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Lymphatic vs blood vascular endothelial growth factors and receptors in human tissues and diseases

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ACADEMIC DISSERTATION

To be publicly discussed, with the permission of
the Medical Faculty of the University of Helsinki,
in the Small Lecture Hall of Haartman Institute, Haartmaninkatu 3, Helsinki
on December 22nd, 2000, at 12 o'clock noon.

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A mind once streched by a new idea, never regains its original dimensions.

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ABBREVIATIONS

aa	amino acid
AIDS	acquired immunodeficiency syndrome
ALPase	alkaline phosphatase
Ang	angiopoietin
AVM	arteriovenous malformations
COX2	cyclooxygenase-2
cDNA	complementary deoxyribonucleic acid
E	embryonic day
EC	endothelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
FGF	fibroblast growth factor
FIGF	c-fos-induced growth factor
FVIIIRA	Factor VIII related antigen
GM-CSF	granulocyte-monocyte colony-stimulating factor
HEL	human erythroleukaemia cell line
HEV	high endothelial venule
HIF-α	hypoxia-inducible transcription factor-α
HGF	hepatocyte growth factor
Ig	immunoglobulin
IGF-1	insulin-like growth factor-1
IL	interleukin
INFγ	interferon gamma;
kb	kilobase
kDa	kilodalton
KS	Kaposi's sarcoma
LYVE-1	lymphatic vessel endothelial hyaluronan receptor
MAPK	mitogen activated protein kinase
MCP-1	membrane cofactor protein-1
MMP	matrix metallo proteinases
mRNA	messenger ribonucleic acid
NP-1	neuropilin-1
NO	nitrix oxide
5'Nase	5'-nucleotidase
PA	plasminogen activator
PAI-1	plasminogen activator inhibitor-1
PAN	puromycin aminonucleoside nephrosis
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PF4	platelet factor-4
PlGF	placenta growth factor
PMA	phorbol myristate 12, 13-acetate
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase polymerase chain reaction
SMC	smooth muscle cell
tek	tunica interna endothelial cell kinase

tie	tyrosine kinase with immunoglobulin and epidermal growth factor homology domains
TIMP	tissue inhibitor of metalloproteinases
TNF-α	tumor necrosis factor- α
TSP-1	trombospondin-1
UEA I	Ulex europaeus I lectin
VCAM	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptors 1, 2, 3
VEGI	vascular endothelial growth inhibitor
VM	venous malformation
vWF	von Willebrand factor

ORIGINAL PUBLICATIONS

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

I Lymboussaki, A.,* Partanen, T.A.,* Olofsson, B., Thomas-Crusells, J., Fletcher, C.D.M., de Waal, R.M.V., Kaipainen, A. and Alitalo, K.: Expression of the Vascular endothelial Growth factor C Reseptor VEGFR-3 in Lymphatic Endothelium of the Skin and in Vascular Tumors. *Am J Pathol*, 153: 395-403, 1998.

II Weninger, W. Partanen, T.A., Breiteneder-Geleff, S., Mayer C., Kowalski, H., Mildner, M., Pammer, J., Stürzl, M., Kerjaschi, D., Alitalo, K. and Tschachler, E..: Expression of Vascular Endothelial Growth Factor Receptor-3 and Podoplanin suggest a lymphatic endothelial Cell Origin of Kaposi's Sarcoma Tumor cells. *Lab Invest* 79: 243-251, 1999

III Partanen, T.A., Mäkinen T., Arola, J., Suda, T., Weich, H.A., Alitalo, K.: Endothelial growth factor receptors in human fetal heart. *Circulation*, 100: 583-586, 1999.

IV Partanen, T.A., Alitalo, K., Miettinen M.: Lack of lymphatic vascular specificity of VEGFR-3 in human vascular tumors. *Cancer*, 86: 2406-12, 1999.

V Partanen, T.A., Arola, J., Saaristo, A., Jussila L., Ora, A., Miettinen, M., and Alitalo, K.: VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3 in fenestrated endothelia in human tissues. *The FASEB J*, 14: 2087-2096, 2000.

* These authors contributed equally to this work.

ABSTRACT

Three different growth factor systems have been described acting via endothelial cell-specific receptor tyrosine kinases (RTKs). These are vascular endothelial cell growth factors (VEGFs), angiopoietins (Angs) and ephrins. Recent studies on endothelial gene targeting suggest that they play a role in embryonic development and contribute to maintaining the integrity and responses to environmental and endogenous factors in the adult vasculature. VEGF-C, VEGF-D and VEGF receptor-3 are thought to control lymphangiogenesis (development of lymphatic vessels). Lymphatic vessel endothelial hyaluronan receptor (LYVE-1), podoplanin and Prox-1 are novel molecular markers of the lymphatic endothelium. Lymphatic vessels have been difficult to study due to a lack of appropriate molecular tools, but antibodies raised against these novel molecules have offered an insight into their gene expression studies in tissues.

Here it was shown that VEGFR-3 identifies a distinct vessel population both in fetal and adult skin, which has properties of lymphatic vessels. The expression of VEGFR-3 was studied in normal human skin by *in situ* hybridization, iodinated ligand binding, and immunohistochemistry. A subset of developing vessels expressed the VEGFR-3 mRNA in fetal skin as shown by *in situ* hybridization and radioiodinated VEGF-C bound selectively to a subset of vessels in adult skin that had morphological characteristics of lymphatic vessels. Monoclonal antibodies against the extracellular domain of VEGFR-3 stained specifically endothelial cells of dermal lymph vessels. These results established the utility of anti-VEGFR-3 antibodies in the identification of lymphovascular channels in the skin.

To define the molecular anatomy of the known endothelial growth factor receptors in the cardiovascular system, their expression patterns were studied. Frozen sections of human fetal heart were stained immunohistochemically with receptor-specific monoclonal or polyclonal antibodies. The results demonstrate differential expression of the endothelial growth factor receptors in distinct types of vessels in the human heart. This information is useful for the understanding of their roles in physiological and pathological processes and for their diagnostic and therapeutic application in cardiovascular medicine.

Fenestrated capillaries of several organs including the bone marrow, splenic and hepatic sinusoids, kidney glomeruli and endocrine glands expressed VEGFR-3. VEGF-C and VEGF-D, which bind both VEGFR-2 and VEGFR-3 were expressed in vascular smooth muscle cells. In addition, intense cytoplasmic staining for VEGF-C was observed in neuroendocrine cells, such as the α-cells of the islets of Langerhans, prolactin secreting cells of the anterior pituitary, adrenal medullary cells and dispersed neuroendocrine cells of the gastrointestinal tract. VEGF-D was observed in the innermost zone of the adrenal cortex and also in certain dispersed neuroendocrine cells. These results suggest that VEGF-C and VEGF-D have a paracrine function in blood vessels and perhaps a role in peptide release from secretory granules of certain neuroendocrine cells to surrounding capillaries.

Despite intensive research over the past decade, the exact lineage relationship of Kaposi's sarcoma (KS) tumor cells has not yet been settled. The expression of two markers for lymphatic endothelial cells (EC), i.e., VEGFR-3 and podoplanin, was investigated in AIDS and classic KS. Both markers were strongly expressed by cells lining irregular vascular spaces in early KS lesions and by tumor cells in advanced KS. Double-staining experiments by confocal laser microscopy established that

VEGFR-3-positive and podoplanin-positive cell populations were identical and uniformly expressed CD31. By contrast, these cells were negative for CD45, CD68, and PAL-E, excluding their hematopoietic and blood vessel endothelial cell nature. Podoplanin expression in primary KS tumor lysates was confirmed by Western blot analysis. Both splice variants of VEGFR-3 were found in KS-tumor-derived RNA by RT-PCR. In contrast to KS tumor cells in situ, no expression of VEGFR-3 and podoplanin was detected in any of four KS-derived spindle cell cultures and in one KS-derived autonomously growing cell line (KS Y-1). Lack of these antigens on cultured cells derived from KS lesions indicates loss of the in vivo phenotype of certain KS tumor cell lines in culture.

VEGFR-3 was also studied by immunohistochemistry in benign, borderline, and malignant vascular tumors. Although normal mesenchymal tissues showed VEGFR-3 only in the lymphatics, benign and malignant vascular tumors and neovascularization of nonendothelial tumors showed widespread VEGFR-3 distribution. All lymphangiomas and KSs showed consistent VEGFR-3 reactivity. The results indicated that although VEGFR-3 shows specificity toward lymphatics in normal tissues, this receptor is distributed extensively in benign and malignant vascular tumors and therefore can be considered a novel marker in the assessment of endothelial cell differentiation of vascular neoplasms.

REVIEW OF THE LITERATURE

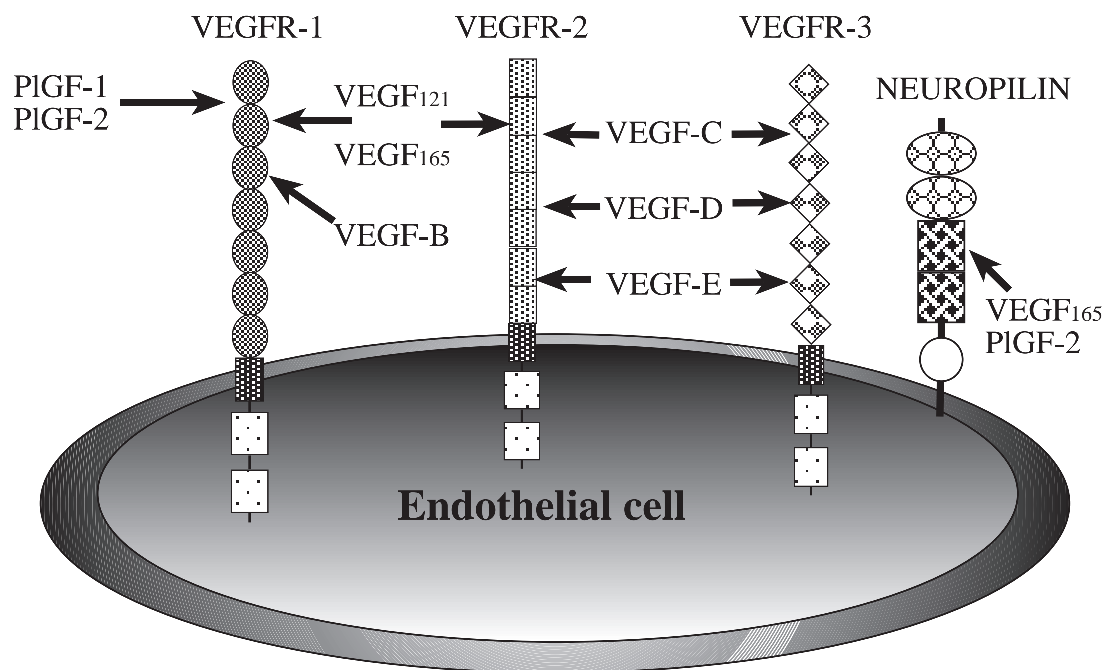
1. The VEGF family

1.1. GROWTH FACTORS

1.1.1. VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

Vascular endothelial growth factor (VEGF) is a highly specific mitogen for vascular endothelial cells (Leung *et al*., 1989). It was originally found to be responsible for glioma-associated brain edema because glioma cells produce it (Bruce *et al*., 1987). Five human VEGF isoforms of 121, 145, 165, 189, and 206 amino acids (VEGF121-206) are generated as a result of alternative splicing from a single VEGF gene (Houck *et al*., 1991; Leung *et al*., 1989; Park *et al*., 1993; Poltorak *et al*., 1997; Tischer *et al*., 1989). These isoforms differ in their molecular mass and biological properties, such as their ability to bind to cell-surface heparin-sulfate proteoglycans (Ferrara and Henzel, 1989). All VEGF isoforms can bind either of two receptor tyrosine kinases, VEGFR-1 (or Flt-1) or VEGFR-2 (or KDR/Flk-1) (Meyer *et al*., 1999) (Figure 1). Neuropilin-1 binds the $VEGF_{164}$ isoform and can potentiate VEGFR-2 activity, acting as a co-receptor (Soker *et al*., 1998).

Figure 1. Schematic representation of VEGFRs and their ligands. Adapted from Ferrara 1999.



The expression of VEGF is potentiated in response to hypoxia (Shweiki *et al*., 1992), by activated oncogenes, and by a variety of cytokines (Pages *et al*., 2000; Park *et al*., 1993). VEGF induces endothelial cell proliferation, promotes cell migration,

and inhibits apoptosis (D'Arcangelo *et al.*, 2000). *In vivo* VEGF induces angiogenesis as well as permeabilization of blood vessels (Roberts and Palade, 1995), and plays a central role in the regulation of vasculogenesis (Millauer *et al.*, 1993).

VEGF contributes to the vascular remodelling that occurs during the ovarian cycle and embryonic implantation (Hazzard *et al.*, 1999; Shweiki *et al.*, 1993) and disorders like endometriosis (Lebovic *et al.*, 2000; Mahnke *et al.*, 2000). Deregulated VEGF expression contributes to the development of solid tumors by promoting tumor angiogenesis and to the etiology of several additional diseases that are characterized by abnormal angiogenesis like diabetes mellitus (Hovind *et al.*, 2000), rheumatoid arthritis (Cho *et al.*, 2000) and psoriasis (Bhushan *et al.*, 1999). Consequently, inhibition of VEGF signaling abrogates the development of a wide variety of tumors (Kim *et al.*, 1993; Melnyk *et al.*, 1996; Millauer *et al.*, 1994). In the light of novel findings VEGF seems to act as an oncogenic factor in endothelial cells when overexpressed (Arbiser *et al.*, 2000).

VEGF is widely expressed in normal adult human tissues, the highest levels were found in normal lung, kidney, heart, and adrenal gland by Northern analysis (Berse *et al.*, 1992). In *in situ* hybridization the signals were strongest in the alveolar walls of the lung and in the renal glomeruli, in the outer cortex epithelium of the adrenal gland and cardiac myocytes. A novel approach, in which VEGF expression was tagged with LacZ, also provided evidence for expression of VEGF in the endothelial cells of the outflow tract of the heart and endocardium (Miquerol *et al.*, 1999).

1.1.2. VEGF-B

VEGF-B has two known isoforms of 167 and 186 amino acid residues, but it has only very low mitogenic potency (Olofsson *et al.*, 1996; Olofsson *et al.*, 1996). The expression of VEGF-B is not regulated by hypoxia (Joukov *et al.*, 1997). VEGF-B is a ligand for VEGFR-1 and neuropilin-1 (Makinen *et al.*, 1999; Olofsson *et al.*, 1999). Although originally cloned from human tumour cell libraries, it has been shown that VEGF-B is expressed in a variety of normal human tissues, primarily in the developing myocardium (Enholm *et al.*, 1997; Joukov *et al.*, 1997). In human tumors, VEGF-B is commonly present in both benign and malignant tumors (Donnini *et al.*, 1999; Salven *et al.*, 1998), although in colon neoplasms VEGF-B mRNA levels have been found unchanged (Andre *et al.*, 2000).

