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HIMADRI ROY

**Vascular Endothelial Growth Factors (VEGFs) -
Role in Perivascular Therapeutic Angiogenesis and Diabetic
Macrovascular Disease**

Doctoral dissertation

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Abstract

Angiogenesis is the process by which new blood vessels are formed from existing vessels. Vascular endothelial growth factors (VEGFs) - VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PlGF) - are key molecules in the process of angiogenesis. The ability of VEGFs to induce angiogenesis can be used for therapeutic purposes but these molecules also play a critical role in many pathological conditions. Recent advancements in vascular research have enhanced our understanding of their functions. However, only limited information is available about therapeutic and pathological roles of VEGFs in vascular compartments. In this study we evaluated the roles of VEGFs in perivascular therapeutic angiogenesis and in pathogenesis of diabetic atherosclerosis.

We used a localized perivascular gene transfer model to study therapeutic angiogenesis produced by VEGFs. Perivascular gene transfer has been used in preclinical and clinical trials for therapeutic angiogenesis, intimal hyperplasia and vein graft survival. Gene transfer was done using silastic collars placed around the carotid arteries of New Zealand White rabbits. VEGF-A, -B, -C, -D, -E, -F, PlGF-2 and Lac-Z gene delivery were carried out using adenoviral vectors. Among the VEGF family members, VEGF-A, VEGF-D, VEGF-D Δ N Δ C and PlGF-2 were most efficient in inducing angiogenesis and VEGF-A, VEGF-D and VEGF-D Δ N Δ C also produced significant intimal hyperplasia. Increased inflammation was seen in VEGF-A, VEGF-B and PlGF-2 transduced arteries. Endogenous VEGF-A expression was increased in the PlGF-2 transduced arteries and PlGF-2 mediated angiogenesis was blocked by sVEGFR-1 and sVEGFR-2.

In vitro studies have suggested that diabetes increases VEGF expression. Using an animal model of diabetes with hyperlipidemia, we studied the effects of diabetes on atherosclerosis, including VEGFs expression in atherosclerotic lesions and plaque angiogenesis. Diabetes was induced in Watanabe heritable hyperlipidemic (WHHL) rabbits by intravenous Alloxan injections. Accelerated atherosclerosis, inflammatory response and significant increases in RAGE, NF κ B, VEGF-A, VEGF-D, VEGFR-1, and VEGFR-2 expression were seen in diabetic animals. An increased tendency towards plaque angiogenesis was also seen in diabetic animals.

We conclude that VEGF-A, VEGF-D, -D Δ N Δ C and PlGF-2 gene transfer significantly increase angiogenesis in adventitia. Endogenous VEGF-A plays a key role in mediating angiogenic responses of PlGF-2. Perivascular therapeutic angiogenesis using VEGFs should be done with a caution. There is significantly increased intimal hyperplasia with VEGF-A, VEGF-D, -D Δ N Δ C and inflammation with VEGF-A, VEGF-B and PlGF-2 gene transfer. Diabetes produced accelerated atherosclerosis and increased VEGF-A, VEGF-D, VEGFR-1 and VEGFR-2 expression in atherosclerotic lesions. The presence of VEGFs in diabetic atherosclerotic plaques is presumably modulated by the macrophages, NF κ B and RAGE.

National Library of Medicine Classification: QZ 52, WK 810, WG 550, QU 107, WG 500

Medical Subject Headings: angiogenesis inducing agents; neovascularization, pathologic; gene therapy; connective tissue; atherosclerosis; Adenoviridae; vascular endothelial growth factors; carotid arteries; tunica intima/pathology; hyperplasia; diabetes mellitus, experimental; inflammation mediators; disease models, animal; rabbits

"Science... never solves a problem without creating ten more."

- George Bernard Shaw

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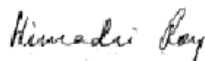
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ABBREVIATIONS

Adv	adenovirus
AGE	Advanced Glycosylation End products
Akt	serine-threonine kinase
ALS	amyotrophic lateral sclerosis
AMD	age-related macular degeneration
ARDS	acute respiratory distress syndrome
AP	activator protein
Asp	aspartate
BPD	bronchopulmonary dysplasia
CAD	coronary artery disease
cDNA	complementary deoxyribonucleic acid
CLD	chronic lung disease
CNV	choroidal neovascularization
CpG	phosphodiester-linked cytosine and guanine pairs
DAG	diacylglycerol
DM	diabetes mellitus
DR	diabetic retinopathy
ECs	endothelial cells
ELISA	enzyme-linked immunosorbent assay
EGR- 1	early growth response factor 1
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal regulated protein kinase
FAK	focal adhesion kinase
FFA	free fatty acids
FGF	fibroblast growth factor
Flk-1	fetal liver kinase-1 (murine VEGFR-2)
Flt-1	fms-like tyrosine kinase-1 (VEGFR-1)
Flt-4	fms-like tyrosine kinase-4 (VEGFR-3)
HDL	high density lipoproteins
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HRE	hypoxia responsive element
IGF-1	Insulin like growth factor
IL-8	interleukin-8
IMCL	Intra-myocellular lipids
IP ₃	inositol 1,4,5-trisphosphate
KDR	kinase domain receptor (human VEGFR-2)
LacZ	β-galactosidase (marker gene)
LDL	low density lipoproteins
MAPK	mitogenactivated protein kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MSR	macrophage scavenger receptor
mTOR	mammalian target of rapamycin
NFκB	nuclear factor Kappa B
NO	nitric oxide
NPDR	non-proliferative diabetic retinopathy
NRP	neuropilin
PAD	peripheral arterial disease
PAI-1	plasminogen activator inhibitor-1
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PDR	proliferative diabetic retinopathy
pfu	plaque forming units

PGI2	prostacyclin
PIGF	placental growth factor
PI3K	phosphatidylinositol-3-OH-kinase
PKB	protein kinase B
PKC	protein kinase C
PLC γ	phospholipase C γ
RA	rheumatoid Arthritis
RAGE	receptor for Advanced Glycosylation End products
ROP	retinopathy of prematurity
ROS	reactive oxygen species
RT-PCR	reverse-transcriptase polymerase chain reaction
SMC	smooth muscle cell
TGF- α	transforming growth factor alpha
Tie	tyrosine kinase with immunoglobulin and epidermal growth factor homology domains
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VHL	von Hippel-Lindau
VLDL	very low density lipoproteins
VSMC	vascular smooth muscle cells

List of Original Publications-

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1. INTRODUCTION

Angiogenesis is the process by which new blood vessels are formed from existing vessels. Vascular endothelial growth factor (VEGF) is a specific mitogen for vascular endothelial cells (EC). VEGF/VEGF-receptor system is a key component in the complex process of angiogenesis that also includes many other stimulators, inhibitors and angiogenic modulators. VEGF family consists of seven members - VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PlGF). They share a common structure of eight characteristically spaced cysteine residues in a VEGF homology domain (Leung et al., 1989; Maglione et al., 1993; Olofsson et al., 1996; Joukov et al., 1996; Achen et al., 1998; Ogawa et al., 1998). These members have different physical and biological properties and act through specific tyrosine kinase receptors - VEGFR-1, VEGFR-2 and VEGFR-3. Neuropilin-1 (Nrp-1) and Nrp-2 are receptors for semaphorins, (Kolkodin et al., 1997; Chen et al., 1997) but they also bind to some members of the VEGF family (Klagsbrun et al., 2002). The ability of VEGFs to induce angiogenesis can be used for therapeutic purposes but these molecules also play a critical role in many pathological conditions. Recent advancements in vascular research have enhanced our understanding of their functions. However, only limited information is available about therapeutic and pathological roles of VEGFs in vascular compartments. In this study we evaluated the roles of VEGFs in perivascular therapeutic angiogenesis and in pathogenesis of diabetic macrovascular disease.

2. REVIEW OF LITERATURE

2.1 Angiogenesis

Hemangioblasts (the common precursor cells for blood vessels and blood) differentiate into blood cell precursors and vascular precursors (angioblasts). These angioblasts migrate, coalesce into cords and form a lumen. This process of vessel formation is called vasculogenesis and is dominant in very early embryogenesis. Angiogenesis consists of two distinct processes, *sprouting* of endothelial cells and splitting of vessel lumens by *intussusceptive microvascular growth* (Risau, 1997; Patan, 2000). Organs derived from the ectoderm-mesoderm, as the brain and neuroectoderm, are vascularized by angiogenesis. Neovascularization in adults also occurs by the process of angiogenesis (Patan, 2000).

The sprouting process consists of several consecutive steps (Folkman, 1986) - 1) New capillaries originate from small venules or from other capillaries. 2) Local degradation of the basement membrane on the side of the venule closest to the angiogenic stimulus (collagenase, plasminogen activators, etc.). 3) Migration of endothelial cells toward the angiogenic stimulus. 4) Alignment of endothelial cells in bipolar mode. 5) Formation of a lumen (intra-cellular from vacuoles or intercellular) and endothelial cell mitosis distant to the leading tip of the sprout. 6) Loop formation by connection of individual sprouts. 7) Flow begins after loops have formed. 8) Pericytes or smooth muscle cells eventually align along the endothelial cells outside the capillary (vessel wall maturation). 9) New basement membrane is formed.

It has been proposed that capillaries might grow by insertion of slender columns of interstitial tissue, termed tissue pillars or posts, thus dividing the vessel lumen'. This process has been called 'intussusceptive microvascular growth' (Caduff et al., 1986). The following steps are seen in intussusceptive microvascular growth (Patan, 2000) - 1) Growth occurs predominantly on the venous side in vessels of all sizes and in the capillary region of the circulation. 2) The endothelial layer retreats successively around an organized unit of interstitial tissue (the pillar core) located in the vessel wall. This results in evagination of the vessel lumen around this unit. 3) Pillar separation from the tissue fold. 4) The synthesis of collagen fibers to stabilize pillar core. 5) Endothelial cell multiplication 6) Peri-endothelial cells ensheath the growing vessels. 7) Finally a basement membrane is formed.

A number of angiogenic growth factors including - fibroblast growth factor (FGF), platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factors (VEGFs), transforming growth factor alpha (TGF- α), interleukin-8 (IL-8), angiopoietins and the ephrins play important roles in process of angiogenesis (Bhardwaj et al., 2005^a).

2.2 Vascular Endothelial Growth Factors

2.2.1 VEGF-A

VEGF-A, which is also referred to as the vascular permeability factor (VPF) was the first VEGF to be discovered. It is an endothelial cell specific mitogen. VEGF-A gene is located at chromosome 6p21.3 and is encoded by 8 exons separated by 7 introns (Podar et al., 2005). VEGF-A is a dimeric 36-46 kd glycosylated protein with a N-terminal signal sequence and a heparin-binding domain. VEGF monomers have a single glycosylation site at Asp-75 of the mature protein. Glycosylation is not necessary for biological activity although it is important for efficient secretion of VEGF-A. Heterodimers have been described in mouse (Brier et al., 1992).

VEGF-A Isoforms

Nine different isoforms, of VEGF-A have so far been identified with varying number of amino acids (121, 145, 148, 162, 165, 165b, 183, 189 and 206 amino acid residues) (Tischer et al., 1991; Houck et al., 1991; Poltorak et al., 1997; Lei et al., 1998). These isoforms have distinct but overlapping functions in angiogenesis due to their differential binding to heparan sulphate and neuropilins. Through alternative mRNA splicing, the VEGF-A isoforms differ by the presence or absence of sequences encoded by exons 6 and 7 (Tischer et al., 1991). In general the longer forms are matrix-bound and the shorter forms are freely diffusible. VEGF-A121 does not bind to

heparin or extracellular matrix (Park et al., 1993) while VEGF-A165 has a heparin binding ability (Park et al., 1993; Cohen et al., 1995). VEGF-A145 contains a heparin binding domain and elements that enable the binding of VEGF-A145 to the extracellular matrix (Lei et al., 1998). VEGF-A189 and VEGF-A206 bind heparin more strongly and are sequestered in the extracellular matrix and at the cell surface (Park et al., 1993; Houck et al., 1992) and these two isoforms are probably less active than either VEGF-A121 or VEGF-A165 in vivo (Cheng et al., 1997). The three secreted VEGF-A splice forms are VEGF-A121, VEGF-A145, and VEGF-A165 while the VEGF-A183, VEGF-A189 and VEGF-A206 are the matrix bound forms. Most VEGF-producing cells express VEGF-A121, VEGF-A165, VEGF-A183 and VEGF-A189, but VEGF-A 145 and VEGF-A206 seem to be restricted to cells of placental origin (Anthony et al., 1994; Cheung et al., 1995).

Homozygous VEGF-A knockout mice die at E8-E9 and mice lacking even a single VEGF-A allele die at E11-E12 (Ferrara et al., 1996; Carmeliet et al., 1996) indicating that VEGF-A expression at appropriate level is essential during embryogenesis. Knockout studies in mice have also suggested that the VEGF165 is probably the major isoform that brings about the VEGF-A actions (Carmeliet et al., 1999).

VEGF-A receptors

VEGF-A mediates its responses primarily by activating VEGFR-1 and VEGFR-2 but it also binds to Nrp-1 and Nrp-2 (Klagsbrun et al., 2002) (Fig.1). Overexpression of VEGF-A produces a pronounced strong angiogenic response in different tissues. However resulting vessels are often large, dilated & leaky (Phillips et al., 1994; Rissanen et al., 2003; Bhardwaj et al., 2003; Rissanen et al., 2004).

Hypoxia and other regulators of VEGF-A expression

The expression of VEGF is potentiated in response to hypoxia (Semenza, 2003). It is now recognized that hypoxia-inducible factor-1 α (HIF-1 α) is a key mediator of the hypoxic responses (Semenza, 2002). In response to hypoxia, HIF-1 α binds to specific enhancer elements, resulting in increased gene transcription. Hypoxia induces binding of HIF-1 α to the Hypoxia responsive element (HRE) in the VEGF-A gene promoter region, which in turn increases VEGF-A transcription. A role of von Hippel-Lindau (VHL) tumor suppressor gene in HIF-1 α dependent hypoxic responses has also been described (Mole et al., 2001). Mutations in the VHL gene are associated with increased angiogenesis, and tumors with VHL mutation display increased VEGF-A expression (Stratmann et al., 1997). While hypoxia is important for VEGF-A regulation, other pathways including growth factors, inflammatory cytokines and hormones also up-regulate VEGF-A mRNA expression (Ferrara, 2004). AGE-RAGE interaction resulting in up-regulation of VEGF-A through an AP-1/NF κ B mediated pathway has been suggested in diabetics (Okamoto et al., 2003).

Role in angiogenesis

It is a key molecule in induction of angiogenesis and vasculogenesis that causes proliferation, sprouting, migration and tube formation of ECs (Ferrara, 2000; Ferrara et al., 2003). VEGF induces angiogenesis in a variety of physiological and pathological conditions including embryogenesis (Breier et al., 1992; Breier et al., 1997), corpus luteum formation (Kaczmarek et al., 2005), tumor growth (Plate et al., 1992), wound healing (Brown et al., 1992; Bates et al., 2003), and compensatory angiogenesis in the heart (Banai et al., 1994; Brier et al., 1997).

Vascular permeability factor

VEGF was initially described as a vascular permeability factor secreted by carcinoma cell lines that enhanced permeability in skin blood vessels and also stimulated the production of ascites (Senger et al., 1983; Criscuolo et al., 1988). Overexpression of VGF-A resulted in increased vascular leakage in rabbit eye after intravitreal adenoviral mediated gene transfer (Kinnunen et al., 2006). Molecular mechanisms by which VEGF induces these effects are not well characterized. It has been postulated that VEGF increases permeability by binding to VEGFR-2 and thereafter activating guanylyl cyclase and cGMP via a nitric oxide dependent pathway.

Increased cGMP levels probably enhance endothelial permeability by increasing the vesico-vascular organelles, fenestrations and transcellular gaps (Bates et al., 2002). VEGF-A mediated extravasations of fluid and plasma proteins, including fibrin might contribute to enhanced migration of ECs in extracellular matrix (Bootle-Wilbraham et al., 2001; Dvorak et al., 1987). VEGF also causes vasodilatation by induction of endothelial nitric oxide synthase (eNOS) and increasing nitric oxide production (Kroll et al., 1999).

α v integrins, such as α v β 3 and α v β 5, were recently shown to be involved in VEGF-A induced vascular permeability response: β 5-null mice show little or no vascular permeability in response to VEGF-A, whereas β 3-null mice show an increased response. The extracellular domain of β 3 integrin was also shown to binds to VEGFR-2 (Weis et al., 2005; Robinson et al., 2004).

Cell survival and proliferation

VEGF-A is also required for the survival of endothelial cells. VEGF induces the expression of antiapoptotic proteins Bcl-2 and A1 in the endothelial cells. This action of VEGF-A might be related to the activation of phosphatidylinositol-3 kinase and Bcl-2 pathways (Gerber et al., 1998(a); Gerber et al., 1998 (b); Zhou et al., 2005) (Fig.1). Most of the studies on VEGF-A have primarily focused on their action on ECs. However, the actions of VEGF-A on other cell types have also been described. VEGF-A is mitogenic for retinal pigment epithelial cells (Guerrin et al., 1995) and Schwann cells (Sondell et al., 1999). VEGF-A also has a neuroprotective effect on hypoxic motor neurons, and is a modifier of amyotrophic lateral sclerosis (Takahashi et al., 2005). Role of VEGF-A in vascular smooth muscle cell proliferation and migration has also been reported (Cucina et al., 2003; Bhardwaj et al., 2005^b).

Hematological effects

VEGF-A is also reported to have hematopoietic effects. It induces colony formation by mature subsets of granulocyte-macrophage progenitor cells (Broxmeyer et al., 1995) and regulates hematopoietic stem cell survival by an internal autocrine loop mechanism (Gerber et al., 2002) and promotes monocyte chemotaxis (Clauss et al., 1990). It also exerts procoagulant activity via its ability to stimulate the production of the potent initiator of coagulation tissue factor in ECs and monocytes (Clauss et al., 1990).

2.2.2 PIGF

Placental growth factor (PIGF) is homodimeric glycoprotein which was first identified in placenta (Maglione et al., 1991) but is also known to be present in heart and lungs. Human PIGF gene has been mapped to chromosome 14q24. PIGF-coding sequence is encoded by seven exons spanning an 800-kb-long DNA interval (Maglione et al., 1993).

PIGF encodes for a protein with an approximately 50% identity to VEGF in the platelet-derived growth factor (PDGF)-like domain. PIGF is a selective ligand for VEGFR-1. PIGF has two N-linked glycosylation sites in each chain and one of the glycosylated residues, Asn84, plays a role in binding VEGFR-1. It has been suggested that VEGF-A and KDR are involved in the first two trimesters of pregnancy in the establishment of the richly branched capillary beds of the mesenchymal and immature intermediate villi, while PIGF and Flt-1 are more likely to be involved in the formation of the long, poorly branched, terminal capillary loops in the last trimester (Ahmed et al., 2000).

PIGF isoforms and receptors

Four isoforms - PIGF-1, PIGF-2, PIGF-3 and PIGF-4 - have been described (Maglione et al., 1993; Tjwa et al., 2003; Cao et al., 1997; Yang et al., 2003). PIGF-1 and PIGF-3 are non-heparin binding diffusible isoforms while PIGF-2 and PIGF-4 have heparin binding domains (Yang et al., 2003). PIGFs mediate their effects through VEGFR-1 (Yla-Herttuala et al., 2003). PIGF-2 is also able to bind Nrp-1 and Nrp-2 due to the insertion of 21 basic amino acids at the carboxy terminus, while both PIGF-1 and PIGF-3 lack this amino acid insert (Migdal et al., 1998; Neufeld et al., 2002).

