibroblast growth factor (FGF-2)
cDNA	complementary deoxyribonucleic acid
CAM	chorioallantoic membrane
CMV	cytomegalovirus
E	embryonic day
ECM	extracellular matrix
FIGF	c-fos induced growth factor (VEGF-D)
Flk1	fetal liver kinase 1 (VEGFR-2)
Flt1	*fms*-like tyrosine kinase 1 (VEGFR-1)
Flt4	*fms*-like tyrosine kinase 4 (VEGFR-3)
HHV	human herpesvirus
HIF	hypoxia-inducible factor
HIV	human immunodeficiency virus
HSPG	heparan sulfate proteoglycan
Ig	immunoglobulin
K14	keratin 14
kb	kilobase
kD	kilodalton
KDR	kinase insert domain containing receptor (VEGFR-2)
KS	Kaposi's sarcoma
LYVE-1	lymphatic vessel endothelial hyaluronan receptor-1
MAPK	mitogen activated protein kinase
mRNA	messenger ribonucleic acid
NP	neuropilin
PDGF	platelet derived growth factor
PDGF-R	PDGF receptor
PI3K	phosphatidylinositol 3'-kinase
PlGF	placenta growth factor
RTK	receptor tyrosine kinase
SC	spindle-shaped cell
SLC	secondary lymphoid chemokine
SMC	smooth muscle cell
Tek	tunica interna endothelial cell kinase (Tie-2)
TGF	transforming growth factor
Tie	tyrosine kinase with Ig and epidermal growth factor homology domains
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 Wise, L.M., Veikkola, T., Mercer, A.A., Savory, L.J., Fleming, S.B., Caesar, C., Vitali, A., Mäkinen, T., Alitalo, K. and Stacker, S.A.: Vascular endothelial growth factor (VEGF)-like protein from orf virus NZ2 binds to VEGFR2 and neuropilin-1. *Proc. Natl. Acad. Sci. USA* 96: 3071-3076, 1999.

II Mäkinen, T., Veikkola, T., Mustjoki, S., Kärpänen, T, Catimel, B., Nice, E.C., Wise, L, Mercer, A., Kowalski, H., Kerjaschki, D., Stacker, S.A., Achen, M.G. and Alitalo, K.: Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J*. 20: 4762-4773, 2001.

III Veikkola, T.*, Jussila, L.*, Mäkinen, T., Kärpänen, T., Jeltsch, M., Petrova, T.V., Kubo, H., Thurston, G., McDonald, D.M., Achen, M.G., Stacker, S.A. and Alitalo, K.: Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J*. 20: 1223-1231, 2001.

IV Saaristo, A.*, Veikkola, T.*, Tammela, T., Enholm, B., Kärkkäinen, M.J., Pajusola, K., Bueler, H., Ylä-Herttuala, S. and Alitalo, K.: Lymphangiogenic gene therapy with minimal blood vascular side-effects. *Submitted for publication*, 2002.

*equal contribution

Abstract

The blood and lymphatic vessels mediate the passage of fluid, metabolites and cells between different parts of the body. Although both vessel systems develop in a parallel manner and share many common features, knowledge of the molecular mechanisms controlling lymphatic growth has lagged behind the more extensively studied blood vascular system. The recent discovery of some of the key factors involved in regulating lymphatic vessel function, as well as the characterization of markers specific for lymphatic endothelium has enabled more detailed studies on the molecular control of lymphatic vascular development.

The growth and maintenance of both the blood vascular and the lymphatic vessel systems is to a large extent mediated by members of the vascular endothelial growth factor (VEGF) family via their tyrosine kinase receptors (VEGFRs) expressed on endothelial cells. While blood and lymphatic endothelial cells mostly express different VEGFRs, overlapping ligand binding patterns have made it difficult to distinguish the signaling pathways specifically involved in angiogenesis and lymphangiogenesis.

This study was undertaken to differentiate between the functions of VEGFR-2 and VEGFR-3. To achieve this goal various *in vitro* and *in vivo* models were developed. Our results show that VEGFR-3 specifically mediates lymphangiogenic signals.

Review of the literature

1. THE BLOOD VASCULAR SYSTEM

The blood vascular system consists of the heart and the blood vessels. The heart muscle pumps oxygenated blood via arteries and arterioles to the capillaries where bidirectional exchange of gases and metabolites occurs between blood and tissues. Venules and veins collect deoxygenated blood from the microvasculature and convey it back to the heart.

Development of the blood vessels

The blood circulatory system is one of the first organ systems to function during vertebrate development. Embryonic blood vessel formation is guided by poorly understood developmental cues, which give rise to a vascular network with remarkable precision and reproducibility with respect to the network basic plan, branching pattern, hierarchy of differently-sized vessels and dichotomous formation of arteries and veins (Weinstein 1999).

Vasculogenesis

The first blood vessels develop *de novo* from endothelial progenitor cells, the angioblasts, in a process called vasculogenesis. Vasculogenesis initiates when the common ancestor cells of both endothelial and blood cells, the hemangioblasts, form aggregates termed blood islands. Cells located in the middle of a blood island develop into hematopoietic precursors, while the outer cell population develops into angioblasts (Luttun et al. 2002). One of the key markers defining the angioblast is expression of VEGFR-2 (Kappel et al. 1999). Studies in quail/chick chimeras have shown that basic fibroblast growth factor (bFGF) mediates the induction of angioblasts from the mesoderm (Poole et al. 2001). In addition, VEGF and VEGFR-2 influence angioblast differentiation (Shalaby et al. 1995; Carmeliet et al. 1996; Ferrara et al. 1996), whereas VEGFR-1 suppresses hemangioblast commitment (Fong et al. 1999). Following commitment to the endothelial lineage, angioblasts may migrate extensively before assembling into a primitive vascular plexus of arteries and veins (Carmeliet 2000). Genetic studies in zebrafish have indicated that there are molecular differences in arterial and venous endothelial cells even before vessel formation. For example, signals mediated by the receptor Notch1 induce expression of the basic helix-loop-helix transcriptional repressor gridlock, which commits angioblasts to arterial fate (Lawson et al. 2001; Zhong et al. 2001). Also members of the ephrin family and their corresponding Eph receptors are important determinants of arterial and venous vessel identity during embryonic development (Luttun et al. 2002). These results indicate that vascular development may be to a large extent genetically predetermined.

Angiogenesis

When the primary vascular plexus has been formed, new capillaries are generated from the pre-existing vessels in a process termed angiogenesis. In developmental angiogenesis, new vessels form either by sprouting, splitting or merging of pre-existing vessels (Risau 1997). After the onset of circulation, the emerging vascular plexus is rapidly remodelled to resemble a more mature system with a hierarchy of large and small vessels.

2. THE LYMPHATIC SYSTEM

The lymphatic system consists of the lymphatic vessels and the lymphoid organs. The function of the lymphatic vessels is to drain protein-rich lymph and immune system cells from tissue spaces and return them to the blood circulation. The lymphatic vessels also absorb lipids from the gut. Lymphatic capillaries are lined with endothelial cells with overlapping junctions. They lack a continuous basement membrane and are therefore highly permeable. From the initial capillaries lymph passes to the collecting lymphatic vessels and finally to the thoracic duct. These larger lymphatics contain contractile smooth muscle cells, connective tissue and valves to prevent backflow. Lymphatic vessels are normally not present in avascular structures like epidermis, cartilage and cornea, nor in some vascularized organs such as brain, retina and bone marrow (Oliver and Detmar 2002). Lymphoid organs have important roles in the immune responses.

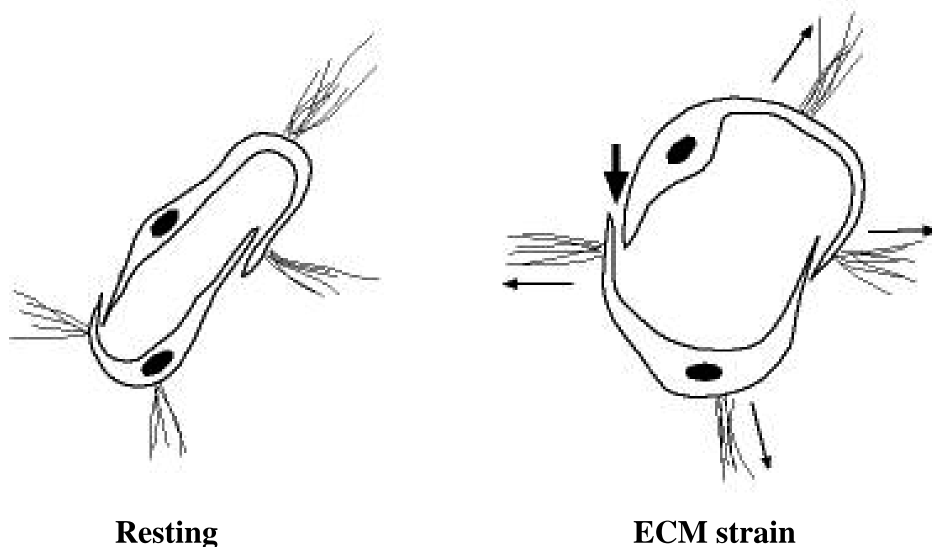

Figure 1. The "tissue pump" that enables lymph formation. Lymphatic capillaries consist of one continuous non-fenestrated endothelial cell layer. They are attached to tissue stroma via elastic fibres called anchoring filaments. When pressure within a tissue increases, these filaments distend and pull on the endothelial cells opening the gaps between adjacent cells and allowing fluid to enter. Modified from (Swartz 2001).

Lymphatic markers

The development of lymphatic vessels starts only after the formation of the blood vascular system (Wigle and Olivier 1999; Wilting et al. 2001). Until recently, the lack of specific markers has hampered the studies of the mechanisms controlling lymphatic vascular development. In the early twentieth century, Sabin proposed that isolated primitive lymph sacs originate from endothelial cells which bud from the veins during embryonic development, and that the peripheral lymphatic system originates from the primary lymph sacs by endothelial sprouting (Sabin 1902; Sabin 1904). This view has been widely

accepted and has recently gained support from genetic analysis of mouse embryos (Wigle and Olivier 1999). An alternative model suggested that the primary lymph sacs arise independently in the mesenchyme and only later establish venous connections (Huntington and McClure 1908). Support for the latter model has been obtained in birds, where it was proposed that the lymphatics of the early wing buds are not exclusively derived by sprouting from the lymph sacs but also incorporate lymphangioblasts from the embryonic mesenchyme (Schneider et al. 1999). A combination of both mechanisms may therefore contribute to the formation of the lymphatic system.

