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JUHA RUTANEN

Vascular Endothelial Growth Factors in Atherosclerosis and Gene Therapy for Restenosis and Myocardial Ischemia

Doctoral dissertation

To be presented by permission on the Faculty of Medicine of the University of Kuopio for public examination in Auditorium, Mediteknia building, University of Kuopio, on Saturday 15th January 2005, at 1 p.m.

Department of Biotechnology and Molecular Medicine
A. I. Virtanen Institute for Molecular Sciences
University of Kuopio

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Research Director Jarmo Wahlfors, Ph.D.
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute

Author's address:
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute
University of Kuopio
P.O. Box 1627
FIN-70211 KUOPIO
FINLAND
E-mail: Juha.Rutanen@uku.fi

Supervisors:
Professor Seppo Ylä-Herttuala, M.D., Ph.D.
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute
University of Kuopio

Docent Juha Hartikainen, M.D., Ph.D.
Department of Medicine
Kuopio University Hospital

Reviewers:
Docent Juha-Pekka Salenius, M.D., Ph.D.
Division of Vascular Surgery
Tampere University Hospital
Finland

Anne Saaristo, M.D., Ph.D.
Molecular and Cancer Biology laboratory, Biomedicum,
University of Helsinki and Päijät-Häme Central Hospital
Lahti, Finland

Opponent:
Professor Markku S. Nieminen, M.D., Ph.D.
Department of Medicine, Division of Cardiology
University Central Hospital
Helsinki, Finland

ISBN 951-781-384-8
ISBN 951-27-0089-1 (PDF)
ISSN 1458-7335

Kopijyvä
Kuopio 2005
Finland

Rutanen, Juha. Vascular Endothelial Growth Factors in Atherosclerosis and Gene Therapy for Restenosis and Myocardial Ischemia. Kuopio University Publications G. - A.I. Virtanen Institute for Molecular Sciences 25. 2005. 81 p.
ISBN 951-781-384-8
ISBN 951-27-0089-1 (PDF)
ISSN 1458-7335

ABSTRACT

Atherosclerosis and its clinical complications, coronary artery disease and peripheral artery disease, are the major cause of morbidity and mortality in the developed countries. Conventional revascularization strategies, such as angioplasty and bypass surgery, are effective in improving both symptoms and prognosis of patients with ischemic disease. However, all patients are not suitable for operative treatment due to their poor general condition or severity of the disease, and the effectiveness of these treatments are limited by post-angioplasty restenosis and bypass graft-failure.

Vascular endothelial growth factors (VEGFs) are a gene family that plays a crucial role in the growth and differentation of the vascular system in physiological and pathological situations. The present study investigated the expression of VEGF-D, a novel member of this gene family, in human atherogenesis using immunohistochemistry. It was found that VEGF-D is present in arteries throughout atherogenesis from early to late lesions. However, in complicated lesions rich in connective tissue, the processing of VEGF-D into its functional form (VEGF-$D^{\Delta N\Delta C}$) is disturbed and the VEGF-D protein remains strictly intracellular.

Next we tested the potential of adenoviral (Ad) VEGF-$D^{\Delta N\Delta C}$ gene transfer to reduce neointima formation in a rabbit model of restenosis. As compared to AdLacZ control, AdVEGF-$D^{\Delta N\Delta C}$ gene transfer led to a substantial reduction in macrophage influx into the vessel wall at six days and protected the vessel from neointimal hyperplasia up to two weeks after gene transfer. Due to the transient nature of adenoviral gene transfer, the therapeutic effect was no longer detectable at four weeks, as was the case in our previous experiments using AdVEGF-C gene transfer. We hypothesized that combining AdVEGF-C gene transfer with platelet derived growth factor (PDGF) inhibition using an anti PDGF-receptor drug, would enhance the therapeutic effect on neointima formation. Indeed, in a rabbit denudation model of intimal hyperplasia this combination treatment led to a sustained reduction in neointima formation which was still detectable at six weeks.

Gene therapy to induce therapeutic vascular growth and relieve the compromised blood flow in tissues is a novel approach to treat ischemic myocardium or skeletal muscle. VEGFs are promising candidates to initiate therapeutic vascular growth. Here we tested the potential of novel electromechanical injection catheter-mediated VEGF-A and VEGF-$D^{\Delta N\Delta C}$ gene transfer to induce angiogenesis in non-ischemic pig heart. Furthermore, we compared two gene transfer vectors used in clinical trials, adenovirus and naked plasmid, and evaluated the side-effects caused by excess VEGF transgene in the myocardium. It was found that catheter-mediated intramyocardial injections induced transient transmural angiogenic effects with up to 2-fold increases in myocardial perfusion six days after adenoviral VEGF-A and VEGF-$D^{\Delta N\Delta C}$ gene transfer. In contrast, naked plasmid –mediated gene transfer with the same growth factors did not induce any detectable vascular effect. Excessive expression of either VEGF after adenoviral gene transfer caused substantial tissue edema which manifested as a pericardial effusion when using the highest doses.

It is concluded that VEGF-D is constututively expressed in human arteries during atherogenesis, which suggests a maintenance role in vascular homeostasis. Adenoviral gene transfer of VEGFs is a promising tool to prevent restenosis after angioplasty and promote therapeutic vascular growth in the myocardium.

National Library of Medicine Classification: QU 107, QW 165.5.A3, QW 504.5, QZ 50, WG 300, WG 550
Medical Subject Headings: adenoviridae / genetics; angiogenesis inducing agents; arteries; arteriosclerosis; coronary restenosis / prevention and control; gene therapy; immunohistochemistry / methods; myocardial ischemia; myocardium; platelet-derived growth factor; receptors, vascular endothelial growth factor; vascular endothelial growth factor A; vascular endothelial growth factor C; vascular endothelial growth factor D; vascular endothelial growth factors

ACKNOWLEDGEMENTS

I had a privilege to work in the department of Molecular Medicine at the A.I. Virtanen Institute where this study was carried out in 1999-2004.

I am deeply grateful to Professor Seppo Ylä-Herttuala for introducing me into an interesting field of molecular medicine. His knowledge, endless enthusiasm and encouragement were the basis of my thesis.

I am grateful to Anne Saaristo and Docent Juha-Pekka Salenius who reviewed my thesis and gave me valuable criticism and suggestions which improved this work. I express my warmest thanks to my colleague and friend Rohit Khurana for revision of the language of the thesis.

Special thanks belong to our collaborators Professor Kari Alitalo, Carl-Henrik Heldin, Arne Östman, Marc Achen and Steven Stacker with their research groups. They have provided tools to complete this work and gave us new refreshing perspectives during meetings.

I owe special thanks to my good friend Tuomas Rissanen for introducing the field of gene therapy to me and for the fun moments we have shared. Without him, I would not have written this text for years. I am grateful to Olli Leppänen for his friendship and tireless work with the combination study. Thanks belong to Johanna Markkanen for her participation to this study and for the artistic view. Jani Räty is acknowledged for his computer skills. Also, every co-author and technician deserves thanks for their contribution that made this study possible.

My warmest thanks belong to the whole SYH-group at A.I.Virtanen Institute for the great atmosphere to work and do research. I am also grateful to people from Kuopio Universal Hospital for their contribution to this study. Especially Antti Kivelä, my second supervisor Juha Hartikainen, Marja Hedman and Ismo Vajanto are acknowledged for providing clinical perspective to the study and also for the non-scientific discussions we have had during these years.

I want to thank all my friends for the quality time. The special thanks go to the members of KePa for fun times and the indoor and outdoor activities. I also want to thank Otto for keeping me company during writing process and for the long walks we have shared. Warm thanks go to my mother Sisko, father Kaino and brother Tomi.

Kuopio, December 2004

Juha Rutanen

This study was supported by grants from Academy of Finland, Sigrid Juselius Foundation, Finnish Medical Foundation, Finnish Cultural Foundation of Northern Savo, Research and Science Foundation of Farmos, Emil Aaltonen Foundation, Kuopio University Hospital EVO grants and Aarne Koskelo Foundation.

ABBREVIATIONS

AAV	adeno-associated virus
Ad	adenovirus
Akt	serine-theonine kinase Akt (PKB)
ALT	alanine aminotransferase
Ang	angiopoietin
AP	alkaline phosphatase
αSMA	α-smooth muscle actin
AST	aspartate aminotransferase
BrdU	bromodeoxyuridine
CABG	coronary artery bypass grafting
CAD	coronary artery disease
CAR	coxsackie/adenovirus receptor
cDNA	complementary deoxyribonucleic acid
CK	creatinine kinase
CK-MBm	creatinine kinase-MBm
Crea	creatinine
CRP	C-reactive protein
EC	endothelial cell
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
FGF	fibroblast growth factor
GM-CSF	granulocyte macrophage-colony stimulating factor
HGF	hepatocyte growth factor
HDL	high-density lipoprotein
HIF	hypoxia-inducible factor
i.a.	intra-arterial
IEL	internal elastic lamina
i.m.	intramuscular / intramyocardial
i.v.	intravenous
*Lac*Z	β-galactosidase producing marker gene
LDH	lactate dehydrogenase

LDL	low-density lipoprotein
L-NAME	L-nitro argenine methyl ester
MAPK	mitogen-activated protein kinase
MCE	myocardial contrast echocardiography
MCP-1	monocyte cehmoattractant protein -1
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MuLV	murine leukemia virus
NF-κB	nuclear factor kappa B
NO	nitric oxide
NRP	neuropilin
OxLDL	oxidized LDL
p	naked plasmid
PAD	peripheral artery disease
PDGF	platelet derived growth factor
PDGFR	PDGF receptor
Pfa	paraformaldehyde
pfu	plaque forming units
PI3K	phosphatidylinosito-3-OH-kinase
PKC	protein kinase C
PlGF	placental growth factor
PTA	percutaneous transluminal angioplasty
PTCA	percutaneous transluminal coronary angioplasty
RT-PCR	reverse-transcriptase polymerase chain reaction
SCID	severe combined immunodeficiency
SMC	smooth muscle cell
TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
TnT	troponine T
VCAM-1	vascular cell adhesion molecule -1
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
vp	viral particles
VPF	vascular permeability factor (VEGF)

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to by their Roman numerals:

I Juha Rutanen, Pia Leppänen, Tiina Tuomisto, Tuomas T. Rissanen, Mikko O. Hiltunen, Ismo Vajanto, Mari Niemi, Tomi Häkkinen, Kari Karkola, Steven A. Stacker, Marc G. Achen, Kari Alitalo and Seppo Ylä-Herttuala. **Vascular endothelial growth factor–D expression in human atherosclerotic lesions.** *Cardiovascular Research; 59:971-979 (2003)*

II Juha Rutanen, Anna-Mari Turunen, Mari Teittinen, Tuomas T. Rissanen, Tommi Heikura, Jonna K. Koponen, Marcin Gruchala, Matias Inkala, Suvi Jauhiainen, Mikko O. Hiltunen, Mikko P. Turunen, Steven A. Stacker, Marc G. Achen and Seppo Ylä-Herttuala. **Gene transfer using the mature form of VEGF-D reduces neointimal thickening through nitric oxide-dependent mechanism.** *Submitted for publication*

III Olli Leppänen, Juha Rutanen, Mikko O. Hiltunen*, Tuomas T. Rissanen*, Mikko P. Turunen, Tobias Sjöblom, Josef Bruggen, Gudrun Bäckström, Marianne Carlsson, Elisabeth Buchdunger, David Bergqvist, Kari Alitalo, Carl-Henrik Heldin, Arne Östman and Seppo Ylä-Herttuala. **Oral imatinib mesylate (STI571/Gleevec) improves the efficacy of local intravascular vascular endothelial growth factor-C gene transfer in reducing neointimal growth in hypercholesterolemic rabbits.** *Circulation; 109:1140-1146 (2004)*

IV Juha Rutanen*, Tuomas T. Rissanen*, Johanna E. Markkanen, Marcin Gruchala, Päivi Silvennoinen, Antti Kivelä, Antti Hedman, Marja Hedman, Tommi Heikura, Maija-Riitta Orden, Steven A. Stacker, Marc G. Achen, Juha Hartikainen and Seppo Ylä-Herttuala. **Adenoviral catheter-mediated intramyocardial gene transfer using the mature form of vascular endothelial growth factor-D induces transmural angiogenesis in porcine heart.** *Circulation; 109:1029-1035 (2004)*

* Authors with equal contribution.

In addition, some unpublished data are presented.

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1. INTRODUCTION

Atherosclerosis is a progressive disease that causes morbidity and mortality in the industrialized nations. Coronary artery disease (CAD) and peripheral arterial disease (PAD) are common atherosclerosis related clinical problems which are often treated with bypass surgery or angioplasty. However, these methods are limited by bypass-graft failure and post-angioplasty restenosis ocurring shortly after the procedures. Furthermore, all patients are not suitable candidates for these conventional treatments and the outcome of these therapies may not be completely satisfactory even after a technically successful procedure.

Better understanding of the molecular pathogenesis of atherosclerosis and related diseases may lead to more specific treatment strategies. Gene therapy, i.e. delivery of a therapeutic gene into target tissue, offers a novel approach to attenuate the progression of cardiovascular disease at the molecular level. The development of viral gene delivery vectors has provided tools to achieve a long expression time with a single treatment and high local concentrations of therapeutic protein without systemic toxicity. In the future, gene transfer vectors may be targeted to certain cell types or organs and the expression of the therapeutic gene may be controlled after the transduction, which may lead to more sustained and regulated effects.

Restenosis after coronary or peripheral artery angioplasty is a clinically important complication that limits the usefulness of this intravascular therapy. Restenosis can be avoided using stents in carefully selected lesions and recently introduced drug-eluting stents have further improved the longer term outcome. However, restenosis still remains a relevant problem, and thus there has been great interest to develop a gene therapy approach to limit it.

When a main artery becomes atherosclerotic and occluded, with diminishing blood flow, the natural response is the enlargement of pre-existing arterial anastamoses to form collateral arteries to bypass the occlusion and enlargement of the capillaries to maintain sufficient tissue perfusion. This natural response in human heart or peripheral muscle is often inadequate for tissue needs and results in ischemic myocardium or peripheral muscle. Thus, a gene therapy approach has been used to take advantage of nature's own tool to restore the compromised circulation in the ischemic areas.

Vascular endothelial growth factors (VEGFs) are a gene family that regulates the growth of blood and lymphatic vessels both in physiological and pathological situations. Thus, VEGFs have been studied widely to induce therapeutic vascular growth in ischemic heart or lower limb and to prevent restenosis in arteries after angioplasty. Although promising preclinical results have been obtained and a gene therapy approach has, this far, been safe and feasible in clinical trials, the basic therapeutic mechanisms of VEGFs are not fully understood. Furthermore, there are crucial differences in the biological effects of different members of the VEGF family which have not been fully defined.