Role in angiogenesis and arteriogenesis

Results available from *in vitro* studies on the angiogenic role of PIGF are inconsistent. In some studies PIGF binding to VEGFR-1 failed to produce EC growth and angiogenesis (Park et al., 1994; Gille et al., 2001), while other studies show that PIGF/VEGFR-1 signaling promotes EC viability and angiogenesis (Cai et al., 2003). In placenta and in PIGF-1 expressing tumors increased PIGF levels inhibit EC growth (Ahmed et al., 2000; Bjorndahl et al., 2004). Knockout studies in mice reveal that PIGF null mice have an apparently normal phenotype but recover poorly from myocardial infarction and have impaired collateral formation in response to hind limb ischemia (Carmeliet et al., 2001). PIGF has direct effects on ECs, both by inducing its own signaling and by amplifying VEGF-driven angiogenesis (Carmeliet et al., 2001; Auterio et al., 2003). PIGF-2 overexpression results in the production of significant angiogenesis in different tissues (Luttun et al., 2002; Roy et al., 2005). Various mechanisms by which PIGFs can enhance angiogenesis include - (a) intracellular signal transduction through VEGFRs (b) Increasing the fraction of VEGF-A available to activate VEGFR-2 by displacing VEGF-A from the 'VEGFR-1 sink', (Carmeliet et al., 2001; Auterio et al., 2003); (c) Activation of VEGFR-1 by PIGFs results in intermolecular transphosphorylation of VEGFR-2 that could increase VEGF-A mediated angiogenesis (Auterio et al., 2003); (d) PIGF/VEGF-A heterodimer formation, which could act through VEGFR-1/VEGFR-2 (Auterio et al., 2003; DiSalvo et al., 1995; Cao et al., 1996). Our recent results show that PIGF-2 overexpression in perivascular tissue increased VEGF-A165 and VEGF-A121 levels and produces significant angiogenesis. These blood vessels were tortuous and well perfused. PIGF-2 mediated angiogenesis was effectively blocked by soluble VEGFR-1 and VEGFR-2 receptors. This data suggests that angiogenic responses to PIGF-2 are also indirectly mediated through VEGFR-2 (Roy et al., 2005). In contrast to the essential role of VEGF in physiological and pathological angiogenesis, the role of PIGF is restricted to pathological vessel formation and is therefore a possible target for therapy. The proposed role of PIGF in the process of arteriogenesis (Pipp et al., 2003; Ferrara et al., 2003, Nagy et al., 2003) is significant and holds promise for the treatment of ischemic diseases.

Inflammatory response

PIGF is also a chemoattractant for inflammatory cells, which are hallmarks of pathological angiogenesis and collateral growth. It has a powerful chemotactic effect on monocytes (Clauss et al., 1996) and increased macrophage accumulation occurs after injections of PIGF protein and adenovirus mediated PIGF gene transfer (Pipp et al., 2003; Roy et al., 2005). Increased infiltration of macrophages presumably contributes to the VEGF-A upregulation in the PIGF-2 transduced arteries (Roy et al., 2005). PIGF expression is upregulated during a number of pathological conditions including non-small cell lung carcinoma (NSCLC) (Zhang et al., 2005), colorectal cancer (Wei et al., 2005) and wound healing (Odorisio et al., 2006), while, PIGF levels are decreased in preeclampsia (Bdolah et al., 2005). A possible role of PIGF in atherosclerosis has also been described (Khurana et al., 2005).

2.2.3 VEGF-B

VEGF-B, which is also called VEGF-related factor/VRF, is expressed beginning early in fetal development. Structurally VEGF-B is closely related to VEGF-A, with a gene that spans about 4000 bp, contains eight exons and six introns, and is located on chromosome 11, band q13 (Grimmond et al., 1996; Paavonen et al., 1996; Olofsson et al., 1996). Cell culture experiments show that VEGF-B is an endothelial cell mitogen. VEGF-B mediated stimulation presumably occurs through direct cell-cell interaction. This cell-cell interaction likely guides migrating endothelial cells and organizes them into developing blood vessels.

VEGF-B expression and isoforms

The promoter region of VEGF-B is different to that of VEGF-A, and this might explain differences in regulation by physiological stimuli (Silinis et al., 1997). While both VEGF-A and VEGF-B promoters are associated with a CpG island and contain transcription factor binding sites for Sp1 and AP-2, the VEGF-B promoter contains Egr-1 sites, but lacks hypoxia-inducible factor-1 and

AP-1 sites found in the VEGF-A promoter. Consequently, stimuli such as hypoxia (Enholm et al., 1997) which can induce VEGF-A expression do not appear to regulate levels of VEGF-B.

VEGF-B gene expression starts early during embryogenesis and by day-14 VEGF-B is expressed by most cells in murine embryo. High levels of VEGF-B are especially seen in spinal cord, brain, heart and brown fat (Lagercrantz et al., 1996). Two splice variants of VEGF-B - VEGF-B167 and VEGF-B186 - are expressed in humans (Li et al., 2001). VEGF-B167 is mostly sequestered in the extracellular matrix while VEGF-B186 is freely diffusible. In vivo, VEGF-B167 is the predominant form and is abundantly expressed in brown fat and in the myocardium and skeletal muscle (Olofsson et al., 1999). The VEGF-B186 isoform is expressed at lower levels and only in a limited number of tissues. Moreover, the VEGF-B186 isoform is up-regulated in mouse and human tumor cell lines and primary tumors compared with their corresponding normal tissues (Li et al., 2001).

VEGF-B receptors

VEGF-B is a ligand for VEGFR-1 and Nrp-1, and it can form heterodimers with VEGF-A (Olofsson et al., 1999). VEGF-B167 binds to heparan sulfates and NRP-1, while the VEGF-B186 which is freely secreted and soluble, requires proteolytic cleavage of the C-terminal region for binding to NRP-1. Neither isoform binds VEGFR-2 or VEGFR-3 (Makinen et al., 1999; Olofsson et al., 1998).

Role of VEGF-B

The precise role of VEGF-B in vivo is not known. Mice lacking a functional VEGF-B gene are healthy and fertile. However, VEGF-B deficiency can result in smaller hearts and impaired recovery after induced myocardial infarction suggesting that formation of coronary collaterals might be partly attributed to VEGF-B (Bellomo et al., 2000). VEGF-B is weakly angiogenic after adenoviral delivery to periadventitial tissue or hindlimb skeletal muscle (Bhardwaj et al., 2003; Rissanen et al., 2003). A protective role of VEGF-B in pulmonary hypertension model has been described (Louzier et al., 2003). Reduced synovial angiogenesis in VEGF-B knockout arthritis models suggest a role of VEGF-B in inflammatory angiogenesis (Mould et al., 2003). VEGF-B reportedly can form stable heterodimers with VEGF-A when coexpressed with VEGF-A. The reported angiogenic activity (Yoon et al., 2003) may be partly due to the heterodimers between VEGF-A and VEGF-B.

2.2.4 VEGF-C

VEGF-C is also referred to as VEGF-related protein. VEGF-C was cloned from human prostate carcinoma cells, and its mature form is 30% identical to VEGF-A165. The VEGF-C gene is located on chromosome 4q34 (Paavonen et al., 1996). VEGF-C genes comprise over 40 kilobase pairs of genomic DNA and consist of seven exons (Chilov et al., 1997). VEGF-C lacks the sequence corresponding to exon 6 of VEGF-A. This sequence confers heparin binding properties to the protein.

VEGF-C receptors

VEGF-C is synthesized as a precursor protein, which is proteolytically processed in the extracellular space by proteases to generate a 23 KD homodimeric protein with high affinity for both VEGFR-2 and VEGFR-3: ~100 and ~10-fold respectively (Joukov et al., 1997; Siegfried et al., 2003). VEGF-C induces mitogenesis, migration and survival of ECs. Mature form contains an unpaired extra cysteine residue in the VEGFR-binding domain that is likely specific to VEGFR-2 binding (Joukov et al., 1998).

VEGF-C expression

VEGF-C is expressed in embryonic and in adult life. It is strongly expressed on day-7 in a murine embryo, before the expression of VEGFR-3/Flt4. This suggests that VEGF-C interacts also with VEGFR-2/KDR during embryonic development (Jukov et al., 1996). VEGF-C is expressed in the heart, small intestine, placenta, ovary and the thyroid gland in adults. Unlike VEGF, the expression of VEGF-C does not appear to be regulated by hypoxia but is increased in response to proinflammatory cytokines. Activation of VEGF-C mRNA transcription by proinflammatory

cytokines suggests a role of VEGF-C in inflammation associated lymphangiogenesis (Chilov et al., 1997; Su et al., 2004).

Role of VEGF-C

Developmental studies, knockout models and gene transfer experiments suggest that VEGF-C is primarily a lymphangiogenic growth factor and its lymphangiogenic effects are mediated by VEGFR-3 (Saharinen et al., 2004; Kukk et al., 1996; Enholm et al., 2001; Veikkola et al., 2001). However, the increase in blood vascular permeability induced by VEGF-C is mediated by VEGFR-2 (Veikkola et al., 2001). Disruption of the VEGF-C gene in mice demonstrates that the growth factor is indispensable in embryonic lymphangiogenesis. Embryos carrying a homozygous deletion of VEGF-C are not viable and fail to form the initial lymph sacs which generate the lymphatic vasculature (Karkkainen et al., 2004). VEGF-C has been shown to induce lymphangiogenesis in transgenic mouse skin and in mature chick chorioallantoic membrane (Jeltsch et al., 1997).

Examination of VEGF-C function in a number of assays has also shown an angiogenic activity (Witzenbichler et al., 1998; Skobe et al., 2001), presumably via activation of VEGFR-2. Recombinant VEGF-C also promotes angiogenesis when applied to early chorioallantoic membrane of chicks, to mouse cornea or to ischemic hindlimbs rabbits. VEGF-C gene transfer produced only moderate angiogenesis in rabbit skeletal muscle (Rissanen et al., 2003) and perivascular tissue (Bhardwaj et al., 2003).

The angiogenic versus lymphangiogenic responses to VEGF-C presumably depends on the proteolytic processing of its precursor and on the expression of its receptors in the target tissue. VEGF-C also has synergistic effects with VEGF-A, during the induction of angiogenesis, and this effect is more prominent in cells expressing both of its receptors. VEGF-C can also compete with VEGF-A in binding to VEGFR-2 (Jussila et al., 2002).

2.2.5 VEGF-D

VEGF-D (also known as c-fos-induced growth factor or FIGF) is a secreted glycoprotein shares 61% sequence identity with VEGF-C, and these two growth factors bind to the same receptors on human endothelial cells. The human VEGF-D gene is 2.0 Kb in size and is located on chromosome Xp22.31. No splice variants have been reported in humans, while two different protein isoforms with distinct C-termini, VEGF-D358 and VEGF-D326 have been identified in mouse (Baldwin et al., 2001^a).

VEGF-D receptors

VEGF-D is proteolytically processed similarly to VEGF-C, and the proteolytic processing also appears to regulate VEGF-D biological activity and receptor specificity. Human VEGF-D is proteolytically processed in its N-terminal and C-terminal ends; the mature form has greater affinity to bind VEGFR-2 and VEGFR-3: ~290- and ~40 fold respectively (McColl et al., 2004). Mouse VEGF-D binds only to VEGFR-3, indicating a different role for VEGF-D in mice (Baldwin et al., 2001). Like mature form of VEGF-C the mature form of VEGF-D also contains an unpaired extra cysteine residue in the VEGFR-binding domain that is likely specific to VEGFR-2 binding (Stacker et al., 1999)

Role of VEGF-D

VEGF-D is expressed in many adult tissues including the vascular endothelium, heart, skeletal muscle, lung, and bowel. VEGF-D has been shown to be responsible for proliferation of ECs, and it shows angiogenic properties in vitro and in vivo. Similar to VEGF-C, it also shows lymphangiogenic potential when overexpressed in skin keratinocytes. The lack of a profound lymphatic vessel defect in VEGF-D deficient mice may reflect a subtle, redundant, or nonexistent role of this growth factor during embryonic development (Baldwin et al., 2005). Nonetheless, VEGF-D may induce lymphatic vessel growth in adult life in response to pathological conditions. In experimental tumors VEGF-D increases lymphatic vessel growth and lymphatic metastasis (Stacker et al., 2001). VEGF-D is expressed by melanoma cells and has been proposed to have

a role in tumor angiogenesis and lymphangiogenesis (Achen et al., 2001). The fully processed form of VEGF-D is able to induce strong angiogenesis in addition to lymphangiogenesis in the rabbit hindlimb muscles (Byzova et al., 2002; Rissanen et al., 2003). Adenovirus mediated gene transfer of VEGF-D and mature VEGF-D in the periadventitial space produces significant activation of angiogenesis and vascular smooth muscle cell (SMC) proliferation (Bhardwaj et al., 2003; Bhardwaj et al., 2005^a).

2.2.6 VEGF-E

VEGF-E was discovered in the genome of the parapoxvirus (Orf virus) that infects sheep, goats, and occasionally humans (Lyttle et al., 1994). Infection by this virus causes proliferative skin lesions in which extensive capillary proliferation and dilation are prominent histological features. Several strains of the virus encode different VEGF-E variants, which bind specifically to VEGFR-2 and Nrp-1 and are able to stimulate EC mitogenesis and vascular permeability (Shibuya, 2003). To date, at least 5 family members including NZ2, NZ7, and D1701 types have been described. VEGF-E is not essential for viral replication but rather plays an important role in modulating the host environment during infection. Gene expression of protein VEGF-ENZ-7 in the basal cell layer of the skin of the mice induces a strong angiogenic response (Ogawa et al., 1998). Edematous lesions and hemorrhagic spots on the ear which were reported as side effects in VEGF-A transgenic mice were not detectable in VEGF-E transgenic mice (Wise et al., 1999; Kiba et al., 2003). VEGF-ENZ7 transgenic mice also show a significant increase in subcutaneous blood vessels with minor side effects like edema and inflammation, compared with VEGF-A transgenic mice (Shibuya M., 2006).

2.2.7 VEGF-F

A seventh exogenous member of the VEGF family, VEGF-F, was recently identified from snake (viper) venom. VEGF-F consists of two VEGF-related proteins designated vammin (110 residues) from *Vipera ammodytes ammodytes* (Western sand viper) venom, and VR-1 (109 residues) from *Daboia russelli russelli* venom. These proteins have a 50% primary structure identity with VEGF-A165 and bind selectively to VEGFR-2 (Suto et al., 2005). They display potent endothelial cell proliferation, hypotensive activities, and vascular permeability. VEGF-F contains a short C-terminal heparin-binding region and the C-terminal peptide of VEGF-F exhibits a specific blockage of VEGF-A165 activity both in vitro and in vivo (Yamazaki et al., 2005).

2.3 VEGF Receptors (VEGFR)

Three VEGF receptors - VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4) have been described till date (Fig.1) . These receptors are highly homologous to each other in overall structure, and closely related to the PDGF receptor (PDGFR) family. Structurally VEGFR have 4 distinct regions; the extracellular ligand-binding domain, transmembrane domain, tyrosine kinase domain, and downstream carboxy terminal region. However, unlike PDGFRs that contains 5 Immunoglobulin (Ig)-like domains the extracellular domain of VEGFR-1 and VEGFR-2 contains 7 Ig-like domains.

2.3.1 VEGFR-1

VEGFR-1 consists of 1338 amino acids in humans and the binding site of VEGF-A is located in the second Ig-like domain. The affinity of VEGFR1 for VEGF-A is very high, with a Kd of about 2 to 10 pM, which is at least one order of magnitude higher than that of VEGFR2. On the other hand, the tyrosine kinase activity of VEGFR1 is relatively weak. VEGFR-1 is expressed in ECs as well as pericytes, placental trophoblasts, osteoblasts, monocytes/macrophages, renal mesangial cells and also in some hematopoietic stem cells (Zachary et al., 2001). VEGFR-1 binds VEGF-A, VEGF-B and PlGF with high affinity. VEGFR-1 knockout mice die at early stages of embryogenesis due to disorganization of blood vessels and overgrowth of EC. VEGFR-1 transmits only weak mitogenic signals in ECs, but it is known to form a heterodimer with VEGFR-2, that has strong signaling properties (Huang et al., 2001). VEGFR-1 activation at least by PlGF can also promote angiogenesis, presumably through intracellular crosstalk with VEGFR-2 (Auterio et al., 2003). VEGFR-1 is associated with monocyte chemotaxis (Barleon et al., 1996).

VEGFR-1 signaling is also involved in the recruitment and survival of bone marrow derived progenitor cells (Hattori et al., 2002). VEGFR-1 expression is up-regulated during angiogenesis and also by hypoxia, unlike that of VEGFR-2 and VEGFR-3 (Gerber et al., 1997). A soluble form of VEGFR-1 (sVEGFR-1), consisting of the extracellular domain of VEGFR-1, is able to inhibit VEGF action in humans and mice and has been linked to preeclampsia (Maynard et al., 2003).

2.3.2 VEGFR-2

VEGFR-2 is the major positive signal transducer for both physiological and pathological angiogenesis. VEGFR-2 gene knock-out mice die at E8.0-8.5 due to a lack of vasculogenesis. This indicates that VEGFR-2 signaling is essential for the differentiation of VEGFR-2 positive endothelial precursor cells into vascular endothelial cells and for their proliferation (Shalaby et al., 1995). VEGFR-2 consists of 1356 amino acids in humans and the binding site of VEGF-A is located in the second/third Ig-like domain. Upon stimulation with VEGF-A, VEGFR-2 is autophosphorylated mostly at the carboxy terminal tail and kinaseinsert region. VEGFR-2 (kinase-insert domain receptor, KDR/fetal liver kinase, Flk-1) binds VEGF-A, VEGF-C and VEGF-D. It also selective binds VEGF-E and VEGF-F. VEGFR-2 is the primary receptor transmitting VEGF signals in ECs (Bjorndahl et al., 2004; Wise et al., 1999; Meyer et al., 1999). The VEGFR-2 signaling pathway is crucial in bringing about the effects of VEGFs including vasodilatation, endothelial cell migration and proliferation. Besides endothelial cells, VEGFR-2 is also expressed by circulating endothelial progenitor cells, pancreatic duct cells, retinal progenitor cells and megakaryocytes (Ferrara et al., 2003). VEGFR-2 may be associated with integrin-dependent migration of ECs, as it forms a complex with integrin α v β 3 upon binding VEGF-A (Hutchings et al., 2003; Soldi et al., 1999).

2.3.3 VEGFR-3

VEGFR-3 (fms-like tyrosine kinase 4, Flt4) binds VEGF-C and VEGF-D (Joukov et al., 1996; Achen et al., 1998). VEGFR-3 comprises extracellular six Ig-like domains. 6th Ig-like domain of VEGFR-3 corresponds to 7th Ig-like domain of VEGFR-1 and -2. VEGFR-3 is present on all endothelia during development but in the adult it becomes restricted to lymphatic ECs and certain fenestrated blood vascular ECs (Kaipainen et al., 1995; Partanen et al., 2000). Knockout and developmental studies suggest that VEGFR-3 signaling is essential for development of blood vessels during embryonic stage but becomes redundant in mature vessels. However, VEGFR-3 is upregulated on ECs of vascular tumors (Partanen et al., 1999). VEGFR-3 signaling pathway is crucial in the process of lymphangiogenesis. Transgenic mice overexpressing a soluble VEGFR-3-Ig fusion protein in the skin lack dermal lymphatic vessels (Makinen et al., 2001).

2.3.4 Neuropilins

The neuropilins, Nrp-1 and Nrp-2, have roles in immunology and neuronal development but they are also involved in angiogenesis (Klagsbrun et al., 2002; Bagri et al., 2002). Neuropilins bind class 3 semaphorins, which are secreted molecules that mediate repulsive signals during neuronal axon guidance (Bagri et al., 2002). Nrp-1 also binds VEGF-A165, VEGF-B and PlGF while Nrp-2 binds VEGF-A165, VEGF-C and PlGF (Klagsbrun et al., 2002) (Fig.1). Nrp-1 acts as a co-receptor enhancing VEGF-VEGFR-2 interactions, forming complexes with VEGFR-1 and augmenting tumor angiogenesis in vivo (Kawasaki et al., 2002). Overexpression of Nrp-1 in chimeric mice leads to excessive formation of capillaries and blood vessels and hemorrhages in addition to cardiac malformations (Kitsukawa et al., 1995). In chick embryos, endothelial Nrp-1 expression is mostly confined to arteries, whereas Nrp-2 primarily marks veins (Herzog et al., 2001). Nrp-2 is expressed also on lymphatic ECs, and mutated Nrp-2 induces abnormalities in the formation of small lymphatic vessels and lymphatic capillaries in mice (Yuan et al., 2002). It is thought that Nrp-1 is required for cardiovascular development because it regulates VEGF-A165 levels (Kawasaki et al., 1999).