VEGFR-3

VEGFR-3 was one of the first molecular markers of the lymphatic vessels to be identified (Kaipainen et al. 1995). VEGFR-3 is a receptor tyosine kinase (RTK) initially expressed at embryonic day (E) 8.5 of mouse development in the posterior cardinal veins and in angioblasts of the head mesenchyme (Kaipainen et al. 1995). At E12.5, VEGFR-3 is expressed both in developing venous and in presumptive lymphatic endothelium, whereas in adult tissues VEGFR-3 is largely restricted to the lymphatic endothelium (Kaipainen et al. 1995; Partanen and Paavonen 2001). In addition to lymphatic endothelial cells, VEGFR-3 is expressed in fenestrated and discontinuous blood vascular endothelia (Partanen et al. 2000; Saaristo et al. 2000), and in blood capillaries of tumors and chronic wounds (Partanen et al. 1999; Valtola et al. 1999; Paavonen et al. 2000).

Podoplanin

Podoplanin, a cell surface glycoprotein, has recently been identified as a marker for the lymphatic vasculature (Breiteneder-Geleff et al. 1999). In humans, podoplanin is expressed in osteoblastic cells, kidney podocytes, lung alveolar type I cells, and lymphatic endothelial cells (Wetterwald et al. 1996; Breiteneder-Geleff et al. 1999). Also cultured lymphatic endothelial cells retain podoplanin expression (Kriehuber et al. 2001).

LYVE-1

LYVE-1 is a receptor for the extracellular matrix glycosaminoglycan hyaluronan (Banerji et al. 1999). Immunohistochemical analyses have demonstrated LYVE-1 expression on the surface of endothelial cells of lymphatic vessels (Banerji et al. 1999; Prevo et al. 2001; Wigle et al. 2002). At E10.5, LYVE-1 is expressed uniformly in the endothelial cells of the cardinal vein, but by E12.5 its expression becomes restricted to lymphatic endothelial cells (Wigle et al. 2002). LYVE-1 expression has also been detected in the sinusoidal discontinuous endothelium and the Kupffer cells of liver (Carreira et al. 2001).

SLC

Secondary lymphoid chemokine (SLC) is a soluble factor released by lymphatic endothelium, which interacts with the CC chemokine receptor 7 on lymphocytes and mediates lymphocyte homing to the lymph nodes and the spleen (Gunn et al. 1998; Gunn et al. 1999). SLC expression is first detectable at around E11.5 (Wigle et al. 2002) and it is expressed uniformly in adult lymphatic endothelia (Gunn et al. 1998).

Prox-1

The homeobox transcription factor Prox-1 was recently shown to be essential for the development of the murine lymphatic system, as Prox-1 null mice were found to be devoid of lymphatic endothelial cells (Wigle and Olivier 1999). In addition to lymphatic endothelial cells, Prox-1 is expressed in the lens, heart, liver, pancreas and the nervous

system (Wigle et al. 1999; Wigle and Olivier 1999; Sosa-Pineda et al. 2000). Both normal and tumor-associated adult lymphatic endothelial cells express Prox-1 (Wigle et al. 2002).

Development of the lymphatic vessels

Detailed analysis of Prox-1 null mice has led to formulation of a model for early lymphatic vascular development (Oliver and Detmar 2002). After the initial formation of blood vascular system, venous endothelial cells become competent to respond to a lymphatic-inducing signal. The first indication that lymphangiogenesis has begun, at around E9.5, is the specific expression of Prox-1 in a restricted subpopulation of endothelial cells located on one side of the anterior cardinal vein. At this stage, LYVE-1 is uniformly expressed in the cardinal vein endothelial cells. Around E10.5, the Prox-1 and LYVE-1 double positive cells start budding and spreading in a polarized manner (Wigle et al. 2002). These budding lymphatic endothelial cells eventually give rise to the primary lymph sacs, from which lymphatic vessels then spread to peripheral tissues of the embryo (Wigle and Olivier 1999). All venular endothelial cells may originally be bipotent, but upon the asymmetric expression of at least Prox-1 in a restricted cell population these cells become committed to lymphatic differentiation (Wigle et al. 2002). Prox-1 activity is not required for initiation of endothelial cell budding from the cardinal vein, but rather for maintaining it (Wigle and Olivier 1999). As the cells bud in a polarized manner, they start expressing additional lymphatic endothelial markers such as SLC (Wigle et al. 2002). Also VEGFR-3 expression is maintained at a high level in the budding lymphatic endothelial cells, while its expression becomes weaker in the blood vasculature. The simultaneous expression of LYVE-1, Prox-1, VEGFR-3 and SLC may indicate irreversible commitment to the lymphatic edothelial cell lineage (Wigle et al. 2002).

Additional lymphangiogenic signals are probably mediated by Ang-2, as genetic deletion of this factor in the mouse results in abnormal lymphatic patterning and function (Dr. George Yancopoulos, personal communication). In addition, mice deficient of Net, a member of the Ets-domain transcription factor family expressed in lymphatic vessels, die neonatally from insufficient lymph drainage and ensuing chylothorax (Ayadi et al. 2001). Furthermore, integrin α9β1 seems to be required for proper lymphatic development, as α9 deficient neonates die because of similar lymph drainage problems (Huang et al. 2000). The latter phenotype may relate to a co-operation between α9β1 and VEGFR-3 signaling (Wang et al. 2001). However, it is not known how the various molecules indicated in lymphatic development interact during the formation of the lymphatic vasculature.

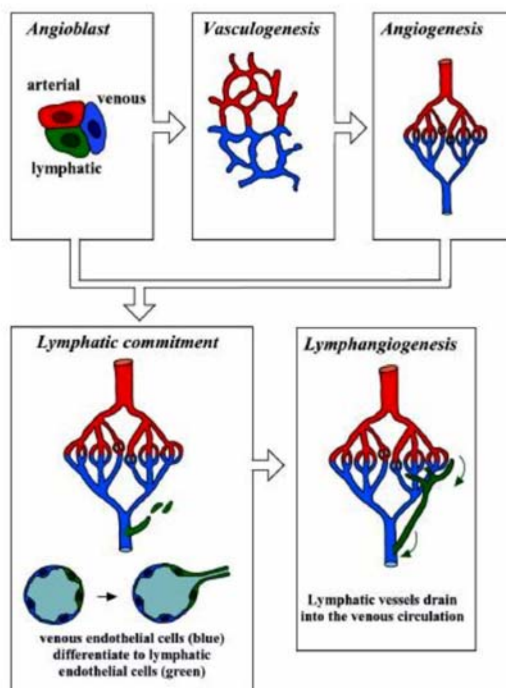

Figure 2. Schematic illustration of the development of the blood and lymphatic vascular systems. Upper panels: Prespecified arterial (red) and venous (blue) endothelial precursors proliferate, migrate and coalesce into a primitive capillary network, which subsequently remodels and expands into a mature hierarchial system of arteries and veins. Lower panels: during embryonic development, venous endothelial cells respond to putative lymphangiogenic signals and differentiate into lymphatic endothelial cells (green). Lymphatic vessels further sprout, expand, and remodel, establishing an open-ended vessel system that connects to the venous circulation. Alternatively, lymphatic angioblasts may differentiate directly into lymphatic endothelial cells and form vessels. Modified from (Alitalo and Carmeliet 2002).

3. MOLECULAR REGULATORS OF ANGIOGENESIS AND LYMPHANGIOGENESIS

The VEGF family

The members of the vascular endothelial growth factor (VEGF) family play critical roles in the growth of blood vascular as well as lymphatic endothelial cells. The VEGF family currently includes six members: VEGF, placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and the orf virus VEGF (also called VEGF-E). The VEGFs are secreted dimeric glycoproteins, all of which contain the characteristic regularly spaced eight cysteine residues, the cystine knot motif.

VEGF

VEGF (or vascular permeability factor, VPF) is a major regulator of both physiological and pathological angiogenesis (Ferrara 1999). In addition, VEGF potently induces vascular permeability, which is considered important for the initiation of angiogenesis (Dvorak et al. 1995). Endothelial cell survival in newly formed vessels is VEGF-dependent (Alon et al. 1995; Benjamin et al. 1999). Consistent with its role in cell survival, VEGF induces expression of anti-apoptotic proteins in human endothelial cells (Gerber et al. 1998).

There are multiple VEGF protein isoforms, the most common human ones having subunit polypeptides of 121, 165, and 189 amino acid recidues (Ferrara 1999). All VEGF isoforms bind to VEGFR-1 and VEGFR-2. In addition, $VEGF_{165}$ also binds neuropilin-1 (NP-1). The splice variants 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. A significant fraction of secreted $VEGF_{165}$ remains bound to the cell surface and extracellular matrix (ECM), and $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the ECM (Park et al. 1993). The longer isoforms can be released by proteolytic cleavage, thus increasing local VEGF concentrations during tissue growth and remodeling.

VEGF is expressed widely in vertebrate embryos, especially in tissues immediatedly adjacent to areas of active vessel formation (Weinstein 1999). VEGF expression is critical for the earliest stages of vasculogenesis, as blood islands, endothelial cells, and major vessel tubes fail to develop in VEGF knockout embryos (Carmeliet et al. 1996; Ferrara et al. 1996). The deletion of even a single *Vegf* allele is lethal, demonstrating a remarkably strict dose-dependence for VEGF during development (Carmeliet et al. 1996; Ferrara et al. 1996).

Isoform-specific knockouts of the VEGF gene have also been generated. Mice expressing exclusively $VEGF_{120}$ have impaired myocardial contractility, enlarged heart and defective angiogenesis, and develop ischemic cardiomyopathy (Carmeliet et al. 1999b). In adddition, endochordal bone formation and retinal vascular patterning are disturbed in these mice, suggesting that the heparin-binding isoforms of VEGF have functions which cannot be compensated by $VEGF_{120}$ (Maes et al. 2002; Stalmans et al. 2002). While mice expressing only $VEGF_{164}$ are normal and healthy, those expressing exclusively $VEGF_{188}$ display impaired arteriolar development (Stalmans et al. 2002).

Transcription of VEGF mRNA is induced by a variety of growth factors and cytokines, including epidermal growth factor, transforming growth factor-beta1 (TGF-β1) and keratinocyte growth factor-1 (Ferrara 1999). Importantly, tissue oxygen tension tightly regulates VEGF expression. Hypoxia rapidly and reversibly induces VEGF expression through both increased transcription and mRNA stabilization (Plate et al. 1992; Shweiki et al. 1992; Levy et al. 1996). Hypoxia-induced transcription is mediated by hypoxia-inducible factor-1 (HIF-1) (Ferrara 1999). Deletion of the hypoxia response element from the *Vegf* promoter results in progressive motor neuron degeneration in adult mice (Oosthuyse et al. 2001).