1.1.3. VEGF-C

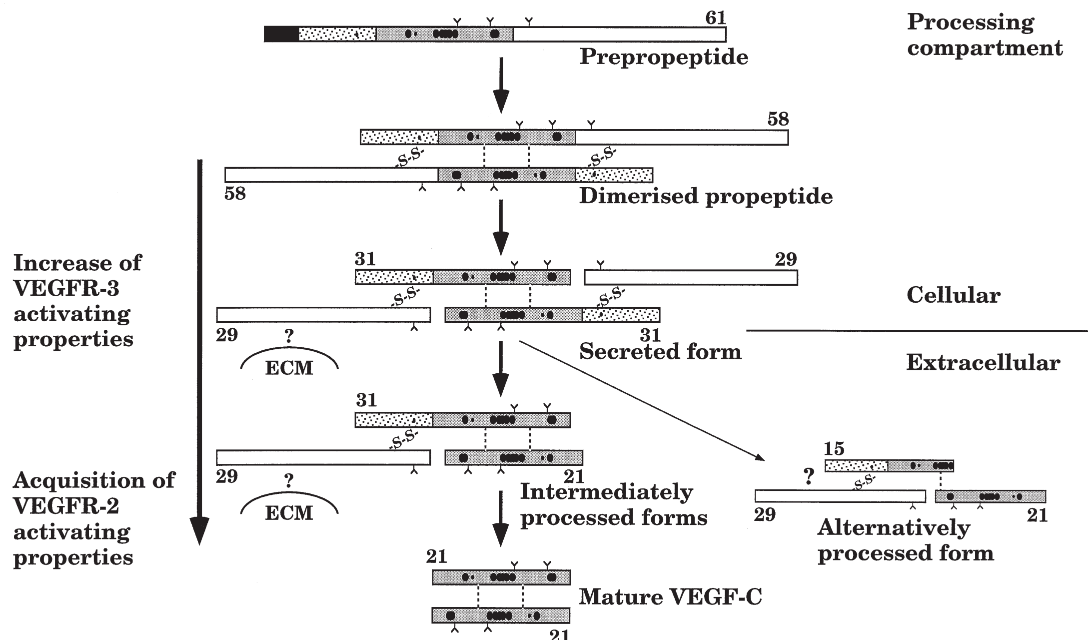
VEGF-C was found in the growth medium of PC-3 prostatic adenocarcinoma cells (Joukov *et al.*, 1996; Lee *et al.*, 1996). It is synthesized as a disulfide-linked prepropeptide dimer of 61 kDa subunit size (Fig. 2) and by proteolytic maturation a homodimer of 21 kDa is formed. Partially processed and mature forms of VEGF-C bind VEGFR-3 with high affinity, while only the fully processed form binds VEGFR-2.

VEGF-C stimulates the migration and proliferation of endothelial cells *in vitro* and *in vivo* (Taipale *et al.*, 1999). Recently, it was shown to stimulate the vasculogenesis and suppress the hematopoiesis in a dose-dependent manner like VEGF-A (Hamada *et al.*, 2000). Compared to VEGF, VEGF-C is 4-5 times less potent in the vascular permeability (Miles) assay (Joukov *et al.*, 1997). VEGF-C mRNA levels are increased

by serum and its component growth factors, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) as well as transforming growth factor-β (TGF-β) and the tumor promoter phorbol myristate 12, 13-acetate (PMA) stimulation (Enholm *et al*., 1997). Conversely, hypoxia, Ras oncoprotein and mutant p53 tumor suppressor do not have an influence on VEGF-C mRNA levels. IL-1 and TNF-α have been shown to stimulate VEGF-C expression in human lung fibroblasts and in human umbilical vein endothelial cells (HUVEC) (Ristimaki *et al*., 1998). Further, the anti-inflammatory glucocorticoid dexamethasone inhibits IL-1-induced VEGF-C mRNA expression. It appears that VEGF-C could be a mediatior in inflammatory reactions (Narko *et al*., 1999). VEGF-C has a dual biological role being able to induce both angiogenesis and lymphangiogenesis (Oh *et al*, 1997; Pepper *et al*., 1998). The latter has been shown by overexpressing VEGF-C in the murine skin under K14 promoter (Jeltsch *et al*., 1997). Under ischemic conditions *in vivo* VEGF-C induces also angiogenesis in a rabbit hindlimb model (Witzenbichler *et al*., 1998).

VEGF-C mRNA is weakly expressed in lymph nodes, heart, placenta, skeletal muscle, ovary, and small intestine tissues (Joukov *et al*., 1996). Its mRNA levels do not correlate with the neoplastic progression of human colonic mucosa (Andre *et al*., 2000). In contrast, some evidence has been presented for VEGF-C association with lymph node metastasis of colorectal carcinoma (Akagi *et al*., 2000). In breast cancer, VEGF-C has been found in the cytoplasm of intraductal and invasive cancer cells and its receptor in blood vessels, suggesting an angiogenic role for it (Valtola *et al*., 1999).

Figure 2. Schematic model of the proteolytic processing of VEGF-C. Adapted from Joukov, 1997.



1.1.4. VEGF-D

Human VEGF-D was isolated as a VEGF-related transcript (Yamada *et al*., 1997) and the mouse homologue, so-called c-fos-induced growth factor (FIGF) was cloned independently (Orlandini *et al*., 1996). VEGF-D is a ligand for VEGFR-2 and VEGFR-3 and a mitogen for endothelial cells (Achen *et al*., 1998). VEGF-D is lymphangiogenic although less potent compared to VEGF-C see (Carmeliet, 2000).

Human VEGF-D mRNA is expressed prominently in the lung, heart and small intestine. Two *in situ* analyses of both mouse fetal (E17) and young adult tissues displayed intense VEGF-D signals in the lung (Avantaggiato *et al*., 1998; Farnebo *et al*., 1999). On the basis of these studies VEGF-D seems to be down-regulated in the course of development.

Table 1 A. Summary of the genetic programs for the VEGF family members as concluded from studies in gene targeted and transgenic mice.

GROWTH FACTOR AFFECTED	SITE OF GENE TARGET	PHENOTYPE	REFERENCE
VEGF	Replacement of the 3rd common VEGF exon with the gene encoding neomycin phospho-transferase.	Dorsal aorta with a smaller lumen. Reduced angiogenic sprouting. Abnormal development of large thoracic blood vessels	(Carmeliet *et al*., 1996)
VEGF	Coding sequence of VEGF exon replaced by a neomycin resistance.	Heterozygous deletion is lethal. Impaired blood-island formation. Severe anomalies (forelimb buds, heart, cranial region).	(Ferrara *et al*., 1996)
VEGF	Removal of exons 6 and 7, encoding basic domains that are only present in $VEGF_{164}$ and/or $VEGF_{188}$.	Impaired myocardial angiogenesis. Ischemic cardio-myopathy.	(Carmeliet *et al*., 1999)
VEGF (Conditional)	Cre-loxP-mediated gene ablation after administration of interferon-α	Impaired postnatal vascular development and endothelial survival.	(Gerber *et al*., 1999)

Table 1B. Summary of the genetic programs for the VEGF family members as concluded from studies in gene targeted and transgenic mice.

GROWTH FACTOR AFFECTED	SITE OF GENE TARGET	PHENOTYPE	REFERENCE
VEGF	Insertion of an IRES-LacZ reporter cassette into the 3' untranslated region of the gene		(Miquerol *et al.*, 1999)
VEGF-B	Replacement of exons 3-7 with a promoter-less β-geo cassette	Smaller hearts. Dysfunctional coronary vasculature	(Bellomo *et al.*, 2000)
VEGF	Expression of murine $VEGF_{164}$ cDNA under the keratin 14 promoter expression cassette	Skin:more numerous, enlarged, tortorous, leaky vessels	(Detmar *et al.*, 1998)
VEGF-C	Expression of VEGF-C cDNA under the human keratin 14 promoter	Skin: hyperplastic lymphatic vessels	(Jeltsch *et al.*, 1997)

1.1.5. VEGF-E

Orf virus is a linear double-stranded DNA virus that causes contagious pustular dermatitis which is characterized histologically by vascular and edematous lesions (Ogawa *et al.*, 1998). As the virus was only found in the keratinocytes and the vascular response in the dermis was VEGF-like, the observations led to the discovery of VEGF-E from the genome of Orf virus strain NZ-7. VEGF-E induces tissue-factor (TF) expression, the proliferation, migration and sprouting of cultured vascular endothelial cells and angiogenesis *in vivo* (Meyer *et al.*, 1999).

1.2. The VEGF receptors

1.2.1. VEGFR-1

VEGFR-1 receptor is expressed predominantly in endothelial cells but it is found in trophoblast cells, monocytes(Barleon *et al.*, 1996) and renal mesangial cells (Takahashi *et al.*, 1995). In addition, there are tumorigenic cell types that express VEGFR-1 (Cohen *et al.*, 1995). The receptor is probably activated by all VEGF isoforms but fulfills somewhat different functions *in vivo*, as targeted gene disruption experiments revealed (Shalaby *et al.*, 1995). VEGFR-1 can transduce signals of other growth factors belonging to the VEGF family, but only the VEGF isoforms can bind to VEGFR-1. The transcription of VEGFR-1 is enhanced by hypoxia (Gerber *et al.*, 1997). Alanine-scanning mutagenesis has revealed that Asp (63), Glu (64), and Glu (64) and Glu (67) are required for the binding of VEGF to VEGFR-1. VEGFR-1 does not induce cell proliferation in response to VEGF. MAP kinase is not activated by VEGF in cells expressing recombinant VEGFR-1 (Seetharam *et al.*, 1995). Therefore

it is possible that VEGFR-1 does not induce cell proliferation, because it does not activate MAP kinase. Finally, activation of VEGFR-1 results in the generation of proteases that are required for the breakdown of the basement membrane of blood vessels in the first steps of angiogenesis (Olofsson *et al*., 1998; Unemori *et al*., 1992).

1.2.2. VEGFR-2

VEGFR-2 is also expressed predominantly in endothelial cells, but also in hematopoietic stem cells, megakaryocytes, and retinal progenitor cells (Katoh *et al*., 1995). In the retina, two functional VEGFR-2 forms are expressed as a result alternative splicing (Wen *et al*., 1998). Malignant melanoma cells express VEGFR-2. Only the final glycosylated form of VEGFR-2 is capable of undergoing autophosphorylation in response to VEGF (Takahashi and Shibuya, 1997). Transcription of VEGFR-2 is not enhanced by hypoxia. VEGFR-2 production is also up regulated under hypoxic conditions, but the mechanism responsible for the induction seems to be post-transcriptional (Waltenberger *et al*., 1996). Activation of the VEGFR-2 receptor by VEGF in cells devoid of VEGFR-1 results in a mitogenic response (Kondo *et al*., 1998). When VEGFR-2 is activated by VEGF, endothelial cells migration is obtained (Yoshida *et al*., 1996).

1.2.3. NEUROPILIN-1

Endothelial cells were also found to contain VEGF receptors possessing a lower molecular weight than either VEGFR-2 or VEGFR-1 (Gitay-Goren *et al*., 1992). It was subsequently found that these smaller VEGF receptors bind to $VEGF_{165}$ but, but not to $VEGF_{121}$. Therefore these receptors are not related to the VEGFR-1 or VEGFR-2 that bind both VEGF isoforms (Gitay-Goren *et al*., 1996), but instead neuropilin-1, a receptor for several types of semaphorins that were initially characterized as repellents of nerve growth cones (He and Tessier-Lavigne, 1997; Soker *et al*., 1998). Neuropilin-1 also functions as a receptor for the heparin binding form of PlGF, PlGF-2, but not for PlGF-1 (Migdal *et al*., 1998). The neuropilins have a short intracellular domain and thus are unlikely to function as independent receptors. On the other hand, gene distruption studies indicate that mouse embryos lacking a functional neuropilin-1 gene die because their cardiovascular system fails to develop properly (Kitsukawa *et al*., 1997).

Neuropilin-1 is considered a $VEGF_{165}$ co-receptor, because VEGFR-2 has been shown to bind $VEGF_{165}$ more efficiently in cells expressing neuropilin-1. In addition, neuropilin potentiates effect endothelial cell migratory response to $VEGF_{165}$.

Table 2. Biological functions of VEGFRs during development concluded from gene targeting experiments.

VEGF RECEPTOR	SITE OF GENE TARGET	PHENOTYPE	REFERENCE
VEGFR-1	The translated portion of the 1st coding exon replaced with LacZ	Disorganized vasculature in early embryos	(Fong *et al*., 1995)
VEGFR-1	As above.	Increase in the number of endothelial cell progenitors	(Fong *et al*., 1999)
VEGFR-2	LacZ into the first exon	Absence of yolk-sac blood islands No organized blood vessels in the embryo or yolk sac Severely reduced hematopoietic progenitors	(Shalaby *et al*., 1995)
Neuropilin-1	Overexpression of NP-1 in ES cells	Excess capillaries and blood vessels Abnormal heart Ectopic sprouting and defasciculation of nerve fibers	(Kitsukawa *et al*., 1995)
Neuropilin-1	Sequence encoding the N-terminal half of the alpha1 domain targeted with neomycin resistance gene (neo)	Agenesis of branchial arch, great vessels and dorsal aorta Transposition of the aortic arch Insufficient septation of the truncus arterious. Disorganized extraembryonic vasculature	(Kitsukawa *et al*., 1997)
VEGFR-3	LacZ in the 1st coding exon	Abnormally organized large vessels with defective lumen. Severe anemia.	(Dumont *et al*., 1998) (Hamada *et al*., 2000)

1.2.4. VEGFR-3

VEGFR-3 is a highly glycosylated, relatively stable, cell surface associated tyrosine kinase of approximately 180 kDa. Its cDNA was cloned from human erythroleukemia cell and placental libraries (Aprelikova *et al.*, 1992; Galland *et al.*, 1993). On the basis of structural similarities VEGFR-1-3 receptors constitute a subfamily of class III tyrosine kinases. Two isoforms of VEGFR-3, have been identified differing in their C-terminal ends.

In the early stages of development VEGFR-3 is widely expressed in the vascular endothelial cells (Kukk *et al.*, 1997). Disruption of the VEGFR-3 gene causes disorganization in the large vessels, resulting in defective lumina. As a result, the pericardial cavity is filled with fluid, and the embryo dies of cardiovascular failure (Dumont *et al.*, 1998). Further studies with this knockout model have shown that the embryos suffer from severe anemia (Hamada *et al.*, 2000). After organogenesis, the VEGFR-3 becomes restricted to lymphatic endothelial cells (Kaipainen *et al.*, 1993) and a missense mutation in VEGFR-3 which creates an inactive tyrosine kinase causes primary human lymphedema (Irrthum *et al.*, 2000; Karkkainen *et al.*, 2000). In the neovasculature of tumors VEGFR-3 is upregulated, which limits the use of the receptor in defining lymphatic vessels (Valtola *et al.*, 1999). Recently, it has been shown that inactivation of VEGFR-3 by blocking it with a monoclonal antibody suppresses tumor growth by inhibiting the neoangiogenesis of tumor-bearing tissues (Kubo *et al.*, 2000). In the light of the findings, VEGFR-3 may be needed for maintaining the integrity of the endothelial lining during angiogenesis.