2. REVIEW OF THE LITERATURE

2.1. Cardiovascular diseases

2.1.1. Pathogenesis of atherosclerosis

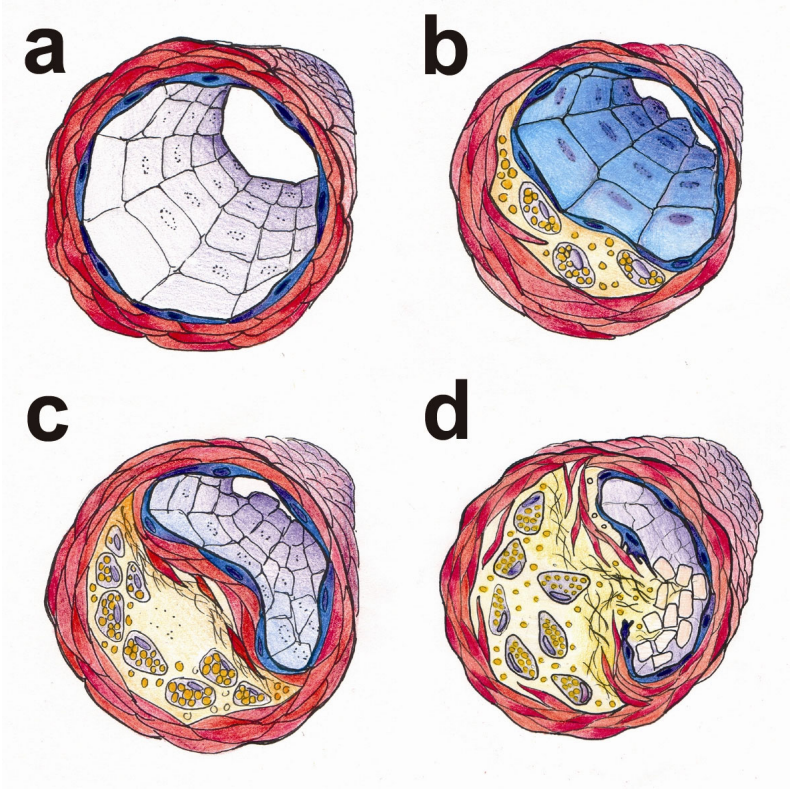
Atherosclerosis is a common cause of morbidity and mortality in western world. Clinical manifestations of atherosclerosis include insufficient blood supply to tissues, i.e. ischemia, affecting the heart, peripheral skeletal muscles and the brain (Dormandy and Rutherford, 2000; Libby, 2002; Grech, 2003). Atherosclerosis is a progressive disease that begins early in life, and is often already apparent in childhood (Ylä-Herttuala et al., 1986; Stary, 2000). The most important risk factors are well known: genetic predisposition, high serum LDL and low HDL cholesterol levels, high blood pressure, diabetes, smoking, low physical activity and male gender. The progression of atherosclerosis can be considered as three steps: *fatty streaks, intermediate lesions* and *advanced lesions* (Stary et al., 1992; Stary et al., 1994; Stary et al., 1995; Ross, 1999). The progression of atherosclerotic lesion is illustrated in Figure 1.

The current consensus on the origin of atherogenesis is that the disease appears to be initiated by the injury of the inner layer of the vessel, endothelium. This "response to injury" –hypothesis was first introduced by Ross and colleagues (Ross and Glomset, 1976; Ross, 1999) suggesting that atherosclerotic lesions are initiated in response to factors that cause injury or dysfunction to the arterial endothelium, such as shear stress, inflammation, viruses and oxidized LDL (OxLDL). Endothelial dysfunction compromises the ability of endothelial cells (ECs) to act as a selective barrier and allows lipid flow to the subendothelial space. Within the arterial wall LDL binds to proteoglycans and is modified by oxidation and glycation. Modified LDL particles cause further damage to the endothelium, attracting monocytes and T-cells to the lesion area (Steinberg, 1997).

Endothelial damage increases adhesion molecule expression which promotes blood monocytes, T-cells and platelets to adhere to the endothelium and enter the subendothelial space (Price and Loscalzo, 1999). Vascular cell adhesion molecule 1 (VCAM-1) is upregulated in endothelial cells in response to OxLDL and is considered important in the pathogenesis of early atherosclerosis (Iiyama et al., 1999; Cybulsky et al., 2001). Also, formation of chemotactic gradients that guide leukocytes to inflammatory sites are crucial to monocyte recruitment into the vessel wall. Chemokines are activators and attractants to leukocytes and their expression is induced by a number of atherogenic stimuli, such as OxLDL, vascular injury, growth factors and cytokines (Murdoch and Finn, 2000; Gerszten et al., 2000). Monocyte chemoattractant protein-1 (MCP-1) expression may be one of the initial steps of atherosclerotic lesion formation. MCP-1 is a chemokine which is expressed in macrophage-rich areas and smooth muscle cells (SMCs) in human atherosclerotic lesions and is actively involved in the recruitment of new monocytes into lesions (Nelken et al., 1991).

As monocytes enter the subendothelial space they differentiate into tissue macrophages. Macrophages accumulate modified lipids becoming foam cells and along with T-cells and small extracellular lipid deposits form fatty streak lesions (Stary et al., 1994). At this stage, the structure of the intima is still organized. As the lesion progresses the normal structure of the intima is replaced by a lipid core that contains extracellular

Figure 1. Development of atherosclerotic lesion. **a.** Normal artery with intact EC lining and organized medial layer composed of SMCs. **b.** Intermediate lesion. Medial SMCs migrate towards intima, start to proliferate and synthesize extracellular matrix. Monocytes have migrated into intima and activated. **c.** Advanced lesion. Plaque contains lipid debris with necrotic compartments and inflammatory cells **d.** Plaque rupture and thrombus formation.



lipids, cholesterol crystals and calcium. These are labelled intermediate lesions which develop into atheroma and finally fibroatheroma (Stary et al., 1994; Stary et al., 1995). Macrophages, ECs and SMCs secrete growth factors and chemokines that further attract monocytes from the circulation and SMCs from the media to the intima thus further modulating the composition of the lesion. SMCs transform from a contractile to synthetic phenotype and contribute to the growing lesion by proliferating and secreting extracellular matrix. Various growth factors participate in the process. Platelet derived growth factors (PDGFs) are a gene family that are likely to play an important role in the development of atheroma. They stimulate SMC proliferation and migration from media. PDGFs and PDGF-β receptor can be detected in atherosclerotic plaques and PDGF-α receptor expression is upregulated on in injured endothelium (Lindner and Reidy, 1995; Ross, 1993).

Eventually, the atherosclerotic lesion forms a fibrous plaque that comprises of a necrotic core filled with cholesterol esters and free cholesterol, covered by a cap of SMCs, fibrotic cells and extracellular matrix which occlude arterial lumen (Stary et al., 1995).

Morphologically these lesions can be classified as stabile or vulnerable plaques. Vulnerable plaques contain a large lipid core and a thin fibrous cap and are prone to rupture resulting in acute ischemic insults to the end-organ. In contrast, stable plaques have a relatively small lipid core and a thick fibrous cap. Shoulder regions of plaques contain macrophages and T-cells and are especially prone to rupture. Furthermore, intra-plaque angiogenesis often occurs in these regions which may make the plaque even more fragile.

2.1.2. Myocardial and lower limb ischemia

Despite significant advances in its prevention, coronary artery disease (CAD) remains the leading cause of death in industrialized nations (Grech, 2003). A significant stenosis of an epicardial coronary artery leads to an impaired myocardial performance and pain under exercise, and eventually also at rest (Grech, 2003). Atherosclerotic vessel wall can accommondate expansion of the neointima by dilating and therefore maintaining the lumen diameter (Glagov et al., 1987). When the lesion mass reaches about 40% of the area encompassed by the internal elastic lamina, adaptive mechanisms are exceeded and further increases in the lesion mass results in a decreased lumen size. When a coronary artery is narrowed by over 50% in diameter or by over 75% in cross sectional area, blood flow through the vessel is reduced so much that angina may be experienced during stress depending upon the existing collateral flow (Glagov et al., 1987; Grech, 2003; Wustmann et al., 2003).

Acute coronary events arise when endothelial injury exposes the thrombogenic core of the plaque to blood, leading to initiation of the coagulation cascade and thrombus formation. Also, a vulnerable plaque may rupture, detach and embolize, thus occluding blood flow downstream. In acute myocardial infarction, occlusion is more complete than in unstable angina, where the occlusion is usually subtotal and may resolve.

Coronary artery bypass grafting (CABG) and percutaneous transluminal coronary angioplasty (PTCA) with or without stenting are the primary interventional therapies for chronic stable angina (O'Toole and Grech, 2003). PTCA is also a prefered to supplement fibrinolysis therapy in acute coronary syndromes (Andersen et al., 2003). The long-term benefit of these treatments is limited by graft failure and post-angioplasty or in-stent restenosis occluding the target vessel. However, the use of the internal mammary artery as a conduit in CABG surgery and the recent introduction of drug eluting stents seem to have alleviated these problems (He, 1999; Moses et al., 2003). Nevertheless, all patients cannot be treated with these interventional therapies. Secondary prevention of CAD includes the use of antiplatelet drugs (acetylsalicylic acid and clopidogrel), anti-hypertensive drugs (β-blockers, diuretics and ACE inhibitors), statins and nitrates in conjunction with risk factor modification (O'Toole and Grech, 2003).

The other common atherosclerosis related clinical problem is peripheral artery disease (PAD). As with CAD, atherosclerotic arteries are incapable of providing sufficient blood flow to the lower limb, which results in skeletal muscle ischemia. First manifest is pain during walking that resolves with rest (claudication). When the situation proceeds to critical limb ischemia, long lasting pain (> 14 days) occurs at rest and may be accompanied by non-healing ischemic ulceration (Dormandy and Rutherford, 2000). Also, acute thrombus formation, plaque rupture or trauma can lead to critical limb ischemia and tissue damage.

The interventional treatments for PAD are surgical treatment either by endovascular or open vascular surgery using vein grafts or synthetic prostheses, or percutaneous transluminal angioplasty (PTA). However, as for CAD, the long-term efficacy of these approaches is limited by the graft failure and occlusion or restenosis. Furthermore, some of the patients cannot be treated by either means due to the severity and extent of the disease or co-morbid illnesses, and thus limb amputation becomes the only remaining option (Dormandy and Rutherford, 2000).

2.1.3. Restenosis and in-stent restenosis

Since first introduced in the 1970s, PTCA/PTA has become a well established technique for the treatment of myocardial and lower limb ischemia. However, as previously mentioned the usefulness of PTCA/PTA is limited by restenosis that occurs despite an apparently successful procedure and patients may need a new revascularization procedure within six months (Bauters et al., 1996; Narins et al., 1998). Restenosis is defined as shrinkage of the vessel lumenal cross-sectional area after vascular intervention. The understanding of events leading to this treatment failure has increased substantially during recent years. Restenosis is a process involving platelet activation, thrombosis, inflammation, monocyte recruitment, vasoconstriction and remodeling, proliferation and migration of neointimal SMCs, and their participation in matrix formation (Bauters et al., 1996; Schwartz, 1998). In response to arterial injury, medial SMCs migrate, proliferate and produce extracellular matrix, leading to the development of neointimal thickening, known as neointimal hyperplasia, and re-occlusion of the artery. Also the inflammatory response after angioplasty and stenting is one of the key elements that leads to neointimal hyperplasia and restenosis (Serrano, Jr. et al., 1997; Kornowski et al., 1998; Brasen et al., 2001; Farb et al., 2002). Numerous agents have been used to prevent restenosis and encouraging preclinical results have been obtained (Rutanen et al., 2002).

Restenosis rates may be decreased by using stents in selected lesions, but in-stent restenosis remains a notable clinical problem (Narins et al., 1998). In-stent restenosis is the result of thrombus formation, acute inflammation and neointimal growth (Komatsu et al., 1998; Farb et al., 1999; Brasen et al., 2001). As negative remodeling is mostly prevented mechanically by stent struts, neointimal growth is the most prominent factor in occluding the treated vessel and should be seen as the main target for the treatment. Importantly, promising clinical results in preventing in-stent restenosis have recently been obtained with sirolimus eluting stents (Sousa et al., 2001; Moses et al., 2003).

2.2. Vascular endothelial growth factors (VEGFs) and their receptors

The family of VEGFs modulate a variety of EC behavior, commencing with embryonic vascular patterning to vasculogenesis and angiogenesis within adults (Ferrara *et al.*, 2003). Five members have been identified in the human VEGF-family: VEGF (VEGF-A), -B, -C, -D and placental growth factor (PlGF) which differ in their ability to bind to three VEGF receptors (Senger et al., 1983; Leung et al., 1989; Maglione et al., 1991; Olofsson et al., 1996; Joukov et al., 1996; Yamada et al., 1997; Achen et al., 1998). Also, viral

VEGF homologues (collectively called VEGF-E) and snake venom VEGFs have been found (Ogawa *et al.*, 1998; Yamazaki *et al.*, 2003).

2.2.1. VEGF receptors

Three high-affinity tyrosine kinase VEGF signaling receptors (VEGFRs) have been isolated (de Vries et al., 1992; Pajusola et al., 1992; Millauer et al., 1993). Although ECs and endothelial progenitor cells (EPCs) are the primary targets of VEGFs (Yamaguchi *et al.*, 1993), other cell types are also known to express VEGFRs (Ferrara *et al.*, 2003). Intracellular signaling and downstream effects of VEGFR activation are illustrated in Figure 2.

VEGFR-1 stimulation by VEGF and VEGF-B results in weak mitogenic signals within ECs but VEGFR-1 appears to play a more important role in monocyte chemotaxis (Waltenberger *et al.*, 1994; Barleon *et al.*, 1996). VEGFR-1 was previously thought serve solely as a negative modulator of angiogenesis because VEGFR-1 also exists as a soluble form (a decoy receptor to "trap" excess circulating VEGF) and its intracellular signaling domain was shown to be unnecessary for normal vascular development (Waltenberger *et al.*, 1994; Hiratsuka *et al.*, 1998; Gille *et al.*, 2001). Furthermore, homozygous knockout mice for the whole VEGFR-1 gene die *in utero* between days 8.5 and 9.5 as a result of excessive angioblast proliferation and failure of organization of EC (Fong et al., 1995). VEGFR-1 activation has been shown to exert even inhibitory effects on VEGFR-2-mediated proliferation (Zeng et al., 2001). However, it was recently shown that the selective VEGFR-1 ligand, PlGF, stimulates angiogenesis, vascular permeability as well as mobilizes endothelial progenitor cells (EPCs) and hematopoietic stem cells (Luttun *et al.*, 2002; Gerber *et al.*, 2002; Hattori *et al.*, 2002). Also, recent results from our lab show that adenoviral PlGF gene transfer induces angiogenic effects in non-ischemic pig myocardium (Markkanen J *et a*l. unpublished observation). Selective activation of VEGFR-1 by PlGF results in phosphorylation of specific tyrosine residues, which causes inter-molecular transphosphorylation of VEGFR-2 (Autiero *et al.*, 2003). Thus, the angiogenic and vascular permeability effects of PlGF induced activation of VEGFR-1 may be due to indirect VEGFR-2 stimulation. VEGFR-1 signaling may also stimulate the release of additional growth factors such as hepatocyte growth factor (HGF), interleukin-6 and other hepatotrophic molecules from sinusoidal ECs in the liver (LeCouter *et al.*, 2003). Thus, it appears that signaling via VEGFR-1 is ligand-dependent; it is a negative modulator of VEGF-induced angiogenesis but in response to PlGF binding, it is capable of promoting proangiogenic effects via indirect VEGFR-2 activation.

VEGFR-2 (Flk-1/KDR) is considered to mediate most of the effects by VEGFs on blood vessel ECs such as proliferation, angiogenesis, survival, and vascular permeability as well as recruitment of bone-marrow derived endothelial progenitor cells (EPCs) (Peichev et al., 2000; Gille et al., 2001; Ferrara et al., 2003). Activation of the mitogen-activated protein kinase (MAPK) pathway via protein kinase C (PKC) increases DNA synthesis, EC migration and proliferation (Kroll and Waltenberger, 1997; Takahashi et al., 1999). The stimulation of the PI3K/Akt pathway promotes EC migration and cell survival, together with upregulation of antiapoptotic pathways (Gerber *et al.*, 1998b; Gerber *et al.*, 1998a; Morales-Ruiz *et al.*, 2000). Nitric oxide (NO) is crucial for VEGFR-2-mediated effects since nitric oxide (NO) synthase inhibition hinders angiogenesis and vascular

permeability (Papapetropoulos *et al.*, 1997; Murohara *et al.*, 1998; Rissanen *et al.*, 2003b). Since activation of VEGFR-2 by VEGF-A or VEGF-D has distinct consequences for endothelial signaling and function, it appears that the downstream effects of VEGFR-2 activation are also ligand dependent (Jia et al., 2004).