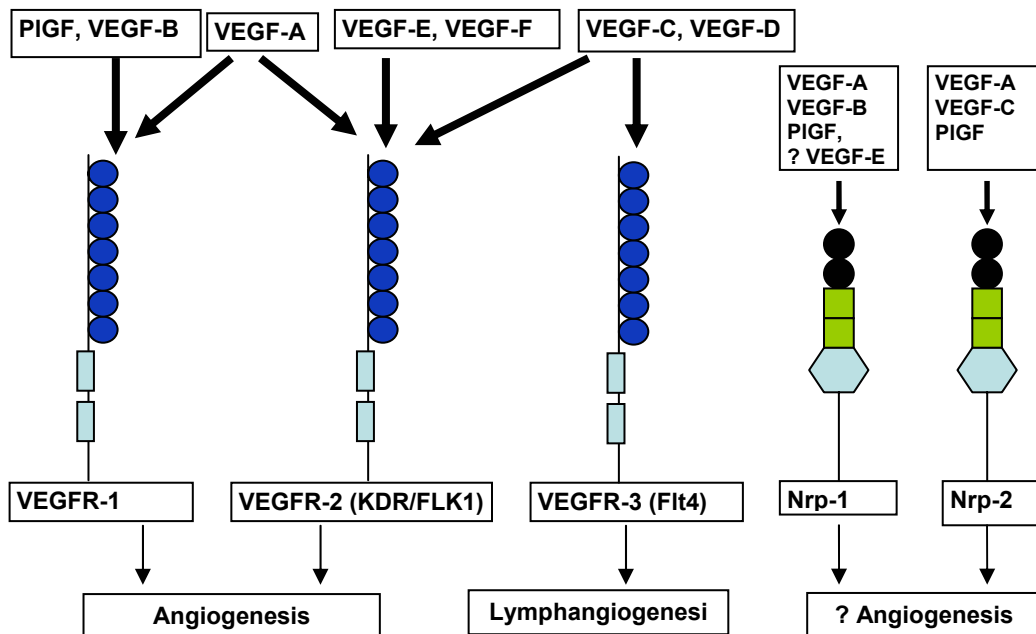


Fig.1 - VEGF receptors and their ligands. VEGFR-1, VEGFR-2 and the NRPs play a role in angiogenesis whereas VEGFR-3 is involved in lymphangiogenesis. VEGFR-1 VEGFR-2 and VEGFR-3 all have IgG domains. The NRPs have an a1/a2 domain, b1/b2 domain & C domain.

2.3.5 VEGF signaling

VEGF promotes cell survival, induce proliferation and enhance migration and invasion of endothelial cells. VEGF /VEGFR-2 signaling via extracellular-regulated kinase-1/2 (ERK1/2), stimulates DNA synthesis and cell proliferation. Activation of ERK 1/2 is mediated by Ras-Raf-MEK-ERK pathway (Fig. 2). The mitogen activated protein kinase (MAPK) pathway is also implicated in cell proliferation in response to VEGF. Under stress conditions VEGF/VEGF interaction activates the phosphatidyl inositol kinase (PI3-Kinase) pathway and Akt/protein kinase B (PKB) phosphorylation. This pathway plays a pivotal role in cell survival (Zachary et al., 2001).

Integrins/cell adhesion receptors such as the endothelium specific adhesion molecule $\alpha\beta_3$ also play a role in VEGF signal transduction. The cell adhesion molecule VE-Cadherin interacts with VEGFR-2 forming a complex with β -catenin and PI3-Kinase to promote cell survival.

VEGF acts as a chemo-attractant for endothelial cells and plays a role in migration and invasion. In addition to endothelial cells, VEGF also stimulates migration of vascular smooth muscle cells, monocytes, mononuclear phagocytes and polymorphonuclear cells and migration and invasion of some tumor cells such as breast and leukemia. The VEGF receptors VEGFR-1, VEGFR-2, VEGFR-3 and the NRPs have all been implicated in VEGF-mediated cell migration and invasion. VEGF induces cell migration by activating factors such as focal adhesion kinase (FAK) and Paxillin and also via the PI3 Kinase/Akt pathway (Fig. 2). VEGF activation of the MAPK pathway is also implicated in cell migration (Zachary et al., 2001; Byrne et al., 2005).

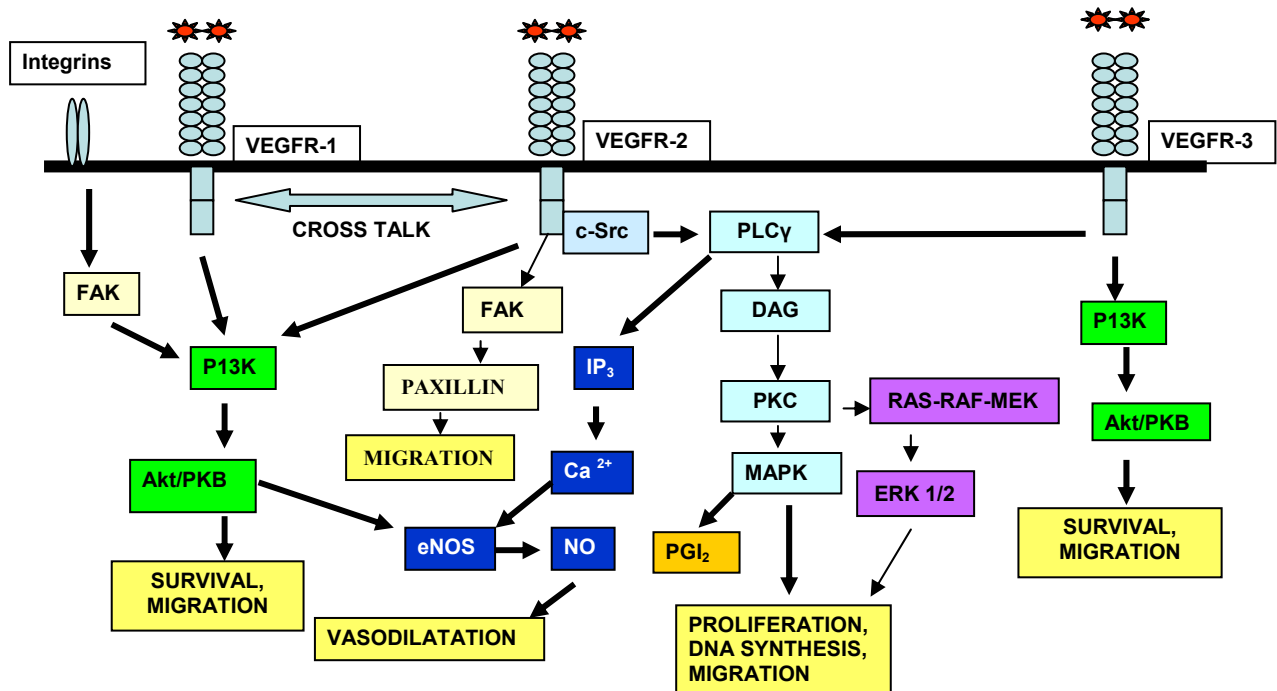


Fig.2 - VEGF receptor signaling - VEGFR-1, VEGFR-2 and VEGFR-3 all have tyrosine kinase activity which mediates VEGF signaling. NRP5 are co-receptors for VEGFs. However, the NRPs have no tyrosine kinase domain and the VEGF signaling pathway via NRPs is currently unknown. PI3K/Akt, MAPK, Ca²⁺ and NO are key mediators of the blood vascular effects of VEGFR-2 signaling. PI3K/Akt pathway phosphorylates Bad, caspase 9 (apoptotic proteins) and eNOS, thereby increasing cell survival. Integrin mediated focal adhesion kinase (FAK) is a point of convergence between integrin and VEGF mediated survival and migration signaling. Signal mechanism for mitogenesis is through PLCγ. The biological role of VEGFR-1 is currently unclear but it can act as a negative modulator of angiogenesis and exists also as a soluble form. VEGFR-1 is associated with monocyte chemotaxis. VEGFR-1 activation at least by PIGF can also promote angiogenesis, perhaps through intracellular crosstalk with VEGFR-2. (modified from Roy et al., 2006) (*MAPK- mitogenactivated protein kinase; FAK- focal adhesion kinase; PLC- phospholipase C; PI3K- phosphoinositide 3-kinase; PKC - protein kinase C; PKB - protein kinase B; IP₃ - inositol 1,4,5-trisphosphate; Akt- Anti-apoptotic kinase; DAG - Diacylglycerol; eNOS - endothelial constitutive nitric oxide synthase; NO - nitric oxide; PGI₂- Prostacyclin)

2.4. VEGFs - role in disease pathogenesis and therapeutics

Angiogenesis is induced in many pathological states, such as wound healing, chronic inflammation, rheumatoid arthritis, ocular neovascularization, tumor progression, endometriosis, and cardiovascular diseases like restenosis, atherosclerosis and chronic ischemia. However, angiogenesis can be used for therapeutic purposes as well. Therapeutic angiogenesis (promoting new vessel growth to treat ischemic disorders) is an exciting frontier of cardiovascular medicine (Roy et al., 2006^a).

2.4.1 Tumor angiogenesis and lymphangiogenesis

Malignant neoplasms are characterized by uncontrolled cellular proliferation. Adequate blood supply and the nutrients are required to sustain their growth. Tumor growth and metastasis are angiogenesis-dependent events. There is extensive data that supports the role of VEGF in tumor angiogenesis, especially in lung, gastrointestinal, ovarian and breast cancers. (Herbst et al., 2005; Yoshiji et al., 1996; Brown et al., 1993; Aggarwal et al., 2005; Hartenbach et al., 1997). Therefore, tumor angiogenesis has become a potential target for cancer therapy (Carmeliet et al.,

2000; Dvorak, 2002). Bevacizumab (Avastin™), a neutralizing monoclonal antibody to VEGF, was the first antiangiogenic agent that was approved for the treatment of metastatic colorectal cancer. Since then it has also been experimentally used for treatment of other cancers (Willet et al., 2004; deGramont et al., 2005; Yang et al., 2003).

VEGF-B and PIGF act through VEGFR-1 and their role in tumor angiogenesis has also been investigated. Increased PIGF expression has been associated with pathological angiogenesis. PIGF expression is significantly increased in non-small cell lung carcinoma tissues and in certain brain tumors (Zhang et al., 2005, Nomura et al., 1998). VEGF-B presumably has a role in early tumor development and in oral squamous cell carcinomas (Hanrahan et al., 2003; Shintani et al., 2004) but there is a paucity of conclusive data to indicate a significant role of VEGF-B in tumor progression.

Lymphatic vasculature provides another route for tumor metastasis. Certain tumors like carcinomas of breast, lung and gastrointestinal tract have a propensity to metastasize through lymphatic vessels. The production of lymphangiogenic growth factors VEGF-C, VEGF-D and their receptor VEGFR-3 stimulate lymphatic growth in the region of the tumor, enabling cancer cells to gain access to the lymphatic vasculature (Stacker et al., 2002). VEGF-C and VEGF-D have been associated with tumor lymphangiogenesis and metastatic spread of tumor cells (Stacker et al., 2001; Karpanen et al., 2001). Recently a role of VEGF-A in peritumoral lymphangiogenesis and lymphatic metastasis has also been proposed (Bjorndahl et al., 2005). In breast carcinomas VEGF-C expression was detected only in node-positive tumors while, VEGF-D expression was detected only in an inflammatory breast cancer (Kurebayashi et al., 1999). VEGF-C, VEGF-D and VEGFR-3 presumably can also act as prognostic markers for various carcinomas (Nakamura et al., 2003^a; Nishida et al., 2004; Nakashima et al., 2004; Yokoyama et al., 2003; Nakamura et al., 2003^b; White et al., 2002; Juttner et al., 2006). Besides their action on lymphangiogenesis the VEGF-C and VEGF-D can also drive tumor angiogenesis through a VEGFR-2 mediated pathway (Zachary et al., 2001).

2.4.2 VEGFs in cutaneous pathologies

Several cutaneous pathological conditions such as psoriasis, rosacea and subepidermal blistering disorders, are associated with increased dermal vascularization. In such diseases, the balance between pro- and anti-angiogenic factors is altered, leading to neovascularization. Angiogenic responses in these diseases occur due to different causes, including hypoxia, inflammatory cytokine derived signals or genetic defects.

Psoriasis

Psoriasis is a chronic inflammatory skin disease clinically characterized by erythematous sharply demarcated papules and round plaques covered by silvery micaceous scales. There is epidermal hyperplasia, impaired epidermal differentiation, and accumulation of distinct leukocyte subpopulations. VEGF is strongly upregulated in psoriatic skin lesions (Detmar et al., 1994) and transgenic delivery of VEGF to mice skin produces lesions resembling human psoriasis (Xia et al., 2003). However, overexpression of VEGF in the adult animal skin by viral gene transfer does not induce psoriasis because short-term VEGF expression is not sufficient to induce the psoriatic phenotype (Xia et al., 2003). Single nucleotide polymorphisms of the VEGF gene occur more frequently in patients with early onset psoriasis and these haplotypes may contribute to the elevated VEGF levels observed in these patients (Young et al., 2004). Thus VEGF plays an important role in the pathogenesis of psoriasis and that therapeutic blockade of the VEGF/VEGF receptor system might represent a novel, pharmacogenomic approach for the future treatment of psoriasis.

Rosacea

Rosacea begins as flushing and redness on the central face and across the cheeks, nose, or forehead but can also less commonly affect the neck, chest, scalp or ears. The principal subtype of rosacea includes erythematotelangiectatic rosacea, which is characterized by uncontrolled

angiogenesis. Anti-angiogenic therapy might prove beneficial in treatment of erythematotelangiectatic rosacea (Cuevas et al., 2005).

Lymphedema

Impaired drainage results in the retention of lymphatic fluid in subcutaneous tissues. Lymphedemas can be classified as hereditary (primary) or acquired (secondary). VEGFR-3 is important for normal lymphatic vascular functions. In some patients with congenital hereditary lymphedema (Milroy disease), missense mutations in the TK domain of VEGFR-3 interferes with the signaling and results in lymphedema (Karkkainen et al., 2000). In an animal model of lymphedema VEGF-C gene transfer led to amelioration of lymphedema (Yoon et al., 2003).

Wound healing

Wound healing consists of a series of overlapping phases, including inflammation, new tissue formation and remodeling. The transient stroma filling the wound, the granulation tissue, has to be richly vascularized to support the regenerating tissue with oxygen and nutrients. The wound healing angiogenic phase is followed by vessel regression when the granulation tissue is reabsorbed. Revascularization of damaged tissue is therefore an important component of wound healing. Inadequate or unregulated vessel growth could result in a delayed healing. VEGF is widely expressed during different phases of wound healing and its role is critical in this process (Bates et al., 2003). Several preclinical studies have been done to study VEGF as a potential therapeutic factor in wound healing (Bates et al., 2003; Guarini, 2003; Galeano et al., 2003). The overexpression of VEGF using gene therapy techniques resulted in an increased vascular density in the wound and a more rapid closure of the wound, but there was a significantly greater deposition of granulation tissue (Deodato et al., 2002). Adenovirus mediated VEGF-A165 gene transfer increased skin flap survival (Giunta et al., 2005) and adeno-associated virus mediated VEGF gene transfer stimulated wound healing in diabetic mice (Galeano et al., 2003). Adenoviral VEGF-C gene transfer at the edges of epigastric skin flaps in mice restores lymphatic flow across incisional wound (Saaristo et al., 2004). In skin, PIGF expression is upregulated during wound healing and PIGF-deficient mice show delayed wound closure, indicating that this factor promotes angiogenesis during skin repair (Odorisio et al., 2006).

2.4.3 VEGFs in neuropathology

The CNS acquires its vascular supply through angiogenesis, rather than vasculogenesis. VEGF which is primarily an angiogenic growth factor has also been recognized as an important signaling molecule in the nervous system. Recent insights into the role of VEGF in a variety of neurological disorders, suggest that VEGF or its downstream effectors may be promising therapeutic targets in these diseases.

Brain Tumors

VEGF-A, VEGF-C and VEGF-D, and their receptors are expressed in some tumor like haemangioblastomas and glioblastomas (Jenny et al., 2006). PIGF has also been reported to be expressed in brain tumors (Nomura et al., 1998). Brain tumors like glioblastoma multiforme are highly vascular. Abnormal blood vessels in these tumors make them prone to bleeding, development of edema and increased intracranial pressure. Anti-angiogenic therapy like soluble VEGF receptors and VEGF anti-bodies therefore prove beneficial in the treatment of certain types of brain tumors.

Brain ischemia

Brain ischemia stimulates angiogenesis (Krupinski et al., 1994). New vessel growth is most pronounced in the ischemic penumbra, where blood flow is reduced but not absent, and where small changes in perfusion might make the difference between cell death and survival. Clinical studies suggest that rescuing tissue within the penumbra improves recovery. VEGF-A can enhance angiogenesis in ischemic brain (Zhang et al., 2000). However, there is a risk of increasing brain edema because of increased vascular permeability with VEGF-A. VEGF-B also seems to exert a neuroprotective effect in cerebral ischemia, infarct volume is increased and

neurological function is more impaired in VEGF-B-knockout than in wild-type mice (Sun et al., 2004).

Neurodegenerative disorders and amyotrophic lateral sclerosis

VEGF also has direct neuronal actions in the form of neurotrophic effects and promotes axonal growth and cell survival through a VEGFR-2 dependent pathway (Sondell et al., 199). VEGF can also protect neurons from a variety of insults (Jin et al., 2000; Matsuzaki et al., 2001). VEGF modifies acute and chronic neurodegenerative processes through effects on both blood vessels and neurons, and probably also glia. VEGF has been also described as a modifier of amyotrophic lateral sclerosis (ALS) (Lambrechts et al., 2003). ALS is characterized by loss of lower motor neurons in the spinal cord and brainstem, as well as upper motor neurons in the motor cortex. Mice with a mutation in a site that confers hypoxia-responsiveness on VEGF developed a disorder manifested by limb weakness and neurogenic muscle atrophy and loss of motor neurons from the anterior horn of the spinal cord and brainstem motor nuclei (Oosthuysen et al., 2001). Injection of a VEGF-expressing lentiviral vector into various muscles delayed onset and slowed progression of ALS in a mice model. VEGF treatment increased the life expectancy of ALS mice by 30 per cent without causing toxic side effects (Azzouz et al., 2004).

2.4.4 VEGF in pulmonary pathologies

VEGF is abundantly present in lung tissue; many different lung cells produce VEGF and also respond to VEGF. VEGF is critical for the development of the lung and serves as a maintenance factor during adult life. There is increasing evidence that VEGF also plays a role in several acute and chronic lung diseases. VEGF not only acts as a growth and morphogenetic factor for lung endothelial cells but also acts on type II pneumocytes.

Bronchopulmonary dysplasia

The realization of the importance of VEGF in fetal lung growth led to a vascular theory of bronchopulmonary dysplasia. Bronchopulmonary dysplasia (BPD) is a chronic lung disease (CLD) that develops in preterm neonates treated with oxygen and positive pressure ventilation. With BPD, there is interstitial fibrosis and inadequate alveolar development for good pulmonary function. There is decreased VEGF, VEGFR-1 and Tie-2 expression in lungs of infants dying with bronchopulmonary dysplasia (Bhatt et al., 2001). Hyperoxia is one of the main contributors to bronchopulmonary dysplasia. Hyperoxia decreases lung levels of VEGF (Maniscalco et al., 1997).

Pulmonary hypertension

Pulmonary hypertension is associated with hypertrophy and hyperplasia in endothelial cells, smooth muscle cells, and adventitial fibroblasts within the small precapillary pulmonary arteries. It is likely that angiogenic factors instrumental in the abnormal growth of endothelial cells in pulmonary hypertension. VEGF is strongly expressed in the angioproliferative plexiform lesions in the lungs from patients with severe primary idiopathic and secondary forms of pulmonary hypertension (Hirose et al., 2000; Tuder et al., 2001; Lassus et al., 2001).

Emphysema

Interalveolar septal damage is one of the characteristic features of emphysema. Inflammation and protease/antiprotease imbalance are the concepts used to explain the pathogenesis of cigarette smoke induced emphysema. Several investigators have shown that a decrease of VEGF and VEGFR-2 expression in the lung tissue was associated with emphysema and the presence of a large number of apoptotic alveolar cells (Kasahara et al., 2001; Kanazawa et al., 2003). A combination of different factors including apoptosis, oxidative stress and matrix proteolysis, lead to lung destruction, facilitating inflammation and further decreasing growth factor availability and action in the emphysematous lungs (Tuder et al., 2003).