2. The Angiopoietin/Tie- family

2.1. GROWTH FACTORS

2.1.1 Angiopoietin-1

Ang-1 is a 70-kDa secreted glycoprotein that induces the phosporylation of the Tie-2 receptor (Davis *et al.*, 1996). It is not mitogenic for endothelial cells in culture, does not it induce tube formation within a collagen substrate, nor cause neovascularization in the corneal micropocket assay (Asahara *et al.*, 1998; Maisonpierre *et al.*, 1997). When Ang-1 was added together with VEGF in the same assay, the effect was, however additive. Ang-1 inhibits apoptosis in endothelial cells dose-dependently during a serum deprivation and this anti-apoptotic effect is increased if VEGF is added (Kwak *et al.*, 1999). Recently it was found that the mechanism by which Ang-1 inhibits EC permeability is through the regulation of junctional complexes, PECAM-1 and VE cadherin (Gamble *et al.*, 2000). After vasculogenesis, the expression pattern of Ang-1 broadens from the myocardial lining of the endocardium to the periendothelial mesenchymal cells as the rest of the vasculature matures. Ang-1 overexpression under the K14-promotor inhibits inflammatory agent [mustard oil, serotonin and platelet activating factor (PAF)] induced permeability (Thurston *et al.*, 1999).

2.1.2. Angiopoietin-2

Ang-2 acts as a Tie-2 receptor blocker on endothelial cells (Maisonpierre *et al.*, 1997). Indeed angiopoietins were the first vertebrate example of a growth factor family consisting of both activatory and inhibitory ligands. In adults, Ang-2 is expressed primaly at sites of vascular remodelling, where it is thought to block the constitutive stabilizing action of Ang-1. Gene targeting studies suggest that Ang-2 participates in lymphatic development see (Carmeliet, 2000).

2.1.3. Angiopoietin-3 and Angiopoietin-4

Human Ang-3 and mouse Ang-4 are structurally more divergent from each other than are the mouse and human versions of angiopoietin-1 and angiopoietin-2 (Nishimura *et al.*, 1999). Angiopoietin-3 and angiopoietin-4 have very different distributions in their respective species, and ang-4 appears to function as an agonist. Ang-3 cDNA was cloned from a human aorta cDNA library. It is a 503 amino acid protein having 45.1% and 44.7% identity with human angiopoietin-1 and human angiopoietin-2, respectively (Nishimura *et al.*, 1999). Additionally, Ang-3 is a secreted protein, but is not a mitogen in endothelial cells. Its mRNA is expressed in the lung and cultured HUVECs (Nishimura *et al.*, 1999). VEGF decreased Ang-3 mRNA expression in HUVECs slightly suggesting that the regulation of Ang-3 mRNA expression differs from that of Ang-2. The NH_2-terminal and COOH-terminal portions of Ang-3 contain the characteristic coiled-coil domain and fibrinogen-like domain that are conserved in other known Angs. Ang-3 has the highly hydrophobic region at the N-terminus (approximately 21 amino acids) that is typical of a signal sequence for protein secretion (Kim *et al.*, 1999). According to the Northern analysis Ang-3 mRNA is most abundant in the adrenal glands, placenta, thyroid gland, heart and small intestine in human adult tissues. The biological properties of Ang-3 are at present unknown. According to the Northern analysis, the highest expression of Ang-4 is exhibited to lung.

2.2. The Tie receptors

2.2.1. Tyrosine kinase with immunoglobulin and EGF homology domains (Tie-1)

Tie-1 is a member of a subfamily of endothelial cell RTKs whose extracellular domains contain three different types of structural motifs: immunoglobulin (Ig)-like loops, cysteine-rich epidermal growth factor (EGF)-like domains and fibronectin type III (FN III) domains (Iwama *et al.*, 1993; Maisonpierre *et al.*, 1993; Partanen *et al.*, 1992; Partanen and Dumont, 1999; Sato *et al.*, 1993). Tie-1 is an orphan receptor, the expression of which is thought to be restricted to endothelial cells and early hematopoietic cells of myeloid differentiation (Armstrong *et al.*, 1993; Batard *et al.*, 1996; Bredoux *et al.*, 1997; Hashiyama *et al.*, 1996; Iwama *et al.*, 1993; Korhonen *et al.*, 1994; Kukk *et al.*, 1997; Partanen *et al.*, 1992; Sato *et al.*, 1993; Suda *et al.*, 1997; Yano *et al.*, 1997). Vasculogenesis happens succesfully without Tie-1 as shown by gene disruption studies (Puri *et al.*, 1995). However, the receptor is crucial for main-

taining the vascular integrity and survival of endothelial cells (Partanen *et al.*, 1996; Sato *et al.*, 1995).

2.2.2. Tie-2 or Tunica interna endothelial cell kinase (Tek)

Tie-2 is a receptor tyrosine kinase which is expressed by the vascular endothelium and primitive hemopoietic cells (Sato *et al.*, 1998) and anti-Tie-2 antibody has proved to be useful in purification of hematopoietic stem cells in the fetal liver (Hsu *et al.*, 2000). As already mentioned, angiopoietins 1-4, have been identified to bind to Tie-2 (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997). Tie-2 is upregulated during the later stages of angiogenesis (maturating wound, ovarian cycle) and is widely expressed and tyrosine phosphorylated in the vascular endothelium of quiescent adult tissues (Asahara *et al.*, 1998). An activating mutation in Tie-2 has shown to cause inherited venous malformations in humans (Vikkula *et al.*, 1996). The Tie-2 signaling pathway may regulate endothelial cell recruitment of the stromal cells required to encase and thereby stabilize endothelial tubes.

3. The Ephrin/Eph Family

The ephrins exist in two classes: GPI-linked [class A(1-8)] or transmembrane [class B(1-6)] proteins (Holder and Klein, 1999). Fourteen genes have been described that are related to Eph by their sequences and by the general characteristics of their kinase and extracellular domains. During embryogenesis Eph signaling contributes to segmentation, axon quidance and fasciculation; and in addition it is also involved in controlling cell migration, development of the vascular system and probably in cellular transformation. At least eight Eph receptor ligands (A1-5 and B 1-3) have been described; they do not function as typical soluble ligands, but must be membrane attached to activate their receptors (Davis *et al.*, 1994; Gale and Yancopoulos, 1997).

When EphrinA1 binds to its receptor, EphA2 on HUVECs it promotes the cell migration but not proliferation (Pandey *et al.*, 1995). The embryonic vasculature of ephrin EphrinB2-deficient mice is severely disrupted, due to a lack of remodeling of the primary capillary network (Wang *et al.*, 1998). Interestingly, ephrinB2 is exclusively expressed in embryonic arteries while EphB4 shows complementary expression in veins. Ephrin-B2/EphB4 expression studies suggest that molecular differences are at least in part genetically programmed genetically in arterial versus venous endothelia, and that these differences may be critical to the normal development of the vasculature (Gale and Yancopoulos, 1999).

4. Other endothelial cell markers

The vascular endothelium has a very different antigenic profile depending on the type of vessel and the tissue in which it lies. Such findings are not unexpected, considering the differing function of endothelial cells in different tissues, such as the maintenance of the blood-brain barrier, hormonal transport in endocrine capillaries (adrenal, thyroid), renal glomeruli and the high endothelial venules of the lymph nodes and spleen. Traditionally patologists have used anti-von Willebrand factor,

anti-CD31, anti-CD34, anti-laminin, anti-type IV collagen etc. antibodies to distinguish vascular structures.

A new era in pathology has begun, with the aid of new molecular techniques, such as serial analysis of gene expression (SAGE) and cDNA microarrays. These have enabled the measurement of the expression of thousands of genes in a single experiment, revealing many new, potentially important cancer genes (Kononen *et al.*, 1998). Instead of using immunohistochemical staining with only one antibody, it is already possible to analyse the profiles of tens or hundreds of antigens on endothelial cells (St. Croix *et al.*, 2000).

As a result of comparing human tumor and normal endothelia using a modification of the SAGE technique, some important conclusions have already been drawn, normal and tumor endothelia seem to be highly related, and share many endothelial cell-specific markers. Secondly, the tumor derived endothelium is qualitatively different from the same type of normal endothelium. The tumor endothelium is generally different from the endothelium in the surrounding normal tissue. Interestingly, most of the genes expressed differentially in tumor endothelia are also expressed during angiogenesis during corpus luteum formation and wound healing (St. Croix *et al.*, 2000).

In the following, established and new antigenic markers of differentiated endothelia are shortly summarized.

4.1. Von Willebrand factor

Von Willebrand Factor (vWF) or Factor VIII related antigen (FVIIIRA) is a multimer produced by endothelial cells and megakaryocytes, it is stored in intracellular organelles, such as the Weibel-Palade bodies and α-granules in endothelial cells and platelets (Spadafora-Ferreira *et al.*, 2000). VWF also plays an important role in platelet aggregation and adhesion to the cell walls of injured vessels (Kuzu *et al.*, 1992). The maturation status of the cell might explain the variability in vWF expression (Bohling *et al.*, 1983; Enzinger and Weiss, ; Zhu and Gu, 1988). In most tissues capillaries and sinuses do not stain for vWF whereas in the large vessels, arteries and veins the staining is very strong (Kuzu *et al.*, 1992). Lymphatic vessels stain for vWF weakly (Marchetti, 1996).

4.2. CD31

CD31 or platelet-endothelial cell adhesion molecule (PECAM)-1 is a 130-kDa glycoprotein commonly used as an endothelium-specific marker (Ilan *et al.*, 2000). Its expression starts early in development and persists through adulthood. Antibodies directed against PECAM-1 have been shown to affect angiogenesis, endothelial cell migration, and polymorphonuclear leukocyte transmigration (Mahooti *et al.*, 2000). PECAM-1 functions as an adhesion and signaling molecule between adjacent endothelial cells and between endothelial cells and circulating blood elements. Generally, CD31 is strongly expressed in all endothelial cells including capillaries, sinuses and larger vessels. Hepatic sinuses and splenic sinusoids are weakly labeled (Kuzu *et al.*, 1992).

4.3. CD34

CD34 is a 110kDa heavily glycosylated transmembrane protein. It is present on immature hematopoietic precursor cells and a ligand for I-selectin involved in leucocyte traffic from blood to a lymph node or sites of inflammation (van de Rijn and Rouse, 1994). Generally, the endothelial staining intensity is not strong and background staining of connective tissue and basement membranes may cause confusion (Kuzu *et al*., 1992). Splenic sinuses do not stain and further, the sinuses in the liver and lymph node show weak but definitive positivity (Natkunam *et al*., 2000).

4.4. Ulex europaeus I lectin

Ulex europaeus I lectin (UEA-I) is specific for alpha-L-fucose-containing glycoconjugates (Gomez *et al*., 1995). UEA-I is a more sensitive marker for endothelial cells than vWF (Holthofer *et al*., 1982). UEA-I also stains many neoplastic cells from endothelial sarcomas (Miettinen *et al*., 1983) and it can also detect sinusoidal endothelial cells from the human liver (Petrovic *et al*., 1989).

4.5. Molecular markers of the lymphatic system vs blood vasculature

According to previously published papers, the lymphatic endothelium can be characterized by using 5'-Nucleotidase (Nase)-alkaline phosphatase (ALPase) double staining (Kato, 1990). The lymphatic endothelium is marked by strong 5'-Nase activity that is significantly lower or absent in blood vessels whereas the blood vessels stain for ALPase. Other possibilities for distinguishing between lymphatic capillaries and blood capillaries are toluidine blue staining following arterial perfusion-fixation and staining of basement membrane components, like laminin, collagen IV (Kubo *et al*., 1990; Otsuki *et al*., 1990).

PAL-E is a monoclonal antibody (IgG2a) that stains the endothelium of capillaries, medium-sized and small veins, and venules in frozen sections (Schlingemann *et al*., 1985). Unfortunatelly, it reacts weakly, or not at all to the endothelia of large, medium-sized, and small arteries, arterioles, and large veins and does not stain the endothelial lining of lymphatic vessels and sinus histiocytes. CD31/PAL-E double staining has been used in the detection of lymphatic vessels (de Waal *et al*., 1997).

4.6. Proposed new lymphatic endothelial markers

4.6.1. Lymphatic vessel endothelial hyaluronan receptor (LYVE-1)

LYVE-1 was found as a result of a homology search of the combined Human Genome databases with the amino acid sequence of full-length CD44 antigen sequence (Banerji *et al*., 1999). LYVE-1 is thus a homologue of the CD44 glycoprotein, but it

is expressed mostly on lymphatic endothelia, where CD44 is almost lacking. Other expressing cells include sinusoidal cells, adrenal zona reticularis cells, pancreatic exocrine cells, syncytiotrophoblasts, cerebral cortex neurons and kidney tubular cuboidal epithelium. The function of LYVE-1 is not known. LYVE-1 displays exquisite specificity for binding hyaluronan which the major component of the extracellular matrix (ECM) (Toole, 1997). Hyaluronan is a key factor involved in cellular migration and adherence and it is expressed nearly everywhere in the body where high swelling capacity is provided. CD44 and LYVE-1, and hyaluronan receptor for endocytosis (HARE) constitute plasma membrane receptors for hyaluronan. Critical data on LYVE-1 expression in vascular tumors and tumor neovasculature is lacking, even though the production of polyclonal anti-LYVE-1 antibodies has been achieved.

4.6.2. Prox-1

The homeobox gene Prox-1 was originally cloned by homology to the Drosophila melanogaster gene prospero (Oliver *et al*., 1993). Prox-1 is expressed in a subpopulation of endothelial cells which by budding and sprouting, give rise to the lymphatic system (Wigle *et al*., 1999). Prox-1-deficient homozygous mice have unaffected vasculogenesis and angiogenesis, but liver and lymphatics do not develop (Wigle and Oliver, 1999). Human Prox-1 gene has been mapped to chromosome 1q32.2-q32.3, and its product shows 94% similarity to the chicken protein (Zinovieva *et al*., 1996). The conserved structure and expression pattern of the Prox-1 gene imply that it may play the same roles in humans as in other vertebrates. The human Prox-1 gene is most actively expressed in the developing lens. Anti-Prox-1 antibodies provide a highly specific marker of lymphatic endothelial cells as shown in the CAM assay (Papoutsi *et al*., 2000).

4.6.3. Podoplanin

A 43-kd membrane protein called podoplanin was identified when glomerular membrane proteins of normal and puromycin aminonucleoside nephrosis (PAN) rats were compared (Breiteneder-Geleff *et al.,* 1997). Glomerular visceral epithelial cell or podocyte foot processes, flatten in human minimal change nephropathy, causing severe proteinuria. PAN provides a model of human minimal change nephropathy.

Affinity-purified rabbit anti-podoplanin were shown to mark lymphatic capillary endothelium. Other cell types positive for the antibody include podocytes, the parietal epithelial cells of Bowman's capsule, the luminal cell membranes of type I alveolar epithelial cells, pleural mesothelial cells, osteocytes, osteoblasts, thymic epithelial cells. Staining positivity was also found in the plexus choroideus, leptomeninges, bone tissue, marginal sinuses of lymph nodes and the mesothelium of spleen, liver, stomach, and intestine.