Recently, the functions of VEGFR-3 signaling have become clearer. VEGFR-3 activation alone is sufficient for the growth, migration and survival of lymphatic ECs in adults (Veikkola *et al.*, 2001; Makinen *et al.*, 2001). Also, heterozygous inactivation of VEGFR-3 causes lymphedema due to a lack of cutaneous lymphatics both in man and in a mouse model (Karkkainen et al., 2000; Karkkainen et al., 2001). In addition to the three tyrosine-kinase receptors, two co-receptors for VEGFs have been recently identified. NRP-1 and NRP-2, originally found to play a role in neuronal guidance, are required for normal embryonic blood and lymphatic vessel development, respectively (Neufeld *et al.*, 1999; Kawasaki *et al.*, 1999; Yuan *et al.*, 2002). NRPs have not been shown to signal after binding VEGFs but they seem to amplify VEGFR-mediated signal transduction (Whitaker *et al.*, 2001; Oh *et al.*, 2002).

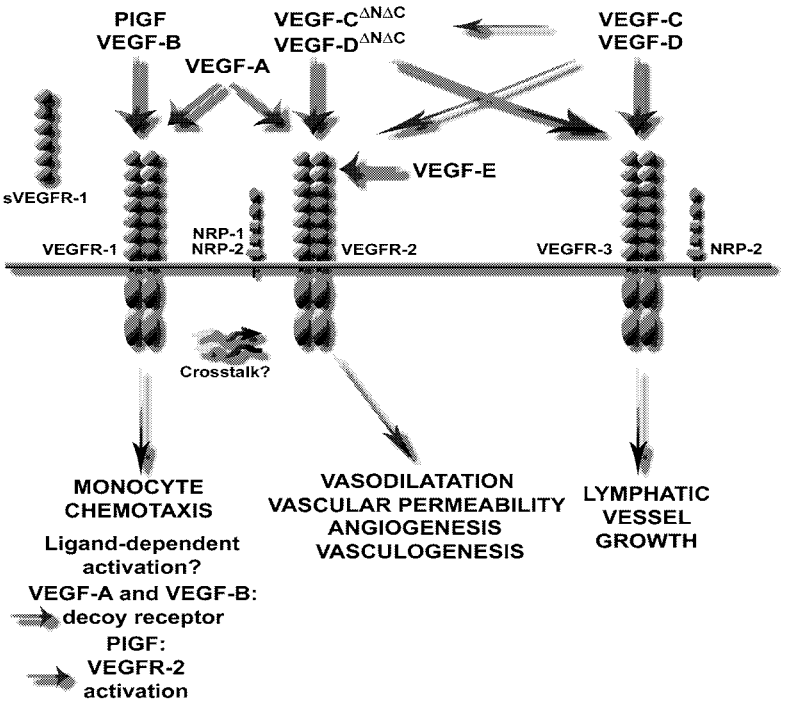


Figure 2. Ligands and receptors of the VEGF gene family. The biological role of VEGFR-1 is currently unclear but it acts as a negative modulator of angiogenesis and exists also as a soluble form. However, there may be crosstalk between VEGFR-1 and VEGFR-2 since also PlGF can promote angiogenesis. VEGFR-2 and VEGFR-3 are the main signalling receptors on ECs of blood and lymphatic vessels, respectively. Modified from: Rissanen TT, Gene Transfer for Blood and Lymphatic Vessel Growth, doctoral thesis, 2004.

2.2.2. VEGF-A

The first member of the VEGF family, VEGF-A, was cloned in 1989 (Leung *et al.*, 1989; Keck *et al.*, 1989; Plouet *et al.*, 1989). However, already in 1983 Senger, Dvorak and colleagues identified a tumor-derived protein, vascular permeability factor (VPF) that was capable of promoting ascitic fluid accumulation (Senger *et al.*, 1983). The cDNA sequences subsequently revealed that VPF and VEGF were the same molecule (Keck *et al.*, 1989).

Alternative mRNA splicing was initially shown to result in four different isoforms consisting of 121, 165, 189 or 206 amino acid residues (VEGF-A_{121} through VEGF-A_{206}) (Tischer *et al.*, 1991; Houck *et al.*, 1991). The corresponding mouse and rat isoforms have one amino acid less than those of humans. Less common splice variants VEGF-A_{138}, VEGF-A_{145} and VEGF-A_{162} have also been reported (Poltorak *et al.*, 1997; Lange *et al.*, 2003). VEGF-A_{121} is acidic and does not bind to heparin or heparan sulfates making it freely soluble in tissues, whereas VEGF-A_{189} and VEGF-A_{206} are highly basic conferring a high affinity towards extracellular matrix (Houck *et al.*, 1992; Rissanen *et al.*, 2003a). VEGF-A_{165} has intermediate properties, because it is secreted but also a significant fraction binds to cell surfaces and extracellular matrix (Rissanen *et al.*, 2003a; Rissanen *et al.*, 2003b). Plasmin cleaves the longer extracellular matrix -bound forms at the C-terminus generating a bioactive and soluble fragment of 110 aminoacids, but which has reduced mitogenic activity (Houck *et al.*, 1992; Keyt *et al.*, 1996).

Actions of VEGF-A, including vasodilatation, vascular permeability and angiogenesis are mediated by VEGFR-2 and subsequent NO production via eNOS (Laitinen et al., 1997b; Matsunaga et al., 2000; Fukumura et al., 2001). VEGF-A promotes angiogenesis in a dose-dependent manner and thus mouse embryos lacking even a single allele show growth retardation and die between embryonic day 11 and 12 (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Binding of VEGF to extracellular matrix is critical for its actions. For example, the extracellular matrix -bound forms of VEGF-A (VEGF-A_{165}, VEGF-A_{189} and VEGF-A_{206}) are essential for vascular branching as mice expressing only VEGF-A_{120} die within two weeks after delivery because of impaired EC organization (Carmeliet *et al.*, 1999). With respect to therapeutic angiogenesis, VEGF-A_{165} may possess the optimal attributes, such as sufficient bioavailability and high biological efficacy (Ferrara *et al.*, 2003). VEGF-A mediated vascular permeability plays a significant role in the deposition of extravascular fibrin, ascites fluid and tissue edema in tissue repair, inflammation and cancer (Dvorak *et al.*, 1995). It has now become evident that tight control of VEGF expression is required for normal development and homeostasis of vasculature and organ function.

Novel DNA array technology is emerging as a powerful tool to gain more information about the endogenous angiogenic response to tissue ischemia. An interesting finding from a DNA array screen covering 8400 human genes was that VEGF-A was the most prominently upregulated growth factor in patients with acute lower limb ischemia together with its major hypoxic regulators, hypoxia inducible growth factors (HIF) -1α and -2α, and its most important signaling receptor, VEGFR-2 (Tuomisto et al., 2004). The important role of VEGF-A in the natural response to skeletal muscle ischemia together with its essential role in vascular development and the robust vascular growth achieved with adenoviral VEGF-A overexpression in skeletal muscle implicate VEGF-A as the main natural

regulator of the "angiogenic switch" (Ferrara et al., 1996; Rissanen et al., 2003b; Tuomisto et al., 2004).

VEGF-A exert protective effects in arteries as it mediates anti-apoptotic effects in endothelium (Spyridopoulos et al., 1997; Zachary et al., 2000), protects against LDL toxicity (Kuzuya et al., 2001), and induces nitric oxide production (Laitinen et al., 1997b). Conversely, the capability of VEGF-A to stimulate monocyte/macrophage influx into the vessel wall (Ferrara, 1999), to increase vascular permeability (Stacker et al., 1999b) and its presence in artery wall during atherosclerotic lesion development (Couffinhal et al., 1997; Inoue et al., 1998) suggests that VEGF-A may contribute to atherogenesis. Also, systemically administered VEGF-A protein induced atherosclerotic plaque formation and neovascularization in mouse and rabbit models of athrosclerosis (Celletti et al., 2001).

2.2.3. VEGF-C and VEGF-D

Human VEGF-C and VEGF-D were cloned in 1996 and 1997, respectively (Joukov et al., 1996; Yamada et al., 1997). VEGF-C and VEGF-D form a subfamily of VEGFs because of the obvious similarities to each another. They are 48% identical at the amino acid level, they enclose N- and C- terminal extensions that are not found in other VEGF family members and both growth factors have a similar receptor binding profile (Joukov *et al.*, 1996; Yamada *et al.*, 1997; Achen *et al.*, 1998). Moreover, VEGF-C and VEGF-D are synthesized as large precursor forms which are then proteolytically processed intra- and extracellularly into mature forms (indicated as $\Delta N\Delta C$) comprising the central VEGF homology domain (Joukov et al., 1996; Achen et al., 1998; Joukov et al., 1997; Stacker et al., 1999a). It was recently found that in addition to many other biologically important proteins, plasmin also cleaves both the full length VEGF-C and VEGF-D into the short mature forms. These mature forms are biologically active and induce diffuse angiogenesis in tissues since they do not bind to extracellular matrix (Rissanen et al., 2003b; McColl et al., 2003).

The unprocessed forms of VEGF-C and VEGF-D signal mainly through VEGFR-3 whilst the mature forms trigger VEGFR-2 signaling efficiently (Joukov et al., 1997; Stacker et al., 1999a). The proteolytically processed mature form of VEGF-D has approximately a 290- and 40-fold greater affinity towards VEGFR-2 and VEGFR-3, respectively, compared to the full length VEGF-D (Stacker et al., 1999a). Thus, the long unprocessed forms are mainly lymphangiogenic whereas the mature short forms are primarily angiogenic and promote vascular permeability (Rissanen *et al.*, 2003b).

Both VEGF-C and VEGF-D promote tumor angiogenesis, lymphangiogenesis, metastatic spread, and their expression in cancer may have prognostic value (Yonemura et al., 1999; Skobe et al., 2001b; Stacker et al., 2001; Achen et al., 2001; White et al., 2002). During embryonic development, VEGF-C is required for normal embryonic and postnatal lymphangiogenesis (Karkkainen et al., 2001; Karkkainen et al., 2004). Thus, the VEGF-C expression pattern during embryonic development is quite similar to VEGFR-3 expression, both colocalizing in regions where lymphatic vessels spout from veins and in the lymphatic vessel-rich mesenterium (Kukk et al., 1996). In adults, VEGF-C is expressed in the lung, heart and kidney, in conjunction with prominent VEGFR-3 expression (Kukk et al., 1996). In addition to lymphangiogenic properties, adenoviral VEGF-C gene transfer has been shown to inhibit restenosis in a rabbit denudation model of intimal hyperplasia

(Hiltunen et al., 2000a) and to promote vascular angiogenic effects in a rabbit hindlimb ischemia model (Witzenbichler et al., 1998).

Little is known about the natural biological role of VEGF-D. So far, the only known mechanisms for VEGF-D regulation involve c-fos and cell-to-cell interactions in fibroblasts (Orlandini et al., 1996; Orlandini and Oliviero, 2001). During embryonic development VEGF-D is expressed in several organs, such as limb buds, heart, kidney, lung, liver and teeth (Achen et al., 1998). In adult human tissues, VEGF-D mRNA is expressed in the heart, lung, skeletal muscle, colon and small intestine (Achen et al., 1998). Furthermore, there may be inter-species variation in its natural biological role because in contrast to human VEGF-D, mouse VEGF-D does not bind to VEGFR-2 at all (Kukk *et al.*, 1996; Baldwin *et al.*, 2001).

2.2.4. Other VEGFs

VEGF-B has about a 43% amino acid sequence homology with VEGF-A and is expressed from the beginning of early development throughout adult life, particularly in the heart but also to a lesser degree in skeletal muscle, pancreas, adrenal gland and SMCs of large blood vessels (Olofsson et al., 1996; Aase et al., 1999). VEGF-B is a ligand for VEGFR-1 and NRP-1. VEGF-B-VEGF-A heterodimers can also bind VEGFR-2 (Olofsson et al., 1996). In contrast to VEGF-A, VEGF-B is not upregulated by hypoxia or serum growth factors (Enholm *et al.*, 1997). VEGF-B has been reported to be an EC mitogen *in vitro* and to regulate plasminogen activator activity within ECs (Olofsson et al., 1996). The significance of VEGF-B *in vivo* remains unclear since studies of VEGF-B knockout mice have yielded conflicting results. VEGF-B$^{-/-}$ mice appear to have smaller hearts, dysfunctional coronary arteries and demonstrate impaired recovery from myocardial ischemia whereas Aase *et al.* showed that these mice have a subtle cardiac phenotype and that VEGF-B is not essential for development of the cardiovascular system (Bellomo *et al.*, 2000; Aase *et al.*, 2001). It was recently reported that recombinant protein administration or overexpression of naked plasmid DNA, VEGF-B induced angiogenesis in the mouse skin, but adenoviral VEGF-B overexpression in normal rabbit skeletal muscle had no such effect (Rissanen *et al.*, 2003b; Silvestre *et al.*, 2003).

The viral VEGFs, collectively called VEGF-E, are encoded by different strains of the parapoxvirus Orf (Lyttle *et al.*, 1994; Ogawa *et al.*, 1998; Meyer *et al.*, 1999; Wise *et al.*, 1999). VEGF-E resembles $VEGF_{121}$ because it does not bind to heparan sulfates and is freely diffusible throughout the extracellular matrix (Ogawa *et al.*, 1998). Different forms of VEGF-E are specific VEGFR-2 ligands and are almost equally potent mitogens for ECs as VEGF-A_{165} both *in vitro* and *in vivo* (Ogawa *et al.*, 1998; Meyer *et al.*, 1999; Wise *et al.*, 1999; Wise *et al.*, 2003). Constitutive VEGF-E overexpression in the mouse skin has been reported to increase vascularization 10-fold (Kiba *et al.*, 2003).

A protein related to VEGF was identified from a placenta cDNA library and accordingly named placental growth factor (PlGF) (Maglione *et al.*, 1991). As with the other selective VEGFR-1 ligand, VEGF-B, the biology of PlGF is not yet fully understood. PlGF appears to exert little or no direct mitogenic or vascular permeability activity (Migdal *et al.*, 1998; Park *et al.*, 1994), although conflicting reports also exist (Landgren et al., 1998; Ziche et al., 1997). PlGF knockout as well as PlGF-VEGF-B double knockout mice do not display any significant vascular phenotype and are fertile (Carmeliet *et al.*, 2001). However, Carmeliet

and colleagues have suggested that impaired blood vessel growth occurs in $PlGF^{-/-}$ mice under pathological conditions such as ischemia and tumor growth (Carmeliet *et al.*, 2001). PlGF has also been reported to promote the recruitment of monocytes and hematopoietic stem cells from the bone marrow (Hattori *et al.*, 2002). The VEGF-VEGFR-2 system appears integral in mediating the effects of PlGF via a number of possible mechanisms. Firstly, excess PlGF can displace endogenous VEGF from VEGFR-1, allowing VEGF binding to VEGFR-2 resulting in angiogenic signals. Secondly, activation of VEGFR-1 by PlGF may cause intermolecular transphosphorylation of VEGFR-2 (Autiero *et al.*, 2003). Thirdly, PlGF may upregulate VEGF expression (Bottomley *et al.*, 2000). Finally, PlGF-VEGF heterodimers are also able to bind VEGFR-2 (DiSalvo *et al.*, 1995; Cao *et al.*, 1996). Although the signaling mechanisms are still ill-defined, PlGF is a promising candidate growth factor for therapeutic angiogenesis since it induces angiogenic effects in non-ischemic pig heart (Markkanen JE *et al.*, unpublished data).