PlGF
Alternative splicing of human PlGF transcripts produces two protein isoforms, PlGF-1 ($PlGF_{129}$) and PlGF-2 ($PlGF_{152}$) (Maglione et al. 1991; Hauser and Weich 1993; Maglione et al. 1993). PlGF-2 contains a basic heparin binding peptide insert near its C-terminus (Hauser and Weich 1993), whereas PlGF-1 is non-heparin binding. In addition to PlGF homodimers, heterodimers of PlGF-1 and $VEGF_{165}$ have been described (DiSalvo et al. 1995; Cao et al. 1996). While PlGF homodimers bind only to VEGFR-1(Park et al. 1994), PlGF/VEGF heterodimers bind to soluble VEGFR-2 with high affinity (Park et al. 1994; Cao et al. 1996). PlGF-2 also binds to neuropilin-1 (Migdal et al. 1998).

PlGF is chemotactic for cultured endothelial cells and monocytes (Clauss et al. 1996), and purified PlGF-1 induces neovascularization in the rabbit cornea assay and in the chick chorioallantoic membrane (CAM) assay either as homodimers or as heterodimers with $VEGF_{165}$ (Oh et al. 1997; Ziche et al. 1997). Migrating keratinocytes upregulate PlGF during wound healing (Failla et al. 2000). Loss of PlGF does not affect vasculogenesis or developmental angiogenesis, but impairs pathological angiogenesis during ischemia, inflammation, wound healing and tumor growth (Carmeliet et al. 2001). PlGF may mediate its effects in pathological angiogenesis by mobilizing bone-marrow derived endothelial precursor cells (Carmeliet et al. 2001).

VEGF-B
VEGF-B (or VEGF related factor, VRF) exists as two alternatively spliced forms, VEGF-B_{167} and VEGF-B_{186}, and like PlGF, it can form heterodimers with VEGF (Olofsson et al. 1996a; Olofsson et al. 1996b). VEGF-B_{186} has a somewhat hydrophobic, but O-glycosylated C-terminus, and is freely soluble, while VEGF-B_{167} has a strongly basic heparin-binding C-terminus and is sequestered into ECM. Both VEGF-B isoforms are ligands for VEGFR-1 and NP-1 (Olofsson et al. 1998; Makinen et al. 1999). VEGF-B is widely expressed with the highest levels in heart and skeletal muscle (Olofsson et al. 1996a; Aase et al. 1999). Deletion of the VEGF-B gene does not affect vasculogenesis or angiogenesis, but may result in heart defects (Bellomo et al. 2000; Aase et al. 2001).

VEGF-C
There are no splice variants of VEGF-C (or VEGF related protein, VRP) (Joukov et al. 1996; Lee et al. 1996). Rather, VEGF-C receptor binding profile and affinity are requlated at the level of proteolytic processing. VEGF-C is produced as a preproprotein with long N- and C terminal propeptides flanking the VEGF-homology domain. Initial proteolytic cleavage of the precursor produces a form with intermediate affinity towards VEGFR-3, but a second proteolytic step is required to generate the fully processed form with a high affinity for both VEGFR-2 and VEGFR-3 (Joukov et al. 1997). VEGF-C stimulates migration and mitogenesis of cultured endothelial cells (Joukov et al. 1996; Lee et al. 1996; Joukov et al. 1997; Cao et al. 1998). Fully processed VEGF-C also induces vascular permeability via VEGFR-2 (Joukov et al. 1997; Joukov et al. 1998).

In the developing mouse embryo VEGF-C mRNA is prominent in regions where lymphatic vessels develop by sprouting from embryonic veins, and in the developing mesenterium (Kukk et al. 1996). The adjacent expression patterns of VEGFR-3 and VEGF-C suggest that VEGF-C acts in a paracrine manner during the formation of the venous and lymphatic vascular systems (Kaipainen et al. 1995; Kukk et al. 1996). When applied to mature CAM VEGF-C works as a highly specific lymphangiogenic factor (Oh

et al. 1997), and overexpression of VEGF-C in the epidermis of transgenic mice results in dermal lymphatic endothelial proliferation and vessel enlargement (Jeltsch et al. 1997). However, recombinant VEGF-C promotes angiogenesis in the early CAM where lymphatic vessels have not yet developed, in avascular mouse cornea and in rabbit ischemic hindlimb (Cao et al. 1998; Witzenbichler et al. 1998).

VEGF-D

VEGF-D (or c-Fos-inducible mitogen for fibroblasts, FIGF) is also produced as a preproprotein, and undergoes proteolytic processing in a manner similar to VEGF-C (Orlandini et al. 1996; Achen et al. 1998; Stacker et al. 1999). Fully processed human VEGF-D binds to both VEGFR-2 and VEGFR-3, and has been shown to be mitogenic for cultured microvascular endothelial cells (Achen et al. 1998) and angiogenic *in vivo* (Achen et al. 1998; Marconcini et al. 1999).

Interestingly, mouse VEGF-D differs from its human counterpart in at least two aspects. Mouse VEGF-D is a specific ligand for mouse VEGFR-3 (Baldwin et al. 2001a), and there are two isoforms of mouse VEGF-D generated by alternative RNA splicing, which differ in their C-termini (Baldwin et al. 2001b). During mouse embryonic development both VEGF-D isoforms are expressed in several structures and organs such as limb buds, heart, lung and skin (Avantaggiato et al. 1998; Farnebo et al. 1999; Baldwin et al. 2001b).

Orf virus VEGF

A gene encoding a VEGF homologue has been discovered in the genome of the parapoxvirus orf (Lyttle et al. 1994). Interestingly, two strains of the orf virus, NZ2 and NZ7, encode different variants of the viral VEGF that are only 43% identical with each other (Lyttle et al. 1994). It is unclear whether the extensive sequence difference between the two viral proteins represents independent acquisition events from different sources, or very extensive divergence from a single progenitor.

The VEGF receptors

The VEGFs mediate their signals via high-affinity RTKs, the VEGFRs. Three such receptors have been identified, VEGFR-1, VEGFR-2 and VEGFR-3. The VEGFRs are structurally and functionally similar to members of the platelet derived growth factor (PDGF) receptor family, and form a subfamily within that receptor class (Neufeld et al. 1999; Karkkainen and Petrova 2000). All VEGFRs have seven immunoglobulin (Ig) homology domains in their extracellular ligand binding part and an intracellular tyrosine kinase signaling domain split by a kinase insert. Like other receptor tyrosine kinases (RTKs), the VEGFRs are thought to dimerize and to undergo transphosphorylation upon ligand binding. Phosphorylated tyrosine residues may serve to control the kinase activity of the receptor, and to create docking sites for cytoplasmic signaling molecules, which provide substrates for the kinase.

VEGFR-1

VEGFR-1 (or Flt1) can exist either as a transmembrane glycoprotein or a shorter soluble form consisting only of the six first extracellular Ig homology domains (Shibuya et al. 1990; De Vries et al. 1992; Kendall and Thomas 1993). Soluble VEGFR-1 has an intron-derived 31 amino acid stretch in its C-terminus, and is highly conserved between mammals and birds (Kendall and Thomas 1993; Yamaguchi et al. 2002). In the adult,

VEGFR-1 is expressed mainly in blood vascular endothelium, vascular smooth muscle cells and monocytes (Shibuya 2001).

During embryonic development VEGFR-1 is first expressed in the angioblasts at E8.5 (Peters et al. 1993; Fong et al. 1995). Targeted inactivation of the VEGFR-1 gene results in increased hemangioblast commitment leading to overgrowth of endothelial-like cells 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 (Hiratsuka et al. 1998), but impairs tumor angiogenesis associated with overexpression of VEGFR-1 –specific ligands (Hiratsuka et al. 2001) in agreement with the results from the PlGF knockout mouse. Also monocyte function is defective in the tyrosine kinase deleted VEGFR-1 mice, indicating that VEGFR-1 signaling is needed to mediate monocyte migration in response to VEGF (Hiratsuka et al. 1998).

The tyrosine kinase activity of VEGFR-1 is usually weak, about one tenth that of VEGFR-2 (Shibuya 2001). This may be due to a repressor sequence in the VEGFR-1 juxtamembrane domain (Gille et al. 2000).

VEGFR-2

There are no reported splice variants for VEGFR-2 (or KDR/Flk1). VEGFR-2 is first expressed at E7.0 in hemangiogenic lateral plate mesoderm, but later becomes restricted to the blood islands (Shalaby et al. 1995). Targeted disruption of the VEGFR-2 gene results in the absence of both blood vessels and blood cells (Shalaby et al. 1995; Shalaby et al. 1997). Also in the adult VEGFR-2 is expressed both in endothelial cells and in hematopoietic stem cells (Ziegler et al. 1999).

Although the binding affinity of VEGF for VEGFR-2 is lower than for VEGFR-1, VEGF is believed to transduce its effects mainly via VEGFR-2. *In vitro*, several signal transduction molecules including phosphatidylinositol 3’-kinase (PI3K), phospholipase Cγ, Src family tyrosine kinases, focal adhesion kinase, Akt/protein kinase B, protein kinase C, extracellular signal-regulated kinase, and p38 mitogen-activated protein kinase (MAPK), are activated or modified by VEGFR-2 in primary endothelial cells (Matsumoto and Claesson-Welsh 2001). *In vivo*, activation of VEGFR-2 leads to expression of proteases required for basement membrane breakdown, upregulation of specific integrins expressed on angiogenic endothelium, and in the initiation of endothelial cell proliferation and migration (Neufeld et al. 1999). VEGFR-2 also mediates vascular permeability, probably via Src and Yes (Eliceiri et al. 1999; Paul et al. 2001).

VEGFR-3

There are two splice variants of human VEGFR-3 (or Flt4) mRNA, a 4.5 kb transcript and a more prevalent 5.8 kb transcript (Pajusola et al. 1993). The smaller one is generated by alternative splicing into the long terminal repeat of a retrovirus integrated between the last two exons of the VEGFR-3 gene (Hughes 2001). The longer transcript encodes 65 additional amino acid residues and is the major form detected in tissues. After biosynthesis glycosylated VEGFR-3 is proteolytically cleaved in the fifth Ig homology domain, but the resulting polypeptide chains remain linked by a disulfide bond (Pajusola et al. 1994; Lee et al. 1996).