A panel of vascular tumors was studied with anti-podoplanin and anti-VEGFR-3 antibodies and the results showed overlapping patterns for these two markers (Breiteneder-Geleff *et al*., 1999). Podoplanin protein was not present in capillary, cavernous or venous hemangiomas whereas it could be detected from lymphangioma, angiosarcoma (variation 0-70%) and KS. A rare intrapericardial lymphangioma has been analyzed with anti-podoplanin antibody and found it suitable for a lymphatic marker (Sinzelle *et al*., 2000). No data on podoplanin expression in tumor neovasculature is available. Expression of podoplanin was found to correlate

with the caliber of lymphatic vessels and be prominent in capillaries while larger lymphatic vessels with smooth muscle cells may lack it.

5. Vasculature

In order to understand the molecular markers that allow the differentiation of blood and lymphatic vessels, one has to understand their functions and the development that leads to the differentiated vessels. In the processes called vasculogenesis and angiogenesis.

Endothelial cells line vessels in every organ and regulate the flow of nutrient substances, diverse biologically active molecules, and the blood cells themselves. This gate-keeping role is effected through the presence of membrane-bound receptors for numerous molecules including proteins, lipid transporting particles, metabolites and hormones. There are also specific junction proteins and receptors, which govern cell-cell and cell-matrix interactions. Such proteins differ greatly in different parts of the vasculature as a result of the developmental processes that lead to their generation.

5.1. Development

The *de novo* organization of endothelial cells into vessels in the absence of any pre-existing vascular system is referred to as VASCULOGENESIS and only occurs in the early embryo. ANGIOGENESIS, the continued expansion of the vascular tree as a result of endothelial cell sprouting from existing vessels, occurs in avascular regions of the embryo and is repeated many times in the mature animal, most commonly during wound healing and tumor metastasis (Carmeliet and Collen, 1998; Hanahan and Folkman, 1996).

Vasculogenesis takes place during embryogenesis in the blood islands of the yolk sac (pictured), and in the embryo through expression of growth factors, in particular FGF and vascular endothelial growth factor (VEGF). The tyrosine receptor kinases, VEGFR-1 and VEGFR-2, are expressed on mesenchymal cells and newly formed ECs, respectively, are essential for the generation and proliferation of new ECs and the formation of tubal EC structures. Angiogenesis, the continued expansion of the vascular tree, is mediated through the expression of additional tyrosine kinase receptors, Tie-2, which binds to angiopoietins 1-2, resulting in the maintenance of mature vessels, the development of new vessels, and the regression of formed vessels in processes dependent on a combination of factors, most notably on the presence or absence of growth factors. Putative EC progenitors or angioblasts have been isolated from adult peripheral blood (Asahara *et al*., 1997) and further these cells have been shown to contribute in vasculogenesis both in physiological and pathological conditions (Asahara *et al*., 1999).

Figure 3. The formation of new vessels during vasculogenesis and angiogenesis.Adapted from Cines, 1998.

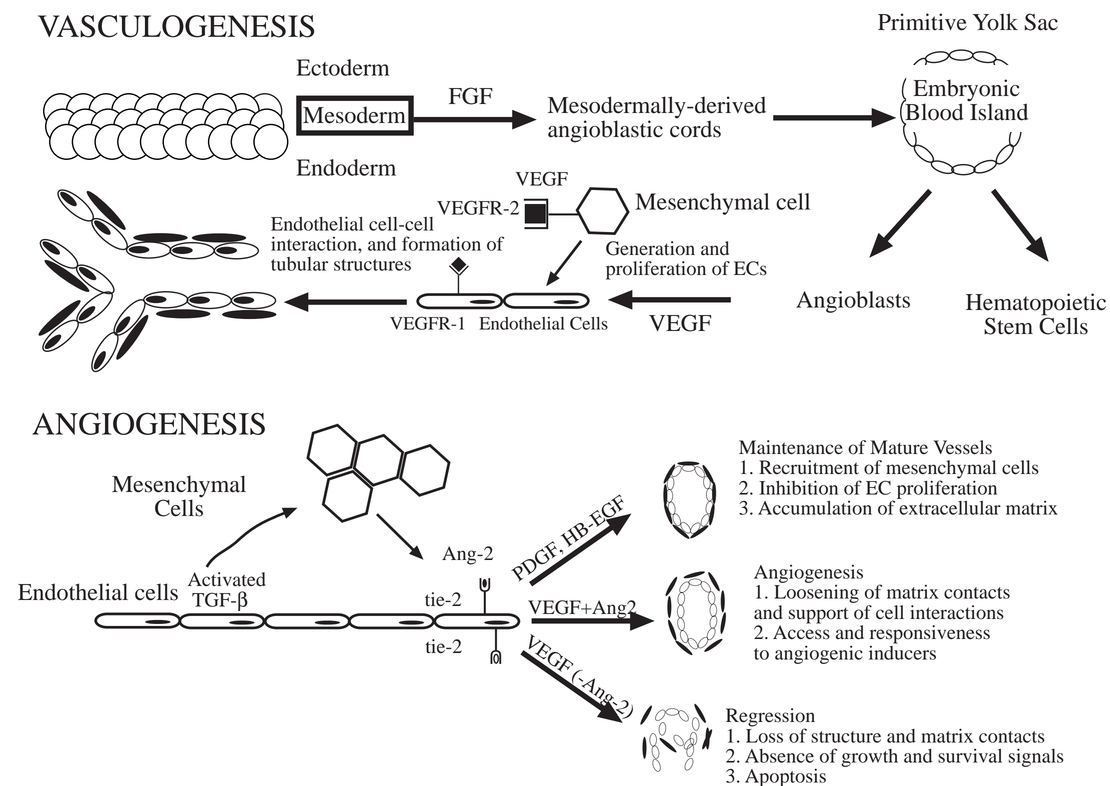


Table 3. A list of known stimulators and inhibitors that contribute to different stages of vascular development. Adapted from Carmeliet, 2000.

STAGE	STIMULATORS	INHIBITORS
VASCULOGENESIS	VEGF, GM-CSF, bFGF, IGF-1	?
ANGIOGENESIS	VEGF, VEGF-B, VEGF-C, VEGF-D, PlGF, VEGFR-1, VEGFR-2, VEGFR-3, Ang-1, Ang-2, Tie-2, FGF, PDGF, IGF-1, HGF, TNFα, TFGβ1, αϖβ3,α5β3, PA, MMP, PECAM, VE-cadh., NO, CXC, HIF-1α, COX2, IL-8	TSP-1, TSP-2, Endostatin, Angiostatin, Vasostatin, PF4, INFγ, INFβ, IL-4, Id1, Id3, Meth, TFPI, VEGI, TIMP, PEX, IP-10, sNP-1
ARTERIOGENESIS	MCP-1, VCAM, GM-CSF, PA, FGF-4, MMP, FGFR1, Selectin, PDGF-B, TGF-β1	PAI-I, TIMP

Abbreviations: GM-CSF, granulocyte-monocyte colony-stimulating factor; IGF-1, insulin-like growth factor-1; HGF, hepatocyte growth factor; MMP, matrix metallo proteinase(s); NO, nitrix oxide; PA, plasminogen activator; HIF-α, hypoxia-inducible transcription factor; MCP-1, membrane cofactor protein-1; VCAM, vascular cell

adhesion molecule-1; TSP, trombospondin; PF4, platelet factor 4; INFγ, interferon gamma; Meth., metallospondin; VEGI, vascular endothelial growth inhibitor; PEX, MMP-2 hemopexin domain TIMP, tissue inhibitor of metalloproteinases, PAI-I, plasminogen activator inhibitor-1.

5.2. Arteries and veins

As mentioned previously (see The Eph family), arterial and venous endothelial cells already show molecular differences even when the embryonic vasculature appears to be a uniform plexus of interconnected tubules. Later these cells can be distinguished by differential lectin staining (Thurston *et al.*, 1996).

5.3. Lymphatic vessels

Like developing blood vessels, the first lymphatics consist only of endothelial cells. The origin of lymphatic endothelial cells is not yet known. Could they be derived from lymphangioblasts of the early mesenchyme (Huntington, 1908), from veins by sprouting (Ranvier, 1895; Sabin, 1909), or by both mechanisms (van der Jagt, 1932)? If lymphatic endothelial cells are derived from veins, and grow exclusively by sprouting, then the main difference between angiogenesis and lymphangiogenesis is the absence of a lymphangioblastic cell lineage. The fact is that lymphatics develop much later than blood vessels. In humans, lymph sacs have been found in 6- to 7-week-old embryos of 10-14 mm total lenght (van der Putte, 1975). This is nearly one month after the development of the first blood vessels. Presumably, there are also endothelial-lined clefts that become integrated into the growing lymphatic system, in addition to lymphatics originating from the veins (Kampmeier, 1912; Rusznyák *et al.*, 1969). Much of the lymphatic system is derived from eight endothelial-lined lymph sacs, which are located immediatelly adjacent to the veins. The jugular (an extension of the jugular lymph sac), subclavian and posterior lymph sacs are paired and single ones are the cisterna chyli and retroperitoneal (mesenterial) lymph sac. With exception of the cisterna chyli, the lymph sacs become transformed into primary lymph nodes.

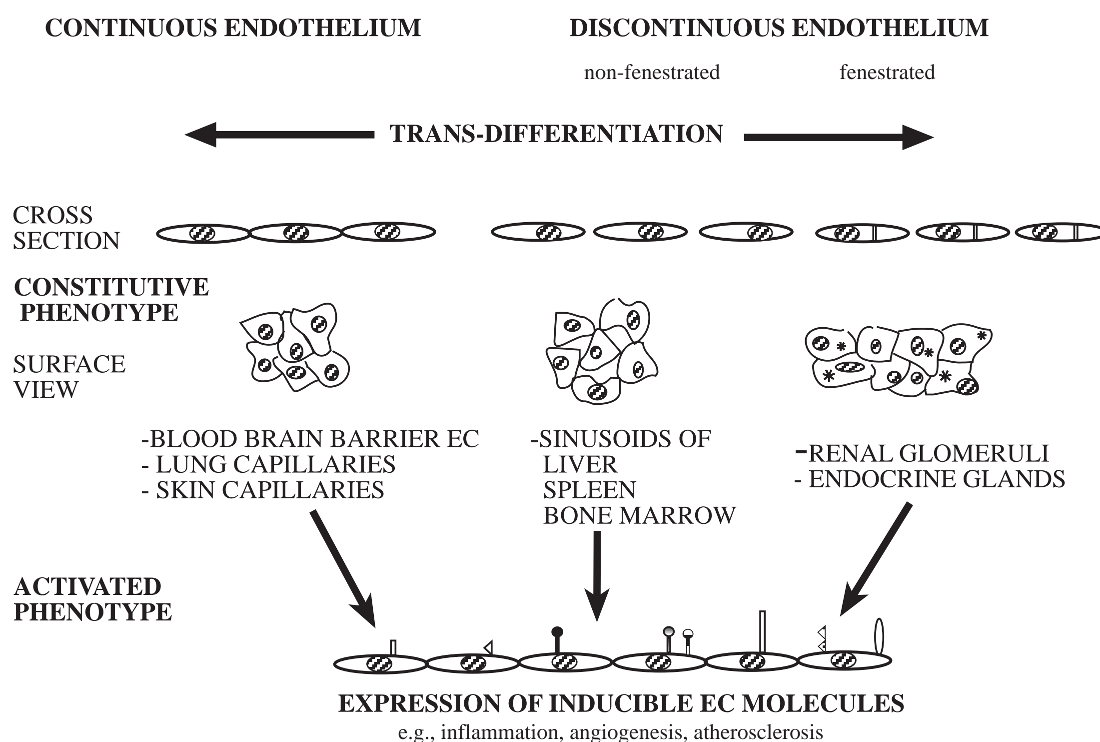
5.4. Endothelial cell heterogeneity and lymphocyte traffic

The trafficking, extravasation, and retention of lymphocytes within certain tissues (e.g., lymph nodes) is mediated by several classes of specialized adhesion glycoproteins that are expressed on the surface of unique endothelial cells (high endothelial venules [HEVs]) that exist in both the blood and lymph microvessels (Roitt *et al.*, 1996). The binding of these endothelial cell adhesion molecules to their corresponding ligands on lymphocytes (L-selectin, [alpha]4[beta]7, [alpha]4[beta]1, leukocyte function– associated antigen-1 [LFA-1], and CLA-1) tightly controls the kinetics and magnitude of lymphocyte margination, rolling, adhesion, and extravasation, thereby allowing these leukocytes to fulfill their immune surveillance functions and, in some tissues, mature into fully differentiated cells (Springer, 1994). To migrate to the extravascular areas, circulating leukocytes have to acquire strong adhesion interactions to the vessel wall while resisting to continuous sheer forces. The traffic and tissue localization of leukocytes is regulated by a series of cell surface adhesion molecules

(CAMs) that recognize specific ligands on endothelial cells and in the extracellular matrix. The most known are the selectins, the super family of the immunoglobulins, the integrins and the proteoglycans, that act in a cascading manner.

Stimulation of resting endothelial cells leads to the situation-specific expression of endothelial cell molecules, which are characteristic for the activated phenotype Likewise, trans-differentiation of endothelial cells characterizes the process through which a specific constitutive endothelial cell phenotype is altered in response to changes in the microenvironmental milieu that controls a specific constitutive phenotype.

Figure 4. Endothelial cell differentiation.



The heterogeneity of endothelial cell phenotype associated with the different types of vessels could give important cues also to the origin of vascular neoplasms or to the characteristics and targets of tumor of vasculature. It is therefore important to know what happens to these properties of endothelial cells in various disease processes.

5.5. Disorders associated with the vasculature

5.5.1. Vascular malformations

Vascular malformations are congenital lesions that are differentiated from hemangiomas on the basis of their normal endothelial cell turnover and lack of excessive proliferation. They are structural anomalies of the vascular system and may be composed of capillaries, veins, lymphatics, arteries, or combinations of the above (Garzon

et al., 2000; Mulliken and Young, 1988; Mulliken and Glowacki, 1982). However, an association between vascular tumors and vascular malformations has been shown.

As previously mentioned, receptor specific evidence of Tie-2 mutations in familiar venous malformations or VEGFR-3 mutation in lymphatic vessel hypoplasia indicates that abnormalities in vasculogenesis are commonly involved in the pathogenesis of vascular malformations. Although the exact mechanism for hemangioma development remains unknown, vascular growth factors seem to play a role in its pathogenesis. EC proliferation in this tumor most likely results from an imbalance between positive and negative angiogenic factors expressed by the hemangioma and the adjacent normal tissue (table 3) (Bielenberg *et al*., 1999).

It has been shown that proliferation markers [proliferating cell nuclear antigen (PCNA), VEGF, bFGF, type IV collagenase and urokinase] can distinguish hemangiomas and vascular tumors (Takahashi *et al*., 1994). Table 4 lists such neoplasms. Unlike normal adult vascular tissue within the cerebral circulation, arteriovenous malformations (AVMs) were shown to stain for VEGF (Rothbart *et al*., 1996). Otherwise comparative immunohistochemical data on vascular malformation versus vascular tumors is scant.

Table 4. Congenital vascular neoplasms and associated conditions.