2.3. Platelet derived growth factors (PDGFs) and their receptors

The family of platelet derived growth factors (PDGFs) comprises four members, PDGF-A, -B, -C and –D, that bind to PDGF receptors PDGFR-α and PDGFR-β (Collins et al., 1985; Bonthron et al., 1988; Li et al., 2000; Bergsten et al., 2001; LaRochelle et al., 2001). The ligands and the receptors form heterodimers that modify the biology of ligand-receptor interaction. PDGFs seem to be crucial for pericyte and SMC recruitment in the maturation of capillaries and bigger vessels via PDGFR-β activation since PDGF-B deficient mice die of microaneurysm formation due to lack of pericyte coverage (Leveen et al., 1994; Lindahl et al., 1997). Also, concomitant targeting of both PDGFRs and VEGFRs with tyrosine kinase inhibitors results in a more efficient inhibition of angiogenesis within a tumor model compared to the effects of either inhibitor alone (Bergers and Benjamin, 2003).

PDGFs play an important role in the pathogenesis of atherosclerosis and restenosis after arterial injury since they are major mediators of arterial SMC proliferation and migration (Libby et al., 1988; Majesky et al., 1990; Lindner and Reidy, 1995; Ueda et al., 1996). With regards of the vascular SMC proliferation and migration that occurs in intimal hyperplasia, most PDGF effects are mediated by PDGFR-β (Sirois et al., 1997; Giese et al., 1999; Davies et al., 2000). Also, PDGF-D induces macrophage recruitment through PDGFR-β during angiogenesis (Uutela et al., 2004). PDGFR inhibition has been succesfully used to prevent restenosis in preclinical in vivo models of intimal hyperplasia (Fingerle et al., 1989; Hart et al., 1999; Ferns et al., 1991; Leppanen et al., 2000).

Recombinant PDGF-B protein given in combination with VEGF results in more mature and stable blood vessels than monotherapy with either factor alone (Richardson et al., 2001). Also, administration of PDGF-B protein together with FGF-2 was shown to produce stable vessel networks in the rat cornea (Cao et al., 2003). Thus, it is evident that PDGFs are needed to develop stable vasculature with sufficient pericyte coverage.

2.4. Mechanisms of vascular growth

Vascular growth can classically be defined as the formation of blood vessels form vascular stem cells during embryonic development (vasculogenesis), sprouting of new capillaries from pre-existing ones (angiogenesis) and collateral artery growth (arteriogenesis) to circumvent the occluded main artery.

2.4.1. Vasculogenesis

Blood vasculature is formed in the beginning of the third embryonic week by a process called vasculogenesis (Risau and Flamme, 1995). The mesoderm-derived stem cells, hemangioblasts, give rise to both vascular and hematopoietic cell lineages (Risau and Flamme, 1995). From these cells angioblasts arise, which further develop into components of the vessel wall, ECs and mural cells such as pericytes and SMCs. ECs and SMCs proliferate and differentiate further to form a vascular plexus, which grows by angiogenic sprouting and remodeling (Carmeliet and Collen, 1999). Finally, the functional vascular network is established by organization of the arterial, venous and capillary circulation (Risau and Flamme, 1995). Recently, vascular stem cells such as EPCs have been found to contribute to vascular growth in adults, but the quantitative significance has not been ascertained (Asahara *et al.*, 1997).

2.4.2. Angiogenesis

Angiogenesis is traditionally defined as the sprouting of new capillaries from preexisting ones (Risau, 1997). However, recently capillary enlargement was also found to occur after VEGF overexpression (Pettersson et al., 2000; Rissanen et al., 2003b). Angiogenesis is a crucial element of various physiological and pathological events, such as wound healing, skeletal growth, hair growth, follicular growth, development of the corpus luteum and tumor growth (Folkman, 1971; Carmeliet, 2003).

Angiogenesis is needed to provide enough oxygen and nutrients to tissues to sene their metabolic demand. The formation and maturation of a capillary network is a complex process involving various steps. Certain growth factors have been shown to be crucial. The most important stimulus for angiogenesis is hypoxia (Risau, 1997; Carmeliet, 2003), usually mediated by VEGF-A expression induced by HIF-1α (Semenza, 2000; Rissanen *et al.*, 2002; Pugh and Ratcliffe, 2003). During angiogenesis, ECs proliferate and migrate towards the stimulus (Semenza, 2000; Vajanto *et al.*, 2002; Pugh and Ratcliffe, 2003). Pericytes also actively participate in the formation of new capillary tubes (Morikawa *et al.*, 2002; Gerhardt and Betsholtz, 2003). Endothelium-derived platelet derived growth factor-B (PDGF-B) is an important factor in mediating pericyte recruitment (Lindahl *et al.*, 1997). Extracellular matrix and the basement membrane are first degraded and later reassembled to provide structural support for the growing vessel (Kalluri, 2003; Jain, 2003). In this process matrix metalloproteinases (MMPs) play a crucial role (Bergers and Benjamin, 2003; Kalluri, 2003; Jain, 2003).

In the maturation process, stabilization of ECs, pericytes, basement membrane and extracellular matrix are needed to form new persistent capillary tubes. For example, vessels with insufficient mural cell coverage are fragile, leaky and prone to regression

(Benjamin et al., 1998; Hellstrom et al., 2001). This is often the case in tumors where the vasculature may be disorganized, dilated, immature, leaky and lacks pericytes (Bergers and Benjamin, 2003; Jain, 2003). In tumors and in inflammatory processes, infiltrating macrophages are an important source of angiogenic growth factors (Barbera-Guillem *et al.*, 2002; Rehman *et al.*, 2003).

2.4.3. Arteriogenesis (collateral artery growth)

After occlusion of the main artery, the natural response is the enlargement of the preexisting arterial anastomoses to form collateral arteries to bypass the occlusion (Schaper and Ito, 1996; Schaper and Scholz, 2003). This process is called arteriogenesis (Schaper and Scholz, 2003). Arteriogenesis involves remodeling of the intima, media and adventitia driven by increased circumferential wall stress against the medial layer and fluid shear stress against the endothelium (Schaper and Scholz, 2003). Ischemia is not a direct trigger for arteriogenesis since collaterals grow upstream to ischemic tissue (Schaper and Ito, 1996; Ito *et al.*, 1997a).

The exact mechanism underlying vascular adaptation to increased blood flow is unclear. The endothelium seems to play a crucial role in this process through NO production and signaling via integrins (Nadaud *et al.*, 1996; Muller *et al.*, 1997; Jin *et al.*, 2003). In an animal model of myocardial ischemia, NO was shown to be a crucial mediator of arteriogenesis (Matsunaga *et al.*, 2000). Also, local inflammation of the vessel wall caused by the rapid increase in fluid shear stress is thought to play an important role in the initial phase of arteriogenesis (Ito *et al.*, 1997b; Arras *et al.*, 1998). Cytokines and adhesion molecules attract monocytes and macrophages to sites of collateral formation where they secrete growth factors such as fibroblast growth factors (FGFs) (Arras *et al.*, 1998). Also, the genetic depletion of tumor necrosis factor-α (TNF-α) or its receptor p55 in mice has been reported to impair collateral growth after femoral artery occlusion (Hoefer *et al.*, 2002).

In ischemic myocardium or skeletal muscle, arteriogenesis is important for the supply of blood to tissue because collaterals provide bulk flow to the ischemic region, whereas capillaries provide blood for the immediate cellular milieu. Unfortunately, endogenous arteriogenesis stops prematurely when the conductance of <50% of normal has been reached. This is caused by the diminished fluid shear stress and circumferential wall stress after collateral enlargement and wall thickening (Hoefer *et al.*, 2001; Buschmann *et al.*, 2003). Also, the tortuous shape of collaterals increases resistance and is a self-limiting factor in arteriogenesis (Schaper and Scholz, 2003). Thus, endogenous collaterals formation is not always sufficient compensation for lose of the native artery.

2.5. Vascular gene transfer

Therapeutic gene transfer can be defined as the transfer of nucleic acids to the somatic cells of an individual with a resulting therapeutic effect. The first human gene transfer was performed in lymphocytes using retroviruses in 1989 (Rosenberg et al., 1990) and the very first therapeutic gene transfer study was performed in patients suffering from adenosinedeaminase deficiency in 1990 (Blaese et al., 1995). Since then hundreds of

clinical gene therapy trials have been performed for different mono- and multifactorial diseases. Gene therapy has several advantages compared to traditional pharmacological therapy: it can be targeted directly to a specific event in the cell cycle, the local expression of protein coded by the transgene is high without necessarily elevating the systemic concentration and a long therapeutic effect can be achieved with a single treatment. In addition to a therapeutic approach, gene transfer of growth factors and cytokines provides mechanistic insight into their biology *in vivo*.

2.5.1. Gene transfer vectors

The effectiveness of gene therapy is determined by the entry of the new genetic material into cells and the expression of the transfected gene within the target tissue. It is often limited by the compromised efficiency of the biological and physical targeting methods (Yla-Herttuala and Alitalo, 2003). The ideal vector for gene transfer would be one that combines efficient transduction with stable and regulated long term gene expression in the target tissue without any side-effects. Unfortunately, such a delivery vector does not yet exist and the choice of the vector is a compromise between their different charasteristics (Table 1).

Even with powerful viral vectors gene transfer efficiency in the target tissue is often low, and there is a risk of transfecting non-targeted organs unaffected by the disease (Hiltunen et al., 2000b; Hackett et al., 2000). Therefore, there is an an active and urging research interest to develop new strategies to deliver gene transfer vectors to the target area, to target vectors to certain cell types and regulate transgene expression (Kim *et al.*, 1997; Keogh *et al.*, 1999; Nicklin *et al.*, 2000; Koponen *et al.*, 2003).

Naked plasmid DNA and complexes

Currently naked plasmid DNA, with or without carrier molecules, is the most widely used non-viral vector for vascular gene therapy. In comparison to viral vectors, plasmids are easy to produce and purify in large quantities. However, only a small fraction of plasmid DNA enters the nucleus, where it remains extrachromosomal and directs transient transgene expression that lasts for a few weeks (Tripathy et al., 1996; Laitinen et al., 1997b; Turunen et al., 1999). The efficiency of gene delivery using plasmid DNA can be improved with liposome complexes or with cationic polymers, but it still remains quite low (Laitinen *et al.*, 1997a; Laitinen *et al.*, 1997b; Turunen *et al.*, 1999).

Non-viral plasmid DNA gene transfer has not been associated with safety concerns. Even high doses of naked VEGF plasmid delivered to the ischemic legs of patients has not lead to any toxic effects (Baumgartner *et al.*, 1998). However, it has been reported that plasmid DNA can cause significant inflammation in skeletal muscle and a transient fever in patients (McMahon *et al.*, 1998; Hedman *et al.*, 2003). Although it has been widely used in clinical trials, the efficiency of plasmid mediated gene transfer falls far behind adenoviral gene therapy (Wright et al., 1998), and may not be sufficient for therapeutic use in humans in its current form.

Table 1. Characteristics of different vectors used for vascular gene transfer. Modified from Rutanen et al., 2002.

Vector	Advantages	Disadvantages
Naked plasmid DNA	Easy to produce Safe	Very low transduction efficiency Transient expression
Adenovirus	High transduction efficiency Relatively high capacity Transient expression Easy to produce in high titers Transducts both proliferative and quiescent cells Tropism for multiple cells	Immunological and inflammatory reactions Transient expression (<2 weeks) Cytotoxic effects at high concentrations
Adeno-associated virus (AAV)	Long transgene expression Low inflammatory and immune responces Transducts both proliferative and quiescent cells Tropism for skeletal muscle and myocardium	Limited DNA capacity (4-5kb) Difficult to produce
Baculovirus	High DNA-capacity Transient expression Easy to produce in high titers Rapid construction of recombinant baculoviruses Wild type does not cause disease in mammals	Transient expression Immunological and inflammatory reactions Moderate transduction efficacy Limited tropism
Herpes simplex-virus (HSV-1)	High transduction efficiency High DNA capacity Easy to produce Tropism for neuronal cells	Unable to transduce non-dividing cells Cytotoxicity and neurotoxicity Limited tropism
Epstein-Barr-virus	High transduction efficiency High DNA-capacity Persistence in the host Extrachromosomal replication	Unable to transduce non-dividing cells Difficult to produce
Lentivirus	High DNA-capacity Transducts both proliferative and quiescent cells Stable gene expression No immune responses	Low transduction efficiency Difficult to produce Low titers Non-specific integration in the chromosomes
Retrovirus	Stable gene expression Relatively easy to produce	Low virus titers Low transfection efficiency Transfects only dividing cells Limited DNA capacity

Adenoviral vectors

Adenoviruses are currently the most widely used viral vectors for therapeutic gene transfer to the vascular system (Laitinen *et al.*, 1998; Rosengart *et al.*, 1999; Makinen *et al.*, 2002; Grines *et al.*, 2002; Yla-Herttuala and Alitalo, 2003). Adenoviruses constitute linear double-stranded DNA of approximately 36 kbp surrounded by a capsid (Kovesdi et al., 1997; St George, 2003). Wild type human adenoviruses exist in at least 47 different serotypes divided in six subgroups and are a general cause of respiratory and other infections. In gene transfer vectors, the sequences essential for replication are replaced by DNA sequences from the gene to be transferred rendering the virus replication deficient, i.e. it cannot elicit an infection in the treated subject.

Adenoviruses enter cells via specific receptors and after entering the nucleus, they remain episomal, not integrating into the host genome (Kovesdi et al., 1997; St George, 2003). First generation adenoviruses cause only transient gene expression, usually lasting from a few days to 2 weeks depending on the target tissue (Hiltunen et al., 2000b; Vajanto et al., 2002). The decline in adenoviral gene expression is due to inactivation or loss of vector DNA within transduced cells. Also, mechanisms other than cellular and humoral attack on transduced cells have been proposed to contribute to the short duration of gene expression (Chen et al., 1999; Wen et al., 2000). Adenoviruses can be produced in high titers and have an ability to transfect both proliferating and non-proliferating cells (Kovesdi *et al.*, 1997; St George, 2003). Their efficiency is dependent upon the presence of coxsackie-adenovirus receptor (CAR) which is expressed at varying degrees in most human tissues (Bergelson *et al.*, 1997).

Adenovirus infection is not associated with malignancy. Indeed, oral adenoviral vaccines have been used in man for decades. These facts, together with the characteristic that adenovirus-mediated gene transfer leads only to a temporary expression of the transgene favour its use in human gene therapy. Although adenoviral gene transfer to the vascular system transduces non-targeted organs and peripheral blood monocytes, it was well tolerated in phase I/II clinical trials (Laitinen et al., 1998; Rosengart et al., 1999; Makinen et al., 2002; Grines et al., 2002; Rajagopalan et al., 2003; Hedman et al., 2003). However, it should be kept in mind that, as with any other drug, higher doses of adenoviral vector may lead to severe side-effects or even death especially in immunocompromised patients (Lehrman, 1999).

Although adenoviral gene transfer promotes efficient gene expression, it is currently thought that in some applications, such as induction of therapeutic angiogenesis, longer gene expression may be needed to achieve a permanent therapeutic effect (Dor *et al.*, 2002). Thus, vectors that provide a longer duration of gene expression combined with a relatively good transfection efficiency are currently being developed but their usefulness for vascular gene therapy need to be further evaluated before planning therapeutic approaches in man (Yla-Herttuala and Alitalo, 2003).