Bronchial asthma

Bronchial asthma is a chronic inflammatory disease that is characterized by increased responsiveness of tracheobronchial tree to a multiplicity of stimuli. Chronic airway inflammation and remodeling are distinctive features of patients with asthma. Airway wall thickening consists mainly of hypertrophy and hyperplasia of smooth muscle, an increase in mucous gland, infiltration of inflammatory cells, extracellular matrix, tissue edema and angiogenesis in the bronchial wall. VEGF and other angiogenic growth factors are therefore relevant to the angiogenic processes within the airways. VEGF is also involved in vascular permeability leading to tissue edema (Hashimoto et al., 2005). Bronchial lymphedema could exacerbate airway obstruction in inflammatory disease. Lymphangiogenesis in inflamed airways is mediated by VEGF-C and VEGF-D and impaired lymphangiogenesis in airway inflammation may interfere with fluid clearance and lead to bronchial lymphedema (Baluk et al., 2005).

Acute Lung Injury

Acute lung injury and acute respiratory distress syndrome (ARDS), involve a disruption of the alveolar-capillary membranes with local inflammation ultimately leading to alveolar flooding with serum proteins and edema fluid. It has been suggested that infiltrating inflammatory cells such as monocytes and local matrix degradation might be sources of VEGF that can increase alveolar capillary permeability (Thickett et al., 2001; Medford et al., 2006; Voelkel et al., 2006). Patients with ARDS have increased plasma VEGF and increased VEGF production by peripheral blood mononuclear cells (Thickett et al., 2001).

Lung Tumors

VEGF is overexpressed in different types of malignant lung tumors and high serum VEGF levels in patients with lung cancer are associated with a poor prognosis (Fontanini et al., 1997; Kaya et al., 2004; Herbst et al., 2005). VEGF-A, -B, -C and -D are expressed in lung adenocarcinoma (Niki et al., 2000). PlGF expression is significantly increased in non-small cell lung carcinoma tissues (Zhang et al., 2005). VEGF-C and VEGFR-3 are also expressed in lung cancer tissues and that they presumably play a role in tumor lymphangiogenesis (Ogawa et al., 2004). A number of anti-angiogenic therapeutic agents including - anti-VEGF monoclonal antibodies and VEGF receptor antagonists - are undergoing at different stages of clinical development for treatment of lung cancers (Herbst et al., 2005).

2.4.5 VEGF in renal Pathology

In kidney VEGF expression is prominent in glomerular podocytes and in tubular epithelial cell, while, the VEGF receptors are primarily found in preglomerular, glomerular and peritubular endothelial cells. VEGF and its receptors are upregulated in diabetic nephropathy especially during the initial stages. VEGF also presumably mediates glomerular recovery in proliferative glomerulonephritis. VEGF is required for growth and proliferation of glomerular and peritubular endothelial cells (Schrijvers et al., 2004).

2.4.6 VEGF in Gastrointestinal tract (GIT) pathology

Growth of new blood vessels is seen in various neoplastic and chronic inflammatory disorders of GIT. Increased VEGF-A, VEGF-C and VEGF-D expression is seen in various GIT malignancies (Brown et al., 1993; Duff et al., 2003) and their expression levels presumably determines tumor prognosis. VEGF serum levels in patients with active Crohn's disease and ulcerative colitis are significantly increased. Angiogenesis is intrinsic to inflammation and is associated with structural changes, including activation and proliferation of endothelial cells and capillary and venule remodeling. There is increased production of VEGF by intestinal mucosa in active inflammatory bowel disease patients (Griga et al., 1999; Kapsoritakis et al., 2003; Danese et al., 2006). It has been suggested that anti-angiogenesis strategies may be beneficial in inflammatory bowel disorders (Danese et al., 2006).

2.4.7 VEGFs in Rheumatoid Arthritis (RA)

Rheumatoid arthritis is a systemic disease characterized by inflammatory polyarthritis, involving small and large joints and constitutional features. Hallmark of joint involvement is the *synovial pannus*, a proliferative synovium infiltrated by inflammatory mononuclear cells. Angiogenesis constitutes an early event of synovial hyperplasia which presumably promotes the destruction of cartilage and bone in later stages of RA (Hirohata et al., 1999). The role of angiogenesis is intricate and varied in RA. Angiogenesis helps in supplying nutrients for hyperplastic synovium but also promotes persistence of synovial inflammation through the influx of inflammatory cells and by producing inflammatory mediators (Pap et al., 2005). The neovascular network in RA is dysfunctional and the joint affected by rheumatoid arthritis is hypoxic (Taylor et al., 2005). VEGF is upregulated by proinflammatory cytokines and by hypoxia in RA. Serum and synovial fluid VEGF concentrations are elevated in RA and correlate with disease activity (Taylor, 2002; Ikeda et al., 2002; Harada et al., 1998). Anti-angiogenic strategies including bevacizumab (a neutralizing monoclonal antibody to VEGF) may have a potential therapeutic role in RA (Lainer et al., 2005).

2.4.8 VEGF in ocular disorders

Diabetic retinopathy

Diabetic retinopathy (DR) becomes clinically apparent several years after the onset of diabetes mellitus (DM). Non-proliferative diabetic retinopathy (NPDR) is characterized by increased vascular permeability, leading to edema, and lipoprotein accumulation (hard exudates) in the outer plexiform layer and small hemorrhages and microaneurysms in retina (Frank, 2004). In later stages there is periretinal neovascularization which typifies the proliferative diabetic retinopathy (PDR). Increased VEGF-A expression has been described in NPDR in humans (Hofman et al., 2001; Boulton et al., 1998) and elevated levels of VEGF-A have been found in the aqueous humor and vitreous of patients with PDR (Shinoda et al., 1999). VEGF receptors are also upregulated in DR (Smith et al., 1999). Increased VEGF-A levels presumably result in vascular leakage and periretinal neovascularization in DR.

Age-related macular degeneration

Age-related macular degeneration (AMD) is the major cause of central vision loss in elderly. There are two forms of AMD, neovascular and non-neovascular. The non-neovascular form of AMD is more common. Visual loss in AMD occurs from photoreceptor damage due to development of choroidal neovascularization (CNV) and related manifestations such as subretinal hemorrhage, detachment of the retinal pigmentary epithelium, and fibrovascular disciform scarring (Gryziewicz 2005; Kulkarni et al., 2005). Vitreous VEGF-A levels were found to be significantly higher in patients with AMD and CNV (Wells et al., 1996). Although the exact mechanisms for development of CNV are poorly understood but tissue hypoxia and VEGF overexpression presumably play a key role in the development of CNV (Saint-Geniez et al., 2004; Compochiaro, 2000; Witmera et al., 2003).

Retinopathy of prematurity (ROP)

ROP is characterized by the proliferation of the retinal blood vessels in premature babies who receive prolonged mechanical ventilation and are therefore exposed to high concentrations of oxygen. Role of VEGF has been clearly established in the pathogenesis of ROP (Saint-Geniez et al., 2004). Hyperoxia causes obliteration of developing retinal vessels. Once the infant returns to normoxic environment retina become hypoxic, resulting in VEGF upregulation and vascular proliferation (Saint-Geniez et al., 2004, Witmera et al., 2003). VEGF is expressed by the astrocytes and the Müller cells which participate in the development of the superficial and deep vascular layers, respectively (Stone et al., 1995). Insulin like growth factor (IGF)-1 also plays an important role in development of ROP. It has been suggested that VEGF may not be able to stimulate vascular growth in absence of IGF-1 (Hellstorm et al., 2001).

2.4.9 VEGFs in cardiovascular pathology

a) Therapeutic angiogenesis

Atherosclerotic narrowing of blood vessels causes decreased tissue perfusion and ischemia. Narrowing or occlusion of coronary arteries and large peripheral arteries due to atherosclerotic lesions could result in coronary artery disease (CAD) and peripheral artery disease (PAD) (Yla-Herttuala et al., 2000; Gruchala et al., 2004). Formation of collateral vessels can improve perfusion in the ischemic tissues (Rissanen et al., 2001; Rosengart et al., 1999). VEGFs have been used to promote development of collateral blood vessels in clinical trials and animal models (Baumgartner et al., 1998; Vale et al., 2000; Makinen et al., 2002; Rutanen et al., 2004). Improvement in exercise tolerance was reported after adenovirus mediated VEGF-A121 gene transfer to ischemic myocardium (Rosengart et al., 1999). In a recent clinical trial patients with PAD were treated with adenoviral delivery of VEGF-A121 but the clinical benefits were not seen (Rajagopalan et al., 2003). Animal experiments using intramuscular or intramyocardial injections of adenovirus encoding VEGF-A and VEGF-D, have shown high angiogenic efficacy (Rissanen et al., Rutanen et al., 2004). VEGF-A and VEGF-D enlarge the preexisting capillaries in skeletal muscle and also enhance collateral growth. PlGF also induces angiogenesis and arteriogenesis in animal models of myocardial infarction and lower limb ischemia (Luttun et al., 2002).

Perivascular gene transfer can be used for therapeutic angiogenesis and to inhibit neointima formation. It has also been used to improve vein graft survival and for the prevention of vascular access stenosis. Perivascular gene delivery of VEGF-A is also useful for promoting therapeutic angiogenesis in ischemic myocardium (Fuster et al., 2001; Nikol et al., 2002). Application methods, such as perivascular collars or sheaths, needle injection catheters and biodegradable gels can be used to deliver vectors directly into the adventitia.

VEGF overexpression increases vascular permeability and may cause substantial tissue edema, pericardial effusion in the heart and angioma formation (Carmeliet, 2000). Use of combination therapy using different growth factors like VEGF and angiopoietin might reduce the side effects (Chae et al., 2000; Bhardwaj et al., 2005^b).

b) Intimal Hyperplasia

Neointima formation occurs following an acute injury to the blood vessels as seen after angioplasty, stent placement and in vein graft stenosis. Multiple factors tend to influence the neointima formation but vascular smooth muscle cell proliferation is perhaps the most important factor responsible for the development of restenosis (Painter, 1991; Hoffmann et al., 1996). VEGF-A, VEGF-D and PlGF can influence smooth muscle cell migration (Ishida et al., 2001; Bhardwaj et al., 2005^a).

The role of VEGFs in intimal hyperplasia has remained controversial. VEGF expression was detected in vascular SMC after balloon injury which suggests that VEGFs may play a role in the development of restenotic lesions (Ruef et al., 1997; Ohta et al., 1997; Shibata et al., 2001). Increased neovascularization has also been observed at sites of intimal hyperplasia (Edelman et al., 1992; Westerland et al., 2000). A correlation between adventitial angiogenesis following VEGF gene transfer and intimal hyperplasia was seen in a collar model of neointima formation (Khurana et al., 2004). However, in a balloon denudation model of neointima formation, transfer of VEGF gene to vessel wall has been shown to decrease neointima formation (Laitinen et al., 1997; Hiltunen et al., 2000; Rutanen et al., 2005). VEGF stimulates endothelial regeneration in injured blood vessels. It has been hypothesized that the rapid regeneration of ECs results in secretion of substances like nitric oxide, C-type natriuretic peptide and prostacyclin- I_2 , which have anti-proliferative effects on smooth muscle cells (Gruchala et al.; 2004).

c) Atherosclerosis

Plaque angiogenesis may be associated with increased atherogenesis and unstable vulnerable plaques (Hayden et al., 2004; Moreno et al., 2004). These vulnerable plaques are more likely to rupture and cause sudden intra-arterial occlusion. An abrupt, coronary artery occlusion following a plaque rupture could result in fatal acute coronary syndrome. VEGFs are potent angiogenic

factors that can affect plaque neovascularization and thereby influence atherosclerotic process. Use of VEGFs for therapeutic angiogenesis in CAD and PAD has been questioned because of the concerns that the VEGFs might enhance the atherosclerotic lesion formation. VEGFs along with other growth factors and cytokines can initiate and/or accelerate atherosclerosis by influencing monocyte activation, adhesion, migration and enhancing vascular permeability. It has also been reported that VEGF-A enhances progression of atherosclerotic plaques (Celletti et al., 2001). VEGF-A and VEGF-D are expressed in medial smooth muscle cells and in macrophages of human atherosclerotic lesions (Rutanen et al. 2003). A role of PlGF in macrophage infiltration and development of early atherosclerotic lesions has also been suggested (Khurana et al., 2005). The debate over the role of VEGFs in atherosclerosis continues with a recent study in an animal model showing that the increased systemic levels of VEGF-A, VEGF-B, VEGF-C, and VEGF-D have no effect on atherosclerotic lesions (Leppänen et al., 2005).

2.4.10 Kawasaki disease

Kawasaki disease (KD) is characterized by systemic vasculitis and increased vascular permeability. It typically occurs in children and clinical symptoms include fever, conjunctivitis, and erythematous induration of the hands and feet, inflammation of the lips, rash and cervical lymphadenopathy. Histopathological studies using specimens of the skin rash in the early acute stage of KD reveal microvascular dilation and subendothelial edema. Serum VEGF levels are markedly increased in KD and VEGF presumably has a role in increased vascular leakage seen in KD (Terai et al., 1999; Terai et al., 2003). VEGF expression is also seen in the coronary artery lesions complicating KD (Hamamichi et al., 2001) and dysregulation of angiogenesis likely contributes to KD vasculopathy (Freeman et al., 2005). Frequency of G allele of VEGF and A1 allele of KDR, was significantly higher in patients of KD with coronary artery lesions suggest that VEGF and its receptor, KDR, genes contributed to the development of coronary artery lesions in KD patients (Kariyazono et al., 2004).

Table 1 - VEGFs in pathogenesis of different diseases

Organ /Tissue	Diseases	Role of VEGF/VEGFR
Lungs	Bronchopulmonary Dysplasia	↓ VEGF & VEGFR-1
	Pulmonary Hypertension	↑ VEGF and proliferation of vascular cells
	Emphysema	↓ VEGF & VEGFR-2 and septal destruction
	Bronchial Asthma	↑ VEGF-A, Increased angiogenesis and edema
	Acute Lung Injury/Acute Respiratory distress syndrome	↑ VEGF and vascular permeability
	Lung cancers	↑ VEGF-A, PIGF, VEGF-B, VEGF-C, VEGF-D & VEGFR-2, VEGF-3. tumor angiogenesis and lymphangiogenesis
Brain	Ischemic stroke	Increased angiogenesis in tissue surrounding ischemic zone; VEGF modifies infarct zone
	Brain tumors	↑ VEGF-A, VEGF-C, VEGF-D, PIGF, VEGFR-2, VEGFR-3; tumor angiogenesis
	Amyotrophic lateral sclerosis	? ↓ VEGF-A; neuronal loss
Gastrointestinal tract	Inflammatory bowel disease	↑ VEGF increased inflammatory and immune response
	GI tumors	↑ VEGF-A, VEGF-C, VEGF-D, VEGFR-2, VEGFR-3; tumor angiogenesis Lymphangiogenesis
Kidney	Diabetic nephropathy,	↑ VEGF-A, VEGFR-1, VEGFR-2 in the initial stages. ? deleterious role in diabetic nephropathy
	Crescentic glomerulonephritis	↑ VEGF-A; Glomerular proliferation
Heart and blood vessels	Myocardial Ischemia	Increased angiogenesis in tissue surrounding ischemic zone; VEGF modifies infarct zone
	Atherosclerosis	↑ VEGF-A, VEGF-D, VEGFR-1, VEGFR-2; vascular cell proliferation, Plaque angiogenesis
	Restenosis	↑ VEGF-A; vascular cell proliferation
Skin	Psoriasis	↑ VEGF-A
	Rosacea	↑ VEGF-A
	Ulcer/wounds	Inappropriate VEGF expression can influence wound healing
	Hereditary Lymphedema	Mutation in VEGFR-3
	Skin cancers	↑ VEGF-A, VEGF-C, VEGF-D, VEGFR-2, VEGFR-3; tumor angiogenesis, lymphangiogenesis
Bones and Joints	Rheumatoid arthritis	↑ VEGF-A; pannus formation
	Healing Fractures	VEGF-A, PIGF influence healing fracture
Eyes	Diabetic Retinopathy	↑ VEGF-A; angiogenesis
	Age-related macular Degeneration	↑ VEGF-A; angiogenesis
	Retinopathy of Prematurity	↑ VEGF-A; angiogenesis
Miscellaneous	Kawasaki disease	↑ VEGF-A; vascular leakage, vasculitis
	Endometriosis	↑ VEGF-A, VEGF-C; abnormal angiogenesis
	Multiple myeloma	↑ VEGF-A; Proliferation and migration of human myeloma cells
	DiGeorge syndrome	VEGF is a modifier of cardiovascular birth defects

Table 2 - Potential clinical applications of VEGFs and VEGF inhibitors

Disease	Therapeutic agent	Potential clinical use	Comments
CAD PAD	VEGF-A, VEGF-D, PIGF	Therapeutic angiogenesis	Neo-angiogenesis/arteriogenesis to restore blood supply to ischemic areas
Wound healing and bone healing	VEGF-A, VEGF-C, VEGF-D, PIGF	Therapeutic angiogenesis, lymphangiogenesis	Angiogenesis to hasten wound/bone healing. Restore lymphatic supply across incisional wounds.
Lymphedema	VEGF-C, VEGF-D	Therapeutic Lymphangiogenesis	Improved lymphatic drainage
Restenosis - post angioplasty, stent	VEGF-A, VEGF-C, VEGF-D	Suppress intimal hyperplasia	Rapid endothelial regeneration
Vein graft	VEGF-D (Trinam™)	Improved vein graft survival	Increased adventitial angiogenesis
Tumor angiogenesis, Lymphangiogenesis	VEGF inhibitors e.g. Monoclonal antibody VEGF-A (Avastin™), soluble VEGF receptors (VEGF Trap)	Inhibit tumor angiogenesis, lymphangiogenesis	Can be used in conjunction with other therapies. Reduce tumor growth and metastasis
Rheumatoid arthritis	VEGF inhibitors	Reduce angiogenesis in pannus	Can be used in conjunction with other therapies to reduce inflammation and angiogenesis
Psoriasis	VEGF-A inhibitors	Decrease angiogenesis and inflammation	Can be used in conjunction with other therapies to reduce inflammation and angiogenesis
Ocular disorders - DR, AMD, ROP	VEGF-A inhibitors, VEGF trap, Macugen™	Suppress angiogenesis	These ocular diseases are characterized by abnormal angiogenesis

*PAD- peripheral arterial disease; CAD- coronary artery disease; DR- diabetic retinopathy; AMD- age related macular degeneration; ROP- retinopathy of prematurity (Roy et al., 2006)

2.5 Vascular dysfunctions in Diabetes

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Although, there are large numbers of different causes of diabetes, the vast majority of cases of diabetes can be classified into two broad categories. Type 1 diabetes is characterized by an absolute deficiency of insulin secretion. In the more prevalent type 2 diabetes, the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response.

The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart and blood vessels. The vascular wall impairment in diabetes is a typical consequence of chronic hyperglycemia. Microvascular complications of diabetes that are well recognized include nephropathy and retinopathy while the macrovascular complications of diabetes include atherosclerotic changes in large vessels like aorta, coronary artery, carotid arteries and peripheral arteries.

There has been an increasing prevalence of coexisting diabetes and hyperlipidemia cases contributing to high morbidity and mortality all over the world. Elevated triglyceride and total cholesterol levels are proven predictors of cardiovascular disease in diabetes (Niemeijer-Kanters et al., 2001) and hypertriglyceridemia is an important feature of diabetic dyslipidemia (Taskinen,

2003). A combination of factors including poorly controlled diabetes (Sosenko et al., 1980; Abraha et al., 1999), familial factors (Abraha et al., 1999) metabolic abnormality and diet often contribute to the development of dyslipidemia that complicates type-1 diabetes. Often dyslipidemia also complicates type-2 diabetes (Brunzell et al., 2003) and Reaven (1988) used the term Syndrome X to describe a relation between several disorders including hypertension, dyslipidemia, impaired glucose tolerance, obesity, and coronary heart disease. Later the term 'Metabolic syndrome' and 'Insulin resistance syndrome' were used to describe this disorder. Nearly 25% of American adults suffer from this disorder.