Hemangiomas
Complicated locations
Cervicofacial
Periorbital
Lumbosacral
Parotid
Associated syndromes
Diffuse neonatal hemangiomatosis
PHACES syndrome
Vascular neoplasms associated with the Kasabach-Merritt syndrome
Kaposiform hemangioendothelioma
Tufted angioma

Abbreviations*:* PHACES, Posterior fossa malformations, most commonly of the Dandy-Walker variant; Hemangiomas (especially large, plaquelike, facial lesions); Arterial anomalies; Cardiac anomalies and coarctation of the aorta; Eye abnormalities; and Sternal cleft and/or supra-umbilical raphe.

5.5.2. Vascular tumors

Vascular tumors consist of broad morphological spectrum from hamartomas to malignant neoplasia (Mentzel *et al*., 1994). Tables 5 and 6 summarize the vascular tumors of the skin and soft tissues. Knowledge about their molecular characteristics is mostly unknown probably, because they are rare (see Tables 7 and 8). Exceptions comprise hemangioma, lymphangioma, KS and angiosarcoma.

Table 5. Classification of vascular tumors of the skin and soft tissues according to C.D.M. Fletcher, 1994.

I BENIGN TUMORS AND TUMOR-LIKE CONDITIONS OF BLOOD VESSELS

1. REACTIVE VASCULAR PROLIFERATIONS
- Papillary endothelial hyperplasia (Masson's tumour)
- Reactive angioendotheliomatosis
- Glomeruloid hemangioma
- Bacillary angiomatosis

2. VASCULAR ECTASIAS
- Naevus flammeus
- Naevus araneus
- Venous lake
- Angioma serpiginosum
- Hereditary hemorrhagic teleangiectasia
- Angiokeratoma

3. CAPILLARY HAEMANGIOMA
- Strewberry naevus
- Tufted hemangioma
- Verrucous haemangioma
- Cherry angioma
- Lobular haemangioma (Pyogenic granuloma)

4. CAVERNOUS HAEMANGIOMA
- Ordinary cavernous haemangioma
- Sinusoidal haemangioma

5. ARTERIOVENOUS HAEMANGIOMA
- Superficial variant (Cirsoid aneurysm)
- Deep variant

6. MICROVENULAR HAEMANGIOMA

7. TARGETOID HAEMOSIDEROTIC HAEMANGIOMA ("HOBNAIL HAEMANGIOMA"

8. EPITHELOID HAEMANGIOMA (ANGIOLYMPHOID HYPERPLASIA WITH EOSINOPHILIA)

9. KIMURA'S DISEASE

10. DEEP HAEMANGIOMA
- Intramuscular (diffuse and circumscribed variant)
- Synovial
- Neural
- Nodal

11. SPINDLE CELL HAEMANGIOMA

12. ANGIOMATOSIS

Table 5. Classification of vascular tumors of the skin and soft tissues according to C.D.M. Fletcher, 1994.

II LOW GRADE MALIGNANT VASCULAR TUMORS

1. **EPITHELOID HAEMANGIOENDOTHELIOMA**
2. **RETIFORM HAEMANGIOENDOTHELIOMA**
3. **MALIGNANT ENDOVASCULAR PAPILLARY ANGIOENDOTHELIOMA (Dabska's tumor)**
4. **KAPOSI-LIKE INFANTILE HAEMANGIOENDOTHELIOMA**
5. **GIANT CELL ANGIOBLASTOMA**
6. **POLYMORPHOUS LOW-GRADE HAEMANGIOENDOTHELIOMA OF LYMPH NODES**
7. **KAPOSI'S SARCOMA**

III MALIGNANT VASCULAR TUMORS

1. **ANGIOSARCOMA**
   - Idiopathic
   - Associated with lymphedema
   - Post-radiotherapy
   - Epitheloid
2. **"INTIMAL SARCOMA"**

IV TUMORS OF LYMPH VESSELS

1. **LYMPHANGIOMA**
   - Lymphangioma circumscriptum
   - Cystic hygroma
   - Progressive lymphangioma (Lymphangioendothelioma)
2. **LYMPHANGIOMYOMA**
3. **LYMPHANGIOMATOSIS**
   - Lymphangiomatosis of the limbs

V TUMORS OF PERIVASCULAR CELLS

1. **GLOMUS TUMOR**
   - infiltrating glomus tumor
2. **GLOMANGIOMA**
3. **GLOMANGIOMYOMA**
4. **GLOMANGIOSARCOMA**
5. **HAEMANGIOPERICYTOMA**

5.5.2.1. Hemangioma

Hemangioma is a benign vascular lesion and is the most common tumor in infancy (Requena and Sangueza, 1997). Infantile hemangiomas are highly proliferative lesions involving aberrant localized growth of the capillary endothelium. They undergo a rapid postnatal proliferative phase that lasts approximately one year followed by a slow involution phase that lasts 5-10 years. The proliferative phase is marked by cellular hyperplasia with and without lumens. It has been suggested that the presence of large nuclei with scant cytoplasm within the endothelial cells of hemangiomas is a

sign for cellular immaturity. In addition, early proliferative cellular hemangiomas have been shown to lack certain differentiation markers spesific to endothelial cells. For example, the cellular regions of hemangiomas lack Weibel-Palade bodies and do not synthesize vWF (Yasunaga *et al*., 1989). Hemangiomas show increased expression of the human hematopoietic progenitor antigen CD34.

The involuted phase is marked by decreased cell turnover and cellularity, an increased number of fully differentiated vessels and more interstitial fibrous and fatty tissue. Based on clinical and histological observations, hemangiomas have been hypothetized to represent angioblastic tissue that undergoes a delayed maturation. Lesions often progress from predominantly unorganized cellular structures during the proliferative phase to ones that have readily identifiable vascular channels at the time of involution.

Histological analyses of tissue specimens have provided important insights into the cellular and molecular interactions within hemangiomas. While hemangiomas are composed of a heterogeneous population of cells, it could be hypothesized that the development of a hemangioma is the product of abnormal endothelial cell proliferation, causing a mis-regulation of angiogenesis. An increase in the number of mast cells has been demonstrated in the proliferative growth phase, and further, mast cells have been shown to be a major source of the angiogenic cytokine, bFGF within hemangiomas (Qu *et al*., 1995). Other promoters of angiogenesis present during the proliverative phase are VEGF (Takahashi *et al*., 1995), type IV collagenase and E-selectin (Kraling *et al*., 1996). In contrast, the involuted phase is marked by a decrease in mast cells and angiogenic factors. Moreover the TIMPs have been shown to be upregulated during the involuting phase. VEGF is produced by a heterogeneous mixture of cells cultured from hemangiomas (Berard *et al*., 1997). Artificial gene manipulation attemps towards angiogenesis therapy have provided additional evidence for the importance of VEGF in the growth of hemangiomas. Murine myoblasts expressing continuously $VEGF_{165}$ induced intramural vascular tumors resembling hemangiomas after injection to mouse myocardium (Lee *et al*., 2000). This kind of hemangioma formation has also occurred when $VEGF_{164}$-transduced myoblasts were introduced to mouse leg muscles (Springer *et al*., 1998).

Usually most hemangiomas occur sporadically and as a single lesions, or as linked to pleiotropic genetic syndromes. The genetic defect for a novel familial form of the infantile hemangioma can be localized to the short arm of chromosome 5 (Walter *et al*., 1999). It is of interest that the defective gene is localized to a region of the chromosome 5 that codes for receptors involved in blood vessel growth, including FGF receptor-4, PDGF-β and VEGFR-3.

5.5.2.2. Lymphangioma

Lymphangiomas result from the abnormal development of the lymphatic system, with prevention of lymph drainage from the affected area. Except for the eye and neural tissue, lymphangiomas can originate in any organ although more than 95% occur in the soft tissues of the head and neck (cystic hygroma) and axilla, with less than 5% occurring in the abdominal cavity.

Macroscopically lymphangioma is a solitary, multicystic mass. The cysts may connect with each other suggesting that they are dilated lymphatic channels. These channels contain serous, serosanguinous, or chylous fluid. The lining of the cysts is smooth and they have thin walls. Histological criteria for lymphangiomas have been

defined by Enzinger: 1) lymphatic spaces lined by endothelium, 2) fascicles of smooth muscle in the septa between the lymphatic spaces, and 3) lymphoid aggregates in the delicate collagenous stroma (Enzinger and Weiss, 1995). Lymphangiomatosis is a rare disorder which affects bones, parenchymal organs, and

Table 7. Summary of the expression of VEGF family members in vascular tumors.

GROWTH FACTOR/ RECEPTOR	VASCULAR LESION	RESULT	REFERENCES
VEGF	Pyogenic granuloma	Strong in proliferating cells without vessel lumen formation	(Bragado *et al*., 1999)
	Hemangioma	Weak/equivocal Proliferative phase positive	(Brown *et al*., 1996) (Takahashi *et al*., 1994)
	Angiosarcoma Aicardi sdr Soft tissue Cutaneous	Focal Strong/moderate High/Intermediate Weak	(Hashimoto *et al*., 1995; McLaughlin *et al*., 2000) (Zietz *et al*., 1998) (Brown *et al*., 1996)
	Kaposi's sarcoma AIDS associated	Mostly weak Not significant	(Brown *et al*., 1996) (Skobe *et al*., 1999)
VEGF-C	Kaposi sarcoma AIDS associated	Not significant	(Skobe *et al*., 1999)
VEGFR-1	Hemangioma	Strong	(Brown *et al*., 1996)
	Angiosarcoma Aicardi sdr	Strong, diffuse	(McLaughlin *et al*., 2000)
	Kaposi sarcoma AIDS associated	Strong	(Skobe *et al*., 1999)
VEGFR-2	Hemangioma	Strong	(Brown *et al*., 1996)
	Angiosarcoma Aicardi sdr	Strong, diffuse	(McLaughlin *et al*., 2000)
	Kaposi sarcoma AIDS associated	Strong	(Skobe *et al*., 1999)

GROWTH FACTOR/ RECEPTOR	VASCULAR LESION	RESULT	REFERENCES
VEGFR-3	Hemangioma	Almost negative	(Fanburg-Smith *et al.*, 1999; Folpe *et al.*, 2000)
	Hobnail hemangioma/Targetoid hemosiderotic hemangioma	Positive	(Mentzel *et al.*, 1999) (Fanburg-Smith *et al.*, 1999)
	Papillary intralymphatic angioendothelioma	Positive	(Fanburg-Smith *et al.*, 1999)
	Angiosarcoma Angiosarcoma -no lymphedema associated	Positive 50% cases positive	(Breiteneder-Geleff *et al.*, 1999) (Folpe *et al.*, 2000) (Fanburg-Smith *et al.*, 1999)
	Kaposi sarcoma - Classic - AIDS associated - not lymphedema associated	Positive Positive Strong positivity	(Jussila *et al.*, 1998) (Folpe *et al.*, 2000)

soft tissues by diffuse proliferation of lymphatic channels (Gomez *et al.*, 1995).

Immunoelectron microscopic studies have demonstrated upregulation of CD31 and CD34 and show type IV collagen expression in lymphangiomas (Sauter *et al.*, 1998). Furthermore, as yet molecularly undefined PAL-E was confined to blood vessels in lymphangiomas. Light microscopy is not always able to detect CD34 immunoreactivity (Paal *et al.*, 1998). Data on the VEGF- and Tie family members in the tumors of lymph vessels is scant. But VEGFR-3 mRNA has been localized to a human lymphangioma (Kaipainen *et al.*, 1995).

A mouse model for lymphangioma was established by Mancardi et al (Mancardi *et al.*, 1999). By injecting incomplete Freund's adjuvant intraperitoneally to nude mice the authors were able induce tumors that fulfill Enzinger's criteria for a lymphangioma. In addition to VEGFR-1 and VEGFR-3, the newly formed tumor endothelium was found to express intracellular adhesion molecule-1/CD54.

5.5.2.3. Angiosarcoma

Recent publications link human herpes virus 8 and two other lympho-proliferative disorders: multicentric Castleman's disease (Cesarman *et al.*, 1995; Soulier *et al.*, 1995) and a newly recognized disease, body-cavity-based lymphoma or primary effusion lymphoma (Knowles *et al.*, 1989). Also an association between angiosarcoma and HHV-8 has been shown (Remick *et al.*, 2000). All of these tumors are characterized by the abnormal proliferation of the vascular endothelium, a putative cellular target of human herpes virus-8 infection.

Cutaneous angiosarcomas can be caterigorized into three types: 1) Wilson Jones angiosarcoma affecting the face and scalp of elderly patients, 2) lymphedema associated AS and 3) radiation-induced AS. Angiosarcoma represents 2% of all soft tissue sarcomas and most typically it appears 10 years after mastectomy in lymphedematous arms (Stewart-Treves syndrome). Lymphedema is thought to modify the biochemical or immunological status of the affected limb, fostering the development of angiosarcoma. It has been noted that skin grafts survive for long periods when transferred to lymphedematous extremities, suggesting that chronically affected lymphedematous sites are "immunologically privileged" (Stark *et al.*, 1960). Tables 7 and 8 show that angiosarcomas have been studied extensively. The data indicate that many of the VEGF and Tie family members are involved.

5.5.2.4. Kaposi's sarcoma

Kaposi's sarcoma (KS) is a multicentric neoplasm of vascular origin and very probably HHV-8 or KS-associated herpes virus etiology (Chang *et al.*, 1994). The kaposin oncogene of the HHV-8 is able to induce tumorigenic transformation, has been found to be expressed in the spindle shaped cells of this tumor (Ganem, 1997; Muralidhar *et al.*, 1998; Staskus *et al.*, 1997). These categories of KS including the "classical" in older males of mainly Mediterranean or Eastern European Jewish backgrounds; "endemic", found in parts of equatorial Africa; "iatrogenic", immunosuppressive drug-associated KS and AIDS associated KS all express the oncogene. Not all HIV-positive patients get KS, and that may be because they lack kaposin as a sexually transmitted agent or co-factor.

Histologically, in early KS lesions, which normally appear on the skin, there is a collection of small, irregular, endothelial-lined spaces that surround normal dermal blood vessels (Gallo, 1998). These are accompanied by a variable, inflammatory infiltrate of lymphocytes, known as the patch stage. This is followed by the expansion of the spindle-cell vascular process throughout the dermis. These spindle cells form slit-like vascular channels containing erythrocytes (the plaque stage). The later, nodular-stage KS lesions are composed of sheets of spindle cells, some of which are undergoing mitosis, and slit-like vascular spaces which have areas of haemosiderin pigmentation. The spindle cells form the bulk of established KS lesions, but based on chromosomal analysis they are not neoplastic (Gallo, 1998).