Other viral vectors

Adeno-associated virus (AAV) is a small single-stranded DNA parvovirus that exists in at least seven different serotypes and is not known to cause disease in humans (Kessler et al., 1996; Pajusola et al., 2002). AAVs integrate into the host genome and provide long-

lasting transgene expression. They can also transduce both dividing and non-dividing cells. AAVs have already been used in the vascular system to promote expression lasting beyond 30 days and a method to target AAV to vascular cells has also been introduced (Eslami et al., 2000; Nicklin et al., 2001; Chu et al., 2003; Arsic et al., 2003; Gruchala et al., 2004). Problems with AAV are related to the difficult production of purified lots and a relatively small capacity to contain transgene inserts (< 5 kbp). However, AAV appears promising for the induction of sustained effects in cardiovascular system (Bohl et al., 1998; Arsic et al., 2003; Gruchala et al., 2004).

Retroviruses (usually murine leukemia virus, MuLV) have been used for gene transfer to the vascular system (Laitinen et al., 1997a; Pakkanen et al., 1999). They enter the cells via specific receptors after which genomic RNA is reverse transcribed to DNA that integrates into the host genome. Hence, retroviral gene transfer leads to long-lasting gene expression. However, retroviruses only transfect proliferating cells and they can be produced only with relatively low titers. Thus, their transfection efficiency remains very low (Laitinen *et al.*, 1997a). This quality makes the practical use of retroviruses limited for extravascular or *ex vivo* approaches (Kankkonen *et al.*, 2004). Furthermore, random integration of the transgene causes a risk of oncogene activation. A major setback affecting the whole gene therapy field was recently faced in a clinical trial to treat SCID when 2 out of 11 children developed leukaemia after otherwise successful treatment of CD34+ bone marrow cells (Marshall, 2003).

Lentiviruses (human, simian and feline immunodeficiency viruses) are likely to replace MuLV-type retroviruses in gene therapy for genetic disorders because they appear safer and also transduce non-dividing cells (Buchschacher and Wong-Staal, 2000; Kankkonen et al., 2004). Lentiviral transduction leads to permanent gene expression in the target cell. Also, a regulated gene expression system may favor the use of lentiviruses for therapeutic purposes (Koponen et al., 2003). Unfortunately, one drawback is that the transduction efficiency of lentiviruses in the vascular system seems to be low (Kang et al., 2002; O'Rourke et al., 2003).

Also, other viruses such as Herpes Simplex viruses, Sendaiviruses, Baculoviruses, Sindbis viruses and Semliki Forest viruses have been used for gene transfer but their usefulness for therapeutic purposes in the vascular system needs further study (Akkaraju et al., 1999; Shiotani et al., 2001; Airenne et al., 2000; Wahlfors et al., 2000).

2.5.2. Gene transfer routes

Various routes for administration of nucleic acids have been used in experimental animal models as well as in clinical settings. Intravascular gene transfer for the prevention of restenosis can be easily performed during angioplasty, stenting or other intravascular manipulations. Limitations of intravascular gene transfer include the presence of anatomical barriers, such as the internal elastic lamina, calcification within atherosclerotic lesions and the presence of virus-inactivating complement system within plasma (Rome et al., 1994; Plank et al., 1996; Laitinen et al., 1998). Perivascular gene transfer can be used for the delivery of therapeutic genes into the arterial wall during by-pass operations, prosthetic graft insertion, anastomosis surgery and endarterectomies (Laitinen et al., 1997b; Pakkanen et al., 2000). When a gene transfer vector is administered to the adventitial surface of the vessels, it can remain in close contact with the target cells for a

long time. The limitation of this method is the adequate uptake of the gene transfer vector or gene product in the inner layers of the vascular wall.

The most commonly used route for gene delivery to induce therapeutic vascular growth is by direct injection into skeletal muscle or myocardium (Baumgartner *et al.*, 1998; Rosengart *et al.*, 1999; Symes *et al.*, 1999). Skeletal muscle is readily accessible for direct injection, but for the heart, thoracotomy is usually required. Thus, traditionally gene transfer directly into the myocardium has been combined with by-pass surgery rather than used as a sole therapy. However, the feasibility of intramyocardial injections has been substantially improved by the introduction of percutaneous catheter-mediated injection systems such as NOGA (Gepstein et al., 1997; Kornowski et al., 1999; Vale et al., 2001). Myocardial gene transfer guided by the NOGA electromechanical mapping-system has been reported to be as efficient as direct injections after thoracotomy (Kornowski *et al.*, 2000).

Intra-arterial delivery has also been used to induce therapeutic vascular growth in humans (Makinen *et al.*, 2002; Grines *et al.*, 2002; Hedman *et al.*, 2003). However, this approach is limited by a low gene transfer efficiency to tissues surrounding the blood vessels (Laitinen *et al.*, 1998; Lee *et al.*, 2000a; Wright *et al.*, 2001; Rissanen *et al.*, 2003a). Furthermore, there is a high risk of transducing distal non-targeted organs when using the intra-arterial approach (Hiltunen et al., 2000b). The efficiency of intra-arterial gene transfer can be increased by interruption of blood flow, prolonged incubation times, permeabilization of the endothelium or modulation of hydrostatic and osmotic pressures (Cho *et al.*, 2000; Logeart *et al.*, 2000; Davidson *et al.*, 2001; Wright *et al.*, 2001). However, these approaches seem relatively complex compared to direct intramuscular/-myocardial injection.

In addition to injections of gene transfer vectors, an *ex vivo* strategy, where cells, blood vessels or organs are isolated and transduced outside the body before returning back to the same subject, can be used for vascular gene transfer. The strategy results in high transduction efficiency and low risk of vector leakage into the circulation (Mann *et al.*, 1999; Lemstrom *et al.*, 2002; Kankkonen *et al.*, 2004). Myoblasts are easy to culture and transduce *in vitro* and are capable of fusing with myofibers *in vivo* post transplantation. Thus, these precursor cells have been used for gene delivery into skeletal muscle and myocardium (Springer *et al.*, 1998; Lee *et al.*, 2000b).

2.5.3. Gene therapy for restenosis

Restenosis is defined as shrinkage of the vessel lumen cross-sectional area after vascular intervention. Understanding of the cellular events leading to this treatment failure has increased substantially during recent years. Restenosis is a complex multi-factorial process involving platelet activation, thrombosis, leukocyte adhesion, vasoconstriction, proliferation and migration of neointimal cells, and their participation in matrix formation (Bauters et al., 1996; Schwartz, 1998). In response to arterial injury, medial SMCs migrate, proliferate and produce extracellular matrix, leading to the development of neointimal thickening. SMC proliferation and migration are key factors in the development of restenosis and most of the gene therapy strategies to date, have been directed towards these processes. However, all contributory aspects of restenosis need to be considered when searching for optimal gene treatment.

Platelet derived growth factor (PDGF) is one of the most potent chemoattractants for vascular SMC. PDGFs mediate neointimal growth after vascular injury in animal models and are expressed in human atherosclerotic lesions (Barrett and Benditt, 1987; Barrett and Benditt, 1988). They also stimulate SMC cell cycle regulating genes, chemotaxis, induction of matrix formation and protection from apoptosis. Restenosis caused by neointimal growth could potentially be blocked by inhibition of PDGF expression with antibodies against PDGF or its receptors (Fingerle et al., 1989; Hart et al., 1999; Ferns et al., 1991; Leppanen et al., 2000). A gene therapy approach utilizing PDGF-receptor decoys or antisense oligonucleotides directed against PDGF mRNA has been introduced (Sirois et al., 1997; Deguchi et al., 1999).

Matrix metalloproteinases (MMPs) are involved in the migration of SMCs from the media and can be inhibited by overexpressing tissue inhibitors of metalloproteinases (TIMPs). Gene transfer of TIMP-1 and TIMP-2 have been shown to reduce neointimal growth in animal models (Cheng et al., 1998; Turunen et al., 2002). Early markers of SMC activation, such as nuclear oncogenes, that lead to the growth factor stimulation are detectable rapidly after arterial injury (Bauters et al., 1996). Thus, antisense oligonucleotides directed against these proto-oncogenes and cell cycle regulators, for example *c-myb, c-myc, cdk-2*, NF-κB and E2F have been used with varying degree of success to decrease neointimal thickening in animal models (Pollman et al., 1998; Morishita et al., 1998). Also, a gene transfer approach to affect these and many other cell cycle regulators have been evaluated in various pre-clinical studies (Rutanen et al., 2002).

Since intravascular manipulation damages the endothelium, it has been hypothesized that rapid re-endothelialization of the luminal surface after balloon dilation should limit the degree of restenosis. VEGFs are a growth factor family that induces endothelial cell proliferation, regrowth and migration (Ferrara, 1999). VEGF-A and VEGF-C gene therapy after arterial injury has been shown to attenuate neointimal growth (Asahara et al., 1995; Laitinen et al., 1997b; Hiltunen et al., 2000a; Khurana et al., 2004). In a controlled human study VEGF-A gene transfer has been shown to be safe and feasible in conjunction with coronary and lower limb vessel angioplasty (Mäkinen et al., 1999; Laitinen et al., 2000; Hedman et al., 2003). Hepatocyte growth factor (HGF) is another gene that reported to reduce neointimal hyperplasia through accelerating re-endothelization (Hayashi et al., 2000).

NO inhibits SMC migration and proliferation. Thus, long term NO production at the lesion may prevent restenosis and in support of this it has been shown that overexpression of NO synthase has an inhibitory effect on neointimal growth (von der Leyen et al., 1995; Kullo et al., 1997; Janssens et al., 1998). Furthermore, the therapeutic effect of VEGF-A gene therapy is attenuated in the presence of NO inhibitor L-nitro argenine methyl ester (L-NAME) (Laitinen et al., 1997b). Thus, some of the effects of VEGF are probably due to increased NO production. VEGF and NO are also cytoprotective compounds because they stimulate endothelial cell re-growth, repair and survival.

Thrombosis is recognized as a major step in initiating restenosis (Bauters et al., 1996). Intravascular interventions cause damage to the endothelium and media exposing thrombogenic molecules (collagen, lipids from plaque), which activate platelets and the thrombotic cascade. Acute thrombus formation has been fought with antiplatelet agents in clinical practice for decades, but thrombotic signals may actually be sustained for periods beyond resolution of the thrombus (Schwartz, 1998). Gene transfer of antithrombotic

agents like tPA, hirudin or tissue factor pathway inhibitor may prevent restenosis by protecting the vessel from thrombus formation shortly after vascular manipulations (Rade et al., 1996; Waugh et al., 1999).

Although the majority of gene therapy interventions are directed against neointimal hyperplasia, it is suggested that the long term success of angioplasty is determined by vascular remodelling rather than intimal hyperplasia (Kakuta et al., 1994; Mintz et al., 1996; Sangiorgi et al., 1999). Also, the alteration in shear stress conditions may exacerbate neointimal hyperplasia (Kumar et al., 1997; Kumar and Lindner, 1997). In supportion of this, prevention of negative vascular remodelling with human tissue kallikrein gene transfer has been shown to reduce neointimal hyperplasia in animal model (Emanueli et al., 2001). Therefore, genes that have an effect on negative remodelling in addition to intimal hyperplasia, such as VEGFs or nitric oxide synthases may be most useful in the prevention of restenosis.

In-stent restenosis is the result of thrombus formation, acute inflammation and neointimal growth (Komatsu et al., 1998; Farb et al., 1999; Brasen et al., 2001). As negative remodelling is mostly prevented mechanically by stent struts, neointimal growth is the most prominent factor in occluding the treated vessel and should be seen as the main target for the treatment. Stents themselves has been used as gene delivery devices. Stents coated with polymers may release gene transfer vectors or oligonucleotides in a controlled fashion (McKenna et al., 1998). Also, ex vivo approaches using genetically engineered endothelial cells attached to the stent surface could prove feasible (Flugelman et al., 1992). Importantly, highly promising clinical results in preventing in-stent restenosis have recently been obtained with sirolimus eluting stents (Sousa et al., 2001; Moses et al., 2003).

In spite of the promising results aimed to prevent neointimal hyperplasia with gene therapy in animal experiments, only a few clinical trials have taken place so far. Laitinen *et. al.* demonstrated the safety and feasilibity of intravascular gene therapy to human peripheral arteries using high adenovirus titers ($1x10^8$ to $4x\ 10^{10}$ pfu), which resulted in a maximum of 5% transfection efficiency within arterial cells (Laitinen et al., 1998). In a controlled study of catheter mediated VEGF-A gene transfer with plasmid or adenovirus to infrainguinal arteries after PTA, increased vasculature was registered in the VEGF treatment groups in the follow-up angiography (Mäkinen et al., 1999). Also, catheter mediated plasmid/liposome and adenoviral VEGF-A gene transfer to human coronaries in conjunction with PTCA was shown to be safe and well tolerated in a randomized, double-blinded, placebo-controlled study (Laitinen et al., 2000; Hedman et al., 2003). There were no differences in restenosis rates compared to placebo groups. However, significant increase in myocardial perfusion was detected in AdVEGF-A treated patients (Hedman et al., 2003).

To date, there is only one successful report in preventing neointimal hyperplasia in clinical trials: peripheral bypass graft failure was reduced using E2F decoy by an *ex vivo* gene transfer approach (Mann et al., 1999).

2.5.4. Gene therapy for vascular growth

Before the development of gene transfer vectors to obtain sustained production of angiogenic growth factors, i.a. or i.m. administration of recombinant proteins was a popular

mode of delivery in animal models and until recently also in clinical trials. Promising results were reported in models of peripheral ischemia with FGF-1, FGF-2 and VEGF given once or repeatedly regardless of the route of administration (Baffour *et al.*, 1992; Pu *et al.*, 1993; Takeshita *et al.*, 1994; Bauters *et al.*, 1995). As always in the development of novel therapies, only large randomized placebo-controlled double-blinded clinical trials definitively test their efficacy in man. Such recombinant protein trials to induce therapeutic angiogenesis have been interpreted as negative trials (Simons et al., 2002; Lederman et al., 2002; Henry et al., 2003). Recombinant protein therapy for angiogenesis is limited by the short half-life of most growth factors *in vivo* and low uptake in tissues after intra-arterial injection (Lazarous *et al.*, 1997). After a single intradermal injection, the biological activity of VEGF protein was significantly attenuated after 60 min (Dafni *et al.*, 2002) which is not enough for the initiation of clinically meaningful vascular growth. Furthermore, high doses of VEGF and FGF-2 induce hypotension via NO production and FGF-2 may cause severe proteinuria (Henry *et al.*, 2003; Cooper, Jr. *et al.*, 2001; Simons *et al.*, 2002; Lederman *et al.*, 2002). Thus, apart from nephrotoxicity the beneficial effects of a single administration of VEGF or FGF-2 seem quite similar to the effects obtained by nitrate treatment for acute coronary ischemia, the vasodilating effects are short-term and high doses cause hypotension.

A continuous i.a. infusion of cytokines such as MCP-1, granulocyte macrophage-colony stimulating factor (GM-CSF) and PlGF have been shown to promote collateral artery growth in animal experiments (Ito *et al.*, 1997b; Buschmann *et al.*, 2001; Pipp *et al.*, 2003). Although continuous infusion is an improvement over a single injection strategy, this approach is hampered by the low uptake of cytokines into the growing collaterals and by the emerging possibility that these cytokines may enhance atherosclerosis by recruiting inflammatory cells from the bone marrow.