Genetic disruption of VEGFR-3 results in defective remodeling of the primary vascular plexus, disturbed hematopoiesis, cardiovascular failure and embryonic death by E9.5

(Dumont et al. 1998; Hamada et al., 2000). However, differentiation of endothelial cells, formation of primitive vascular networks and vascular sprouting are normal in VEGFR-3 null embryos, indicating that VEGFR-3 function is not necessary for these early stages of vascular development (Dumont et al. 1998). VEGFR-3 signaling is required for the formation of the lymphatic vasculature, as lymphangiogenesis can be inhibited with a soluble VEGFR-3 that competes for ligand binding with the endogenous receptors (Makinen et al. 2001). Missense mutations in the VEGFR-3 gene resulting in inactivation of the tyrosine kinase have been linked to primary lymphedema both in humans and in the mouse (Karkkainen et al. 2000; Karkkainen et al. 2001b).

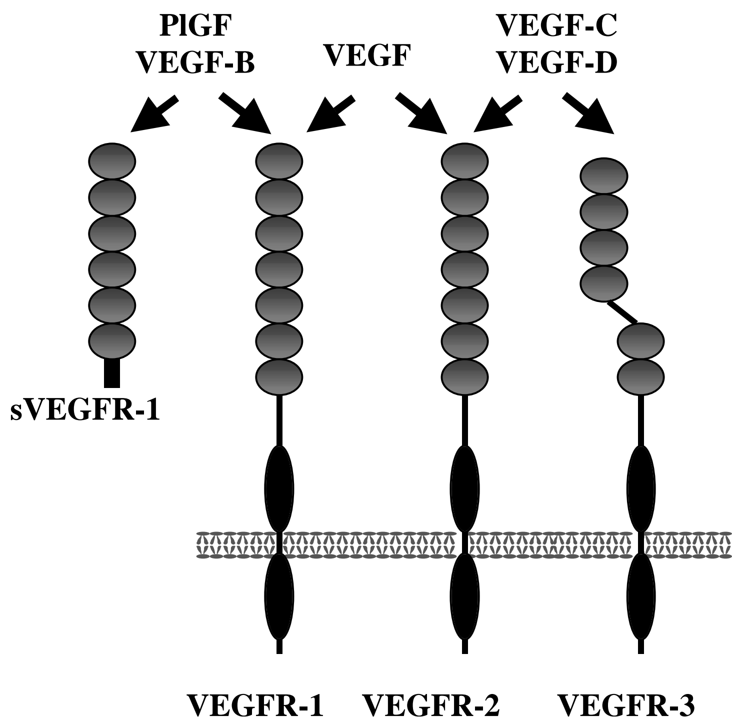

Figure 3. The VEGFs and the VEGFRs. VEGFR-1 and VEGFR-2 are primarly expressed in blood vascular endothelium, while VEGFR-3 is mostly specific for lymphatic endothelia.

Other VEGF binding molecules

Neuropilins

NP-1 and NP-2 are widely expressed cell surface glycoproteins with short cytoplasmic tails. They bind class 3 semaphorins, secreted molecules mediating repulsive signals during axon guidance, as well as several VEGF family members (Matsumoto and Claesson-Welsh 2001). The NPs are believed to be non-signaling and therefore to require the presence of a signal-transducing receptor in order to mediate their effecfts. By binding to various VEGFs the neuropilins may act as coreceptors for the VEGFRs and present the growth factors to their tyrosine kinase receptors in a favourable manner (Soker et al. 1998).

Results from various genetically modified mouse models have revealed that the NPs have important functions during blood vascular development. Constitutive overexpression of NP-1 in chimeric mice results in excess capillary formation, blood vessel dilation and extensive hemorrhaging (Kitsukawa et al. 1995). Mice deficient of NP-1 die at around E13 with both severe neuronal defects and disturbed blood vascular development (Kitsukawa et al. 1997; Kawasaki et al. 1999). While no vascular phenotype has been reported for the NP-2 deficient mice (Chen et al. 2000; Giger et al. 2000), the double knockout of NP-1 and NP-2 resuls in death at E8.5 with early defects in blood vessel development, suggesting that compensatory mechanisms operate in the single knockouts (Takashima et al. 2002). Interestingly, in developing chick embryos endothelial NP-1 expression is mostly restricted to arteries, whereas NP-2 preferentially marks veins, indicating that the NPs may play a role in determining endothelial arterial and venous identity (Herzog et al. 2001).

Heparan sulfate proteoglycans

Certain splice isoforms of the VEGFs also bind heparin and heparan sulfate proteoglycans (HSPG) via distinct heparin binding domains (Gitay-Goren et al. 1992; Olofsson et al. 1996b; Persico et al. 1999). Interestingly, a direct heparin binding site has been indentified in the extracellular domain of VEGFR-2, and HSPGs are required for $VEGF_{121}$ binding to VEGFR-1 (Cohen et al. 1995; Dougher et al. 1997), indicating that these receptors may also associate with cell surface HSPGs. The role of heparin and heparan sulfates in VEGF-VEGFR function is likely to be complex and to depend on the interacting molecules and the endothelial cell type involved (Matsumoto and Claesson-Welsh 2001).

Molecules interacting with VEGF receptors

In addition to receptors that directly bind to the VEGFs, certain cell surface molecules may participate in VEGF-mediated signaling by interacting with the VEGFRs and modulating their activity.

VE-cadherin

Vascular endothelial (VE) cadherin is an endothelium-specific transmembrane adhesive protein involved in forming adherens junctions between endothelial cells. *In vitro*, VE-cadherin participates in density-dependent inhibition of endothelial cell growth (Caveda et al. 1996) by negatively regulating VEGFR-2 signaling (Rahimi and Kazlauskas 1999). VE-cadherin mediates interactions between β-catenin, PI3K, and VEGFR-2, leading to activation of Akt and increased expression of the antiapoptotic protein Bcl2 (Carmeliet et

al. 1999a). In mouse embryos targeted inactivation of VE-cadherin or truncation of its intracellular domain impairs remodeling and maturation of the primary vascular plexus due to increased endothelial cell apoptosis, and results in embryonic lethality at E9.5 (Carmeliet et al. 1999a). Interestingly, also VEGFR-3, but not VEGFR-1, associates with VE-cadherin.

Integrin $\alpha_v\beta_3$

Integrin $\alpha_v\beta_3$ is an adhesion molecule that binds to ECM proteins containing an arginine-glycine-aspartic acid (RGD) peptide motif, such as vitronectin and fibronectin (Eliceiri 2001). $\alpha_v\beta_3$ interacts with VEGFR-2 in primary endothelial cells and $\alpha_v\beta_3$ ligation increases VEGFR-2 kinase activity and enhances VEGF-mediated mitogenic signals (Soldi et al. 1999; Borges et al. 2000). $\alpha_v\beta_3$ is expressed in growing, but not in quiescent blood vessels, and various $\alpha_v\beta_3$ antagonists, such as blocking antibodies or cyclic RGD peptides, inhibit angiogenesis (Eliceiri 2001). Surprisingly, pathological angiogenesis is enhanced in mice lacking integrin β_3 alone or in combination with β_5, demostrating that the functions of α_v integrins in the regulation of angiogenesis are still incompletely understood (Reynolds et al. 2002).

Molecules regulating blood vessel remodeling and stabilization

In addition to the endothelial cells lining the vessel lumen blood vessels contain mural cells. Depending on their morphology and density the latter are referred to as either pericytes or smooth muscle cells (SMC).

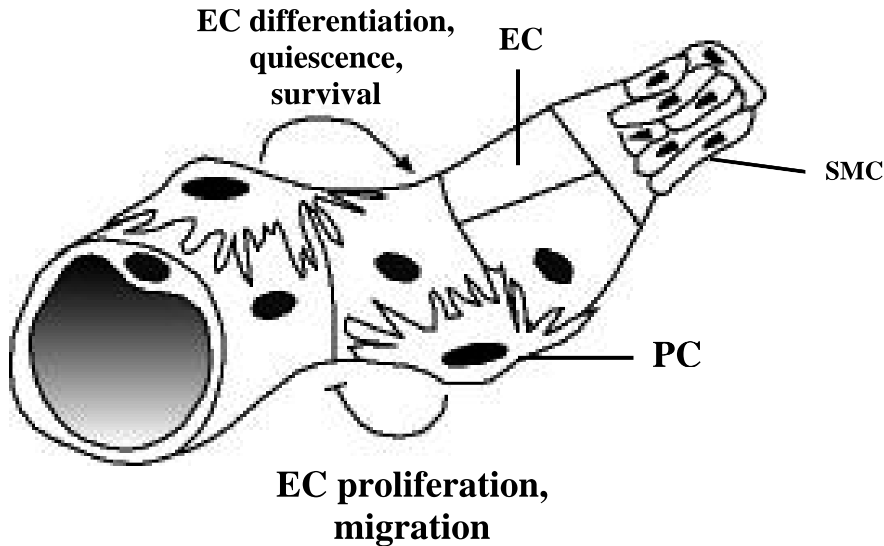

Figure 4. Endothelial-mural cell interactions. Periendothelial cells (pericytes/PC or smooth muscle cells/SMC) inhibit proliferation and migration, and induce differentiation, quiescence, and survival of endothelial cells (EC). In addition, they participate in the control of vascular tone, establish structural integrity of the vessel wall, mediate hemostasis, control vascular permeability, and determine vessel remodeling and plasticity. Modified from (Carmeliet and Collen 2000).

Pericytes are solitary SMC-like cells associated with arterioles, capillaries and venules, whereas SMCs form concentric layers around large arteries and veins (Hirschi and D'Amore 1996). The initial formation of vascular networks proceeds independently of perivascular cells, but subsequent vessel remodeling and stabilization relies on mesenchymal-endothelial short range signaling (Lindahl et al. 1998). Several gene knockout studies have demonstrated that the perivascular cells are essential for maintaining vascular integrity. These include the disruption of the endothelial cell-specific RTKs Tie-1 and Tie-2 (Dumont et al. 1994; Puri et al. 1995; Sato et al. 1995), the Tie-2 ligand angiopoietin (Ang)-1 (Suri et al. 1996), and the PDGF-B/PDGF-B receptor (PDGF-Rβ) system (Leveen et al. 1994; Soriano 1994; Lindahl et al. 1997; Hellstrom et al. 1999).

Tie receptors and angiopoietins

There are two members of the Tie class of RTKs, Tie-1 and Tie-2 (or Tek). Both have extracellular domains consisting of two Ig homology domains, three epidermal growth factor repeats and three fibronectin-type III homology domains (Loughna and Sato 2001a). The cytoplasmic region of the Tie receptors contains a tyrosine kinase domain interrupted by a kinase insert. Tie-1 and Tie-2 are predominantly expressed by vascular endothelial cells (Loughna and Sato 2001a).