The majority of the spindle cells stain for endothelial cell markers, though evidence for smooth muscle cell, macrophage, dendritic cell and multipotential cell origin also exists (Kaaya *et al.*, 1995). High local levels of cytokines such as interleukin 6 (IL-6), basic fibroblast growth factor (bFGF), tumour necrosis factor- α (TNF-α), interferon-γ and VEGF have been isolated from KS lesions (Ensoli *et al.*, 1989; Miles *et al.*, 1990) (Nakamura *et al.*, 1988). Interestingly, the HIV Tat protein, that transactivates transcription of HIV, specifically binds and activates VEGFR-2 (Albini *et al.*, 1995). It has been shown that the Tat basic domain contains an arginine- and lysine-rich sequence that is similar to that of the potent angiogenic growth factors, VEGF and FGF (Albini *et al.*, 1996). KSHV encodes a G protein-coupled receptor (GPCR) that switches on the angiogenesis by inducing the production of VEGF (Munshi *et al.*, 1999). VEGF-C has been found to be strongly expressed by the blood vessels surrounding blood vessels the KS, but not the spindle shaped cells (Skobe *et al.*, 1999). As table 7 shows VEGFR-1, VEGFR-2 and

VEGFR-3 are strongly expressed in the spindle cells of KS. However, no study was available in which these factors had been analysed from the same sample.

Table 8. Summary of the expression of Tie family members in vascular tumors.

GROWTH FACTOR/ RECEPTOR	VASCULAR LESION	EXPRESSION	REFERENCES
Tie-1	Angiosarcoma Cutaneous	 Strong	 (Brown *et al*., 2000)
	Kaposi's sarcoma AIDS associated	 Mostly strong	 (Brown *et al*., 2000)
Tie-2	Angiosarcoma Cutaneous	 Strong	 (Brown *et al*., 2000)
	Kaposi's sarcoma AIDS associated	 Mostly strong	 (Brown *et al*., 2000)
Angiopoietin-1	Angiosarcoma Aicardi sdr Soft tissue Kaposi's sarcoma HIV-positive	 Not clear Low Strong	 (McLaughlin *et al*., 2000) (Brown *et al*., 2000)
Angiopoietin-2	Angiosarcoma Aicardi sdr Soft tissue Kaposi's sarcoma HIV-positive	Very strong,diffuse Low Strong	 (McLaughlin *et al*., 2000) (Brown *et al*., 2000)
Angiopoietin-4	Angiosarcoma Aicardi sdr Cutaneous Kaposi sarcoma HIV-positive	 Not clear Equivocal Low/equivocal	 (McLaughlin *et al*., 2000) (Brown *et al*., 2000)

AIMS OF THE PRESENT STUDY

We wanted to elucidate the specificity of the recently cloned growth factors and receptors of the lymphatic vasculature and to explore their usefulness in molecular identification of lymphatic vascular structures, for which no molecular antigenic markers have been previously presented. Also, no proper attempt to distinguish different types of vascular tumors and vascular malformations has been made by assessing molecular markers for angiogenic growth factors and receptors. Instead, the widely used classification of the International Society for the Study of Vascular Anomalies is still based on the clinical and histological observations. The general aim of this study was thus to investigate angiogenic molecules as endothelial cell markers.

The specific aims of the study were:

1. *In vivo* analysis of VEGF-C and VEGF in human skin and verifying VEGFR-2 and VEGFR-3 in the dermal vasculature.

2. Assessment and comparison of VEGFR-3 and podoplanin as lymphatic markers in Kaposi's sarcoma, and in Kaposi's sarcoma-derived cell cultures.

3. Locate the known endothelial growth factor receptors in human fetal heart vasculature.

4. Analyze the expression of VEGFR-3 in vascular tumors and analyze the conditions where anti-VEGFR-3 antibodies can be used as markers for endothelia of lymphatic origin.

5. Establish the normal expression patterns of VEGFR-3 and its ligands, VEGF-C and VEGF-D in various human tissues.

MATERIALS AND METHODS

1. Materials

1.1. Tissue specimens

All fetal tissues included in this study have been obtained from legal abortions of healthy women induced with prostaglandins. The gestational age was estimated from the foot length (Munsick, 1984). Tissue samples had been fixed in 4% paraformaldehyde for 20 hours, dehydrated, and paraffin-embedded for sectioning.

All the other specimens, histologically normal adult tissues and tumor samples were fixed in 4% phosphate–buffered formaldehyde immediately after removal, and transported to the Department of Pathology, University of Helsinki, or in the case of KS samples (for details, see original publication II) to the Institute of Clinical Pathology and the Department of Dermatology, University of Vienna or in case of vascular tumors (for details, see original publication IV) to the Department of Soft Tissue Pathology, the Armed Forces Institute of Pathology, Washington, DC. The vascular tumors were classified according to Enzinger and Weiss (Enzinger and Weiss, 1995).

1.2. Antibodies for immunocytochemistry and Western analysis

The antibodies used in this study are listed in tables 9 and 10. In the previously unpublished studies the following antibody concentrations/dilutions were used: VEGF-C (hybridoma culture fluid of clones #9H7F10, 2C1D11, 9H7C12 and 9H7H3) 1:2; VEGF-C (882) 1.5µg/ml; VEGF-D (#78939.11, 78923.11 and 78935.11) 10µg/ml; VEGF-D (VD1) 2.9µg/ml; VEGF-D (#N19) 1:250-300, VEGF-D (749-1AP) 0,2µg/ml; VEGFR-1 1:200 dilution of the supernatant of clone 19 and VEGFR-2 1:800.

Table 9. Summary of the antibodies used specifically in detecting VEGFs and Tie-receptors.

ANTIBODY	SOURCE	REFERENCE	PEPTIDE	USED IN
VEGF Monoclonal Clone# 26503.11	Santa Cruz Biotechnoloy, CA			V
Polyclonal N19	SantaCruz Biotechnoloy, CA			V
VEGF-C Monoclonal clone# 2C1D11	Dr K. Alitalo		Baculo	V
9H712	Dr K. Alitalo		Baculo	V
9H7H3	Dr K. Alitalo		Baculo	V
9H7F10	Dr K. Alitalo		Baculo	V
Polyclonal 882	Dr K. Alitalo	(Joukov *et al.*, 1997)	Aa residues 104-120 of the VEGF-C prepropeptide	V

C20	Santa Cruz		Corresponding to aas 400-419	V
VEGF-D				
Monoclonal clone#				
78923.11	R&D			V
78935.11	R&D			V
78939.11	R&D			V
VD1	Dr M. Achen	(Achen *et al.*, 2000)		V
VEGF-D				
Polyclonal				
N19	Santa Cruz	Corresponding to aas 22-40		V
749-1AP	R&D	(Barleon *et al.*, 1997)		V
VEGFR-1	R&D	(Simon *et al.*, 1998)	Human soluble Flt1 protein (domain 1-5)	V
VEGFR-2	Dr H. Weich	(Simon *et al.*, 1998)	Baculovirus	III
Neuropilin-1	Dr H. Kitsukawa	(Kitsukawa *et al.*, 1995)	aas 483-856 of mouse neuropilin	III
VEGFR-3				
Monoclonal clone # 9D9F9	Dr. K. Alitalo	(Jussila *et al.*, 1998)	EC domain of VEGFR-3 in a Baculovirus	I, II, III, IV, V
Tie-1				
Monoclonal clone #				
7E8	Dr. K. Alitalo	(Salven *et al.*, 1996)	EC domain of Tie	III
10F11	Dr. K. Alitalo	-“-	-“-	III
Tie-2/Tek				
Monoclonal clone #	Dr T. Suda	(Yano *et al.*, 1997)		III
Podoplanin	Dr D. Kerjaschki	(Breiteneder-Geleff *et al.*, 1997)		II

Table 10. Summary of the commercial antibodies used in this study.

COMMERCIAL ANTIBODIES	SOURCE	STUDY
CD31 (platelet/endothelial cell adhesion molecule 1)	DAKO Immunoglobulins, Glostrup, Denmark	I, II, III & V
CD34 (clone QBEND)	DAKO Immunoglobulins, Glostrup, Denmark	IV
CD34 (clone QBEND)	Immunotech, Westbrook, Maine	II
CD31 (clone JC/70)	DAKO, Carpinteria, CA	IV
CD68	Becton Dickinson, San Jose, California	II
vWF (von Willebrand factor/factor VIII-related antigen)	DAKO Immunoglobulins, Glostrup, Denmark	I, III
PAL-E (an as yet molecularly undetermined vascular marker)	Serotec, Oxford, United Kingdom	II
PAL-E	Sanbio, Uden, The Netherlands	III
laminin	Sigma Chemical Co, St.Louis, MO	I
Desmoplakin 1&2	Progen Biotechnik GmbH	III
α-smooth muscle actin, clone 19	Sigma Chemical Co, St.Louis, MO	III
α-smooth muscle actin, clone 1A4	Sigma Chemical Co, St.Louis, MO	V
Insulin	DAKO, Carpinteria, CA	V
Glucagon	DAKO, Carpinteria, CA	V
Somatostatin	DAKO, Carpinteria, CA	V
Chromogranin A	DAKO, Carpinteria, CA	V
Gastrin	DAKO, Carpinteria, CA	V
Serotonin	DAKO, Carpinteria, CA	V
Glial fibrillary acidic proteon	DAKO, Carpinteria, CA	V
Adrenocorticotropin	DAKO, Carpinteria, CA	V
Growth hormone	DAKO, Carpinteria, CA	V
S-100	DAKO, Carpinteria, CA	V
Neurofilament 200	Boehringer Mannheim, Germany	V

1.3. Probes for *in situ* hybridization and Northern analysis

Each tyrosine kinase receptor or ligand cDNA sequence was subcloned into an appropriate transcription vector with RNA polymerase promoters on either side of the insert. The plasmids were linearized by restriction endonuclease digestion and purified. Sense and antisense radiolabelled RNA probes were obtained via the incorporation of (^{35}S)UTP on addition of specific polymerases. Synthesis of radioactive RNA was followed by treatment with DNAase I and partial alkaline hydrolysis of RNA to

obtain fragments of appropriate length. The probes thus produced are listed in table 11.

Table 11. Summary of the probes used in this work.

cDNA	Plasmid	cDNA source	Nucleotides	Used in
hVEGFR-2	pBS K II SK+	Dr Arja Kaipainen	6-715	I, V
hVEGFR-3	pGEM 3Z (f+)	Dr Arja Kaipainen	1-595	I, V
hVEGF-C	pREP7	Dr Vladimir Joukov	494-1661	V
hVEGF-D	pcDNA3.1	Taija Mäkinen	411-1685	V

1.4. Primary cell culture

Cultures of spindle cells (table 12) derived from KS tissue were established by explant culture method (M7/2, M12/4) or by enzymatic dissection (M7Col12, M12T8) of KS biopsies from skin of two male patients with AIDS (M7, M12). Cells were maintained in Dulbecco's minimal essential medium with 10% fetal bovine serum as previously described (Roth *et al.*, 1988) (Roth *et al.*, 1989). KS spindle cells were characterized by cytochemical staining and were positive for Ulex europaeus antigen-1 and BMA120.

KS Y-1 is a autonomously growing KS-derived tumor cell line and a kind donation from Dr. M.Reitz. Human umbilical vein endothelial cells were cultured as described earlier (Weninger *et al.*, 1998).

Table 12. The primary cell cultures used in this work.

CELL LINE	DESCRIPTION	SOURCE	USED IN
M7/2	human KS *	(Pammer *et al.*, 1996)	II
M12/4	human KS*	(Pammer *et al.*, 1996)	II
M7Col 12	human KS†	(Pammer *et al.*, 1996)	II
M12T8	human KS†	(Pammer *et al.*, 1996)	II
KS Y-1	human KS	(Lunardi-Iskandar *et al.*, 1995)	II
HUVEC	Umbilical vein EC	(Weninger *et al.*, 1999)	II

*Abbreviations: KS, Kaposi's sarcoma; * explant culture; †enzymatic dissection.*

Table 13.

Northern blot	Description	Source	Used in
Human endocrine system	Multiple tissue mRNA	Clontech	V

1.5. Receptor-binding analysis using iodinated growth factors

The growth factors listed in table 13 were labeled with ^{125}I using the Iodo-Gen reagent (Pierce, Rockford, IL) and purified by gel filtration on PD-10 columns (Pharmacia, Uppsala, Sweden). The specific activities were 2.2x105cpm/ng and 1.0x105cpm/ng for rh-VEGF and rh-VEGF-C, respectively. The iodinated growth factors were tested for specific binding using PAE-VEGFR-1 and VEGFR-3 cells (Joukov *et al.*, 1997) and soluble receptor proteins (Achen *et al.*, 1998). For details, see the original publication (I).

Table 14.

GROWTH FACTORS	SOURCE OR REFERENCE	USED IN
Rh VEGF165	R & D Systems	I
VEGF-C 21-kd	(Joukov *et al.*, 1997)	I

2. METHODS

2.1. Immunocytochemistry

In all studies indirect immunocytochemistry was used, using a secondary antibody against the primary antibody, and the antibody complexes were detected by the avidin-biotin-peroxidase complex (ABC)-technique (Hsu and Raine, 1981).

In both formalin and paraformaldehyde-fixed paraffin-embedded tissues, VEGFR-3 antigenicity had to be recovered before staining by high-temperature heating of sections. The citrate-mediated high-temperature antigen retrieval alone gave only a very weak staining result but when it was combinated with commercial tyramide signal amplification system (TSA™ by NEN® Life Science Products, Inc., Boston, Mass.) staining result of the 4% paraformaldehyde fixed material was successful. The best staining result was achieved by heating the slides for 20 min in 0.05M Tris-Hcl buffer containing 0.1% Tween (DAKO®antibody diluent with background reducing components) at 98°C and using DAKO's antibody diluent. The latter protocol has turned out to be the most useful one especially in formalin-fixed material. For details, see the original publications (I-V). Negative controls were done by omitting the primary antibody, using irrelevant primary antibody of the same isotype, or blocking the anti-VEGFR-3 and the anti-VEGF-C by overnight incubation with a 10-fold molar excess of the immunogen (Joukov *et al.*, 1997; Jussila *et al.*, 1998). VEGF-D blocking was done with a 40-fold molar excess of the immunogen for 1 h at room temperature.

Table 15. Summary of the methods used in this work.

METHODS	USED IN
Immunohistochemical staining	I, II, III, IV, V
Preparation of human fetal and adult tissues	I, III, V
RNA isolation	II
RNA in situ hybridization	I
RT-PCR	II
Northern blotting	II, V
Immunofluorescence staining	II, V
Protein radioiodination	I
Confocal laser microscopy	II
Cell culture	II
Flow cytometry	II

2.2. *In situ* hybridization (I)

For in situ hybridization, 4μm cryostat sections were cut onto sterile glass slides pretreated with 2% 3-aminopropyltriethoxysilane in acetone, and fixed in 4% paraformaldehyde. The hybridization was performed as described by Wilkinson et al (Wilkinson *et al.*, 1987) with some modifications. After the hybridization the sections were washed at low stringency conditions (2x saline-sodium citrate buffer (SSC), 20 mmol/L dithiotreitol) for 30 min at 65°C. The slides were then coated with the NTB2 Kodak emulsion, incubated for 2-8 weeks and developed with Kodak D19 developer for 4 min. Post-fixation was done with Kodak Unifix for 4 min, and thereafter the slides were counterstained with Mayer's hematoxylin, dehydrated and finally mounted in Permount. The controls included hybridization with sense probes, as well as unhybridized slides..