Because of the disadvantages of recombinant protein therapy, gene transfer of angiogenic molecules may potentially yield more sustained growth factor levels in tissues and be more efficacious. The first gene transfer vehicle to be used was naked plasmid DNA. In animal models, naked plasmid DNA encoding various factors including VEGF, FGFs, Ang-1, HIF-1α and HGF was reported to promote therapeutic vascular growth (Tsurumi *et al.*, 1996; Shyu *et al.*, 1998; Vincent *et al.*, 2000). Although these studies reported increases in capillary density, collateral growth, blood pressure and perfusion in the treated muscle, they did not significantly contribute to our understanding of angiogenesis because the mechanisms of vascular growth were not addressed. Furthermore, vascular permeability and edema, both of which indicate efficient VEGF production in tissue (Poliakova *et al.*, 1999; Pettersson *et al.*, 2000), have not been reported to occur after naked plasmid VEGF gene transfer in animals (Tsurumi *et al.*, 1996). Nevertheless, the uncontrolled phase I clinical studies also suggested that naked plasmid DNA-mediated gene transfer of VEGF is effective (Losordo et al., 1998; Baumgartner et al., 1998; Symes et al., 1999; Laitinen et al., 2000; Vale et al., 2001; Losordo et al., 2002; Kastrup et al., 2003; Shyu et al., 2003). However, the substantial placebo effects observed in angiogenesis trials precluded useful conclusions being made about the efficacy of the treatment.

Viral vectors are replacing naked plasmid DNA gene transfer in efforts to stimulate blood vessel growth because of the extremely low gene transfer efficacy of the latter. It is now clear that growth factor production lasting for a few days is required for efficient

induction of blood vessel growth. Preclinical experiments using viral vectors have provided evidence for the usefulness of VEGFs and FGF-1 and -2 towards vascular growth *in vivo* (Muhlhauser et al., 1995; Ueno et al., 1997; Mack et al., 1998; Pettersson et al., 2000; Gowdak et al., 2000; Rissanen et al., 2003a; Rissanen et al., 2003b). In contrast to naked plasmid VEGF gene transfer, adenoviral VEGF induces vascular permeability and edema approximately a week after gene transfer both in rabbits and mice showing active angiogenesis (Poliakova *et al.*, 1999; Pettersson *et al.*, 2000). Also, clinical trials using adenoviruses encoding VEGFs or FGFs in human ischemic heart have been safe and well tolerated (Rosengart *et al.*, 1999; Laitinen *et al.*, 2000; Grines *et al.*, 2002; Hedman *et al.*, 2003).

VEGFs and FGFs are the most extensively studied growth factors for therapeutic vascular growth in the myocardium. In preclinical studies adenoviral VEGF-A_{121} have improved local myocardial perfusion and collateral circulation (Lee et al., 2000a). Experimental studies with adenoviral FGFs have demonstrated histological angiogenesis and improvement in myocardial function in models of chronic ischemia as detected by echocardiography (Safi *et al.*, 1999; Horvath *et al.*, 2002). Also, gene therapy using HGF has shown promising results in preserving myocardial function after infarction in small rodent models (Aoki *et al.*, 2000; Li *et al.*, 2003).

In addition to vector development, approaches using cocktails of growth factors have been suggested to generate more stable vasculature than what is achieved by a single growth factor alone. For example, strategies have been designed to stimulate growth of more functional vessels with VEGF combined with PDGF to induce pericyte recruitement or Ang-1 to reduce vascular leakage (Thurston et al., 2000; Richardson et al., 2001). However, the true potential of these combinations for therapeutic angiogenesis is currently unclear. Also, the mechanism by which Ang-1 decreases plasma protein extravasation is poorly understood (Thurston *et al.*, 1999).

3. AIMS OF THE STUDY

The aim of this thesis was to study the expression of VEGF-D, a novel member of the VEGF gene family, during human atherogenesis and to evaluate the therapeutic potential of different growth factor gene transfer for myocardial ischemia and post-angioplasty restenosis in animal models. This goal was divided into specific aims as follows:

1. Study the expression of VEGF-D in normal and atherosclerotic human arteries (I).

2. Determine the potency of gene therapy using the functional form of VEGF-D (VEGF-$D^{\Delta N \Delta C}$) for post-angioplasty restenosis in a rabbit-denudation model (II).

3. Test the efficacy of combination therapy for post-angioplasty restenosis using adenoviral VEGF-C gene transfer in conjunction with a PDGF receptor blocking drug, in a rabbit-denudation model (III).

4. Test the feasibility and efficacy of catheter-mediated intramyocardial VEGF gene transfer for vascular growth in pig heart guided by a NOGA electromechanical mapping system (IV).

5. Compare the efficacy of naked plasmid DNA and adenoviral vector for gene transfer into myocardium (IV).

6. Study the duration and side-effects of adenoviral gene transfer of VEGFs (II and IV).

4. MATERIALS AND METHODS

4.1. Human samples (I)

Human arterial samples were obtained from both medicolegal autopsies and lower limb amputation operations. The total number of samples included in the study was 39. The autopsy samples were collected within 12h after death. The samples from lower limb were collected in the operation theatre immediately after the limb was detached. Each sample was divided into two parts. One part was immersion-fixed in 4 % paraformaldehyde (Pfa) / 15 % sucrose (pH 7.4) for 4 h, rinsed in 15 % sucrose (pH 7.4) and embedded in paraffin (Ylä-Herttuala et al., 1990). Serial 6-10μm sections were used for in situ hybridization and immunohistochemistry studies. The other part was snap frozen in liquid nitrogen and stored at –70 °C for RNA extraction and analysis. The study protocol was approved by the Ethics Committee of the University of Kuopio.

4.2. Gene transfer models

4.2.1. Rabbit balloon-denudation restenosis model (II and III)

New Zealand White (NZW) rabbits (n=4-9 in each group) were maintained on a 0.25% cholesterol diet for two weeks prior to balloon denudation. The whole aorta was denuded twice by using a 3.0 F arterial embolectomy catheter (Sorin Biomedical, CA, USA) (Hiltunen et al., 2000a). Three days later the gene transfer was performed using a 3.5 F Dispatch™ local drug delivery catheter (Boston Scientific, MA). Under fluoroscopical control the catheter was positioned caudal to the left renal artery in a segment free of side branches (Figure 3) and the gene transfer was performed at 6 ATM pressure for 10 min. $2.0x10^{12}$ (II) or $1.15x10^{12}$ (III) viral particles (vp) of Ad*LacZ* or therapeutic gene [AdVEGF-$D^{\Delta N\Delta C}$ (II) and AdVEGF-C (III)] were used in a total volume of 2ml (II) or 10ml (III) of sterile saline.

In study II, animals were sacrificed and the samples harvested 6, 14 or 28 days after the gene transfer. To study the role of NO in the downstream signaling of VEGF-$D^{\Delta N\Delta C}$, two additional groups were fed with NO synthase inhibitor L-nitro arginine methyl ester (L-NAME, N-5751, Sigma, 100 mg/kg/day) and sacrificed at the two week time point (Laitinen et al., 1997b). In study III, imatinib mesylate (STI571/Gleevec; 10 mg/kg daily; Novartis) dissolved in sterile water (2 mg/mL) was given with an orogastric sond 1 hour before gene transfer and every 12 hour thereafter for the following 3 weeks. The 4 treatment groups (VEGF-C/ STI571: -/-, +/-, -/+, +/+) were analyzed at 3 and 6 gene transfer.

Three hours before sacrifice animals were injected i.v. with 50 mg of bromodeoxyuridine (BrdU) dissolved in 40 % ethanol. After sacrifice the transduced segment of aorta was removed, flushed gently with saline and divided into four equal parts (Hiltunen et al., 2000a; Ylä-Herttuala et al., 1995). The proximal part was immersion-fixed in 4 % Pfa/15 % sucrose (pH 7.4) for 4 h, rinsed in 15 % sucrose (pH 7.4) overnight and embedded in paraffin. The next part was fixed in 4 % paraformaldehyde/phosphate buffered saline (PBS) (pH 7.4) for 10 min, rinsed in PBS, embedded in OCT compound (Miles) and stored at –70 °C. The third part was fixed in 70 % ethanol overnight and

embedded in paraffin. The distal part was snap frozen in liquid nitrogen and stored at –70 °C. Tissue samples from the liver, lung, spleen, kidney, and testis were also collected for subsequent safety analyses. The study protocols were approved by Experimental Animal Committee of the University of Kuopio and they conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

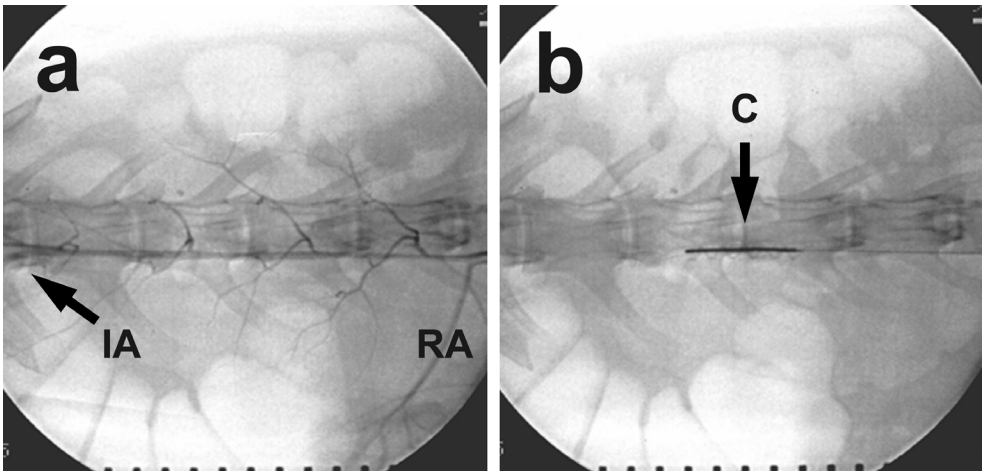


Figure 3. Gene transfer to rabbit aorta using Dispatch-catheter. **a.** Angiogram from rabbit abdominal aorta. **b.** Dispatch-catheter in rabbit aorta. RA=renal artery. IA=iliac artery. C=Dispatch-catheter

4.2.2. Pig myocardium (IV)

Electromechanical mapping of the left ventricle of domestic pigs weighing 25-30kg (n=45) was performed with the NOGA system and an 8F NOGA injection catheter (Biosense-Webster, Johnson & Johnson, USA)(Gepstein et al., 1997; Kornowski et al., 1999; Vale et al., 1999; Vale et al., 2001; Kornowski et al., 2000). After mapping at least 70 points, ten intramyocardial injections (Figure 4. 0.2 ml each, total volume 2.0 ml, at least 5 mm apart from each other) were performed to the anterolateral wall. Human GMP-grade first generation adenoviruses (Hedman et al., 2003) using two viral doses [$2x10^{11}$ or $2x10^{12}$ viral particles (vp)] or naked plasmids (1mg) encoding *Lac*Z marker gene, human VEGF-A_{165} (Ferrara, 1999) or the mature form of human VEGF-D (VEGF-$D^{\Delta N \Delta C}$) (Achen et al., 1998) driven by the CMV promoter were administered. The total dose of naked plasmids used was five times higher than in a recently reported clinical trial (Vale et al., 2001). The needle was advanced 5-6 mm into the anterolateral wall of the left ventricle and each injection was completed in 30s.

Before sacrifice, six days or three weeks after gene transfer, perfusion was studied with myocardial contrast echocardiography (MCE) and microspheres. Vascular permeability and pericardial effusion induced by the VEGFs, was assessed using the Modified Miles assay and echocardiography, respectively. Plasma was collected

immediately before injections and before sacrifice. After sacrifice, the hearts were perfusion fixed and the myocardial samples were collected for histological analysis. The study protocol was approved by the Experimental Animal Committee of Kuopio University.

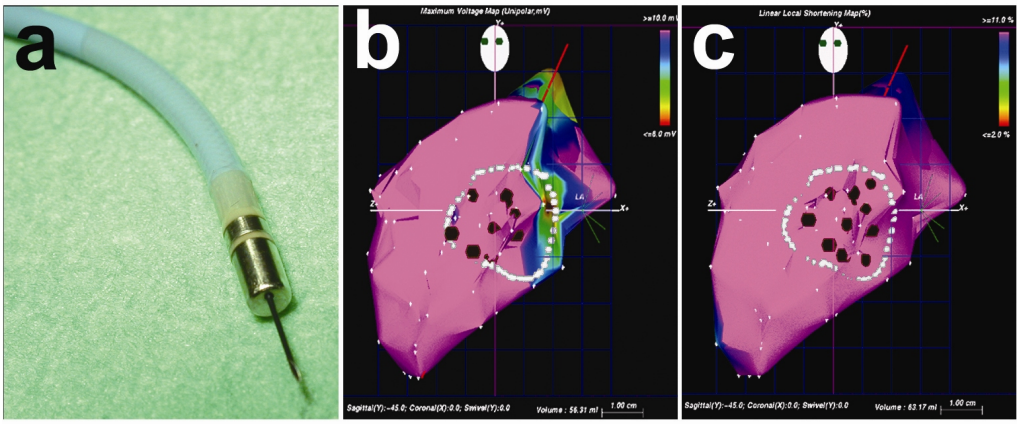


Figure 4. NOGA-electromechanical catheter mediated gene transfer into anterolateral wall of the left ventricle. **a.** Tip of the catheter **b.** Voltage map of the pig left ventricle **c.** Linear shortening map. Black spots indicate the injection sites in the anterolateral wall.

4.3. RNA analyses

4.3.1. RT-PCR (I, II and III)

Total RNA was extracted from the snap frozen tissue samples using Trizol® reagent (Gibco BRL). After DNase treatment cDNA synthesis was performed using random hexamer primers (Promega) with 3 μg of total RNA.

RT-PCR for human VEGF-A (I) was performed as described earlier (Inoue et al., 1998). For human endogenous VEGF-D (I) RT-PCR primers were 5'-GTTGCAATGAAGAGAGCCTT-3' and 5'-TCCCATAGCATGTCAATAGG-3', and for β-actin RT-PCR primers were 5'-CCCTGAAGTACCCCATCGAG-3' and 5'-GGGAGACCAAAAGCCTTCATA-3'.

Expression of transduced VEGF-$D^{\Delta N \Delta C}$ in rabbit aortic samples was confirmed with RT-PCR (II). Primers for PCR amplification were as follows; forward: 5'-TTGCCAGCTCTACCACCAG-3' and reverse: 5'-TTCATTGCAACAGCCACCAC-3' resulting in 292 bp product. Transduced human VEGF-C (III) was detected from arterial segments using the following primers: 5'-CTGCTTACTGGCTTATCG-3' and 5'-CCTGTTCTCTGTTATGTTGC-3'. Five μl of the first PCR product was used for the second PCR with primers 5'-TCTCCAAAAAGCTACACCG-3' and 5'-CAAGTGCATGGTGGAAGG-3'.Controls where reverse transcriptase had been omitted, were included in each run.

4.3.2. In situ –hybridization (I)

The localization of VEGF-D, VEGFR-2, and VEGFR-3 mRNA in human arteries was studied by *in situ* hybridization in serial paraffin sections. All antisense and control sense riboprobes (nucleotides 789-1113 of VEGF-D, 1723-2365 of VEGFR-2 and 1-595 of VEGFR-3) were synthesized from pBluescript (VEGF-D) or pGEM (VEGFR-2 and –3) plasmids using T7, T3 or SP6 polymerases in the presence of [^{33}P]UTP. *In situ* hybridizations were performed on pretreated (Proteinase K 0.5 mg/ml at 37°C for 30 min) tissue sections (1x10^6 cpm per section) as previously described (Ylä-Herttuala et al., 1990).