Metabolic abnormalities that characterize diabetes, including hyperglycemia, insulin resistance, increased levels of free fatty acids (FFA) and dyslipidemia (Creager et al., 2003; Renard et al., 2004) provoke cellular and molecular mechanisms that can alter the function and structure of blood vessels and have discrete effects on the initiation and progression of atherosclerotic lesions.

2.5.1 Pathophysiology of Diabetic vascular disease

Abnormalities in endothelial and vascular smooth muscle cell function, as well as a predisposition to thrombosis, contribute to atherosclerosis and its complications. Multiple pathogenic mechanisms like formation of Advanced Glycation Endproducts (AGEs), sorbitol pathway, activation of protein kinase C (PKC), oxidative stress and also hemodynamic changes are involved in generation of the endothelial dysfunction (Fig. 3). Endothelial cells synthesize important bioactive substances like nitric oxide (NO), prostaglandins, endothelin and angiotensin II which regulate blood vessel functions. Chronic hyperglycemia in diabetes is perhaps the single most important factor responsible for vascular dysfunction in diabetes. Studies have confirmed that improved diabetes control characterized by blood glucose levels lower than in uncontrolled patients is accompanied by decreased development of microangiopathy. It is a typical feature in both type 1 and type 2 diabetic patients although in the latter hyperglycemia is combined more often with atherosclerotic changes (Skrha et al., 2003). The impact of hyperglycemia in the development of diabetic macroangiopathy may be both direct and indirect. Direct effects of hyperglycemia like accumulation of sorbitol in SMCs of arterial walls and increased oxidative stress due to reduced NADH/NAD⁺ ratio (Yasunari et al., 2000). An indirect consequence of elevated blood glucose is the generation of advanced glycation endproducts that are signal transduction ligands for receptor for AGE (RAGE) (Yan et al., 2003).

2.5.2 Advanced Glycation Endproducts (AGEs) and Receptor for AGEs (RAGE) in diabetes

AGEs are the products of non-enzymatic glycation and oxidation of proteins and lipids. The term 'AGE' is used for a broad range of advanced products of the Maillard reaction. The formation of AGEs in vitro and in vivo is dependent on the turnover rate of the chemically modified target, time and sugar concentration (Sano et al., 1999). In diabetes increased AGE accumulation occurs in the vasculature, and they likely participate in the vascular dysfunction of diabetes. In addition to hyperglycemia and oxidant stress, AGEs may form in multiple milieus, such as inflammation and renal failure.

AGEs can effects blood vessels by receptor independent or receptor-dependent pathways. AGEs can directly influence the structural integrity of the vessel wall by excessive cross-linking of matrix molecules and disrupting matrix-matrix and matrix-cell interactions (Haitoglou et al., 1992). Non-enzymatic glycation of intracellular molecules can also impair their function. AGEs exert their cellular effects on cells by interacting with specific cellular receptors. A number of receptors have been identified, such as macrophage scavenger receptor (MSR) type II, OST-48, 80K-H, galectin-3, CD36, and RAGE (Yan et al., 2003). These receptors remove and detoxify AGEs, as well as modify cellular properties by receptor-triggered signal transduction. RAGE signal transduction likely propagates inflammatory responses. The AGE-RAGE interaction induces the expression of a range of proinflammatory molecules and activation nuclear factor of NFκB (Kislinger et al., 1999). Besides AGEs, RAGE also interacts with various other ligands, such as S100/calgranulins, amphoterin, amyloid-β peptide and β-sheet fibrils and MAC-1. RAGE

mediated activation of oxidant stress also triggers a p21^{ras} dependent Mitogen-activated protein kinase (MAPK) pathway (Lander et al., 1997). Increased expression of these molecules might contribute to the development of vascular complications in diabetes.

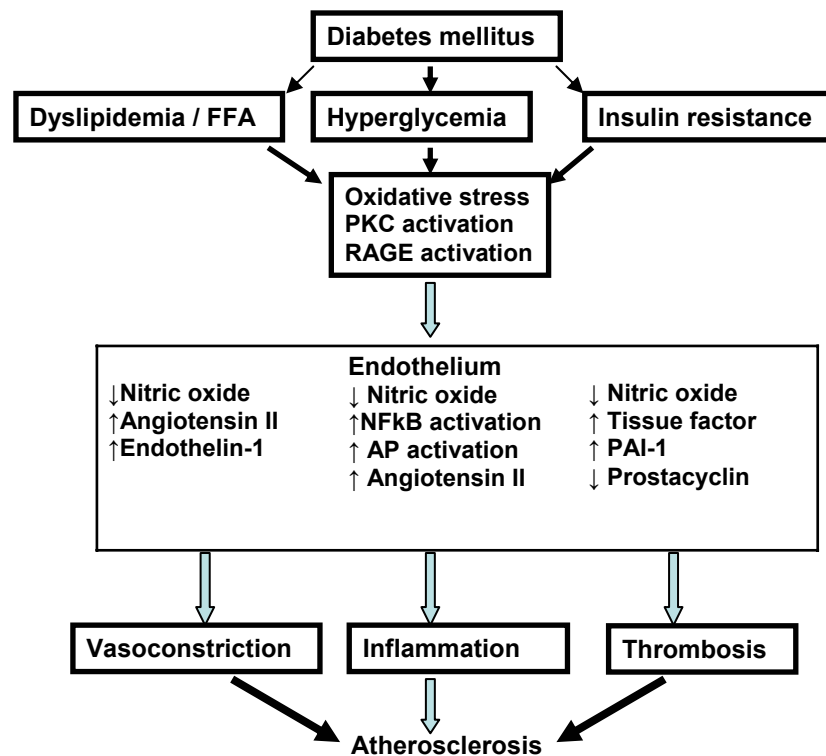


Fig.3 - Hyperglycemia, free fatty acids, and insulin resistance, provoke molecular mechanisms that alter the function and structure of blood vessels. These include increased oxidative stress, disturbances of intracellular signal transduction (such as activation of PKC, and activation of RAGE). Consequently, there is decreased availability of NO, increased production of endothelin-1, activation of transcription factors such as NFkB and AP-1, and increased production of prothrombotic factors such as tissue factor and plasminogen activator inhibitor- 1 (PAI-1)

2.5.3 Endothelial dysfunction in diabetes

Incubation of endothelial cells with the AGE and infusion of AGEs in mice, resulted in increased cell surface expression of vascular cell adhesion molecule 1 (VCAM-1) and activation of NFkB. RAGE blockade, reduced inflammatory cell binding to stimulated ECs (Kislinger et al. 1999). These findings demonstrate that AGEs and RAGE can modulate endothelial cell function.

Nitric oxide (NO) plays an important role in mediating vascular relaxation. It protects the blood vessel from injury by mediating molecular signals that prevent platelet and leukocyte interaction with the vascular wall and inhibit vascular smooth muscle cell proliferation and migration (Fig.3) (Verma et al., 2001; Sarkar et al., 1996; Kubes et al., 1991). NO dependent vasodilatation is impaired in diabetes due to the reduced bioavailability of nitric oxide. Hyperglycemia inhibits production of nitric oxide by blocking eNOS activation and increasing reactive oxygen species production in endothelial cells and smooth muscle cells (De Vriese et al., 2000). Superoxide anion binds with nitric oxide and forms peroxynitrite ion, which oxidizes NOS co-factor tetrahydrobiopterin. This uncouples eNOS, which then preferentially increases superoxide anion production over NO production (Milstien et al., 1999). Superoxide anion also increases intracellular production of advanced glycation end products (AGEs) (Nishikawa et al., 2000). These glycated proteins activate the receptor for AGEs (RAGE) (Schmidt et al., 1999). Oxygen-

derived free radical production is increased by AGEs and the intracellular superoxide production is further enhanced by RAGE activation (Schmidt et al., 2000; Tan et al., 2002).

Hyperglycemia also stimulates the production of the lipid second messenger diacylglycerol, which causes activation of PKC (Xia et al., 1994). Activation of PKC inhibits phosphatidylinositol 3 kinase (PI3 kinase) pathway. Inhibition of PI3 kinase limits the activation of Akt kinase and subsequent phosphorylation of NOS, resulting in less NO production. Diminished endothelium-dependent relaxation of rabbit aorta exposed to elevated glucose levels is restored by PKC inhibition (Tesfamariam et al., 1991). Decreased levels of endothelium-derived NO also results in increased activity of the NFkB. There is increased expression of leukocyte adhesion molecules and production of chemokines and cytokines (Zeiber et al., 1995).

2.5.4 Dyslipidemia and endothelial dysfunction

In diabetes there is excess liberation of fatty acids from adipose tissue and diminished uptake by skeletal muscle resulting in elevated levels of circulating free fatty acids (FFA) (Boden, 1999; Kelley et al., 1994). Elevated FFA in diabetes can produce endothelial dysfunctions through several mechanisms, including increased production of oxygen-derived free radicals, activation of PKC, and exacerbation of dyslipidemia (Dresner et al., 1999; Inoguchi et al., 2000). Increased FFA levels activate PKC (*Fig.3*) and decrease insulin receptor substrate-1- associated phosphatidylinositol-3 kinase activity thereby decreasing NOS activity (Dresner et al., 1999; Griffin et al., 1999). An increase in the delivery of fatty acids to muscles or a decrease in intracellular metabolism of fatty acids, leads to the accumulation of intracellular fatty acid metabolites that could influence insulin resistance (Shulman, 2000).

Increased FFA delivery to liver also results in an increased production of triglyceride-rich VLDL and cholesteryl ester from the liver. This increased production of triglyceride-rich proteins and the diminished clearance by lipoprotein lipase contributes to the development of hypertriglyceridemia in diabetes (Verges, 1999; Sniderman et al., 2001; Cummings et al., 1995; Taskinen, 2003). Hypertriglyceridemia is an important feature of diabetic dyslipidemia. Elevated triglyceride concentrations lower HDL by promoting cholesterol transport from HDL to very-low density lipoprotein (Sniderman et al., 2001). VLDL activates transcription factor NFkB in endothelial cells, which presumably contributes to endothelial dysfunction (Dichtl et al., 1999). LDL morphology also changes and there is an increase in amount of the more atherogenic, small, dense LDL (Dimitriadis et al., 1995). Low HDL and hypertriglyceridemia have been associated with endothelial dysfunction (de Man et al., 2000; Kuhn et al., 1991) and elevated triglyceride and total cholesterol levels are proven predictors of cardiovascular disease in diabetes (Niemeijer-Kanters et al., 2001).

2.5.5 Insulin resistance and atherogenesis

Insulin resistance is diagnosed when a defined amount of insulin stimulates glucose uptake by peripheral tissues to a lesser degree than it does in normal (insulin-sensitive) individuals. Insulin resistance can occur in both, type 1 and type 2, diabetes and is one of the factors contributing to the development of diabetic atherosclerotic disease (Beckman et al., 2002). Insulin promotes vasodilatation by stimulating NO synthesis from endothelial cells by increasing the activity of eNOS via activation of phosphatidylinositol-3 kinase and Akt kinase (Kuboki et al., 2000; Zeng et al., 2000). Insulin resistance is associated with an impairment of insulin signal transduction via the phosphatidylinositol-3 kinase pathway and decreased NO production (Montagnani et al., 2002). The mitogen-activated protein kinase pathway which is associated with increased endothelin production and a greater level of inflammation and thrombosis remains intact in insulin resistance states (Montagnani et al., 2002; Oliver et al., 1991). Drugs such as metformin and the thiazolidinediones, which increase insulin sensitivity, also improve endothelium-dependent vasodilatation (Mather et al., 2001; Watanabe et al., 2000)

Increased Intra-myocellular lipids (IMCL) have been found to be closely associated with insulin resistance in both type-1 and type-2 diabetes. Although hyperinsulinemic euglycemic clamp study is considered to be the gold standard for diagnosing insulin resistance, IMCL levels can be used as a marker for insulin resistance (Kuhlmann et al., 2003; Jacob et al., 1999). Insulin resistance

has also been linked with elevations in free fatty acid levels. Activating lipoprotein lipase to metabolize these free fatty acids increases insulin sensitivity (de Souza et al., 2001). Free fatty acid-induced alterations in intracellular signaling, may also contribute to decreased NOS activity and reduced production of NO in insulin-resistant states.

2.5.6 Effect of diabetes on vascular smooth muscle cells

Diabetes also increases migration of vascular smooth muscle cells into atherosclerotic lesions, where they produce extracellular matrix contributing to the formation of a mature lesion (Suzuki et al., 2001). A role of RAGE-AGE has been proposed in the smooth muscle cell migration and proliferation in diabetic atherosclerotic lesions. AGE is a key mediator of neointimal development in the injured vessel wall. It has been demonstrated that proliferation or migration of smooth muscle cells (SMCs) is significantly attenuated by blocking the RAGE (Ramasamy et al., 2005).

Diabetes also increases the production of vasoconstrictor substances like endothelin-1 and prostanoids (Hopfner et al., 1999; Tesfamariam et al., 1990). Endothelin-1 activate endothelin-A receptors on smooth muscle cells to increase vascular muscle tone and also produces vascular smooth muscle cell hypertrophy (Hopfner et al., 1999). Diabetes increases PKC activity, NFκB production, and generation of oxygen-derived free radicals in vascular smooth muscle, similar to these effects in endothelial cells (Inoguchi et al., 2000).

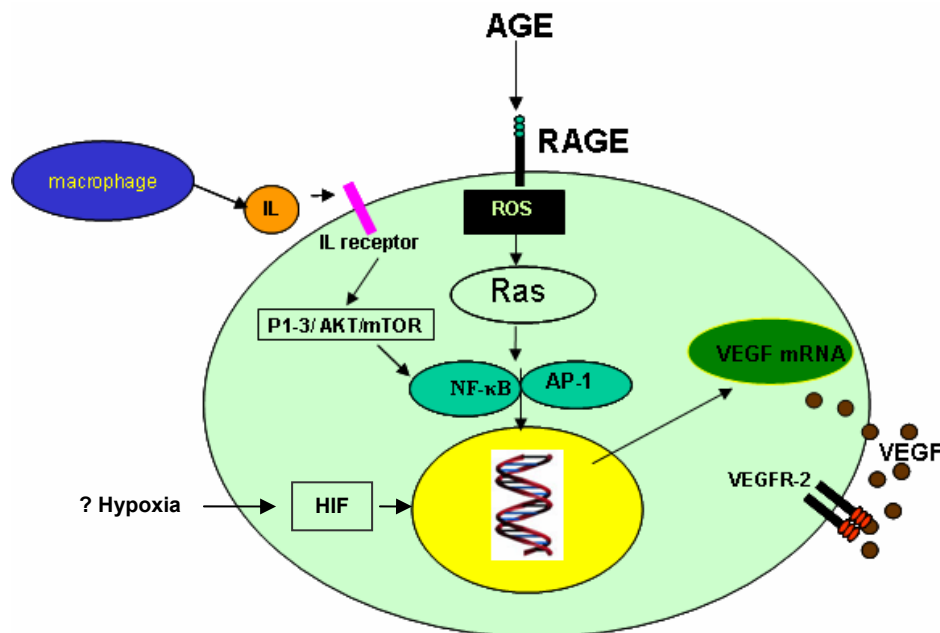


Fig.4 - Increased AGE produced in diabetes binds to RAGE. Reactive oxygen species (ROS) generated in diabetes from AGE/RAGE interaction and hyperglycemia modulates VEGF through a Ras-NFκB/AP-1 mediated pathway. Inflammatory pathways that include macrophages and interleukins (IL) also contribute to VEGF upregulation in diabetes. Another possible mechanism of increased VEGF expression could be - diminished blood supply in growing atherosclerotic plaque resulting in hypoxia and upregulation of HIF-1.

2.5.7 Plaque angiogenesis and plaque rupture in diabetes

Several studies have demonstrated that angiogenesis plays a role in plaque progression (Sueishi et al., 1997; Moulton et al., 1999). In experimental models during the initial stages of atheroma formation new vasa vasorum is formed in the areas of subsequent atherosclerotic plaque development. In subsequent stages there is extension of adventitial neovessels to the media and the enlarging plaque (Herrmann et al., 2006). Vascular endothelial growth factors (VEGFs) are potent angiogenic factors that can affect plaque neovascularization and these angiogenic effects are largely brought about by their action on endothelial cells. VEGF also produce monocyte

activation and migration. Increased numbers of macrophages occur in regions of increased angiogenesis (Leek et al., 2000). Macrophages are the primary inflammatory cells in the atherosclerotic lesions and they elaborate large number of growth factors and cytokines including VEGFs (Ross, 1993; Celletti et al., 2001). Multiple factors including - hypoxia, oxidative stress and inflammation can modulate VEGF expression in atheroma (Herrmann et al., 2006). Tissue hypoxia could be a cause of plaque neovascularization. Areas of hypoxia were demonstrated in atherosclerotic plaques (Bjoernheden et al., 1999) and increased HIF-1 α expression has been reported in some experimental models of atherosclerosis (Kuwahara et al., 2002). Other studies however could not validate these findings. Role of hypoxia in induction of VEGF expression in atherosclerosis requires further confirmation. Induction of VEGFs through a pathway involving RAGE, ras, NF κ B/AP-1 (Fig. 4) could result in increased expression of VEGFs in the diabetic atherosclerotic lesions (Okamoto et al., 2002, Roy et al., 2006^b).

VEGF enhances atherosclerotic plaque progression (Celletti et al., 2001) and VEGFs (Inoue et al., 1998; Rutanen et al., 2003) have a role in plaque angiogenesis. Plaque angiogenesis may be associated with increased atherogenesis and unstable vulnerable plaques (Hayden et al., 2004; Moreno et al., 2004). Increased elaboration of cytokines in plaques, decreased collagen synthesis and increased elaboration of matrix metalloproteinases are all likely to contribute towards an unstable plaque in diabetics (Uemura et al., 2001). Rupture of atherosclerotic plaque could result in, fatal events, such as a myocardial infarction or a cerebral stroke.

Diabetes also results in platelet dysfunction and there is increased platelet-von Willebrand factor and platelet-fibrin interaction (Vinik et al., 2001). Increased superoxide anion formation and PKC activity and decreased platelet-derived NO in diabetes contribute to the platelet dysfunction (Vinik et al., 2001; Assert et al., 2001). Hyperglycemia further changes platelet function by impairing calcium homeostasis (Li et al., 2001). In diabetes, plasma coagulation factors and tissue factors are increased and endogenous anticoagulants decreased (Hafer-Macko et al., 2002; Ceriello et al., 1995). Platelet dysfunction along with an increased tendency for coagulation increases the risk of thrombosis following a plaque rupture.

2.5.8 VEGF-inhibition strategies

VEGF is a key mediator of angiogenesis and several VEGF inhibition strategies have emerged for treating the diseases associated with pathological angiogenesis. The most common strategies have been receptor-targeted molecules and VEGF-targeting molecules. The best-studied and most advanced approach to VEGF inhibition is the humanized monoclonal antibody bevacizumab (Avastin), which is the only anti-angiogenic agent approved for treatment of cancer. Other anti-VEGF strategies in different stages of trials include:

- Vatalanib - receptor tyrosine kinase inhibitor of VEGFR-1 and VEGFR-2
- ZD6474 - receptor tyrosine kinase inhibitor of VEGFR-2, VEGFR-3 and epidermal growth factor receptor
- Sorafenib - receptor tyrosine kinase inhibitor of VEGFR-2 and PDGFR
- Sunitinib - receptor tyrosine kinase inhibitor of VEGFR, PDGFR, Flt-3, and c-kit
- VEGF-Trap- Binds VEGF-A
- IMC-1C11 - Monoclonal antibody against VEGFR-2
- AE-941- Inhibitor of VEGFR-2, MMP-2, MMP-9, and MMP-12 (Zakarija and Soff 2005)

Adverse effects including mild hypertension, proteinuria and thrombosis are seen in some patients receiving VEGF inhibition therapy.