2.3. Iodinated growth factor binding (I)

Frozen sections were cut at 7μm from adult human skin. The sections were mounted onto silane-coated slides and stored in air-tight boxes at –70°C. After thawing, the sections were incubated for 30 min at room temperature in the blocking solution, [MEM (Gibco), 0.5mg/ml BSA, 20mM Hepes pH 7.4, 1mM PMSF and 4 μg/ml leupeptin]. The blocking buffer was then removed and the sections were covered by a droplet of the same buffer containing 10 pM ^{125}I-rh VEGF or 10pM ^{125}I-rh-VEGF-C. To define non-specific binding, adjacent sections were incubated in the same concentration of iodinated growth factor in the presence of 1nM of the corresponding non-labeled growth factor. After 90-minute incubation in a humidified chamber at room temperature, the sections were rinsed for 5x3 minutes on ice, once with the binding buffer and four times with phosphate-buffered saline (PBS). Sections were the fixed for 10 min in 2% paraformaldehyde, 2% glutaraldehyde in 0.1M phosphate buffer pH 7.4, rinsed for 2-5 s in dH_2O, and dried at room temperature for approximately 2 h.

The dried sections were covered with NTB-2 emulsion (Eastman Kodak Co., Rochester, NY) and stored at 4°C for 2 weeks, developed and stained.

2.4. Fluorescence microscopy (II, V)

Expression of VEGFR-3 and podoplanin was studied by fluorescence microscopy on acetone fixed cryosections of two HIV-1-positive and two HIV-1-negative KS samples. For double staining the sections were sequentially incubated with anti-VEGFR-3 or anti-podoplanin antibodies at 4°C overnight, followed by tetrarhodamine isothiocyanate (TRITC)- and FITC-labeled second step reagents, respectively. After incubation with 10% normal mouse serum, the anti-VEGFR-3 sections were reacted with FITC-labeled anti-CD31 mAb (1,5 mg/ml, Alexis Corp., San Diego, California) or FITC-labeled anti-CD68 mAb (1:2000), Beckton Dickinson, San Jose, California; kindly provided by Dr. O.Majdic, Vienna). The podoplanin labeled sections were incubated with anti-CD31 (1:250), Becton Dickinson), anti-PAL-E (1:200) or anti-VEGFR-3 (1μg/ml) followed by TRICHH-labeled goat anti-mouse serum (1:100, Jackson Immuno Research Laboratories, Westgrove, Pennsylvania). Sections were evaluated by using a confocal laser microscope (LSM 410, Zeiss, Oberkochen, Germany). Appropriate isotope matched controls were run in parallel, and these consistently showed no reactivity.

2.5. Northern analysis (V)

The probes listed in table 11 were labelled by the random priming method and incubated with the blot (table 12) in ULTRAhyb solution at 55°C overnight, followed by washes in high stringency conditions and exposure in PhosphoImager.

RESULTS AND DISCUSSION

1. Expression of the Vascular endothelial Growth factor C Receptor VEGFR-3 in Lymphatic Endothelium of the Skin and in Vascular Tumors (Study I)

The microvasculature of the skin was characterized by early morphological studies in the 1950s and 1960s. Remarkably, the cutaneous lymphatic network has been rather neglected, probably due to the lack of specific markers. A limited number of ultrastructural studies showed that the structure of the cutaneous lymphatics to be markedly different from that of vascular capillaries (Berens von Rautenfeld *et al.*, 1987; Leak and Burke, 1966). Lymphatics are flattened tubes lined by an extremely attenuated endothelium encompassed only by sub-endothelial “basal lamina-like” material, whereas surrounding pericytes are lacking (Ryan, 1989). The lymphatic endothelium contains very few pinocytotic vesicles and lacks Weibel-Palade bodies and fenestration.

Based on previous observations of VEGFR-3 expression by *in situ* analysis, we assumed that the monoclonal antibody against the receptor EC domain detected only the lymphatic endothelium, after organogenesis (Jussila *et al.*, 1998; Kukk *et al.*, 1996). In the normal human skin this turned out to be true. Furthermore, the endothelium of the skin or soft tissue affected by lymphangiomatosis was strongly stained for VEGFR-3, compared to that of normal dermal lymphatics, indicating up-regulation of the receptor in that rare disorder.

In vivo analysis VEGF-C by protein radio-iodination, showed the growth factor binding to the lymphatic vessels of the upper dermis whereas VEGF bound throughout the extending dermal vascular endothelium. Indeed, over-expression of VEGF-C under the keratin 14 promoter has been shown to cause hyperplasia of lymphatic vessels (Jeltsch *et al.*, 1997), a condition that mimics lymphangiomatosis in humans (personal communication with C.M.D. Fletcher). It would thus be of interest to determine if patients with lymphangiomatosis suffer from genetic alteration in the coding region of VEGF-C. This would not be surprising considering the early occurrence of this disease mainly in children or during the first two decades of life and the fact that lymphatics differentiate probably as the last endothelium lined structure of vascular tree (Gomez *et al.*, 1995).

The findings documented here indicate that VEGFR-3 is distributed in a manner consistent with the known lymphatic vascular pattern in human skin as shown by specific radioactive ligand binding, receptor *in situ* hybridization, and immunohistochemistry.

2. Expression of Vascular Endothelial Growth Factor Receptor-3 and Podoplanin Suggests a Lymphatic Endothelial Cell Origin of Kaposi's Sarcoma Tumor Cells (Study II)

One of the most asked questions about the pathogenesis of KS is: Which cell type gives rise to the spindle shaped cells that characterize the late stage of the disease? KS is commonly thought to be derived from endothelial cells because of the predominant expression of endothelial markers, CD34, VE-cadherin, vWF and endothelial leukocyte adhesion molecule type 1 in KS lesions (Samaniego *et al.*, 1998). However, the heterogeneity of the spindle-cell compartment makes the precise lineage relationship of KS tumor cells unclear.

Some cultured KS-derived spindle cells constitutively overexpress antiapoptotic proteins and exhibit invasive properties, which suggest that they may adequately represent the tumor cells of KS (Mori *et al.*, 1999). Interestingly, KS-derived spindle four cell cultures did not express VEGFR-3 or podoplanin in our studies. This may reflect generally the problem that in the course of cell culture, the original antigenic phenotypes are easily lost or as the second explanation the cultured cells are not related to the tumor cells in KS lesions. Absence of other KS markers, CD31 and CD34 supports the former alternative. HHV-8 is lacking from the KS- cell cultures which gives further support to that (Lunardi-Iskandar *et al.*, 1995). However, there are also VEGFR-2 and VEGFR-3 expressing KS-cell cultures, too, that can be stimulated by VEGF-C (Liu *et al.*, 1997; Skobe *et al.*, 1999). The mitogenic effect of VEGF-C on KS-cells is mediated through VEGFR-2 because a recombinant point mutant of VEGF-C that binds and activates selectively VEGFR-3 did not induce endothelial cell migration (Joukov *et al.*, 1997). Until now, no data is available about the role of VEGF-D, the other ligand for VEGFR-2 and VEGFR-3, in the pathogenesis of KS.

Recently, it was suggested that spindle cells are derived from myofibroblasts (Simonart *et al.*, 2000). The authors concluded that in histological sections the prominent growth of endothelial cells is a reactive hyperplasia and showed by myofibroblast antigens in their cultured KS-cells. Our immunohistochemical results are in accordance with this view. The consistent expression of VEGFR-3 on both the very early stage of KS, which is characterized by endothelial cell activation and proliferation and inflammatory cell infiltration, and KS tumor cells in nodular lesion deepened earlier data from our laboratory where we showed the antigen in spindle cells (Jussila *et al.*, 1998). In the light of present knowledge about VEGFR-3's role in the maintenance of endothelial lining integrity in tumor vasculature, the antigen is needed for survival signaling in KS cells as well (Kubo *et al.*, 2000). By immunohistochemical staining of serial sections and immunofluorescence double labeling, VEGFR-3 immunopositive cells of early and late lesions shared an identical phenotype and were negative for CD68, CD45, and PAL-E, but were positive for CD31 and CD34. This antigenic profile excluded that VEGFR-3 positive cells represent sinus lining cells of lymph node, macrophages, and cells of hemopoietic lineage in general. Northern hybridization and RT-PCR assessment confirmed VEGFR-3 mRNA in KS lesions. Further, KS had been shown to be immunopositive for VEGFR-3, irrespective of origin (Jussila *et al.*, 1998).

Our study was based on the assumption that anti-VEGFR-3 antibody detects only the lymphatic endothelium. Previous studies on the antigen have changed that view (Valtola *et al.*, 1999). Additional evidence for the relationship of KS tumor cells to lymphatic EC came from human podoplanin expression studies (Breiteneder-Geleff *et al.*, 1999). This membrane glycoprotein, which was originally identified on podocytes in rat glomerular cells, is expressed on lymphatic but not blood vessel EC. It is present on lymphangiomas, early intestinal KS, variable present on angiosarcomas, but absent from tumor cells in hemangiomas. All KS tumor cells and cells lining vascular slits in early as well as in late KS lesions homogeneously express podoplanin. Staining patterns for podoplanin and VEGFR-3 were overlapping, as detected by confocal laser microscopy.

Even though several authors have suggest a lymphatic derivation of KS spindle cells from lymphatic endothelia, the confirmation of this hypothesis has been impossible because of the lack of specific lymphatic markers. Based on present knowlegde about VEGFR-3 we can not be sure if KS-cells are of lymphatic endothelial origin. On the other hand, the information about podoplanin contribution to vasculogenesis and angiogenesis is lacking completely and not very much is known about its biological function in normal vasculature. One possibility is that the KS clonogenic cells have circulating mesenchymal stem cell properties.

3. Endothelial Growth Factor Receptors in Human Fetal Heart (Study III)

Transfecting genes encoding angiogenic cytokines, particularly those naturally secreted by cells, may constitute an alternative treatment strategy for patients with extensive tissue ischemia, in who contemporary therapies (anti-anginal medication, angioplasty, bypass surgery) have previously failed or are not feasible (Ferrara and Alitalo, 1999). Therapeutic angiogenesis is designed to promote the development of supplemental collateral blood vessels, which will constitute endogenous bypass conduits around occluded native arteries. When using such techniques, it is essential to map the ligand binding receptors, and thus estimate the consequences, or side effects of the given growth factor. The former was done for human fetal heart tissue.

The immunohistochemical approach supported previous findings of VEGFR1, VEGFR-2 and VEGFR-3 receptor tyrosine kinases in the human fetal heart, except that in the present study, a minor population of the myocardial capillaries also contained VEGFR-3 in 13- to 30-week fetuses (Kaipainen *et al.*, 1993). However, while the analysis with *in situ* hybridization suffers from morphological insensitivity, with immunohistochemistry the structures could be better recognized. This allowed us to report the specificity of these important receptors in different types of heart vessels in great structural detail. Furthermore, besides VEGFR-3, we were able to demonstrate that VEGFR-2 and both of the Tie-receptors are present in lymphatic vessels. The more intense staining of VEGFR-3 in 5-week embryos suggest that the expression is downregulated in the blood vessels during the first trimester. VEGFR-1 and NP-1 showed overlapping expression patterns. A notably strong co-expression of VEGFR-1, VEGFR-2, NP-1, Tie-1, and Tie-2 was associated with the endocardial endothelium, which is subject to considerable degree of hemodynamic stress. Conversely, VEGFR-3 was not detected in the endocardium or other subject to shear stress.

The assembly and maintenance of a functional vasculature likely requires the orchestration of a variety of endothelial growth factors and receptors, which play roles at different stages of the process. Even though VEGFR-1 and VEGFR-2 were expressed in the same structures, only the former located to coronary arteries and myocardial capillaries. Unlike all the other receptors VEGFR-3 was not detected in the blood vessels or in the endocardium. Although VEGFR-3 is abundant in the early stages of cardiovascular development, it later seems to take part in more specialized biological functions preferably those of lymphatic endothelia. When compared with fetal frozen sections, the adult heart samples showed qualitatively same results. Further studies will need to be established to find out if these endothelial growth factor receptor expression patterns are altered in an ischemic adult heart.

4. Lack of Lymphatic Vascular Specificity of Vascular Endothelial Growth Factor Receptor 3 in 185 Vascular Tumors (Study IV)

The origin of various vascular lesions, lymphatic or blood endothelium has prompted much debate. The staining with anti-VEGFR-3 antibodies also suggested the lymphatic origin of KS spindle shaped cells (Jussila *et al.*, 1998). In the subsequent study both classical and AIDS-associated KS forms were therefore studied along with a panel of 185 vascular endothelial tumors, both benign and malignant. This revealed that the VEGFR-3 antibody is not suitable for distinguishing the blood vascular or lymphatic origin of tumor endothelium. The receptor was widely expressed among benign and malignant vascular tumors in adults as well as children. Moreover, the VEGFR-3 is switched on the newly developed blood capillaries around the tumor which makes it even a bigger problem in the identification of lymphatic vessels. Thus anti-VEGFR-3 antibody detects both the lymphatic vessels and blood capillaries induced by the tumor. Considering its expression, it is of interest that recently, the biological function of VEGFR-3 in the tumor neovasculature was associated with the maintenance of the endothelial cell lining during tumor angiogenesis (Kubo *et al.*, 2000).

Most capillary hemangiomas, tumors with blood-filled vessels, were positive for VEGFR-3, highlighting the extensive distribution of VEGFR-3 in proliferating vessels of non-lymphatic nature. Although VEGFR-3 expression was present in most infantile capillary hemangiomas, it was equally seen in capillary hemangiomas of adults, including pyogenic granulomas, reactive vascular proliferations related to lobular capillary hemangiomas. In contrast, in a recent study only 2/13 hemangiomas stained for VEGFR-3 that gives a view of more spesific marker for lymphatic endothelial cells (Folpe *et al.*, 2000). The difference between our study and the one by Folpe et al can be explained by the antigen retrieval process.

Among strongly positive vascular tumors were spindle cell hemangiomas (hemangioendotheliomas) and Kaposiform hemangioendotheliomas, specific types of proliferative vascular lesions the former now being interpreted as benign and the latter belonging to the category of hemangioendotheliomas implying borderline malignant status (Mills *et al.*, 1980; Perkins and Weiss, 1996). Although the strong expression of VEGFR-3 would be consistent with lymphatic differentiation, the extensive erythrocyte content in the vascular lumina of these lesions supports the idea that VEGFR-3 expression in these tumors reflects a proliferative vascular phenotype rather than their lymphatic phenotype.

The reactivity of VEGFR-3 with the majority of angiosarcomas, including the poorly differentiated ones with limited or no vasoformation, indicates that this receptor is generally conserved upon malignant transformation. It appears to be an equally sensitive marker for endothelial differentiation of malignant vascular tumors as CD31, especially in the group of non-epithelioid vascular tumors which were positive in 87% of cases. However, VEGFR-3 is more specific for endothelia than CD31, which also reacts with platelets and subsets of leukocytes. VEGFR-3 also has advantage over CD34 and BNH9 in specificity, as the latter markers are positive in a variety of fibroblastic tumors and carcinomas, respectively. The absence of VEGFR-3 in non-endothelial cell tumors such as malignant melanoma, undifferentiated carcinomas and sarcomas further indicates that VEGFR-3 is a useful supplementary lineage marker to identify endothelial differentiation in human sarcomas and should be included in the immunohistochemical panel to evaluate the possible endothelial cell differentiation of poorly differentiated neoplasms.