Deletion of Tie-2 results in embryonic death between E9.5 and E10.5 as a consequence of insufficient expansion and maintenance of the primary capillary plexus (Dumont et al. 1994; Sato et al. 1995). In embryos lacking Tie-1, endothelial cell integrity is compromised, leading to edema, hemorrhages and death between E13.5 and birth (Puri et al. 1995; Sato et al. 1995). Interestingly, while Tie-2 deficient embryos lack periendothelial cells pericyte numbers are increased in Tie-1 deficient embryos, indicating that these receptors are required for establishing normal endothelial cell-pericyte interactions (Patan 1998). In Tie-1 and Tie-2 double knockout embryos vasculogenesis proceeds normally, but both receptors are required for angiogenesis during late embryogenesis and in the adult (Puri et al. 1999). An activating mutation in Tie-2 has been shown to cause hereditary venous malformations characterized by dilated blood vessels with deficient smooth muscle cell covering (Vikkula et al. 1996). Abnormally high Tie-2 activity may also be responsible for the formation of enlarged capillaries in the skin of mice overexpressing Ang-1 under the keratin 14 (K14) promoter (Suri et al. 1998; Thurston et al. 1999).

The angiopoietins are ligands for Tie-2 (Davis et al. 1996; Suri et al. 1996; Maisonpierre et al. 1997; Valenzuela et al. 1999). Whereas Ang-1 and Ang-4 activate Tie-2, Ang-2 and Ang-3 appear to function as specific antagonists and inhibit Tie-2 signaling. In the developing embryo, Ang-1 is expressed in the mesenchyme and smooth muscle cells surrounding the developing vasculature (Davis et al. 1996). Deletion of Ang-1 results in angiogenic defects very similar to those seen in mice lacking Tie-2, including the absence of perivascular cells (Suri et al. 1996). Importantly, double knockout of Ang-1 and Tie-1 results in asymmetrical disruption of the right-hand venous system, indicating that distinct genetic programs control the left-right polarity of the blood vascular system (Loughna and Sato 2001b). Ang-2 is highly expressed at sites of active vessel remodeling, such as the ovary and the placenta (Maisonpierre et al. 1997). These expression patterns and the phenotypes of Tie-2 and Ang-1 knockout mice have led to a model for angiogenic vessel remodeling, in which Ang-2 is required to block a constitutive stablilizing action of Ang-1, leading to detachment of perivascular cells that inhibit endothelial cell proliferation,

and allowing the blood vessels to revert to a more plastic state for initiation of angiogenesis (Maisonpierre et al. 1997).

PDGFs

Growth factors of the PDGF family are VEGF-related disulfide-bonded dimeric molecules consisting of homo- and heterodimers of A- and B-polypeptide chains (PDGF-AA, PDGF-AB and PDGF-BB), or homodimers of C- and D-chains (PDGF-CC and PDGF-DD) (Heldin et al. 2002). The PDGF isoforms exert their effects by binding to their RTKs, which consist of α and β subunits. The α subunit binds PDGF A, B and C chains, while the β subunit binds the B and D chains (Heldin et al. 2002). The different PDGF isoforms induce different receptor complexes depending on the receptor subunit types expressed by the target cell.

During the development of the blood vascular system, PDGF-B is expressed in the endothelial cells of arteries and angiogenic vessel sprouts, while its receptor PDGF-Rβ is expressed in the perivascular cells of arteries, arterioles and capillaries (Lindahl et al. 1997; Hellstrom et al. 1999). Targeted disruption of PDGF-B or PDGF-Rβ results in loss of pericytes in several organs, including the central nervous system (Lindahl et al. 1997; Hellstrom et al. 1999). As a consequence, the blood capillaries of PDGF-B null mice are dilated, contain increased numbers of endothelial cells and hemorrhage lethally in late embryogenesis (Lindahl et al. 1997). However, the dependence on PDGF-B/ PDGF-Rβ-mediated signaling for pericyte and SMC recruitment varies in a tissue-specific manner, indicating that some pericyte/SMC progenitors are likely to be formed independently of PDGF-B by endothelial cell-mediated induction of perivascular mesenchymal cells (Hellstrom et al. 1999). It is therefore believed that newly formed vessels signal to the surrounding mesenchyme and induce the formation of PDGF-Rβ positive progenitor cells. Once induced, the PDGF-Rβ positive cells respond to PDGF-B secreted by endothelial cells by proliferating and migrating along the sprouting capillaries. Differentiated perivascular cells downregulate PDGF receptors and become refractory to PDGF activity, but they may retain the ability to revert to their developmental phenotype and be resensitized to PDGF during physiological or pathological angiogenesis (Lindahl et al. 1998).

Ephrins

In contrast to VEGFs and angiopoietins, the ligands of the large Eph RTK family, ephrins, do not function as soluble molecules, but are membrane attached via either a transmembrane domain or a glycolipid anchor (Adams et al. 1999). Ephrin-Eph signaling between endothelial cells and the surrounding mesenchymal cells has been shown to be necessary for the correct formation of the cardiovascular system, both in *Xenopus laevis* and in the mouse (Adams et al. 1999; Helbling et al. 2000). In early mouse development ephrin-B2 specifically marks arterial endothelial cells, whereas its receptor EphB4 is expresed in the venous endothelium (Wang et al. 1998; Gerety et al. 1999). Full deletion of ephrin-B2, the deletion of its cytoplasmic domain only, or disruption of EphB4 results in embryonic death with defects in the remodeling of arteries and veins from the primary capillary plexus (Wang et al. 1998; Gerety et al. 1999; Adams et al. 2001). Bidirectional signaling between prespecified arterial and venous endothelial cells therefore seems to be required for normal angiogenesis.

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

Table 1. Summary of knockout studies of ligands and receptors regulating the development of the circulatory system. For references, see the text.

Later in embryonic development as well as in the adult Ephrin-B2 continues to selectively mark the arterial side of the blood vasculature. However, at these later stages its expression spreads from the endothelial cells to the surrounding smooth muscle cells and pericytes (Gale et al. 2001; Othman-Hassan et al. 2001; Shin et al. 2001). Interestingly, ephrin-B2 expression also extends into the capillaries midway between terminal arterioles and postcapillary venules, thereby challenging the classical conception that capillaries have neither arterial nor venous identity (Gale et al. 2001; Shin et al. 2001). Ehprin-B2 is also detected in microvessels in several settings of adult neovascularization including tumor angiogenesis, contrary to earlier views that such vessels arise exclusively from postcapillary venules (Gale et al. 2001; Shin et al. 2001).

4. BLOOD VESSEL GROWTH IN THE ADULT

Physiological angiogenesis

A number of physiological processes such as ovulation and menstruation require new blood vessel growth. Angiogenesis is considered to be the principal mechanism of new vessel formation in the adult. In addition, recent studies have revealed that endothelial cell precursors can be incorporated into sites of active blood vessel growth in a vasculogenesis-type process (Asahara et al. 1997; Asahara et al. 1999a; Takahashi et al. 1999), but the relative contribution of such cells to the forming neovasculature is unclear.

Angiogenesis initiates with vasodilation, a process involving nitric oxide (Carmeliet 2000). Vascular permeability increases in response to angiogenic growth factors, and extravasating plasma proteins lay down a provisional matrix for migrating endothelial cells (Dvorak et al. 1995). Periendothelial support cells detach and interendothelial connections loosen. Activated endothelial cells produce proteases that degrade the basement membrane. Proteinase action also activates or liberates growth factors sequestered within the extracellular matrix such as bFGF, VEGF and insulin-like growth factor-1, thereby further stimulating the angiogenic response (Carmeliet 2000). As a consequence, endothelial cells migrate into the tissue, proliferate and organize to form new vessels. The endothelial cells also upregulate expression of PDGF-B to attract pericytes and SMCs (Lindahl et al. 1998). Signaling by Angiopoietin-1 via Tie-2 stabilises the interaction between the endothelial cells and SMCs, and other factors such as TGF-β1 further promote vessel maturation by inhibiting endothelial cell proliferation and stimulating the production of extracellular matrix components (Pepper 1997). Vessels surrounded by basement membrane and mural cells are regarded as mature.

Pathological angiogenesis

A wide range of diseases, including neoplastic diseases, chronic inflammatory diseases and diabetic retinopathy are characterized by excessive 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 inflamed sites (Carmeliet 2000). These cells produce angiogenic factors, which stimulate the growth and migration of endothelial cells and SMCs.

Tumor angiogenesis

Mammalian cells require oxygen and nutrients for survival and therefore need to be located within the diffusion limit of oxygen (100 to 200 μm) from the nearest blood

vessel. Therefore, in order to grow to a size beyond a few cubic millimeters tumors must promote new blood vessel growth. Tumors are believed to switch on angiogenesis by increasing the expression of angiogenic factors to overcome the inhibitory effects of endogenous anti-angiogenic factors (Hanahan and Weinberg 2000). Hypoxia plays an important role in the initiation of tumor neovascularization by upregulating the expression of many angiogenic growth factors and their receptors (Semenza 2001). Circulating endothelial precursors may also contribute to tumor angiogenesis (Rafii 2000).

In contrast to normal vessels, tumor vasculature is highly disorganized and tumor blood flow is chaotic and variable (Baish and Jain 2000). Consequently, hypoxic and acidic areas often develop within tumors (Helmlinger et al. 1997). Tumor endothelial cells can be abnormally shaped, overlap each other and have large openings between the cells (Hashizume et al. 2000). Pericytes may be absent or only losely associated with the endothelial cells (Morikawa et al. 2002). These defects make tumor blood vessels leaky.

Several different strategies have been designed to inhibit tumor angiogenesis using agents that interfere with angiogenic ligands, their receptors or downstream signaling, utilize endogenous angiogenesis ingibitors (Cao 2001), or directly target tumor vasculature (Arap et al. 2002). Many of these are currently in clinical trials for the treatment of cancer. However, the efficacy of such therapeutics in human patients is still unclear.

Hypoxia-driven angiogenesis

Hypoxia-stimulated angiogenesis takes place in disorders such as myocardial infarction and peripheral ischemia. Hypoxia activates HIFs, transcription factors that function as master switches to induce the expression of several angiogenic factors including VEGF, nitric oxide synthase, PDGF, Ang-2 and others (Semenza 2001). Hypoxia-driven angiogenesis may protect the ischemic myocardium (Carmeliet and Jain 2000). However, it can also cause blindness in premature newborns (Alon et al. 1995) and in diabetic patients, or hemorrhagic rupture of atherosclerotic plaques.