4.4. Protein analyses

4.4.1. Immunohistochemistry, histological analyses and X-Gal staining (I-IV)

Paraffin embedded sections (6-10μm) were used for immunohistochemical analyses and hematoxylin-eosin (HE) stainings. Immunohistochemistry was performed using the avidin-biotin-horseradish peroxidase system (Vector Laboratories) with 3'-5'-diaminobenzine (DAB, Zymed) as a color substrate (Ylä-Herttuala et al., 1990). For double immunostainings, the alkaline-phosphatase system with Vector Blue color substrate (Vector) was also used. The primary antibodies used for immunohistochemistry are listed in Table 2. Controls for immunostainings included incubations with class- and species matched immunoglobulins and incubations where primary antibodies were omitted (Ylä-Herttuala et al., 1995; Hiltunen et al., 2000a). All morphometry analyses were done from randomly selected multiple sections in a blinded manner using analySIS® software (Soft Imaging System, Germany) and an Olympus AX70 microscope (Olympus Optical, Japan).

Human arterial lesions in study I were histopathologically classified into four categories according to Stary *et al* (Stary et al., 1992; Stary et al., 1994; Stary et al., 1995): no lesion n = 5, fatty streak (lesions type I and II in classification), plaque (lesions III and IV), and complicated lesion (lesions V and VI). Semiquantitave microscopical evaluation of the sections was done in a random order without prior knowledge of the origin of the samples. VEGF-D and VEGF-A analyses were done from sections stained with antibodies VD1 and sc-7269, respectively. The specimens were graded using the following criteria: no detectable staining (-); weak staining (+), meaning that less than 10% of the lesion area was positive for the studied signal; moderate staining (++), meaning that 10-50% of the area was positive for the studied signal; strong staining (+++), meaning that more than 50% of the area was positive for the studied signal (Hakkinen et al., 2000).

In studies II and III the gene transfer efficiency was evaluated using X-Gal staining of OCT embedded tissue sections as previously described (Hiltunen et al., 2000b). Proliferation marker BrdU-positive cells were detected with a mAb (DAKO, 1:100) using ethanol fixed paraffin sections. Measurements for histological parameters (intima-media ratio, intact endothelium, macrophage count, intact internal elastic lamina and proliferating cells) were done using paraffin embedded sections by one observer from randomly selected multiple sections (4/rabbit). TUNEL reaction (III) was performed on serially cut

cryosections from the transfected vessel segment as previously described (Leppanen et al., 2000).

In study IV, blood vessels in myocardial samples were immunostained with a mouse mAb against α-smooth muscle antigen (α-SMA). Myocardial capillary density (capillaries/mm^2), capillary mean area (μm^2) and total capillary lumen area in two transduced and one intact apical samples were measured from α-SMA immunostained sections at 200X magnification. Measurements were done in a blinded manner from five fields in each section. In the transduced samples, capillaries were measured from regions adjacent to the needle track which were thought to represent the maximal angiogenesis effects of the treatment.

Table 2. Antibodies used in immunohistochemistry

Antibody	Specificity	Code / clone	Species	Ig isotype	Dilution	Company / producer
αSMA	α-smooth muscle actin	clone 1A4	mAb mouse anti-human	IgG_{2a}	1:250	Sigma
BrdU	proliferating cells	clone Bu20a	mAb mouse anti-human	$IgG_{1,\kappa}$	1:50-100	DAKO
CD31 / PECAM1	endothelium	JC/70A	mAb mouse anti-human	$IgG_{1,\kappa}$	1:50	DAKO
CD68	macrophages	KP1	mAb mouse anti-human	$IgG_{1,\kappa}$	1:150	DAKO
HHF35	skeletal, cardiac and smooth muscle α/γ-actin	HHF-35	mAb mouse anti-human	IgG_1	1:50	DAKO
MCP-1	MCP-1	AF-279-NA	pAb goat anti-human	IgG	1:5	R&D
PCNA	proliferating cells	PC10	mAb mouse anti-human	$IgG_{2a,\kappa}$	1:500	Neomarkers
RAM11	macrophages	RAM11	mAb mouse anti-rabbit	$IgG_{1,\kappa}$	1:50	DAKO
VEGF-A	VEGF-$A_{121-206}$	sc-7269	mAb mouse anti-human	IgG_{2a}	1:500	Santa Cruz
VEGF-D	VEGF-D	VD1 / 78923.11	mAb mouse anti-human	IgG / IgG_1	1:100 / 1:25	(Achen et al., 2001) / R&D
VEGF-D	VEGF-D, c-terminal end	sc-7602	pAb goat anti-human		1:100	Santa Cruz
VEGFR-2	VEGFR-2	sc-6251 / MFLK1 Cabm	mAb mouse anti-human	IgG_1	1:500 / 1:200	Santa Cruz/R&D
VEGFR-3	VEGFR-3		mAb mouse anti-human	IgG	1:100	(Joukov et al., 1996)

mAb=monoclonal antibody, pAb=polyclonal antibody

4.4.2. Elisa analyses and clinical chemistry (II-IV)

For quantitative measurement of transduced human VEGF-$D^{\Delta N \Delta C}$ and VEGF-A proteins in myocardium and plasma, respective ELISA assays were performed according to the manufacturer's instructions (cat no: DVED00 and DVE00, respectively; Quantikine, R&D Systems). Snap-frozen myocardial samples were homogenized as described(Rissanen et al., 2003a). The results are expressed as pg of growth factor/mg of total protein. According to the manufacturer, the sensitivities of human VEGF-A and VEGF-D ELISAs are 9 pg/ml and 11 pg/ml, respectively. Plasma imatinib mesylate (STI571/Gleevec) drug concentrations (III) were determined by HPLC.

Plasma Troponin T (TnT), creatinine (Crea), creatinine kinase (CK), creatinine kinase-MBm (CK-MBm), C-reactive protein (CRP), alanine aminotransferase (ALT), alkaline phosphatase (AP) and lactate dehydrogenase (LDH) were measured at the Department of Clinical Chemistry, Kuopio University Hospital.

4.5. Perfusion measurements

4.5.1. Microspheres (IV)

Myocardial perfusion was assessed from tissue samples using colored microspheres. The perfusion ratio between the transduced anterolateral and intact apical control regions was calculated at rest and during dobutamine stress (10-80 μg/kg/min until a heart rate x 2 of rest, typically 180-200 beats/min, was achieved; Dobutrex, Lilly) using red and yellow-green fluorescent microspheres ($6x10^6$, 15μm in diameter, FluoSpheres, Molecular Probes) (Rissanen et al., 2003b; Rissanen et al., 2003a). Microspheres were injected into the left ventricle near the mitral valve via a 5F left coronary catheter (Cordis, Johnson & Johnson). After sacrifice, microspheres were extracted from myocardial samples with the sedimentation method according to the manufacturer's instructions.

4.5.2. Myocardial contrast echocardiography (IV)

Myocardial contrast echocardiography for the assessment of perfusion within the injected region of the heart was performed at 3.5 MHz and for receiving the second harmonic echo using Acuson Sequoia 512 and 3V2c transducer (Siemens). Real-time reperfusion images (22 Hz, power -19dB, gain -3, MI 0.16) were obtained with short-axis mid-papillary level after destruction of the i.v. administered contrast agent (1.0 ml, Sonovue, Bracco) with a high-energy Doppler wave. The images which represent maximal reperfusion of the treatment area, compared to untreated segments of the left ventricle, were chosen for analysis.

4.6. Vascular permeability and pericardial effusion (IV)

The modified Miles assay was used for quantitative determination of plasma protein extravasation resulting from increased microvessel permeability within transduced

myocardium. Evans Blue dye (30 mg/kg) was injected i.v. 30 min prior to sacrifice, either six days or three weeks after gene transfer. Pig hearts were harvested from the mediastinum and perfusion fixed. Extravasated Evans Blue dye was extracted from transduced (anterolateral wall of the left ventricle) and intact control (apex) region by incubation of the samples in formamide at 60°C for 24-48h. The amount of Evans Blue was determined by the absorbance at 610 nm, and the ratio between the transduced and control samples was calculated.

Plasma protein extravasation resulted in pericardial effusion in some groups. This was detected by echocardiography using modified long-axis views for qualitative analysis.

4.7. Cell culture experiments

In study II, rabbit aortic smooth muscle cells (RAASMC) were incubated for 30 min in serum-free medium containing VEGF-$D^{\Delta N\Delta C}$ or *LacZ* adenoviruses using a multiplicity of infection (MOI) of 1000. The ability of VEGF-$D^{\Delta N\Delta C}$ to induce human endothelial cell (EA hy926) tube formation was analyzed with the conditioned medium in matrigel (Jones et al., 1998; Hiltunen et al., 2000a). Tube formation was measured by counting the number of connected cells per well (n=4 wells in both groups).

To document the ability of STI571 to the block rabbit PDGF receptor in study III porcine aortic endothelial cells transfected with PDGF β-receptor (PAE/PDGFβR) or PDGF α-receptor (PAE/PDGFαR), were maintained in F12 medium, and rabbit abdominal aortic smooth muscle cells (RAASMCs) were cultured in DMEM. Receptor expression and tyrosine phosphorylation were analyzed as described (Pietras et al., 2001). Receptor phosphorylation was determined by scanning and analysis with the NIH ImageJ free-ware.

4.8. Statistical analyses (I-IV)

Results are expressed as mean±SEM. Statistical significance was evaluated using one-way ANOVA or Kruskal-Wallis test followed by Mann-Whitney U-test with Bonferroni's correction, independent sample *t*-test or Dunnett's post-hoc analysis where appropriate. Correlation analyses were performed by the Pearson test. $P<0.05$ was considered statistically significant. Microsoft Excel, Stat View 5.0 (Abacus Concepts) and SPSS (SPSS Inc) software were used to perform statistical analyses.

5. RESULTS

5.1. VEGF-D expression during atherogenesis

5.1.1. VEGF-D and VEGF-A expression in human lesions

The expression of VEGF-D and VEGF-A was studied using immunocytochemistry (I). Two different monoclonal antibodies used for VEGF-D staining showed similar expression patterns (data not shown). Means of the VEGF-D and VEGF-A expression semiquantitative analysis in both ECs, intima and media were calculated (Figure 5). For VEGF-D the majority of the samples showed at least weak staining in ECs. VEGF-D was present in ECs at all stages of atherosclerosis and there were no significant differences between the groups. The expression of VEGF-A in ECs changed during atherogenesis. There were more VEGF-A positive ECs in plaques and complicated lesions than in normal arteries or fatty streaks.

VEGF-D was abundant the in intima of every type of lesion studied. The majority of the samples showed strong expression. However, the staining for VEGF-D was significantly lower in complicated lesions when compared to either normal arteries or plaques, and there was a trend towards a decrease in staining when compared to fatty streaks. Whereas the immunostaining of VEGF-A was mainly localized inside the cells, VEGF-D was also found in the extracellular space. In normal arteries, fatty streaks and plaques rich in SMCs, diffuse VEGF-D staining was found mostly around SMCs. In plaques and complicated lesions rich in connective tissue, VEGF-D staining was most prominent in macrophage-rich areas, whereas the staining in SMCs was mostly intracellular. The expression of VEGF-A within the intima increased during atherogenesis. The majority of normal arteries were devoid of expression but there was at least weak expression in the majority of samples graded as fatty streak, plaque, and complicated lesions. Whereas the positive cells in normal arteries and fatty streak lesions were mostly SMCs, in advanced lesions the positive cells were located almost exclusively in macrophage-rich areas. Both VEGF-A and VEGF-D were found in macrophages in association with plaque neovascularization. Furthermore, both VEGFs were abundant in medial layers of all studied arteries and no differences in immunostaining patterns were found between the study groups.

Double immunostaining confirmed that the VEGF-D protein was localized to ECs, SMCs, and macrophages. In situ hybridization showed that the VEGF-D RNA was present in the same cell types and RT-PCR verified that the VEGF-D and VEGF-A RNAs were present in the vessel wall. The proteolytic processing of VEGF-D was analyzed by comparing immunostainings with different antibodies: VD1 recognizes all forms of VEGF-D, whereas sc-7602 recognizes the C-terminal end of VEGF-D (i.e. unprocessed or partially processed VEGF-D). VD1 staining was abundant in all samples apart from complicated lesions which were rich in connective tissue. Sc-7602 staining was notably less abundant than VD1 and was localized almost exclusively inside the cells.

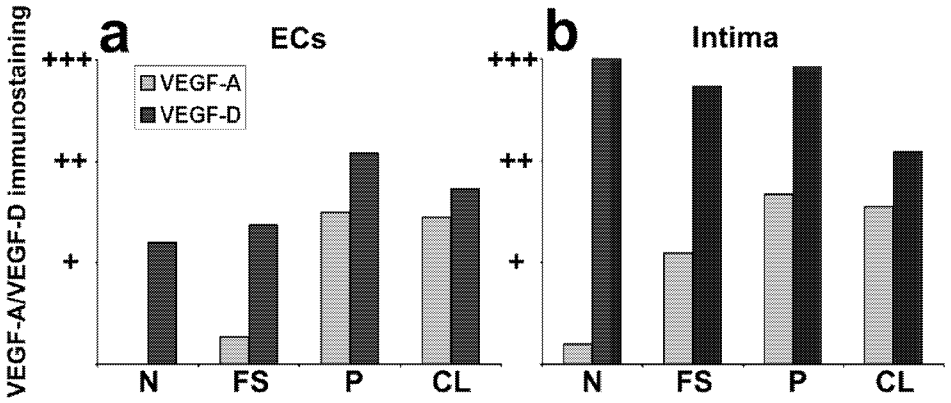


Figure 5. VEGF-A and VEGF-D expression in **a.** ECs and **b.** intima of the human atherosclerotic lesions. N=normal artery; FS=fatty streak; P=plaque; CL=complicated lesion

5.1.2. VEGFR-2 and VEGFR-3 expression in human arteries

Immunostaining for VEGFR-2 showed it to be localized to SMCs within the intima and media in every study group. Positive cells were also detected in the endothelium of fatty streaks and more advanced lesions, and strong expression was localized to the ECs of adventitial vessels. Both antibodies used gave similar expression patterns. Thus, the expression of VEGFR-2 in SMCs was not related to the stage of atherosclerosis. With *in situ* hybridization VEGFR-2 mRNA was found in ECs and intimal SMCs in the same areas which were positive for immunostaining.

VEGFR-3 was undetectable in ECs, intima, or media with either immunostaining or *in situ* hybridization techniques. Only some ECs of the adventitial small vessels stained positive for VEGFR-3 immunostaining.

5.2. Gene therapy for restenosis in a rabbit denudation model

5.2.1. Gene transfer efficacy and biodistribution of adenoviral vector (II and III)

Gene transfer efficiency was determined by analyzing β-galactosidase activity with X-gal staining (Hiltunen et al., 2000b). Transduced cells were located within the neointima and media adjacent to the internal elastic lamina (IEL). At the six day time point, the gene transfer efficiency was 1.91±1.32% within the intima and 0.21±0.08% within the media. Two weeks after gene transfer, 0.30±0.09% of the intimal cells and 0.15±0.03% of the medial cells were positive for X-Gal staining. Only sporadic X-Gal positive cells were found at three the week (III) and four week (II) time points. The transduced VEGF-$D^{\Delta N \Delta C}$ mRNA was readily detectable in the target area with RT-PCR at each time point (II). VEGF-C

mRNA was detectable in the VEGF-C–transfected animals at the three week time point (III).