VEGF/VEGF-receptor inhibition strategies have been used for the treatment of different cancers. Similar VEGF/VEGF-receptor inhibition strategies can be used for treatment of other diseases with pathological angiogenesis (Table 2) (Roy et al., 2006). It has been suggested that angiogenesis in atheroma is similar to angiogenesis in malignant tumors and anti-angiogenesis strategies could be beneficial in treatment of atherosclerosis (Herrmann et al., 2006, Moulton, 2006). High dose statins also have an anti-angiogenic action which is produced by modulating VEGF and MMP production (Weis et al., 2002; Luan et al., 2003). Paclitaxel a drug that is used for inhibiting neointima formation reduces VEGF induced neointima formation (Celletti et al., 2002). The exact role of anti-angiogenesis therapy in atherosclerosis awaits further confirmation.

3. AIMS OF THE STUDY

The study was carried out to:

1. To evaluate the angiogenic potential of VEGF-A,-B,-C,-D and processed forms of VEGF-C and -D in perivascular space using collar model.
2. To investigate the angiogenic potential of PlGF-2 *in vivo* after adenovirus mediated PlGF-2 gene transfer in perivascular tissue and to evaluate the potential role of endogenous VEGF-A, VEGFR-1 and VEGFR-2 in PlGF-2-mediated angiogenesis.
3. To determine the intimal hyperplasia induced by various members of the VEGF family.
4. To develop a diabetic hyperlipidemic animal model which would allow studies of the combined effects of these risk factors on atherogenesis and (b) to investigate the effects of these risk factors on the expression of VEGF-A, VEGF-D, VEGFR-1, VEGFR-2 and angiogenesis in atherosclerotic lesions.

4. MATERIAL AND METHODS

4.1 Experimental Animals

The Animal Care and Use Committee of the University of Kuopio, Finland approved all animal procedures. Male New Zealand White (NZW) rabbits were used for the Collar model study and male WHHL rabbits were used in the atherosclerosis study.

4.2 Periadventitial gene transfer - Collar Model

New Zealand White rabbits were used to study the VEGFs delivered to periadventitial surface of carotid artery. The rabbits were fed 0.25% cholesterol diet for two weeks prior to operation. A midline neck incision was used to expose both the left and right carotid arteries. The benefit of using collar model is that it allows efficient local delivery of transgene to the adventitial surface of the large arteries. Biologically inert silastic collar (2 cm in length; Ark Therapeutics oy, Kuopio, Finland) was placed around each carotid artery (Booth *et al.*, 1989; Laitinen *et al.*, 1997). Gene transfer was done 3 days after the collar installation by surgically exposing the collars. Arteries were analyzed for the effects of gene transfer 7 days after the transduction.

4.3 Diabetic hypercholesteremic animal model

Diabetes was induced in male WHHL rabbits by a single intravenous injection of alloxan (Sigma Aldrich), 100 mg/kg dissolved in 10 ml of 0.9% NaCl. Blood glucose was monitored before alloxan injection and thereafter every 2 h for 8 h after the alloxan injection. Blood glucose levels were then measured every morning using a standard Glucometer (ELITA XL- Bayer). In order to prevent life-threatening hyperglycemia, diabetic rabbits were treated with injections of Ultratard Insulin (1-2 units/kg, Novo Nordisk), whenever the morning blood glucose level was >18 mmol/l. All rabbits received standard chow and water *ad libitum*. Diabetic animals were divided into two groups that were sacrificed after two months and six months period respectively.

4.3.1 Oral glucose tolerance test (OGTT)

Rabbits were fasted overnight and a fasting blood glucose level was measured. Thereafter the rabbits were given orally 1.2g of glucose/kg body weight. Blood glucose levels were measured at 15, 30, 60, 90 and 120 minutes using a glucometer (ELITA XL- Bayer).

4.3.2 Serum lipids

Arterial blood was collected at baseline and before sacrificing the animals. Total serum cholesterol and triglyceride levels were measured by standard enzymatic procedures using Ecoline[®] reagent kits (E. Merck, Darmstadt, Germany). FFA were measured by an enzymatic colorimetric method (Wako NEFA C test kit; Wako Chemicals GmbH, Neuss, Germany)

4.3.3 ¹H NMR spectroscopy

Two months after the induction of diabetes, ¹H NMR spectrum was obtained from the *biceps femoris* muscles of the diabetic rabbits and non-diabetic controls. MRS was performed with a horizontal bore 4.7 T magnet equipped with actively shielded field gradients interfaced to a Varian ^{UNITY} INOVA console. A quadrature surface coil was used both as a transmitter and a receiver. Gradient-echo images were acquired to place the voxel to the muscle. For metabolite analysis, LASER method incorporating water suppression was used (repetition time TR=6s, echo time TE=34ms and number of scans NS=128). Non-water-suppressed spectra were obtained similarly (NS=8) to provide a reference. Spectral analysis was performed in the time domain using jMRUI software. Metabolite concentrations were calculated based on the water content.

4.4 Lectin angiography

To verify perfusion of the newly formed vessels we injected rhodamine ricinus communis agglutinin (Vector laboratories; dose-0.3mg/kg dissolved in 10ml of saline) in the left ventricle of the rabbits after thoracotomy ten minutes before sacrifice. Fluorescence by rhodamine ricinus communis injected systemically was observed in a number of neovessels formed in the adventitia of transduced arteries.

4.5 Immunohistochemistry

In collar experiments, transduced segment of the carotid artery was dissected after sacrificing the animal and the samples were obtained for paraffin embedding, frozen sections and electron microscopy. In diabetes experiment the lower thoracic aorta and upper abdominal aorta were dissected, processed and embedded in paraffin for further histological evaluation. Immunohistochemistry for paraffin sections was done with avidin-biotin HRP, DAB was used as substrate (Vector laboratories). Fluorescein Avidin D and DAPI (Vector laboratories) were used in fluorescent staining. The antibodies used for immunostainings are described in Table 3.

Table 3. Antibodies used for immunohistochemistry

TARGET	ANTIBODY	DILUTION
Endothelial cells	CD31 (Dako)	1:50
Macrophages	RAM-11 (Dako)	1:100
Basement membrane	Laminin (Zymed)	1:200
Lymphocytes	MCA-800 (Serotec)	1:5
Leukocytes	CD45 (Serotec)	1:20
Pericytes	α -SMA, clone 1A4 (Sigma-Aldrich)	1:800
Smooth muscle cells	HHF (Enzo Diagnostics)	1:50
Proliferating cells	PCNA (Neomarkers)	1:500
VEGF-A	VEGF-AC-1 (Santa Cruz Biotechnology)	1:200
VEGF-B	VEGF-B (N-19) (Santa Cruz Biotechnology)	1:50
VEGF-C	anti-hVEGF-C (R&D Systems)	1:50
VEGF-D	anti-hVEGF-D (R&D Systems)	1:100
VEGFR-1	antiVEGFR-1 (Santa Cruz Biotechnology)	1:50
VEGFR-2	antiVEGFR-2 (Santa Cruz Biotechnology)	1:500
VEGFR-3	antiVEGFR-3 (Santa Cruz Biotechnology)	1:200
RAGE	anti RAGE (Chemicon International)	1:500
NFkB	anti NFkB-c-terminus of p65 (Pharmingen)	1:50

4.6 Enzyme histochemistry

Alkaline phosphatase staining for capillaries was demonstrated by a modification of the azo dye method. Sections were counterstained with nuclear fast red. Blue staining indicated presence of capillaries. Dipeptidyl peptidase IV (DPPIV) staining was done for venous end of capillaries. Positive reaction was depicted by a red color. 5'Nucleotidase staining was done to detect lymphatic vessels in the adventitia.

4.7 Transmission electron microscopy (TEM)

Prefixation of the carotid artery samples was done in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, 2-4 h or overnight. Embedding was done in freshly prepared resin using suitable moulds. Polymerisation was done at + 37°C for 24 h and + 60°C for 48-72 h. Ultrastructural changes occurring in carotid artery after VEGF gene transfers were studied in three groups with maximum neointima formation (VEGF-A, VEGF-D, and VEGF-D Δ N Δ C), using TEM. LacZ transduced carotid arteries served as controls.

4.8 Adenoviruses

Adenoviral vectors (1×10^9 pfu) encoding human VEGF-A165, -B167, -C, - Δ N Δ C, -D, -D Δ N Δ C, sVEGFR-1, sVEGFR-2 and mouse PIGF-2 under CMV promoter were used for the study. Human clinical grade E1-partial E3 deleted adenoviruses were produced in 293 cells as described (Mäkinen *et al.*, 2002). Adv-CMV-LacZ was used as control. Although we have used same concentration of adenoviral vector in our study (1×10^9) the level of expression of various growth factors may differ in vascular tissue.

4.9 ELISA assays

For quantitative measurement of the transduced human VEGF-A proteins in exudates collected from the collar, specific ELISA assays were performed (Quantikine, R&D Systems). Similarly, constitutive VEGFA in the exudates collected from AdvLacZ transduced arteries were measured. An enzyme-linked immunosorbent assay (ELISA) (Quantikine; R&D Systems, Minneapolis, MN) was used to detect the presence of mPIGF-2 protein in tissue fluids collected from collars of animals transduced with Adv-PIGF-2.

4.10 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from transduced carotid artery segments using Trizol reagent (Gibco-BRL) and 2 μ g of RNA was used for cDNA synthesis. Dynazyme polymerase (Finnzymes, Finland) was used for the amplification. Nested PCR was used for the amplification of VEGF-A and VEGF-C. Details of PCR are given in Table-4.

4.11 Quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from carotid artery segments with TRIZOL reagent (GIBCO; Invitrogen, Carlsbad, CA). SYBR Green (Applied Biosystems, Foster City, CA) real-time polymerase chain reaction (PCR) using an ABI PRISM 7700 sequence detection system (Applied Biosystems) was used to quantify mRNA expression levels of endogenous VEGF-A121 and VEGF-A165. A common VEGF-A forward primer and specific reverse primers to amplify VEGF-A121 and VEGF-A165 isoforms were used (Table 5). 18S was selected as a reference gene. Reactions contained 100 ng of cDNA, 15 μ l of SYBR Green master mix (Applied Biosystems), and 7.5 pmol of primers. All samples were done as duplicates under the following conditions: 30 sec at 50°C and 10 min at 95°C followed by 42 cycles of 15 sec at 95°C and 1 min at 60°C. Ratios of target gene and 18S expression (relative gene expression numbers) were calculated as instructed in ABI PRISM 7700 Sequence Detection System User Bulletin #2.

4.12 Morphometry and image analysis

Morphometry and image analysis were done using analySIS (Soft Imaging System GmbH, Germany) software with Olympus AX70 microscope (Olympus Optical, Japan). Capillaries and macrophages were counted in four randomly selected non-overlapping high power (20x) fields and data expressed as number of cells/mm². Area of intima and media was measured using analySIS software and then expressed as a ratio. PCNA index was defined as the number of cells in each layer of artery with strong unequivocal nuclear staining, divided by the total number of cells. Area with positive staining for MMP-2 and MMP-9 in intima was calculated and expressed as percentage of total area of intima. Total area of atherosclerotic plaques was measured from five standardized sections (stained with hematoxylin-eosin) of each animal using the imaging software and the mean value of the affected area is reported. The total number of CD-31 positive capillaries in the intima was counted and data expressed as a number of capillaries/mm². Semiquantitative immunohistochemical analysis was performed by the image processing software that was programmed to select immunostained or immunofluorescent areas based on preset thresholds. The positively stained areas were then calculated as percentages of the total intimal cross-sectional area.

4.13 Statistics

Results were expressed as mean +/- SD; Statistical significance was evaluated using analysis of variance (ANOVA) with Bonferroni or Dunnett's post hoc tests was used for multiple comparisons. Mann-Whitney U test or *t*-test was used for comparison of two variables. Correlation analyses were performed by the Pearson test. $P < 0.05$ was considered statistically significant. Graphpad prism 4.0, Statistica or SPSS software were used to perform statistical analysis.

Table 4. Oligonucleotide primers used for RT-PCR

Primer Sequence (5', 3')	Annealing Temperature	Cycles
VEGF-A		
5'-TCGATCCATGAACTTTCTGC-3'	(outer)	56°C/45s 34
5'-TTCGTTTAACTCAAGCTGCC-3'		
5'-GAGCCTTGCCTTGCTGCTC	(Inner)	32
5'-GGAACATTTACACGTCTGCG-3'		
VEGF-B		
5'-TCCACAGGTGAGGGTCCAG-3'	53°C	40s 36
5'-GGCTTCACAGCACTGCCTT-3'		
VEGF-C		
5'-CTGCTTACTGGCTTATCG-3'	(outer)	51°C/40s 34
5'-CCTGTTCTCTGTTATGTTGC-3'		
5'-TCTCCAAAAAGCTACACCG-3'	(inner)	39
5'-CAAGTGCATGGTGAAGG-3'		
VEGF-D		
5'-GTGCATTGGAAGCTTGGTAC-3'	55°C	45s 39
5'-GTTCTCCAAACTAGAAGCA-3'		
VEGF-ΔNΔC		
5'-TTGCCAGCTCTACCACCAG-3'	56°C	40s 39
5'-TTCATTGCAACAGCCACCAC-3'		
VEGF-CΔNΔC		
5'-CGGGAGGTGTGTATAGATGTGGGG-3'	58°C	40s 30
5'-ATAATGGAATGAACTTGTCTGTAAAC-3'		
VEGFR-1		
5'-TACAAGAGGCTTAGTGCAAG - 3'	50°C	40s 38
5'-AAGAGATTAAGGTGGCATGC - 3'		

Table- 5. Oligonucleotide primers used for real-time RT-PCR

Primer	Sequence
VEGF-sense	5'-CATGCGGATCAAACCTCA-3'
VEGF121-antisense	5'-CTCGGCTTGTCACATTTTTTC-3'
VEGF165-antisense	5'-CAAGGCCACAGGGATTTTC-3'
18S-sense	5'-CGCCGCTAGAGGTGAAATTC-3'
18S-antisense	5'-CTTGGCAAATGCTTTGCTC-3'
VEGFR-2 Sense	5'- ATGTACCAGACCATGCTTGA - 3'
VEGFR-2 Anti sense	5'- AGTCTCTGACATCGGAAGAA - 3'

5. RESULTS

5.1. Periadventitial Angiogenesis (I, II)

5.1.1 Angiogenic responses to VEGFs

Gene transfer of different VEGF family members in periadventitial space resulted in varying degree of angiogenesis. *VEGF-A* gene transfer induced formation of large dilated and tortuous capillaries in the periadventitial tissue. *VEGF-B* and *VEGF-C* gene transfer produced only a limited number of new capillaries that were small and poorly perfused. *VEGF-D* and *VEGF-DΔNΔC* showed a similar potential for capillary formation. *VEGF-CΔNΔC* resulted in the formation of capillaries of smaller size as compared to *VEGF-A*, -D and *VEGF-DΔNΔC*. Large numbers of neovessels were seen in the adventitia of PIGF-2 transduced arteries. Some large vascular channels with branching were also observed. In *LacZ* transduced arteries only a few vasa vasorum were seen (Fig. 5A). *VEGF-A*, -D, -DΔNΔC and PIGF-2 have high, -CΔNΔC has moderate and *VEGF-B* and *VEGF-C* have a mild angiogenic potential. All members of VEGF family produced a significant ($p < 0.05$) increase in the number of capillaries as compared to the *LacZ* controls (Fig 5B).

Capillaries that were formed in *VEGF-A*, -D, *VEGF-DΔNΔC* and PIGF-2 transduced tissue were well perfused as indicated by lectin angiography. Transmission electron microscopy also showed presence of RBCs inside these capillaries. By CD-31 and -SMA double immunostainings we were able to identify the presence of pericytes in the newly formed channels. Enzyme histochemistry showing alkaline phosphatase enzyme activity further confirmed that these neovessels were capillaries.

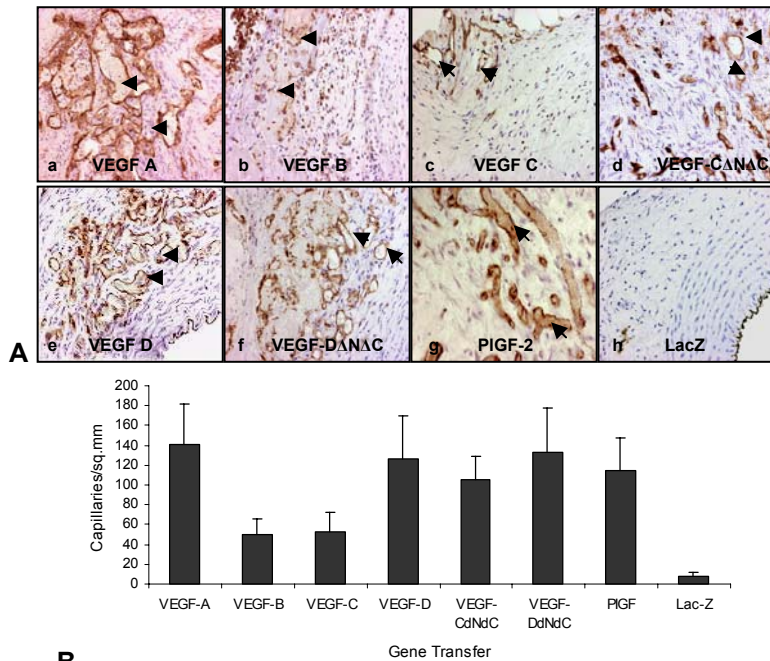


Fig.5 - (A) Angiogenesis in the adventitia of carotid arteries, after adenoviral gene transfer. (a) Vascular endothelial growth factor (VEGF)-A-, (b) VEGF-B-, (c) VEGF-C-, (d) VEGF-D-, (e) VEGF-CΔNΔC-, (f) VEGF-DΔNΔC- and (g) PIGF-2 transduced arteries. (h) Vasa vasorum seen in the adventitia of Adv lacZ-transduced artery (B) Significantly increased formation of capillaries was seen with VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-DΔNΔC, VEGF-CΔNΔC, PIGF-2 transduced arteries ($p < 0.05$)

5.1.2 Absence of lymphangiogenic response in vascular tissue

Immunostainings for desmoplakin, VEGFR-3, Laminin and CD31-SMA double immunostaining were used to detect lymphatic vessels in the adventitia of carotid artery. In addition,

5'Nucleotidase enzyme histochemistry was also used for the detection of lymphatic vessels. However, histological analysis by the above methods did not reveal any well formed lymphatic vessels in the adventitia of carotid arteries.

5.1.3 Expression of VEGF receptors

Signaling through VEGFR-2 has been primarily implicated in angiogenesis. On immunohistochemistry we found the expression of VEGFR-2 in the adventitia of VEGF-A, -C, -CΔNΔC, -D, -DΔNΔC and PlGF-2 transduced carotid arteries. VEGFR-1 was upregulated in VEGF-B and PlGF-2 transduced arteries. VEGFR-3 expression was found in VEGF-C, -CΔNΔC, and -D transduced arteries.

5.1.4 Upregulation of endogenous VEGF-A in PlGF-2 transduced arteries

Significant increases in VEGF-A121 and VEGF-A165 mRNA levels were seen in PlGF-2 transduced arteries on real-time quantitative RT-PCR (Fig. 6A, B) compared with LacZ controls. Increased VEGF-A immunostaining was seen in the adventitia of PlGF-2-transduced arteries (Fig. 6C). Some VEGF-A immunostaining was also seen in medial cells of PlGF-2- as well as LacZ-transduced arteries.