Arteriogenesis

The term arteriogenesis refers to the growth of pre-existing arteriolar connections into true collateral arteries that takes place in obstructive arterial disease. Arteriogenesis differs from angiogenesis both at the cellular and the molecular level (van Royen et al. 2001). Although angiogenesis probably precedes and accompanies arteriogenesis (Ito et al. 1997), capillary sprouting alone cannot provide enough blood circulation to the underperfused tissue. Arteriogenesis initiates when shear stress increases in the collateral pathways upon narrowing of a main artery (van Royen et al. 2001). As a result of increased collateral flow, endothelial cells are activated, start expressing monocyte chemotatctic protein and upregulate cell surface receptors involved in monocyte tethering, rolling and transmigration. Monocytes are subsequently recruited to the site and infiltrate the vessel wall. They produce cytokines and growth factors which stimulate the proliferation and recruitment of endothelial cells and SMCs, such as tumor necrosis factor α, bFGF, PDGF-B and TGF-β1 (Arras et al. 1998). Arteriogenesis results in the formation of functionally and structurally normal arteries, which are able to sustain circulation and adapt to changes in physiological demands of blood supply.

Angioproliferative disease associated with virus infection

Kaposi's sarcoma

Kaposi's sarcoma (KS) is an angioproliferative disease occurring in several different forms that share the same histological traits and are all associated with infection by the KS-associated herpesvirus or human herpesvirus 8 (HHV8) (Ensoli et al. 2001). KS generally arises on the skin of the extremities as multiple patches or plaques, but mucous membranes or internal organs may also be affected. The most aggressive form of KS is found in human immunodeficiency virus type 1 (HIV-1)-infected individuals.

KS starts as an inflammatory process initiated with T cell activation and systemic production of inflammatory cytokines (Ensoli et al. 2001). Early stage KS lesions are infiltrated by immune cells and characterized by intense angiogenesis and proliferating spindle-shaped cells (SC) of endothelial and macrophage origin, which are considered to be the tumor cells of KS (Schulz et al. 2002). Inflammatory cytokines reactivate latent HHV8 in lymphocytes and monocytes. When HHV8-infected leukocytes migrate into KS lesions, they release the virus, establishing a persistent latent infection of the SC and endothelial cells (Schulz et al. 2002). These cells start expressing viral latent gene products that provide growth and antiapoptotic signals. The long-lasting expression of HHV8 latency genes together with the deregulated expression of cellular oncogenes and/or tumor suppressor genes, including *c-myc*, *bcl-2* and *p53*, are likely to participate in KS progression toward a real tumor. HHV8 may play a role in KS initiation by inducing immune responses that paradoxically enchance inflammatory cytokine production and lesion growth (Ensoli et al. 2001). The Tat protein of HIV-1 acts as a progression factor for AIDS-KS, and may be responsible for the higher frequency and aggressiveness of KS in the setting of HIV-1 infection (Barillari and Ensoli 2002).

Orf virus lesions

The orf virus is a double stranded DNA parapoxvirus that infects damaged skin of sheep, goats and humans causing contagious pustular dermatitis (Haig and Mercer 1998). The virus replicates in regenerating epidermal keratinocytes and is maintained locally. The dermal lesions show extensive vascular proliferation, blood vessel dilation and dermal swelling (Haig and Mercer 1998). Generally, orf virus infections resolve within weeks, but severe infections that fail to resolve without surgical intervention develop in immune compromised individuals (Haig 2001). In these cases, the large proliferative lesions have a tumor-like appearance.

The orf virus genome contains a gene encoding a VEGF homologue (Lyttle et al. 1994). The viral VEGF gene is expressed early after infection and its removal attenuates the disease (Savory et al. 2000). Therefore, viral VEGF is a true virulence factor. The function of viral VEGF is not fully understood, but it may participate in maintaining or expanding epidermal cells for infection (Haig and Mercer 1998).

Therapeutic angiogenesis

In most clinical settings the natural adaptive responses to poor perfusion are insufficient and unable to halt the progression of ischemic disease in the myocardium or in the extremities. The term therapeutic angiogenesis refers to the induced growth of new blood vessels as a treatment for such diseases. Several angiogenic molecules including various FGFs and VEGF isoforms have been tested in animal models for treatment of ischemic

disease, either in the form of recombinant proteins, plasmid DNA or viral vectors (Ferrara and Alitalo 1999). Also a modified constitutively active form of HIF-1α and a peptide inhibitor of HIF-1α degradation have been shown to induce angiogenesis in the setting of experimental ischemia (Li et al. 2000; Vincent et al. 2000).

While VEGF is a potent inducer of angiogenesis it also mediates vascular permeability. Lower extremity edema is the most commonly reported side-effect after VEGF gene transfer (Baumgartner et al. 2000). Intravenous injection of adenovirus encoding VEGF into mouse circulation resulted in widespread tissue edema and death (Thurston et al. 2000). However, VEGF-induced vascular leakage was blocked by simultaneous administration of adenoviral Ang-1, indicating that multifactorial therapy may be required for the generation of non-leaky vessels (Thurston et al. 2000).

Ischemia, VEGF and specific cytokines have been shown increase the mobilization of endothelial progenitor cells from the bone marrow (Asahara et al. 1999b; Takahashi et al. 1999; Kalka et al. 2000a). Endothelial progenitor cells in the systemic circulation home to sites of neovascularization (Asahara et al. 1999a; Crosby et al. 2000). *Ex vivo* expanded endothelial progenitor cells incorporate into angiogenic vessels both in rat myocardium and in mouse ischemic hindlimb, and VEGF gene transfer into the progenitor cells in culture augmented the therapeutic effect in a hindlimb ischemia model (Kalka et al. 2000b; Kawamoto et al. 2001; Iwaguro et al. 2002). Endothelial progenitor cell transplantation, possibly in combination with *ex vivo* gene transfer, might thus be used to supplement the endogenous angiogenic response.

5. LYMPHATIC VESSEL GROWTH IN THE ADULT

Physiological lymphangiogenesis

In adult tissues lymphangiogenesis appears to follow but lag behind angiogenesis. The requirement for lymphatic growth in the setting of angiogenesis can be understood by the need to drain fluid leaking out of the immature blood vessels. For example, during wound healing VEGFR-3 positive lymphatic vessels sprout from pre-existing lymphatics into the granulation tissue (Paavonen et al. 2000). Therefore, lymphangiogenesis is probably an essential feature of tissue repair and inflammatory reactions in most organs.

Pathological lymphangiogenesis

Tumor lymphangiogenesis and lymphatic metastasis

In many human cancers the lymphatic system serves as the primary route for metastatic spread of tumor cells, and metastasis to the regional lymph nodes is one of the most important indicators of tumor aggressiveness (Karpanen and Alitalo 2001). Whereas lymphatic vessels containing clusters of tumor cells are frequently observed at the periphery of malignant tumors, it is generally believed that functional lymphatics are absent within solid tumors (Jain and Fenton 2002). However, intratumoral lymphatic vessels have been documented in tumor xenografts overexpressing VEGF-C or VEGF-D (Skobe et al. 2001a; Skobe et al. 2001b; Stacker et al. 2001), and in head and neck squamous cell carcinomas (Beasley et al. 2002). On the other hand, no intra-tumoral lymphatic vessels were detected in invasive breast cancer (Jackson et al. 2001) or in mice overexpressing VEGF-C as a transgene in tumor cells (Mandriota et al. 2001). This

discrepancy may at least in part be explained by the trapping of pre-existing lymphatic vessels between the rapidly growing tumor foci in the xenografts.

A critical issue is whether the intratumoral vessels expressing lymphatic markers are functional lymphatics. Lymphangiography by intravital microscopy has been used to study lymphatic function in experimental tumors. The results indicate that while lymphatic vessels in the tumor periphery are perfused and often enlarged, those inside the tumor mostly appear compressed and non-functional (Leu et al. 2000; Padera et al. 2002). This may be due to the mechanical stress generated by the neoplastic cells grown within a confined space, which would compress the lymphatic channels inside the tumors.

VEGF-C-induced tumor lymphangiogenesis and lymphatic metastasis can be inhibited by adenoviral expression of soluble VEGFR-3, which "traps" available VEGF-C and VEGF-D (Karpanen et al. 2001). Inhibition of VEGFR-3 with neutralizing antibodies also suppressed the growth of experimental tumors by destabilizing tumor-associated angiogenic vessels without affecting the established blood and lymphatic vasculature (Kubo et al. 2000). Furthermore, tumor lymphatic spread induced by VEGF-D was blocked with an antibody specific for VEGF-D (Stacker et al. 2001). Therefore, drugs specifically targeted to peritumoral lymphatic vessels might be used to inhibit lymphatic metastasis. However, destruction of these vessels could further elevate interstitial pressure inside the tumor, which is known to interfere with the delivery of anti-cancer agents (Jain 2002).

Lymphedema

Lymphedema is a disorder caused by insufficient lymph drainage, which results in the build-up of protein rich fluid in tissues causing chronic swelling of the extremities (Witte et al. 2001). Lymphedema patients also suffer from dermal fibrosis, accumulation of adipose tissue, impaired wound healing and increased susceptibility to infections (Witte et al. 2001). Etiologically lymphedema can be divided into two main categories. Primary lymphedema is a rare developmental disorder characterized by hypoplasia of the cutaneous lymphatic vessels. Missense mutations in the VEGFR-3 gene that interfere with VEGFR-3 tyrosine kinase function have been identified in some cases of hereditary, early-onset lymphedema (Milroy's disease) (Karkkainen et al. 2000). However, primary lymphedemas are a heterogenous group of disorders and several other genetic loci have also been implicated in Milroy's disease and other lymphedema syndromes. For example, inactivating mutations in the transcription factor FoxC2 have been reported in lymphedema distichiasis characterized by congenital lymphedema, a double row of eyelashes and, in some cases, additional complications such as cardiac defects (Fang et al. 2000). Secondary or acquired lymphedema can develop when lymphatic vessels are blocked or damaged by infection or radiation therapy, or when lymph nodes are surgically removed. Secondary lymphedema is a relatively common disorder with estimated 3 to 5 million patients in the USA alone.

A mouse model for Milroy's disease has been obtained by chemical mutagenesis. Like some of the human lymphedema patients these Chy mice are heterozygous for a germline inactivating mutation in the VEGFR-3 tyrosine kinase domain (Karkkainen et al. 2001b). The Chy mice have hypoplasia of the dermal lymphatic vessels, develop subcutaneous fluid and demostrate swelling of the paws. In addition, the function and integrity of the intestinal lymphatic vessels are compromized resulting in accumulation of chylous fluid in the peritoneal cavity (Karkkainen et al. 2001b).

Therapeutic lymphangiogenesis

At present, therapeutic options for the management of lymphedema are limited, consisting mainly of physiotherapy, massage and external compression (Witte et al. 2001). The discovery of specific genes involved in the regulation of lymphatic function and in the pathology of lymphedema has led to experimental approaches for treating this disease. Adenoviral VEGF-C gene transfer into normal mouse 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 the Chy mice (Karkkainen et al. 2001b). However, high levels of VEGF-C also caused blood vascular effects such as increased vessel leakiness, presumably through the interaction of VEGF-C with VEGFR-2 expressed on blood vascular endothelium (Saaristo et al. 2002).