Post-mastectomy angiosarcomas, often also referred to as lymphangiosarcomas were consistently VEGFR-3 positive, similar to the majority of other angiosarcomas (Aygit *et al*., 1999). Considering the wide distribution of VEGFR-3 in benign and malignant vascular tumors, its expression in post-mastectomy angiosarcoma cannot be specifically interpreted to indicate lymphatic origin. Usually angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries (Breiteneder-Geleff *et al*., 1999). Based an assumption of podoplanin as a specific marker for lymphatic endothelium angiosarcomas could be divided into three categories (low, moderate and high) according to their podoplanin expression. In a study by Folpe et al, 50% (8/16) of angiosarcomas were strongly positive VEGFR-3; of the positive cases, six had either a prominent lymphocytic infiltrate or hobnail endothelial cells (Folpe *et al*., 2000).

Epithelioid vascular tumors form a spectrum of benign, borderline (low grade malignant) and high grade malignant tumors as represented by epithelioid hemangioma, epithelioid hemangioendothelioma and epithelioid angiosarcoma. Common to all these lesions is the epithelioid appearance of the lesional endothelial cells by their ample cytoplasm previously described as “histiocytoid”. While the benign epithelioid hemangiomas and borderline/low grade epithelioid hemangioendotheliomas were positive in a third of the cases, the epithelioid angiosarcomas were positive in 50% of the cases. If these lesions are assumed to represent a spectrum of transformation of the same phenotype, the increasing frequency of VEGFR-3 expression from benign to malignant epithelioid vascular tumors may suggest that VEGFR-3 is upregulated during malignant transformation. Another finding that suggests upregulation of VEGFR-3 upon transformation is the strong immunoreactivity commonly seen in angiosarcomas.

A consistent finding in this study was the common VEGFR-3-reactivity in the cytologically benign capillary tumor neovascularization seen in both vascular and non-vascular tumors, including carcinomas, sarcomas and lymphomas. This finding suggests that VEGFR-3 is important for the genesis of tumor-induced neovascularization, and raises the question of its role in angiogenesis of physiological (wound healing) and pathological but nonmalignant diseases, such as diabetic retinopathy, atherosclerosis, psoriasis, and rheumatoid arthritis. In cutaneous wound healing, angiogenic blood vessels lack the antigen (Paavonen *et al*., 2000). Immunohistological data about inflammatory conditions is absent but proinflammatory cytokines, TNF-α and IL-1β induced increase in VEGF-C in human

lung fibroblasts whereas VEGFR-3 mRNA did not alter by IL-1β (Ristimaki *et al.*, 1998).

Studies on other VEGFRs have shown strong VEGFR-2 mRNA expression in Kaposi's sarcoma and angiosarcoma, whereas capillary hemangiomas express both VEGFR-1 and VEGFR-2 (Brown *et al.*, 1996; Hashimoto *et al.*, 1995). In the pathogenesis of angiosarcoma VEGF, a ligand for VEGFR-1 and VEGFR-2 has also been demonstrated to be expressed by high amounts (Zietz *et al.*, 1998).

For surgical pathologists, vascular invasion is an important prognostic factor in malignant disease. In addition, the examination of vessels invaded by tumors is essential for the study of the metastasis of malignant cells and the origin of tumors (Welch and Rinker-Schaeffer, 1999). Importantly, lymphatic invasion seems to be of greater prognostic importance for the survival of patients than blood vessel invasion (Lauria *et al.*, 1995). In general, markers of metastasis fall into two categories: the first category predicts the metastatic propensity based on expression and/or activity of a molecule with an established role in metastasis. The second category includes markers for which there is no established mechanistic association with metastasis. Each of these categories can be further divided on the basis of whether the amount of marker, increases or decreases. This criterion impacts upon the clinical utility of the marker. Certainly VEGFR-3 needs to be classified in the first category as it is likely to correlate with a metastatic tendency.

In summary, we immunohistochemically demonstrated unexpected, widespread VEGFR-3-expression in the majority of benign and malignant human vascular tumors. The expression of this marker is less consistent in malignant epithelioid vascular tumors. Because VEGFR-3 is absent in non-endothelial malignancies and positive in most angiosarcomas, this marker is a useful adjunct in the immunohistochemical identification of human endothelial cell neoplasms. In particular, this antibody could serve to distinguish various fibrovascular proliferations (e.g., stasis changes, chronic ulcers with surrounding reactive fibrosis) from difficult cases of KS.

5. VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3 in fenestrated blood vessels in human tissues (Study V)

In the last study we wanted to complete VEGFR-3 expression studies after becoming suspicious due to unexpectedly broad distribution of the antigen in pathological vasculature. The availability of novel antibodies against the ligands, VEGF-C and VEGF-D at the same time made it possible to analyze if the receptor and its ligands co-operate in the immediate vicinity of each other.

Even though the lymphatic system develops from large central veins in the embryonic jugular, retroperitoneal and perimesonephric regions surprisingly few adult tissues retained VEGFR-3 expression in their venous endothelia (Dumont *et al.*, 1998; Sabin, 1909). Such endothelia were seen in the vessels of the cartilage channels in vertebral bodies, in venous canals of the adrenal medulla and in the splenic venous sinuses.

The capillary endothelia vary structurally according to their location and functions; the major groups include continuous and discontinuous endothelia (Fig.4).

Continuous endothelium is present in tissues such as connective tissue, lung and brain, whereas discontinuous endothelium is typical of tissues undergoing high molecular exchange across the vessel wall, such as secretion of hormones from endocrine glands and filtration in glomeruli and in the choroid plexus. Electron microscopic studies have established that for example the capillaries of the anterior pituitary and glomerulus are fenestrated (Ross *et al.*, 1995). In this study, we found differences in VEGFR-3 expression between continuous and discontinuous endothelia, the former typically being negative and the latter positive. This finding suggests that VEGFR-3 plays a role in the transport functions of the discontinuous and more permeable endothelia in locations such as in the endocrine organs and kidney glomeruli.

Non-endothelial expression of VEGFR-3 was apparent in notochordal cells of 5 wk old embryos in accordance with earlier observations from avian embryos (Wilting *et al.*, 1997). This data indicates completely different type of role for the receptor from the present. Cytotrophoblast layer and intermediary trophoblasts gave positive staining reaction in the first and second trimester placenta, but not in term placenta. Indeed, VEGFR-3 is no longer needed for maintaining the integrity of endothelial cell lining in a term placenta. Cytotrophoblasts, lining blood containing placental spaces, are related to endothelial cells. A study with a commercial antibody against VEGFR-3 showed different results from ours (Vuorela *et al.*, 2000). Among placentas from both miscarriages and healthy controls the antigen was found faintly in cytotrophoblasts of "missed abortion" pregnancy tissue. Stromal cells in placental villi and decidua expressed the receptor constantly in both of the groups. In addition, vWF negative structure resembling a blood vessel displayed some positivity. Recently, it has become clear that invading cytotrophoblasts transform their receptor phenotype to resemble the endothelial cells they replace (Damsky and Fisher, 1998). Interestingly, we found that the cytotrophoblasts were stained for both VEGFR-3 and VEGF-C. It is as yet unknown if VEGF-C is produced in these cells or just adsorbed to its receptor on their surface.

A close association for VEGFR-3 and for its ligands, VEGF-C and VEGF-D was found in the endocrine organs. However, we could not show a positive result for VEGFR-3 in the capillaries in close proximity of VEGF-C/VEGF-D positive NE cells. We concluded that if these growth factors have a function in the fenestrated vessels, they must act via VEGFR-2. The distribution of VEGF-C and VEGF-D in the normal tissues was, surprisingly, limited and in accordance with earlier RNA expression data.

After finding the association of VEGF-C and VEGF-D with NE cells it is reasonable to hypothesize that these growth factors might contribute to tumorgenesis of NE cells. NE cells have histologically common features represented by argyrophilic cytoplasm containing NE granules. In particular, carcinoid tumors are rare tumors that are thought to arise from NE cells (Caplin *et al.*, 1998). They may be found anywhere in the human body, but are traditionally described as originating from the foregut, midgut, or hindgut. We proceeded to analyse carcinoid tumor material from the gastrointestinal tract, and found both VEGF-C and VEGF-D positivity from well-differentiated tumors (unpublished data of Taina A. Partanen, Johanna Arola and Kari Alitalo). VEGF-C was distributed palisade-like in the tumor cells lining the edge of the tumor. The strongest staining intensity could be seen in the smooth muscles invading cell clusters. These data indicate that at least in some malignant tumors, the tumor cells retain their cell specific expression pattern of VEGF-C. How such tumors

overexpressing VEGF-C differ in terms of lymphatic metastasis will be interesting to know.

VEGFR-3 was also expressed at major sites of hematopoiesis or blood cell trafficking, such as in the sinusoids of the liver, spleen and bone marrow. The endothelium at these sites is capable of regulating the translocation of hematopoietic cells, which may be related to the function of VEGFR-3 in these locations.

In a relatively short time four distinct markers for lymphatic endothelium have been characterized. These include VEGFR-3, podoplanin, Prox1 and LYVE-1 (Banerji *et al.*, 1999; Breiteneder-Geleff *et al.*, 1997; Jussila *et al.*, 1998; Wigle and Oliver, 1999). Podoplanin has been localized also to podocytes, parietal epithelial cells of Bowman's capsule, lung, choroid plexus, leptomeninges, osteocytes and osteoblasts (Breiteneder-Geleff *et al.*, 1997). Podoplanin and VEGFR-3 antigens were found to overlap in lymphatic endothelium, benign vascular tumors, and in angiosarcomas [Breiteneder-Geleff, 1997 #37]. At least during development, Prox1 is also expressed in the lens, heart, liver, pancreas and in the central nervous system (Wigle and Oliver, 1999). Our unpublished results have also demonstrated differences between the distributions of the VEGFR-3 and LYVE-1 markers and it is as yet unknown how useful these antigens would be for the detection of lymphatic vessels in e.g. malignant processes. Antibodies against LYVE-1 demonstrate that the receptor seem to posses a more prominent localization in lymphatic endothelium compared to that of VEGFR-3 which makes it easier to detect from a histological section. Further, preliminary data shows that the tumoral neovasculature lacks it that is an advantage when analyzing lymphatic vessels.

The ligands for VEGFR-3, VEGF-C and VEGF-D showed a narrow tissue distribution. Results obtained from the frozen tissue sections where smooth muscle cells and occasionally endothelial cells were stained with antibodies against these ligands suggest paracrine functions for these growth factors in e.g. the arterial wall. The high level of expression of VEGF-C in NE cells of respiratory and especially of digestive mucosas and endocrine organs lined by fenestrated endothelial cells suggests that it could play a role in the interaction between the NE cells and capillary endothelia and perhaps in hormone secretion. Alternatively, VEGF-C and VEGF-D may have roles unrelated to the vasculature in the NE system. Previously, VEGF has been located in the NE cells in the prostatic vermontanum epithelia (Guy *et al.*, 1998) and in the digestive mucosa (Terris *et al.*, 1998). Interestingly, VEGF and VEGF-C, which are both potent vascular permeability factors, were partly colocalized in the anterior pituitary gland. Although most of the VEGF-C positive cells were prolactin producing, such cells may also include the follicular stellate cells of the pituitary gland.

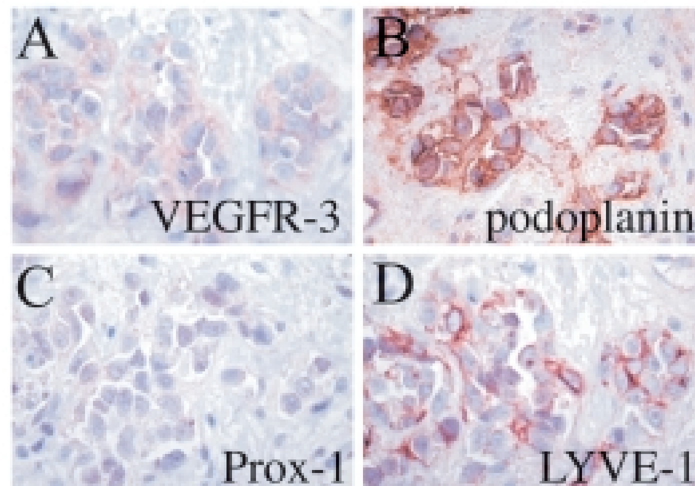
In conclusion, we have evaluated the distribution of VEGFR-3 and its ligands, VEGF-C and VEGF-D in human fetal and adult tissues. Although our results confirm the consistent presence of this receptor in lymphatic endothelia, they also reveal its expression in discontinuous capillary endothelia, and production of its two ligands by several types of NE cells. These data suggest that VEGFR-3 signals participate in the maintenance on filtration or secretion functions across fenestrated capillary endothelia.

Table 16. VEGFR-3 expression outside the lymphatic endothelium.

ORGAN	FETAL	ADULT
HEART		
- Myocardial capillaries	+-++	ND
- Vasa vasorum of aorta	ND	+++
CARTILAGE		
- cartilage channel blood vessel endothelium, venous ?	+++	ND
LYMPHOHEMATOPOIETIC		
- bone marrow sinusoids	+++	+++
- splenic -"-	+++	+++
- liver -"-	+++	+++
GENITOURINARY & REPRODUCTIVE		
- glomerular capillaries	+++	+++
- cytotrophoblasts	+++	+++
CNS		
- notochorda	+++	-
- capillaries of choroid plexus	+++	+++
ENDOCRINE		
- adenohypophyseal, thyroid, parathyroid and adrenal capillaries	+++	+++
- medullary venous channels in adrenal gland	+++	-
VASCULAR TUMORS	+-+++	+-+++
TUMORAL NEOVASCULATURE	ND	+-+++

Abbreviations: ND = not determined

Figure 5. Adjacent sections from an angiosarcoma sample (classical Stewart-Treves syndrome) stained with proposed new lymphatic endothelial markers.



CONCLUSIONS

Anti-VEGFR-3 antibodies are useful tools in detecting lymphatic vessels in normal skin as well as in other human tissues. However, VEGFR-3 is upregulated in the tumor neovasculature which poses a limitation when identifying and counting lymphatic vessels and "hot spots" (endothelial-stained vessels in clusters). VEGFR-3 expression in tumor vessels reflects the immature nature of tumor vascular endothelium.

VEGFR-3 is widely distributed in vascular tumors, in both benign and malignant – thus it does not correlate with tumor malignancy.

VEGFR-3 and podoplanin colocalize specifically on lymphatic endothelia and they are associated with the tumor cells of all stages of AIDS-associated and classic KS.

Our results also demonstrate differential expression of the endothelial growth factor receptors in distinct types of vessels in the human heart. This information is useful for the understanding of their roles in physiological and pathological processes and for their diagnostic and therapeutic application in cardiovascular medicine.

Expression of VEGF-C and VEGF-D is associated mainly with neuro-endocrine and smooth muscle cells in adult tissues. Their receptor also appears in some fenetrated blood vessel endothelia. Paracrine functions for VEGF-C and VEGF-D are suggested by the studies where arterial wall smooth muscle cells and endothelial cells were shown to stain for both of ligands. In the endocrine glands these growth factors may contribute to the secretion of hormones into the circulation.

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