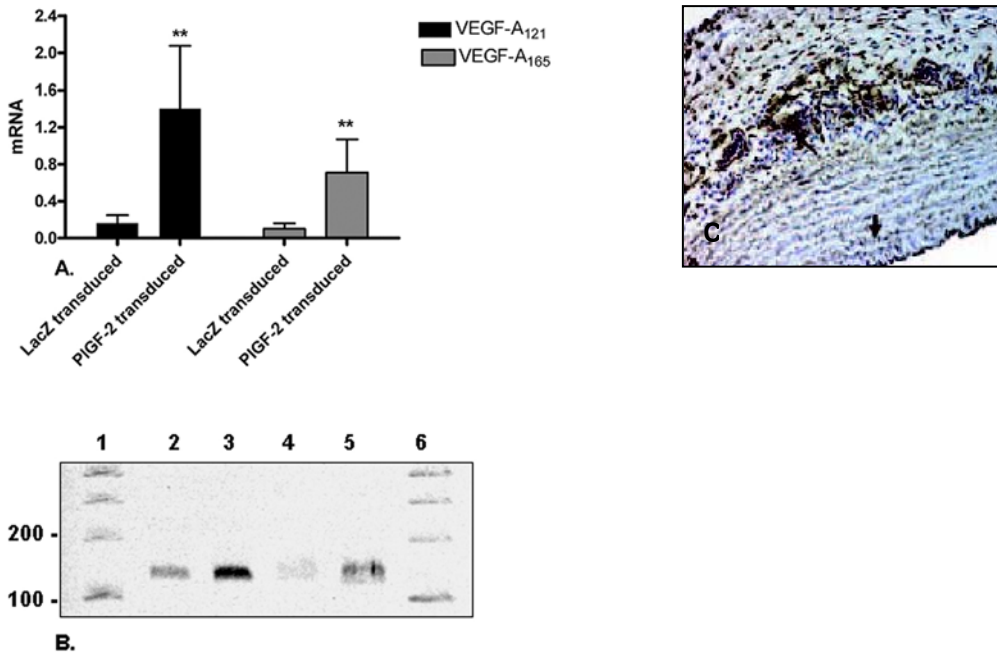


Fig.6 - RT-PCR analysis of transduced arteries. (A) Expression of mRNA levels of endogenous VEGF-A isoforms measured by quantitative RT-PCR. A significant increase in mRNA levels of VEGF-A121 and VEGF-A165 isoforms was seen in PlGF-2-transduced arteries compared with LacZ controls (** $p < 0.01$). (B) RT-PCR showing expression of VEGF-A121 and VEGF-A165 isoforms in PlGF-2- and LacZ transduced arteries. From left: (1) molecular weight marker; (2) VEGF-A121 expression in LacZ-transduced artery; (3) VEGFA121 expression in PlGF-2-transduced artery; (4) VEGF-A165 expression in LacZ control; (5) VEGF-A165 expression in PlGF-2-transduced artery; (6) molecular weight marker. (C) Immunohistochemical analysis of transduced arteries-Increased VEGF-A immunostaining was seen in PlGF-2-transduced arteries

5.1.5 sVEGFR-1 and sVEGFR-2 inhibit PlGF-2-mediated angiogenesis

In PlGF-2+sVEGFR-1-transduced vessels, only some small and poorly developed CD-31-positive neovessels were present in the adventitia. sVEGFR-1 effectively prevented the angiogenic effect of PlGF-2. In arteries co-transduced with PlGF-2+sVEGFR-2, a few small capillaries were seen in

the adventitia. The number of capillaries in the adventitia of arteries transduced with PIGF-2+sVEGFR-1 or PIGF-2+sVEGFR-2 was significantly lower than in PIGF-2 transduced arteries (Fig.7). The number of neovessels in PIGF-2+VEGFR-2-transduced arteries tended to be higher than in PIGF-2+sVEGFR-1-transduced arteries, but the difference was not statistically significant.

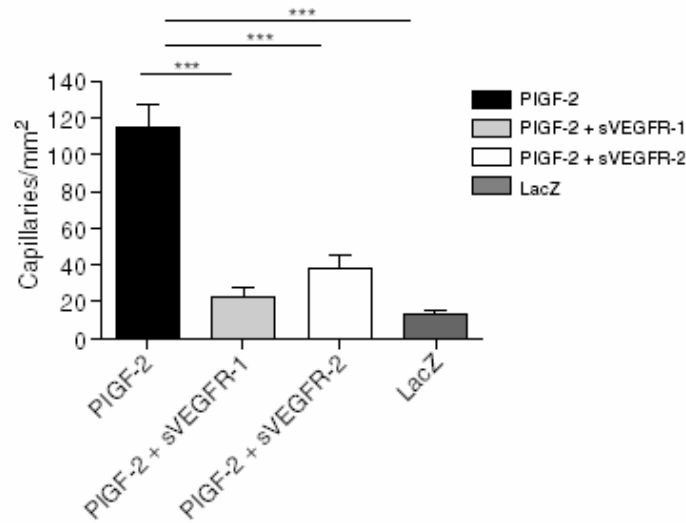


Fig.7 - Number of neovessels in the adventitia of PIGF-2-transduced arteries was significantly higher ($p < 0.001$) than in arteries transduced with PIGF-2+sVEGFR-1, PIGF-2+s VEGFR-2, or LacZ.

5.2 Inflammatory responses of VEGFs (I, II)

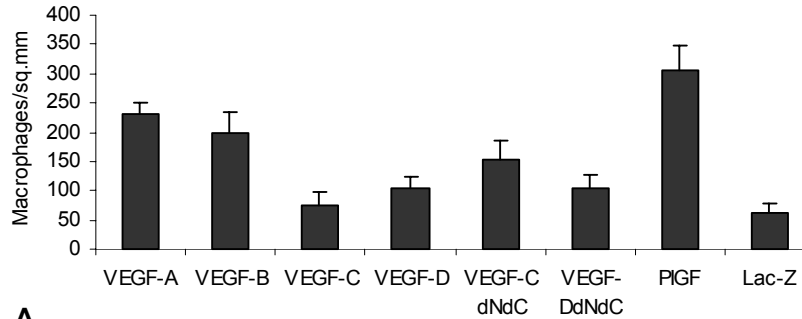
Macrophages were identified in the transduced arteries by RAM-11 immunostaining. There was a significant increase in the number of macrophages ($p < 0.01$) in the adventitia, media and intima of the VEGF-A, VEGF-B and PIGF-2 transduced arteries as compared to LacZ control (Fig.8A). VEGF-A, VEGF-B and PIGF-2 bind to VEGFR-1, which is also present on macrophages, this might be responsible for their recruitment. VEGF-C, -C Δ N Δ C,-D and -D Δ N Δ C transduced arteries showed inflammatory response comparable to LacZ controls. The macrophage infiltration in PIGF-2+sVEGFR-1-transduced arteries was significantly lower ($p < 0.001$) than in arteries transduced with PIGF-2 alone. The inflammatory response in arteries transduced with PIGF-2+sVEGFR-2 was less than that of PIGF-2-transduced arteries, but the difference was not statistically significant (Fig. 8B). No polymorphonuclear leukocytes were seen in the intima of the transduced arteries.

5.3 Neointimal hyperplasia (III)

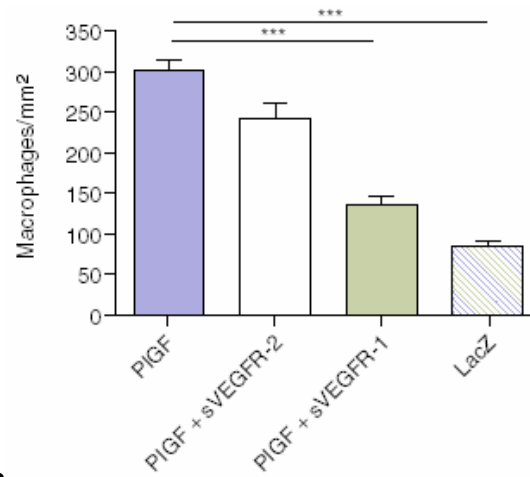
5.3.1 Neointima formation by VEGFs

Neointima formation was significantly increased ($p < 0.05$) in VEGF-A, VEGF-D and VEGF-D Δ N Δ C transduced arteries as compared with controls. Immunohistochemistry with α -SMA antibodies indicated that the majority of cells in neointima of the VEGF transduced arteries were SMC (Fig.9a, b). There was no significant difference in the intima/media ratio of VEGF-B, VEGF-C and VEGF-C Δ N Δ C transduced arteries and LacZ controls (Fig.9b). Cellular proliferation was studied by immunohistochemical detection of PCNA, an auxiliary protein of DNA delta polymerase. An increase in the number of proliferating cells was indicated by a significant increase ($p < 0.01$) in the PCNA index of intima, media and adventitia in the VEGF-A, VEG-D and VEGF-D Δ N Δ C transduced arteries as compared to LacZ controls. In VEGF-C Δ N Δ C transduced arteries a significant increase ($p < 0.05$) in PCNA index was seen only in the adventitia. Vascular

remodeling requires tightly controlled extracellular matrix degradation mediated by extracellular proteolytic enzymes including MMPs. Immunohistochemistry indicated a significant increase ($p < 0.01$) in the MMP-2 and MMP-9 staining in the VEGF-A, VEGF-CΔNΔC, VEGF-D and VEGF-DΔNΔC transduced arteries.



A



B

Fig.8 - Macrophages counted from RAM-11 immunostainings as number of immunopositive cells per square millimeter of the carotid artery sections seven days after the gene transfers. Significantly increased infiltration ($*p < 0.05$) of macrophages was observed in vascular endothelial growth factor (VEGF)-A- and VEGF-B-transduced arteries compared to LacZ controls. (B) Number of macrophages in the adventitia of PIGF-2-transduced arteries was significantly ($p < 0.001$) higher than in PIGF-2+sVEGFR- 1-transduced arteries and in LacZ-transduced arteries

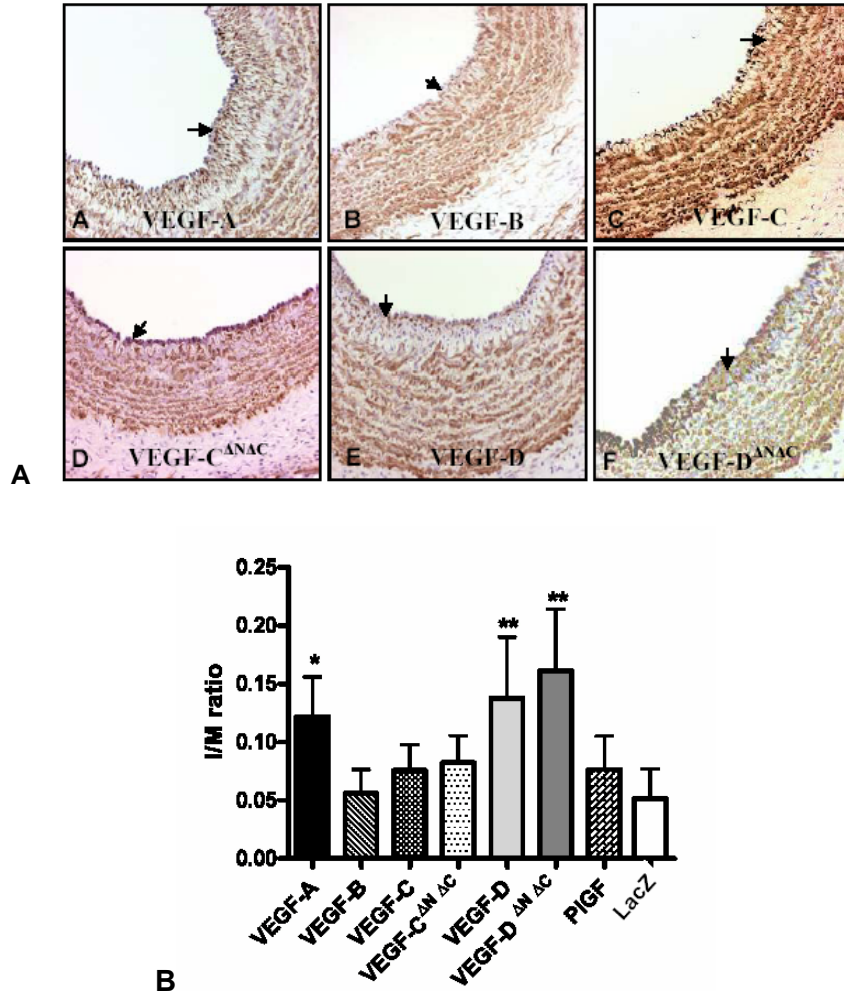


Fig. 9 - (A) α -SMA staining shows intimal hyperplasia in carotid arteries 7 days after gene transfer. (A) VEGF-A gene transfer shows a significant intimal proliferation. (B) VEGF-B, (C) VEGF-C and (D) VEGF-C Δ N Δ C show less intimal proliferation. (E) Significant intimal proliferation was observed after VEGF-D and (F) VEGF-D Δ N Δ C gene transfers. **(B)** Effect of VEGFs on intimal hyperplasia 7-days after the gene transfer. (a) A significant increase in the intimal hyperplasia was observed in the VEGF-A ($P < 0.05$), VEGF-D and VEGFD Δ N Δ C ($P < 0.001$) transduced arteries as compared with the LacZ controls.

5.3.2 Ultrastructural changes - Transmission electron microscopy (TEM)

Ten days after inserting a collar we analyzed the transduced and control carotid arteries by TEM. Endothelial cells were activated in all transduced arteries (Fig11B). These cells were hypertrophic with electron-dense cytoplasm, increased number of vesicles and abundant endoplasmic reticulum. In normal resting state the medial smooth muscle cells are of contractile phenotype, having increased contractile bodies and responsible for maintaining the tone of vasculature (Donetti *et al.*,2002). On stimulation by various stimuli they develop into synthetic phenotype. Synthetic types have increased cytoplasmic organelle, surface receptors for chemotaxis and are capable of migration and neointima formation. Both synthetic and contractile phenotypes were present in the medial layer of the transduced arteries. The number of SMC with synthetic phenotype was significantly higher ($p < 0.01$) in the VEGF-A, VEGF-D and VEGF-D Δ N Δ C transduced arteries as compared with the LacZ controls (Fig 10). Extensive neointima formation was seen in the VEGF-A, VEGF-D and VEGF-D Δ N Δ C transduced arteries. Infiltrating SMCs were seen between the internal elastic lamina and endothelial cells, forming the neointima.

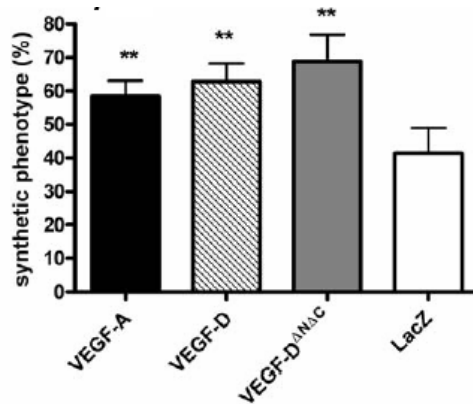


Fig.10 - Significant ($P < 0.01$) increase in the number of SMCs showing synthetic phenotype in the VEGF-A, VEGF-D and the VEGF-D Δ N Δ C transduced arteries.

5.3.3 Co-relation between VEGFs mediated adventitial angiogenesis and neointima formation

Newly formed capillaries were seen in the adventitia of the arteries transduced with VEGFs. We found significant angiogenic response produced by VEGF-A, VEGF-D and VEGF-D Δ N Δ C. Similarly; there was significant degree of neointimal hyperplasia in these vessels. On comparing all the treatment groups together we found positive correlation ($r=0.52$) between adventitial blood vessel formation and intima/media ratios in collared arteries (Fig.11). These results suggest that the induction of angiogenesis in adventitia could contribute to neointimal growth.

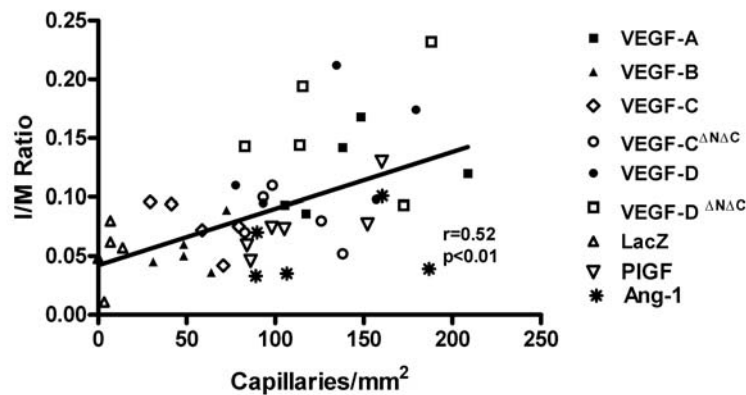


Fig.11 - A significant correlation ($P < 0.001$) was observed between adventitial angiogenesis (capillaries/mm²) and intima/media ratio in the transduced arteries.

5.4 Changes in atherosclerotic lesions of Diabetic WHHL rabbits (IV)

5.4.1 Biochemical parameters in diabetic WHHL rabbits

Mean blood glucose in the diabetic WHHL rabbits was significantly higher ($p < 0.001$) in the two months and the six months diabetic subgroups, as compared to the non-diabetic controls. OGTT showed high fasting blood glucose levels (17.96 ± 5.14 mmol/l) in the diabetic groups, with high levels of blood glucose (24.98 ± 5.16 mmol/l) persisting two hours after the initial glucose load. In the control group the fasting blood glucose levels were in the normal range (4.86 ± 0.30 mmol/l) and the blood glucose level returned back to normal (5.2 ± 0.20 mmol/l) two hours after the initial glucose load. Serum cholesterol levels in the diabetic rabbits tended to be higher at the two month and the six month time points but the differences did not reach statistical significance.

Serum triglyceride levels were significantly increased ($p < 0.001$) in the two months and the six months diabetic subgroups.

5.4.2 Intramyocellular lipids and serum free fatty acids

Two months after the induction of diabetes a significant increase ($p < 0.05$) was found in the serum FFA levels of the diabetic rabbits. Proton Magnetic resonance spectroscopy ($^1\text{H NMR}$) was used to assess intramyocellular lipids (IMCL). We observed a significant increase ($p < 0.05$) in the IMCL levels in the diabetic rabbits.

5.4.3 Enhanced atherogenesis in diabetic rabbits

Atherosclerosis was significantly increased in the two months ($p < 0.01$) and the six months ($p < 0.001$) diabetic subgroups. There was a significant ($p < 0.001$) increase in the immunostained area positive for macrophages in the diabetic rabbits at six months. Macrophages and foam cells were present in the atherosclerotic lesions of both the diabetic and the control rabbits. Macrophage infiltration was most prominent in the subendothelial region (Fig. 12 a-d) of the plaques. Large numbers of smooth muscle cells were seen in the atherosclerotic lesions in both groups (Fig. 12 e-h).

A strong RAGE and NF κ B p65 immunoreactivity was seen in atherosclerotic plaques of the diabetic animals. There was a significant increase in RAGE and NF κ B p65 staining in both the two months and the six months diabetic subgroups. RAGE localized predominantly in the endothelial cells, subendothelial region, macrophages and foam cells (Fig. 12 i-l). NF κ B staining was most prominent in macrophages, but positive immunostaining was also observed in some endothelial cells and smooth muscle cells (Fig. 12 m-p).

5.4.4 Angiogenic growth factors and angiogenesis in atherosclerotic lesions

Real time quantitative RT-PCR from aortas showed that VEGF-A165 mRNA expression was significantly increased in the diabetic rabbits. The VEGF-A121 mRNA expression levels were comparable in the aortas of the diabetic and the control animals.

VEGF-A immunostaining was significantly increased both in the two months and the six months diabetic subgroups. VEGF-A immunostaining was observed in some lesion macrophages and smooth muscle cells. VEGF-A staining was also observed in the subendothelial region (Fig. 13 a-d).

VEGF-D immunostaining tended to be increased in the two months diabetic subgroup but the difference with the controls was not significant. On the other hand, in the six months diabetic subgroup a significant increase was found. VEGF-D was detected in macrophages, smooth muscle cells and endothelial cells. Positive VEGF-D staining was also seen in the adjacent extracellular area (Fig. 13 e-h).

RT-PCR and immunohistochemistry confirmed VEGFR-1 expressions in aortic samples. VEGFR-1 immunostainings were detected in the endothelial cells, macrophages and SMCs (Fig. 11i-l). VEGFR-1 immunostainings were significantly increased in the atherosclerotic lesions of the six months diabetic rabbits. A significant increase in the VEGFR-2 m-RNA expression was seen the aortas of the diabetic rabbits. VEGFR-2 immunostaining was detected in smooth muscle cells and also in some endothelial cells (Fig. 13 m-p). VEGFR-2 staining in the atherosclerotic plaques of the two months diabetic subgroup was not significantly different whereas in the six months diabetic rabbits a significant increase was found.

Few small CD-31 positive blood vessels were present in the subendothelial area. The number of vessels in the atherosclerotic lesions of the diabetic rabbits tended to be higher but the difference with the controls was not significant. Some medium-sized CD-31 positive blood vessels were also seen in the subendothelial area in the six months diabetic subgroup (Fig. 13u). No evidence of plaque rupture or thrombosis was seen in the lesions.