Aims of the study

I Biochemical characterization of the orf virus VEGF variant NZ2

Orf virus lesions feature angiogenesis, vasodilation and dermal swelling. These characteristics may result from the action of orf virus encoded VEGF homologue, but the biochemical properties of the viral VEGF were not known. We produced recombinant orf virus VEGF variant NZ2 and tested it in a variety of assays to study its receptor specificity and functions.

II Isolation and culture of primary lymphatic endothelial cells

In adult tissues VEGFR-3 is mainly expressed in lymphatic endothelium, but the signaling pathways activated by VEGFR-3 have been incompletely characterized due to lack of cultured lymphatic endothelial cells. We isolated primary lymphatic endothelial cells from mixed cultures of dermal microvascular endothelial cells and studied VEGFR-3 –mediated signals in these cells.

III Characterization of VEGFR-3 *in vivo* function

VEGF-C and VEGF-D are ligands for VEGFR-3, but they also bind to and activate VEGFR-2. In order to differentiate between the signals mediated by VEGFR-2 and VEGFR-3 *in vivo*, we generated transgenic mice overexpressing VEGF-D or the VEGFR-3 specific mutant form of VEGF-C, VEGF-C156S (Joukov et al. 1998), under the K14-promoter and analysed their dermal blood vascular and lymphatic phenotypes.

IV Lymphangiogenic gene therapy specifically targeting VEGFR-3

VEGF-C is known to stimulate lymphangiogenesis *in vivo*, but high levels of VEGF-C also induce blood vascular effects (Saaristo et al. 2002). In order to develop a lymphatic-specific gene therapy approach, we compared lymphangiogenesis induced by VEGF-C and VEGF-C156S in different *in vivo* models.

Materials and Methods

An overview of the methods used in these studies is presented here. For more detailed information, please refer to the original articles.

Expression and purification of recombinant orf virus VEGF variant NZ2 (I)
A DNA fragment containing nucleotides 4-401 of the VEGF-like gene from orf virus strain NZ2 was prepared by PCR and inserted into the pEFBOS-I-Flag expression vector. The protein was expressed transiently in COS cells and recovered from cell culture medium by anti-Flag chromatography.

Bioassay for VEGFR stimulation (I, II)
Ba/F3 cells expressing chimeric receptors containing the extracellular domains of either VEGFR-1, VEGFR-2 or VEGFR-3 and the transmembrane and cytoplasmic domains of the mouse erythropoietin receptor were distributed to 96-well microtiter plates containing dilutions of the test reagent or medium alone. After 48 h, cell viability was determined.

Endothelial cell apoptosis and migration assays (II)
For the apoptosis assay, 70 000 cells were seeded into 24-well plates. Treatments were carried out in duplicate and apoptosis was detected by measuring cytoplasmic histone-associated DNA fragments.

Migration assays were performed in a 48-well chemotaxis Boyden chamber. The cells were suspended in serum-free medium, 10 000 cells per well were allowed to migrate for six hours at +37°C, and the number of migrated cells was counted.

Receptor binding and activation analysis (I, III, IV)
The binding of various VEGFs to human and mouse receptors was assessed by precipitation from metabolically labelled transfected cell conditioned medium using soluble recombinant receptor-Ig fusion proteins. The complexes were adsorbed to protein-A sepharose and analysed by SDS-PAGE and autoradiography.

Receptor stimulation was carried out using serum-starved cells. The growth factors were diluted in serum-free media and the cells were stimulated for ten minutes at +37°C. Cell lysates were immunoprecipitated with antisera specific for VEGFR-2 or VEGFR-3 and analysed for tyrosine phosphorylation with Western blotting using phosphospecific antibodies.

Isolation of lymphatic and blood vascular endothelial cells (II)
Monoclonal antibodies against VEGFR-3 (clone 2E11) or polyclonal affinity-purified anti-podoplanin antibodies were used to isolate lymphatic endothelial cells from cultures of human microvascular endothelial cells. MACS colloidal super-paramagnetic MicroBeads conjugated to rat anti-mouse IgG1 or goat anti-rabbit IgG antibodies, MACS-MS separation columns and MiniMACS separator were used for cell sorting.

Generation of recombinant viruses (IV)
For the adenovirus constructs, full length human VEGF-C and VEGF-C156S cDNAs were cloned under the CMV promoter. Replication-deficient, E1-E3 deleted adenoviruses were produced in 293 cells and concentrated by ultracentrifugation. Adenovirus encoding nuclear-targeted *LacZ* was used as a control virus. For the AAV constructs, full length

human VEGF-C and VEGF-C156S were cloned under the CMV promoter and recombinant AAVs (serotype 2) were produced as previously described (Karkkainen et al. 2001b). AAV encoding enhanced green fluorescent protein was used as a control.

Mouse models (III, IV)
For generation of transgenic mice, the cDNAs encoding full-length human VEGF-D and VEGF-C156S were cloned into a human K14 promoter expression cassette (Vassar et al. 1989) and injected into fertilized mouse oocytes of the strain FVB/NIH. Several independent inbred transgenic lines were generated and those with high levels of transgene mRNA expression were used for phenotype analysis. The generation of the K14-VEGF-C mice has been described previously (Jeltsch et al. 1997).

Immunodeficient athymic NMRI nu/nu mice were used for the initial studies of viral VEGF-C and VEGF-C156S gene transfer. The Chy lymphedema mice were used for analyzing the therapeutic potential of VEGF-C156S. VEGFR-2+/LacZ (Shalaby et al. 1995) and VEGFR-3+/LacZ (Dumont et al. 1998) mice were used for the analysis of VEGFR-2 and VEGFR-3 expression in the lymphatic vessels.

Analysis of blood and lymphatic vessels (III, IV)
Blood vessels in whole mount preparations of mouse skin were visualised either by biotinylated *Lycopersicon esculentum* lectin or by immunohistochemical staining using antibodies against PECAM-1. Lymphatic vessels were visualised either with antibodies against VEGFR-3 or by X-gal staining in heterozygous VEGFR-3+/LacZ mice. Whole mount analysis results were confirmed by staining tissue sections with antibodies against mouse podoplanin, VEGFR-2, VEGFR-3, LYVE-1, and PECAM-1.

The effects of VEGF-C and VEGF-C156S on blood vessel permeability were studied by comparing vascular leakage in infected ear skin after an intravenous injection of Evans blue dye. Statistical analysis was performed using Student's *t*-test. The function of skin lymphatic vessels was analysed by fluorescent microlymphangiography or by Evans blue injection.

Results and discussion

I. Orf virus VEGF variant NZ2 is a ligand for VEGFR-2 and NP-1

Amino acid sequence alignment of the orf virus VEGF variant NZ2 with other VEGF family members revealed that it carries the characteristic cysteine knot motif present in all mammalian VEGFs. Recombinant Flag-tagged orf virus VEGF was produced in mammalian cells and determined to be a glycosylated disulfide-linked homodimer with a monomeric molecular mass of approximately 25 kD. Orf virus VEGF bound to soluble VEGFR-2 and induced VEGFR-2 tyrosine phosphorylation in transfected cells, but failed to bind to VEGFR-1 or VEGFR-3. In addition, it bound to soluble NP-1. Orf virus VEGF was mitogenic for primary endothelial cells in culture and induced vascular permeability in the Miles assay.

These results demonstate that orf virus VEGF is a biologically active member of the VEGF family with a unique receptor binding profile. Interestingly, the amino acid residues which in VEGF have been determined to be critical for VEGFR-1 binding are partially conserved in orf virus VEGF, whereas those involved in binding to VEGFR-2 are not (Keyt et al. 1996). This suggests that the VEGFR-2 binding sites for orf virus VEGF and VEGF differ.

The orf virus VEGF variants NZ7 and D1701 have also been biochemically characterized (Ogawa et al. 1998; Meyer et al. 1999). Both were shown to be specific ligands for VEGFR-2, and to induce endothelial cell proliferation and vascular permeability (Ogawa et al. 1998; Meyer et al. 1999). Orf virus VEGF resembles $VEGF_{121}$ in that both lack heparing binding affinity, but whereas $VEGF_{121}$ has much reduced mitogenic activity in comparison to $VEGF_{165}$, orf virus VEGF potency was similar to that of $VEGF_{165}$. This may be due to orf virus VEGF-induced interaction between NP-1 and VEGFR-2 resulting in enhanced VEGFR-2 mitogenic signaling. Phylogenetically orf virus VEGF variants constitute a novel subfamily of the VEGF growth factors, distinct but most closely related to VEGF (Meyer et al. 1999). All orf virus VEGFs have a conserved C-terminal threonine- and proline-rich motif that is not present in any mammalian VEGF (Meyer et al. 1999). The variants NZ2 and D1701 are closely related, but NZ7 represents an exceptionally divergent sequence.

II VEGFR-3 mediates growth, survival and migration in cultured lymphatic endothelial cells

Based on differential expression of cell surface markers, cultured human dermal microvascular endothelial cells were shown to consist of distinct populations of lymphatic and blood vascular endothelial cells. Antibodies against the lymphatic-specific markers VEGFR-3 and podoplanin were used to isolate lymphatic endothelial cells from these cultures. In the absence of added growth factors the lymphatic endothelial cells did not proliferate whereas VEGF and VEGF-C promoted the growth of these cells. In apoptosis assays VEGF, VEGF-C and VEGF-C156S protected the lymphatic endothelial cells from serum starvation-induced apoptosis, while only VEGF and VEGF-C mediated survival of the VEGFR-3 negative blood vascular endothelial cells. Both cell populations were shown to express VEGFR-2. VEGFR-3 signaling was found to induce via PI3K the activation of the serine-threonine kinase Akt, a major mediator of cell survival. VEGFR-3 stimulation also induced p42/p44 MAPK activation, although MAPK phosphorylation was much

stronger in cells treated with the VEGFR-2 ligands VEGF and VEGF-C. MAPK activation was mediated by PKC, as a specific inhibitor of PKC completely abolished p42/p44 activation induced by VEGF, VEGF-C and VEGF-C156S. Whereas both blood vascular and lymphatic endothelial cells migrated towards VEGF and VEGF-C, only the VEGFR-3 positive lymphatic endothelial cells migrated towards VEGF-C156S in the Boyden chamber assay.