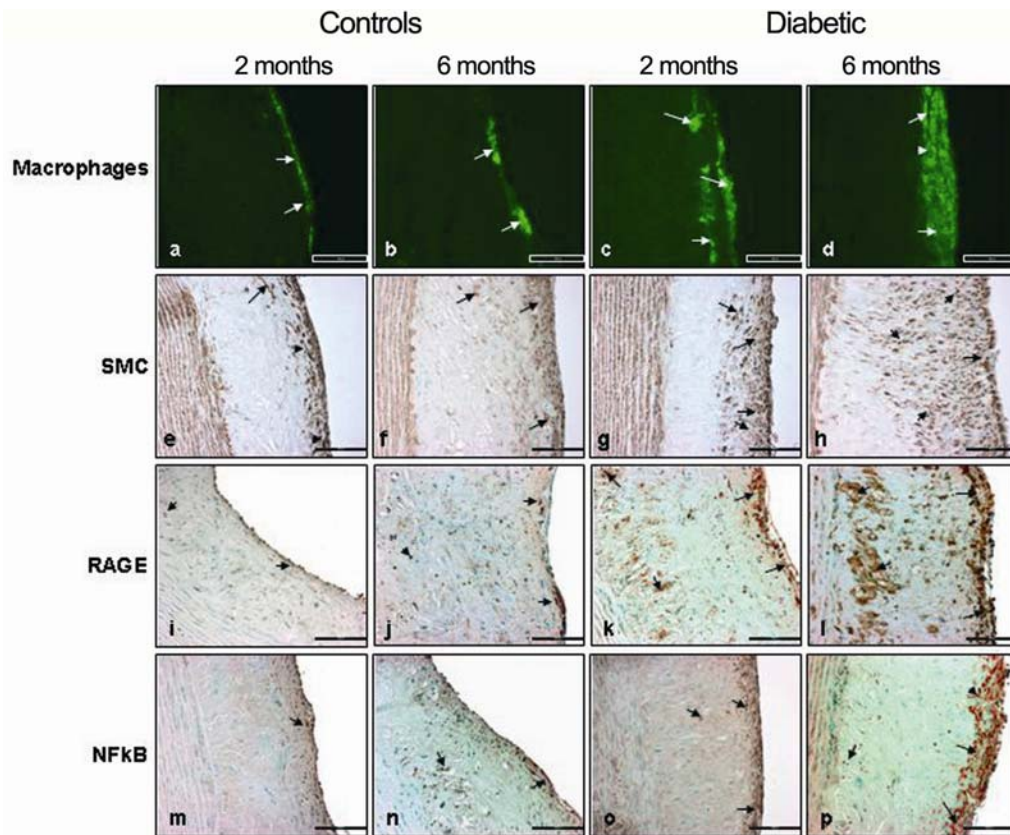


Fig.12 - Representative examples of atherosclerotic lesions in diabetic and control rabbits: (a-d) Macrophages (RAM-11 immunostaining). (e-h) HHF-35 (SMC) immunostaining (i-l) RAGE Immunostaining. (m-p) In NFkB p65 immunostaining. Arrows indicate a positive signal

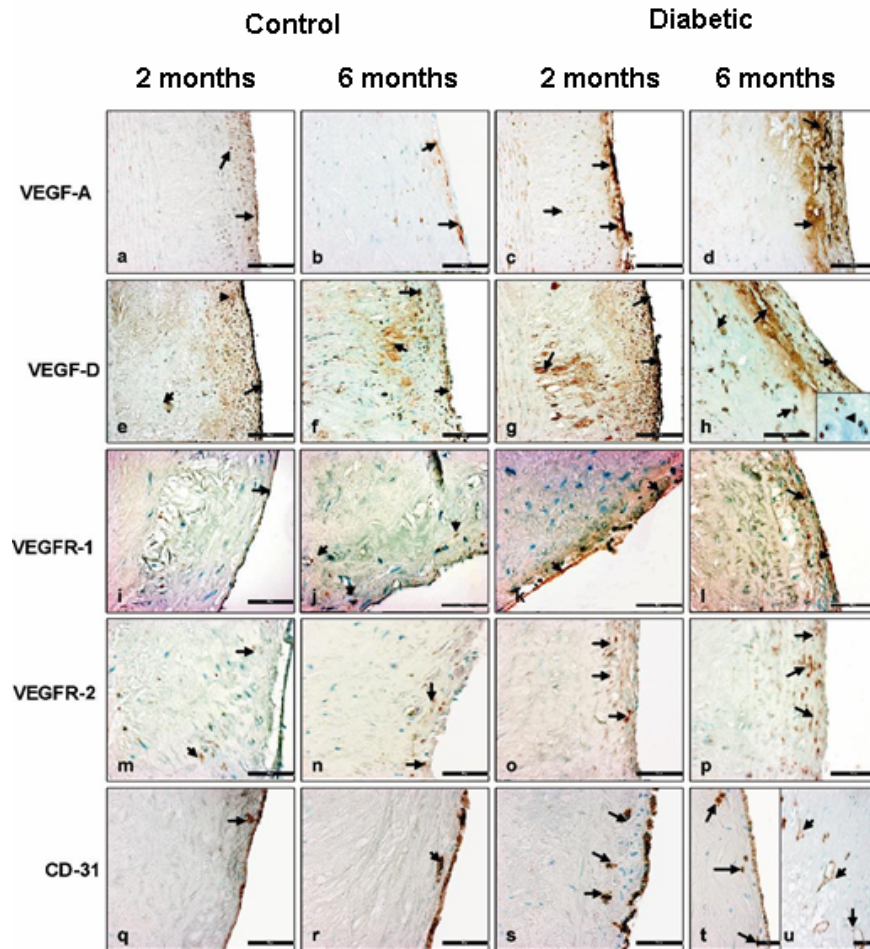


Fig. 13 - (a-d) VEGF-A immunostaining in atherosclerotic lesions. (e-h) VEGF-D immunostaining in atherosclerotic lesions. (i-l) VEGFR-1 staining in atherosclerotic lesions. (m-p). VEGFR-2 staining in atherosclerotic lesions. (q-u) CD-31 staining for endothelium was used as a marker for blood vessels in the atherosclerotic plaques

6. DISCUSSION

6.1 Angiogenic potential of VEGFs (I, II)

We found that VEGF-A, VEGF-D, VEGF-D Δ N Δ C and PIGF-2 are more angiogenic than other members of the VEGF family. VEGF-C Δ N Δ C also induced significant angiogenesis. VEGF-B, which is strongly expressed in cardiac tissue and is known to be a weak mitogen (Olofsson *et al.*, 1996^a) induced only a few small capillary structures in the vascular tissue along with clumps of CD31 positive endothelial cells. Most of the neovessels formed after the gene transfer had α -SMA-positive pericytes and a basement membrane. Electron microscopy showed well-differentiated capillaries with red blood cells in their lumen. Enzyme histochemistry was positive for alkaline phosphatase in these capillaries. Positive VEGFR-2 expression was seen in VEGF-A, -C, -C Δ N Δ C, -D, -D Δ N Δ C and PIGF-2, transduced arteries, which supports the role of VEGFR-2 signaling pathway in angiogenesis. The VEGFR-1 expression was seen in PIGF-2 and VEGF-B transduced arteries, suggesting a role of VEGFR-1 in angiogenic response seen with these two growth factors. According to previous studies proteolytically processed forms VEGF-C Δ N Δ C and VEGF-D Δ N Δ C are biologically more active than their respective long forms with intact N and C terminals (Joukov *et al.*, 1997; Stacker *et al.*, 1999). In agreement with these findings we found enhanced angiogenic responses with the proteolytically cleaved VEGF-C Δ N Δ C. However, angiogenic responses of VEGF-D and VEGF-D Δ N Δ C were relatively similar. Presumably there is efficient proteolytic processing of VEGF-D to VEGF-D Δ N Δ C in the periadventitial tissues resulting in similar angiogenic responses. The perfusion of the newly formed capillaries indicated that these vessels were functionally mature and capable of delivering oxygen and nutrients to the vascular tissue.

6.2 VEGFR-1 as a mediator of inflammatory response (I, II)

VEGF-A, -B and PIGF-2 produced significant macrophage infiltration. Binding of VEGF-A, -B and PIGF-2 to VEGFR-1 presumably results in the recruitment of macrophages by the activation of the VEGFR-1 signaling pathway. Expression of VEGFR-1 in macrophages has been reported previously (Sawano *et al.*, 2001). VEGF-A stimulates monocyte migration (Heil *et al.*, 2000) and VEGFR-1 deficient mice show defects in VEGF-A dependent migration of peritoneal macrophages, indicating that the VEGFR-1 activity is important for the VEGF-A induced migration of macrophages. In the present study, macrophage infiltration was significantly reduced in PIGF-2+sVEGFR-1-transduced arteries. This is in line with previous observations, in which anti-Flt1 inhibited recruitment of macrophages into ischemic limbs and atherosclerotic plaques (Carmeliet *et al.*, 2001^b; Lutun *et al.*, 2002).

6.3 Endogenous VEGF-A, VEGFR-1 and VEGFR-2 in PIGF-2-mediated angiogenesis (II)

Significant increases in VEGF-A121 and VEGF-A165 mRNA levels and increased VEGF-A immunostaining were seen in the adventitia of PIGF-2-transduced arteries. It is likely that the increased infiltration of macrophages contributed to the VEGF-A upregulation in PIGF-2-transduced arteries. Soluble decoy VEGFR-1 and VEGFR-2 were used to identify the signaling receptors associated with PIGF-2 mediated angiogenesis. sVEGFR-1 can bind both PIGF and endogenous VEGF-A. The suppression of PIGF-2-mediated angiogenesis by sVEGFR-1 in this model suggests that by binding to both PIGF-2 and endogenous VEGF-A, sVEGFR-1 effectively blocked the mechanism through which PIGF promotes angiogenesis. Antibody-based inhibition of VEGFR-1 has also been successful in reducing pathological angiogenesis (Lutun *et al.*, 2002). sVEGFR-2 coexpression with PIGF-2 also produced a significant suppression of PIGF-2-induced angiogenesis, indicating that endogenous VEGF-A contributes to the PIGF-2 effects. sVEGFR-2 binds only to VEGF-A, and overexpression of sVEGFR-2 presumably reduced effects mediated

via endogenous VEGF-A and possibly also via PIGF/VEGF-A heterodimers. sVEGFR-1 and sVEGFR-2 can significantly reduce PIGF-2-mediated angiogenesis and can be potentially used to block PIGF and VEGF-A overproduction in pathological conditions.

6.4 Intimal hyperplasia and VEGFs (III)

Multiple factors including mechanical injury, inflammatory cells, adventitial angiogenesis, SMC proliferation and migration and matrix alteration appear to influence the neointima formation. Placement of a collar around carotid artery causes intimal thickening and the present study shows that the adventitial delivery of VEGFs using collar model, increased the activation and proliferation of SMCs. Overexpression of the VEGFA, VEGF-D and VEGF-D Δ N Δ C in the adventitia through gene transfer produced significant increases in the proliferation and migration of SMCs. We found significant increase in PCNA labeling index in the intimal, medial and adventitial layer of these arteries. TEM revealed that there was a significant increase in the number of activated SMCs with synthetic phenotype in these arteries. Some members of the VEGF family decrease neointima formation by promoting endothelial regeneration when given through intraluminal route after balloon denudation (Hiltunen *et al.*, 2000). The disparity in the results from these two different models suggests that VEGFs have diverse roles in different compartments of the vascular tissue and in damaged and intact arteries. The vascular compartment where the VEGFs are expressed and their concentrations could be crucial in determining the net effects *in vivo*.

MMPs produced by SMCs degrade extracellular matrix and facilitate migration of these cells. We found that the MMP-2 and the MMP-9 immunostainings were significantly increased in the neointima of the VEGF-A, VEGF-D and VEGF-D Δ N Δ C transduced arteries. Our results corroborate the findings of an earlier *in vitro* study showing that VEGF increases SMC migration by upregulating matrix metalloproteinases (Wang *et al.*, 1998). SMC can alter the surrounding matrix when stimulated by appropriate growth factors and cytokines and this helps in their migration into the neointima (Dollery *et al.*, 1995). The increased MMP-2 and MMP-9 presence in the VEGF-A, VEGF-D and VEGF-D Δ N Δ C transduced arteries presumably contributes to the development of neointima.

Vasa vasorum present in the adventitia are responsible for maintaining nutrient and oxygen supply to the outer surface of arteries. There is some evidence that dysfunction of vasa vasorum plays a role in the development of arterial aneurysms, hypertension, atherosclerosis, deep vein thrombosis, and restenosis (Inci and Spetzler, 2000, Pels *et al.*, 1997). Increased adventitial angiogenesis also contributes to neointima formation (Khurana *et al.*, 2004, Scott and Wilcox, 1998). The present study reveals a correlation between adventitial angiogenesis and intimal hyperplasia. At least two mechanisms can explain the close association between adventitial neovascularization and intimal thickening in response to angiogenic stimuli. The local supply of nutrients and oxygen to the inner layers of the artery may become rate limiting after the initial phase and the growth of adventitial new vessels may create a microenvironment permissive of vascular remodeling (Stupack *et al.*, 2002). Also, local production of growth and chemotactic factors, such as VEGF, FGFs and PDGF-BB directly stimulate VSMC accumulation (Carmeliet *et al.*, 2003, Cao *et al.*, 2003). Recent studies have shown that migration of myofibroblasts that are either resident within the adventitia or recruited as progenitors from the circulation, contribute significantly to neointimal VSMC accumulation (Sartore *et al.*, 2001, Wilcox *et al.*, 2001). A careful assessment of the role of VEGFs in neointima formation is needed before using them for therapeutic angiogenesis.

6.5 Enhanced atherogenesis in diabetes (IV)

In diabetes, excess FFAs, insulin resistance and hyperglycemia generate adverse metabolic events in endothelial cells that result in endothelial dysfunction, augmented vasoconstriction, inflammation and thrombosis (Beckman *et al.*, 2002). Elevated triglyceride and total cholesterol levels are also known to increase the risk of cardiovascular diseases in diabetes (Niemeijer-

Kanters et al., 2001). The results of the current study demonstrate that diabetes augments inflammatory reactions in atherosclerotic lesions. Diabetes in WHHL rabbits accelerated atherogenesis. Macrophages were more prevalent together with an increase in NFkB expression in the atherosclerotic lesions of the diabetic rabbits. Increased expression of RAGE - a multi-ligand member of the immunoglobulin superfamily of the cell surface molecules - was seen in this study. RAGE and NFkB overexpression in atherosclerotic lesions is associated with enhanced inflammatory reactions in the vessel wall (Cipollone et al., 2003). It has also been hypothesized that the ligand-RAGE axis amplifies vascular stress and accelerates atherosclerosis (Naka et al., 2004).

6.6 VEGFs and angiogenesis in atherosclerotic lesions in diabetes (IV)

The early presence of angiogenic factors in atheromas that lack neovascularization is analogous to dormant tumors that have abundant angiogenic factors before the onset of tumor angiogenesis and angiogenesis in atherogenesis is reminiscent of angiogenesis in carcinogenesis (Moulton, 2006, Herrmann et al., 2006). It has been reported that VEGF-A enhances progression of atherosclerotic plaques (Celletti et al., 2001). In the present study, VEGF-A₁₆₅ and VEGFR-2 expressions were significantly increased in the aortas of diabetic rabbits. VEGF-A, VEGF-D, VEGFR-1 and VEGFR-2 immunostainings were also increased in the atherosclerotic plaques of the diabetic rabbits. The presence of VEGFs in macrophages and in the macrophage-rich subendothelial region of the atherosclerotic plaques in the diabetic rabbits was observed. This indicates that macrophages, RAGE and NFkB modulate the presence of VEGFs in atherosclerotic lesions. Induction of VEGF-A and VEGF-D through a pathway involving RAGE and NFkB/AP-1 (Fig.4) (Okamoto et al., 2002) could explain the increased expression of VEGFs in the atherosclerotic lesions of the diabetic rabbits. VEGF-A is expressed predominantly in aggregating macrophages in atheroma. Endogenous VEGF-A from atherosclerotic lesions induces direct migration of monocytes in atherosclerotic lesions and that could further augment the presence of VEGFs in the plaques. This results in an increasing cycle of inflammation, VEGF production and atherogenesis. VEGF-A and VEGF-D promote smooth muscle cell proliferation/migration and VEGF-A enhances macrophage infiltration (Bhardwaj et al., 2005, Bhardwaj et al., 2003). Increased presence of VEGF-A and VEGF-D could therefore also contribute to accelerated atherogenesis by enhancing SMC and macrophage recruitment. VEGFs also enhance atherogenesis via stimulation of vasa vasorum growth that can recruit inflammatory cells (Matsumoto and Mugishima, 2006). The present study shows that there is a significant increase in the presence of VEGF-A, VEGF-D, VEGFR-1 and VEGFR-2 and a trend towards increased vascularization in diabetic atherosclerotic lesions. Thus it is likely that increased expression of VEGFs contribute to the enhanced atherogenesis seen in diabetes.

7. SUMMARY AND CONCLUSIONS

Overexpression of VEGFs in vascular compartments - for therapeutic purpose or during the course of a disease process - could produce diverse manifestations. The expression levels of VEGFs and the vascular compartment where the VEGFs are expressed could be critical in determining the net effects *in vivo*. Periadventitial gene delivery could be beneficial during prosthesis and anastomosis surgery, bypass operations and endarterectomies (Laitinen *et al.*, 1998). Perivascular gene transfer also improves collateral formation around occluded coronary arteries and peripheral arteries (Nikol *et al.*, 2002). Thus perivascular delivery of VEGFs could be used to improve vascularity in ischemic tissues. Our results show that VEGF-A, VEGF-D, VEGF-D Δ N Δ C and PlGF-2 are the best candidates for periadventitial therapeutic angiogenesis. However, VEGF-D appears to have an added advantage in causing less inflammatory reactions than VEGF-A and PlGF-2. Conversely significant intimal hyperplasia that occurs with VEGF-A, VEGF-D and VEGF-D Δ N Δ C adventitial gene transfer is a major detrimental factor. Multiple factors including mechanical injury, inflammatory cells, adventitial angiogenesis, SMC proliferation and migration, and matrix alterations appeared to influence the neointima formation in the collar model. Adenovirus mediated adventitial gene transfer leading to high concentrations of VEGF-A, VEGF-D and VEGF-D Δ N Δ C in the periadventitial space produced a significant activation of SMCs and up-regulates MMPs, thereby increasing the neointima formation in the collar model. It is likely that proliferation of vasa vasorum in the adventitia of the arteries, after the gene transfer of VEGFs also contributes to the neointima formation.

Diabetes enhances the atherogenesis by increasing the inflammatory reaction in the atherosclerotic lesions. There was an increased expression of VEGF-A, VEGF-D, VEGFR-1 and VEGFR-2 in atherosclerotic lesions of diabetic animals. Increased presence of VEGF-A, VEGF-D, VEGFR-1 and VEGFR-2 in diabetic atherosclerotic lesions is therefore enhances the risk of plaque angiogenesis. Induction of VEGF-A and VEGF-D in diabetic atherosclerotic lesions presumably occurs through a pathway involving RAGE and NF κ B/AP-1.

PlGF-2 gene transfer results in upregulation of endogenous VEGF-A, and soluble VEGF receptors - sVEGFR-1 and sVEGFR-2 can block the angiogenic responses of PlGF-2. This observation support the concept that at least some of the angiogenic responses of PlGF-2 are mediated through endogenous VEGF-A. sVEGFR-1 and sVEGFR-2 are therefore appropriate candidates for blocking angiogenic responses of some of the VEGFs.

Strategies inhibiting VEGF expression in atherosclerotic plaques could be beneficial in protecting against the complications like plaque angiogenesis and rupture. Anti-VEGF strategies like receptor tyrosine kinase inhibitors, anti-VEGF monoclonal antibodies and soluble VEGF receptors could be useful in reducing plaque angiogenesis. Identification of at risk groups; site specific delivery of agents and adverse effects due to prolong VEGF suppression are some of the issues that should be critically examined before using anti-angiogenic strategies to treat plaque angiogenesis. Advances in the understanding of molecular mechanism of plaque angiogenesis will likely lead to development of more selective anti-angiogenesis regimes.

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