This was the first time when specific molecular markers were used to isolate lymphatic endothelial cells. Our results demonstrate that VEGFR-3 stimulation alone is sufficient to protect lymphatic endothelial cells from apoptosis, and to increase their growth and migration. VEGFR-3 also induced two signaling cascades important for cell proliferation and survival, the PI3K mediated Akt phosphorylation and the PKC-dependent p42/p44 MAPK activation. *In vivo*, inhibition of VEGFR-3 signaling causes regression of the developing lymphatic vessels due to increased lymphatic endothelial apoptosis, further demonstrating the importance of VEGFR-3 mediated survival signals (Makinen et al. 2001).

An independent study demonstrating the isolation of primary lymphatic endothelial cells using podoplanin as a specific marker was also published (Kriehuber et al. 2001). In accordance with our findings, Kriehuber and co-workers demonstrated that only the lymphatic cell population expresses substantial levels of VEGFR-3. In addition, they showed that the lymphatic endothelial cells constitutively secrete SLC. On the other hand, while in our study LYVE-1 was expressed only in a subset of the lymphatic endothelial cells, Kriehuber and co-workers observed uniform LYVE-1 expression on the lymphatic endothelial population. Importantly, in both our work and in that of Kriehuber and co-workers', blood vascular endothelial cells were the sole source of VEGF-C. *In vivo*, the regeneration of lymphatics after injury follows that of the blood vessels with a delay (Paavonen et al. 2000), but the reason for this has remained enigma. In the light of these data it seems that the re-established blood vasculature may directly induce lymphatic endothelial proliferation and lymphangiogenesis by secreting VEGF-C.

III VEGFR-3 mediates lymphangiogenesis in transgenic mice

Human VEGF-C156S was shown to be a specific ligand for mouse VEGFR-3, whereas human VEGF-D binds to both mouse VEGFR-2 and VEGFR-3. We established transgenic mouse lines expressing high levels of human VEGF-D or VEGF- C156S under the K14 promoter, which directs transgene expression to the basal keratinocytes. In both the K14-VEGF-D and the K14-VEGF-C156S mice, large spaces were detected in the upper epidermis. These spaces were lined with endothelium positive for the lymphatic markers VEGFR-3 and LYVE-1, and were devoid of red blood cells. In whole mount analysis, the skin blood vasculature of both the K14-VEGF-D and the K14-VEGF-C156S mice appeared normal, whereas the superficial lymphatic capillaries were greatly dilated. These hyperplastic lymphatic capillaries were, however, functional in that they took up and transported fluorescent dextran and Evans blue dye after an intradermal injection. We did not detect transgene-encoded proteins in systemic circulation, and the lymphatic phenotype of distant organs remained unchanged. Mating the K14-VEGF-D and the K14-VEGF-C156S mice with mice expressing soluble VEGFR-3 under the K14 promoter completely abolished the lymphatic hyperplasia.

Our results demostrate that signaling via VEGFR-3 alone is sufficient for lymphangiogenesis *in vivo*. Lymphatic dilation in the skin of the K14-VEGF-D and K14-VEGF-C156S mice is unlikely to be the result of increased vascular leakage, as both VEGF-D and VEGF-C156S have been reported to be inactive in the Miles permeability assay (Achen et al. 1998; Joukov et al. 1998). Importantly, even though VEGF-D has been reported to be angiogenic in the rabbit avascular cornea (Marconcini et al. 1999), we observed no VEGF-D-induced blood vascular effects. The lack of angiogenesis in our transgenic model may relate to incomplete proteolytic processing of VEGF-D in the skin, and therefore reduced affinity towards VEGFR-2. As no systemic effects were detected in either mouse strain, VEGF-D and VEGF-C156S can be considered for use in local lymphangiogenic gene therapy .

VEGF isoforms $VEGF_{164}$ and $VEGF_{120}$ have also been transgenically overexpressed in the epidermis (Detmar et al. 1998; Larcher et al. 1998). In these mice the numbers of cutaneous blood capillaries and vascular permeability were highly increased. Despite substantial edema, no changes in the skin lymphatic vasculature were detected in the K14-$VEGF_{164}$ mice (Detmar et al. 1998; Thurston et al. 1999). These findings indicate that VEGFR-2 activation even in combination with increased intestitial fluid pressure is not sufficient for new lymphatic vessel growth, but that VEGFR-3 mediated signals are required.

IV VEGFR-3 mediates therapeutic lymphangiogenesis

In order to compare the consequences of VEGF-C156S and VEGF-C gene transfer *in vivo*, the lymphatic phenotypes of K14-VEGF-C156S and K14-VEGF-C transgenic embryos heterozygous for LacZ in the VEGFR-3 locus were first analysed. At E14.5, the cutaneous lymphatics of both types of embryos were clearly hyperplastic, but whereas VEGF-C156S mainly induced enlargement of the pre-existing lymphatics, VEGF-C strongly stimulated lymphatic sprouting. Recombinant adenoviruses (Ad) and adeno associated viruses (AAV) encoding human VEGF-C or VEGF-C156S were generated and shown to produce active transgene-encoded proteins in infected cell cultures. To detemine whether the effects of VEGF-C156S and VEGF-C are recapitulated in adult lymphangiogenesis, viruses encoding VEGF-C156S or VEGF-C were injected intradermally into ears of immunocompromised mice. Both factors mediated lymphatic vessel growth, but as in the transgenic mouse models, the response to VEGF-C156S was mostly lymphatic hyperplasia whereas VEGF-C induced intensive lymphatic sprouting. Analysis of the blood vascular phenotypes after AdVEGF-C156S or AdVEGF-C injection revealed that whereas VEGF-C induced blood vessel tortuosity, enlargement, and leakage, minimal blood vascular changes resulted from VEGF-C156S. The consequence of an intravenous injection of AdVEGF-C was dose-dependent accumulation of thoracic fluid and in some cases death, but systemic AdVEGF-C156S had no adverse effects. 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. In the adult lymphatic vasculature VEGFR-3 was most strongly expressed by the initial lymphatic capillaries, while VEGFR-2 was present in highest amounts in the collecting lymphatic vessels. Although the lymphatic capillaries were highly hyperplastic in the K14-VEGF-C mice, their collecting lymphatic vessel phenotype was normal.

Figure 5. Skin lymphatic vessels of E14.5 VEGFR-3+/LacZ embryos visualized by β-galactosidase staining. Overexpression of VEGF-C156S or VEGF-C increases skin lymphatic vascularity, but their lymphangiogenic mechanisms differ.

The mechanisms of lymphangiogenesis appear to be very similar to angiogenesis in that both involve vessel enlargement, sprouting and splitting. Although both VEGF-C156S and VEGF-C induced an increase in skin lymphatic vascularity, their effects on the lymphatic vessels differed. Our results indicate that VEGFR-2 activation may be required for efficient induction of lymphatic sprouting. While systemic injection of AdVEGF-C led to greatly and sometimes fatally increased vascular permeability, we observed minimal blood vascular effects with AdVEGF-C156S. Gene transfer using AAV vectors resulted in a more controlled and longer-lived lymphangiogenic response than was achieved with acute high-level transgene expression from the adenoviral vectors, indicating that AAV may be more suitable for future human lymphangiogenic gene therapy. The differential expression of VEGFR-3 and VEGFR-2 on lymphatic capillaries and collecting vessels may indicate differences in lymphatic endothelial cell function, and underscores the importance of developing strategies to specifically target various types of lymphatic vessels.

Angiogenic gene therapy has raised concerns about the potential stimulation of growth of dormant tumors due to increased tumor angiogenesis in response to elevated systemic VEGF. Some animal experiments have also indicated that instead of stimulating angiogenesis constant delivery of high levels of VEGF can result in the development of vascular malformations or hemangiomas (Springer et al. 1998; Lee et al. 2000; Pettersson et al. 2000; Dor et al. 2002). Therefore brief duration and low level of localised transgene expression are requirements for safe angiogenic gene therapy. For lymphatic-specific therapy the safety margin will probably be wider, and VEGF-C156S gene transfer thus seems like an attractive choice for patients with lymphatic hypoplasia, dysfunction and edema. However, as tumor induced lymphangiogenesis has been associated with enhanced metastasis to the lymph nodes, the risk of tumor dissemination needs to be carefully evaluated.

Concluding remarks

The blood and lymphatic vessel systems co-operate in a tightly regulated manner to maintain tissue homeostasis. The VEGFRs govern many aspects of the growth and maintenance of these two vessel systems, both during embryonic development and physiological and pathological responses in the adult. While VEGFR-1 and VEGFR-2 mainly orchestrate blood endothelial cell responses in angiogenesis, VEGFR-3 plays a major role in lymphangiogenesis.

There is reason to believe that different endothelial cells express specific sets of receptors depending on their organ of origin, vessel type and endothelial activation state. Therefore, they are likely to respond differently to specific stimuli. Physiological angiogenesis involves a wide spectrum of agents with roles in certain aspects of vessel formation, such as branching or fusion. Better understanding of the molecular processes involved in regulation of the various stages of blood and lymphatic vessel growth will undoubtedly contribute to the design of improved treatment strategies for disorders involving angiogenesis and lymphangiogenesis.

To date, VEGF-C and VEGF-D are the only known growth factors capable of promoting lymphatic vascular growth, and are therefore being considered for use in therapeutic lymphangiogenesis. Understanding the molecular mechanisms behind various forms of lymphatic disease will allow the development of specific treatments. The results on experimental therapeutic lymphangiogenesis for hereditary lymphedema using VEGF-C156S are encouraging, but as for proangiogenic therapy, combinations of growth factors may be required for the creation of a functional and stable lymphatic vessel network. Future clinical trials will judge the therapeutic potential of these molecules in man. Also the existence of circulating or tissue resident lymphangioblasts is still an unresolved issue. Such lymphatic endothelial precursors could potentially provide a major input for the generation of new lymphatic vessels.

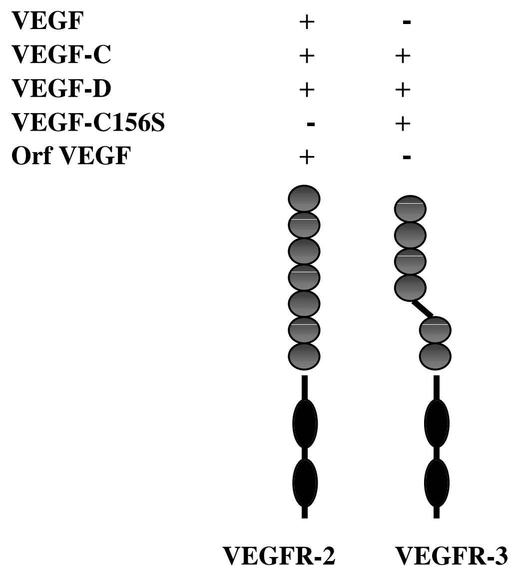

Figure 6. VEGFR-2, VEGFR-3 and their ligands.

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