Intravascular adenoviral gene transfer results in biodistribution of the vector to non-targeted tissues (Hiltunen et al., 2000b). Positive cells were found in all tested organs with X-Gal staining at the six day time point (II). The spleen and liver were the most prominently transduced organs. Consequently, a transient increase was detected in serum levels of transduced human VEGF-$D^{\Delta N\Delta C}$ after adenoviral gene transfer. Although we used growth factors which are associated with strong angiogenesis and induce edema formation (Rissanen et al., 2003b), such effects were not detected in ectopic organs (II and III). No significant differences were found in serum values for C-reactive protein (CRP), Creatinine (Crea), aspartate aminotransferase (AST), alkaline phosphatase (AP) and lactate dehydrogenase (LDH) (II) and no signs of toxicity to internal organs were reported at autopsy performed by a veterinarian pathologist (III).

5.2.2. Adenoviral VEGF-$D^{\Delta N\Delta C}$ gene transfer reduces intimal thickening (II)

The capability of VEGF-$D^{\Delta N\Delta C}$ secreted from transduced vascular SMCs to induce EC tube formation was tested in matrigel assay. Endothelial tube formation was significantly enhanced In VEGF-$D^{\Delta N\Delta C}$ treated wells compared to *Lac*Z controls.

Balloon-denudation of the rabbit aorta results in intimal thickening and SMC proliferation. The intima/media ratios (I/M) were determined from animals sacrificed 6, 14 or 28 days after the gene transfer. Two weeks after the gene transfer, AdVEGF-$D^{\Delta N\Delta C}$ group showed a 52% decrease (P=0.039) in I/M as compared to the Ad*Lac*Z control group. However, no differences were found in I/M between the study groups six days and four weeks after the gene transfer. The therapeutic mechanism of AdVEGF-$D^{\Delta N\Delta C}$ gene transfer was studied further by inhibiting NO production with NO synthase inhibitor (L-NAME) two weeks following AdVEGF-$D^{\Delta N\Delta C}$ or AdLacZ gene transfers. L-NAME completely blocked the reduction in neointimal growth two weeks after the gene transfer. There were no differences in macrophage influx, damage to the internal elastic lamina, endothelial regrowth or proliferating cells between these two groups.

AdVEGF-$D^{\Delta N\Delta C}$ gene therapy resulted in reduced macrophage influx into the vessel wall, as studied by immunohistochemistry. At six day, there was an 85% reduction (P=0.016) in the number of macrophages per mm^2 of arterial wall in AdVEGF-$D^{\Delta N\Delta C}$ group compared to the Ad*Lac*Z group. The damage to the internal elastic lamina (IEL) was equal at each time point showing that the initial injury to the aorta did not differ between the study groups. There were no significant differences in lumenal endothelial regrowth or in the number of proliferating cells between the study groups.

5.2.3. Combination treatment leads to a more permanent therapeutic effect than lone therapy

To confirm the ability of STI571 to inhibit the phosphorylation of the rabbit PDGF receptor, we performed experiments with rabbit aortic SMCs. Analysis revealed that cultured rabbit aortic SMCs expressed PDGF ß-receptors but not PDGF α-receptors. Pretreatment of rabbit cells with STI571 resulted in a dose-dependent inhibition of PDGF-BB–induced PDGF β-receptor phosphorylation. Scanning of immunoblots was performed to provide

semiquantitative data on PDGF receptor inhibition. At 3 µmol/l STI571, 70.7% and 13.6% inhibition of the human and rabbit PDGF β-receptor was observed, respectively. After treatment with10 µmol/l STI571, 89.1% inhibition of the human receptor and 53.5% inhibition of the rabbit receptor were achieved.

Three weeks after gene transfer, control-treated rabbits displayed I/M of 0.38±0.05. At this time point, the groups subjected to VEGF-C gene transfer alone or VEGF-C gene together with the kinase inhibitor demonstrated a significant reduction in lesion formation, with I/M of 0.23±0.02 and 0.24±0.05, respectively (P<0.05). No significant reduction was seen after treatment with STI571 alone. A decrease in the number of vessel wall macrophages, as determined by counting RAM-11–positive cells, was seen in STI571-treated animals at the end of drug therapy. No significant differences in reendothelialization were detected at this time point.

At the 6-week end point, theI/M in the control group was 0.51±0.08. Single therapy with VEGF-C or STI571 failed to induce a persistent reduction in lesion formation with no statistically significant differences as compared to the control group. In contrast, the group that had received the combined treatment displayed a decreased I/M (0.23±0.05, P<0.05). This corresponds to a 55% reduction in lesion size. The effect was completely attributable to a decrease in neointima size at both time points and did not involve vascular remodeling. At 6 weeks, an increase in endothelial cell coverage was observed in both groups that had received VEGF-C gene transfer. No differences between the groups were observed in internal elastic lamina (IEL) damage, animal weights, blood lipid profiles, and basic hematological parameters. At the six week time point, significantly fewer vessel wall macrophages were seen in both groups that had received VEGF-C.

A reduction in intimal SMC population occurred in the 2 VEGF-C–treated groups at 3 weeks and in the combination group at 6 weeks, in line with the I/M data. None of the treatment groups displayed, at either end point, any difference in medial SMC number. To additionally characterize the effects on SMCs, we determined the fraction of SMCs undergoing apoptosis and proliferation. At 3 weeks, an increase in SMC apoptosis was observed within the intima after treatment with STI571 alone or in combination with VEGF-C gene transfer and a reduction in the fraction of proliferating SMCs was seen in both groups treated with VEGF-C. No effects on the fraction of SMCs undergoing proliferation or apoptosis were observed within the intima or media at the later time point.

5.3. Myocardial angiogenesis

5.3.1. Gene transfer efficacy

AdVEGF-$D^{\Delta N\Delta C}$, but not pVEGF-$D^{\Delta N\Delta C}$, gene transfer induced a dose-dependent protein production within the myocardium and plasma leakage as measured by ELISA six days after gene transfer. Transduced human VEGF-A_{165}, which remains strongly bound to the matrix (Ferrara, 1999), was not detectable by ELISA from myocardial or plasma samples. However, high gene transfer efficacy was demonstrated by immunohistochemistry after adenoviral gene transfer. VEGF-$D^{\Delta N\Delta C}$ production in the myocardium became undetectable three weeks after adenoviral gene transfer. Consequently, there were also no detectable angiogenic effects or side-effects at that time point.

5.3.2. Transmural angiogenesis in myocardium

AdVEGF-D$^{\Delta N\Delta C}$ and AdVEGF-A stimulated a remarkable transmural angiogenic effect in the injected regions six days after gene transfer. The angiogenic effects also comprised an enlargement of pre-existing microvessels, as measured from αSMA-immunostained sections. Apical control and Ad*Lac*Z transduced regions had similar microvessel mean areas showing that adenovirus by itself did not induce microvessel enlargement. The low and high doses of AdVEGF-A caused a 1.7-fold (P=0.017) and a 2.3-fold (P=0.004) increase, respectively, in the microvessel mean area compared to the Ad*Lac*Z control six days after gene transfer. Both doses of AdVEGF-D$^{\Delta N\Delta C}$ increased the microvessel mean area 2.1-fold over Ad*Lac*Z (P=0.003 and P=0.02, respectively). The angiogenic effect after adenoviral gene transfer was transmural and, interestingly, was strongest in the epicardium despite the intraventricular route of injections, as demonstrated by a modified Miles assay. The microvessel enlargement, at its best, led to a 2.0-fold increase in the total area of the myocardium covered by microvessel lumens (from 6.7% in Ad*Lac*Z group to 13.5% in AdVEGF-A 10^{12}vp group, P=0.004). No changes in αSMA-positive microvessel density were detected between the study groups.

AdVEGF-A-induced angiogenesis was oriented along the muscle bundles and involved clusters of αSMA-positive vessels, but the effect of AdVEGF-D$^{\Delta N\Delta C}$ tended to be more diffuse. Immunostaining for proliferating cell nuclear antigen (PCNA) demonstrated that the enlargement of αSMA-positive microvessels was accompanied by proliferation of both ECs and pericytes. Three weeks after AdVEGF-D$^{\Delta N\Delta C}$ gene transfer, angiogenic effects were no longer detected. In contrast to adenovirus, which showed a high efficacy of gene transfer, only sporadic positive cells were observed with X-Gal staining, six days after p*Lac*Z gene transfer. In line with the low gene transfer efficiency, pVEGF-A or pVEGF-D$^{\Delta N\Delta C}$ did not induce any changes in microvessel morphology.

5.3.3. Angiogenesis results in myocardial perfusion increases

Regional myocardial perfusion was quantitatively assessed using colored microspheres. Compared to Ad*Lac*Z, the higher dose of AdVEGF-A and the lower dose of AdVEGF-D$^{\Delta N\Delta C}$ resulted in statistically significant 2.0- and 1.6-fold increases (P=0.006 and P=0.037), respectively, in the perfusion ratio between the transduced and apical control areas at rest. In addition, the higher dose of AdVEGF-D$^{\Delta N\Delta C}$ also elicited a trend towards increased perfusion (1.6-fold, P=0.064). During dobutamine stress, the perfusion ratios between the transduced and apical control regions were the same in all groups. Qualitative analysis with myocardial contrast echocardiography revealed marked increases in perfusion in the corresponding regions six days after AdVEGF-A and AdVEGF-D$^{\Delta N\Delta C}$ gene transfers.

5.3.4. Changes in vascular permeability and pericardial effusion

Ad*Lac*Z did not change vascular permeability in the myocardium. In contrast, AdVEGF-D$^{\Delta N\Delta C}$ and AdVEGF-A increased vascular permeability in the target areas as shown by the modified Miles assay six days after gene transfer. Whereas the 10^{11}vp dose of AdVEGF-A

did not significantly induce any permeability change, the same dose of AdVEGF-D$^{\Delta N\Delta C}$ caused a 4.8-fold increase compared to Ad*Lac*Z (P=0.032). Higher doses of AdVEGF-A and AdVEGF-D$^{\Delta N\Delta C}$ caused a 4.3- and 3.8-fold increase (P=0.008 and 0.003), respectively, in vascular permeability. pVEGF-A or pVEGF-D$^{\Delta N\Delta C}$ gene transfer did not increase vascular permeability compared to the p*Lac*Z control. A positive correlation was found when the vascular permeability ratio of each heart was plotted against the respective microvessel mean area (Pearson correlation 0.687, P<0.01)

Microvessel hyperpermeability with the higher dose of AdVEGFs resulted in substantial pericardial effusion detectable by echocardiography. The total protein and fibrinogen concentration of the effusate was smaller than in the plasma of the same animals (total protein concentration 38±3 vs 49±4 g/l, P=0.022). Using specific ELISAs, high amounts of VEGF-D$^{\Delta N\Delta C}$ (6320±687 pg/ml) and VEGF-A (796±108 pg/ml) were found in the effusate after adenoviral gene transfer with the respective adenoviruses. There was no effusion after either the higher dose of Ad*Lac*Z or after the lower doses of AdVEGFs. Despite the myocardial edema and pericardial effusion, there were no increases in TnT, CK and CK-MBm levels in any of the study groups.

Table 3. Summary of primary findings of the thesis

Study	Model / Study Aim	Methods	Primary Findings
I	VEGF-D expression in human arterial samples	Immunohistochemistry	VEGF-D is constitutively expressed during atherogenesis. In complicated lesion its secretion to the extracellular space is disrupted and the expression in lesion is diminished
II	Adenoviral intra-arterial gene transfer of VEGF-$D^{\Delta N\Delta C}$ in a rabbit balloon-denudation model of restenosis	Histological measurements, immunohistochemistry	AdVEGF-$D^{\Delta N\Delta C}$ gene transfer reduces neointimal thickening and macrophage influx to the vessel wall
		NO inhibition by per oral L-NAME	Therapeutic effect is mediated through NO
III	Intra-arterial AdVEGF-C gene transfer in conjunction with oral anti-PDGFR drug in a rabbit balloon-denudation model of restenosis	Histological measurements, immunohistochemistry	Combination treatment leads to more persistent therapeutic effect than sole treatments
IV	Catheter-mediated intramyocardial VEGF-$D^{\Delta N\Delta C}$ and VEGF-A gene transfer to pig heart using adenoviral vector and naked plasmid	Immunohistochemistry, Microspheres, modified Miles assay, Myocardial contrast echocardiography	Adenoviral VEGF-$D^{\Delta N\Delta C}$ and VEGF-A gene transfers induce microvessel enlargement with significant perfusion increase
			As a side-effect vascular permeability is increased resulting in pericardial effusion with higher adenoviral VEGF doses
			VEGF gene transfer using naked plasmid DNA does not induce any vascular changes

6. Discussion

6.1 VEGF-D expression in human arteries

We investigated the expression patterns of VEGF-D and VEGF-A in normal human arteries and atherosclerotic lesions using immunohistochemistry. In ECs and intima, the expression of VEGF-A increased with the progression of atherosclerosis. Meanwhile, VEGF-D was present in ECs and intima at all stages of atherosclerosis. Only within the intimas of complicated lesions a clear reduction of VEGF-D staining was observed.

Immunostainings indicated that the expression of VEGF-D in normal arteries and fatty streaks was mainly localized to SMCs. In plaques and complicated lesions the protein was also detected in macrophages. The positive VEGF-D staining was diffuse in each studied sample, except in complicated lesions rich in connective tissue. In macrophage-rich lesions, VEGF-D staining was found in macrophages in close proximity to regions of plaque neovascularization. VEGF-D is initially produced as a prepropeptide and further processed to its functional form by extracellular proteases (Stacker et al., 1999a). The staining with antibody that recognizes all forms of VEGF-D (VD1) was substantial both intracellular and also in the extracellular space, whereas the staining with antibody that binds to the C-terminal end of the unprocessed VEGF-D (sc-7602) was strictly intracellular. This suggests that within the vessel wall, lymphangiogenic long form of VEGF-D is effectively processed to its mature form (VEGF-$D^{\Delta N \Delta C}$) which has strong effects in the vascular system (Rissanen et al., 2003b).

The presence of VEGF-D in normal arteries suggests that it may have a constitutive or homeostatic role within the artery wall. In complicated lesions, SMCs present in lacunae of connective tissue seem to be unable to secrete VEGF-D to extracellular space. This results in a reduction of VEGF-D within the intima of complicated lesions. On the other hand, with the influx of macrophages into progressively mature lesions the high focal expression of VEGF-D in macrophages within the plaque shoulder regions suggests that VEGF-D may play a role in plaque neovascularization. Our results suggest that VEGF-D plays a dual role in atherosclerotic human arteries. When produced by SMCs of the normal arteries and early atherosclerotic lesions it is most likely to be homeostatic, whereas in macrophage-rich inflammatory regions it may contribute to the pathological changes observed in advanced atherosclerosis.

In this study it was seen that the expression of VEGF-A in the artery wall increased during atherogenesis and that VEGF-A was found in macrophages, in close to regions of plaque neovascularization. This is consistent with previous studies that have demonstrated increased VEGF-A expression during atherogenesis (Couffinhal et al., 1997; Inoue et al., 1998). Recent studies have also shown that VEGF-A promotes atherogenesis when administered systemically to small rodents (Celletti et al., 2001) and is chemotactic for macrophages (Ferrara, 1999). On the other hand, VEGF-A has also protective effects in arteries as it mediates anti-apoptotic effects in endothelium (Spyridopoulos et al., 1997; Zachary et al., 2000), protects against LDL toxicity (Kuzuya et al., 2001), and induces nitric oxide production (Laitinen et al., 1997b). Still, it is unclear whether the net effect of local production of VEGF-A in the artery wall is protective or harmful.

We demonstrated constitutive VEGFR-2 expression within arterial SMCs in all arteries studied. This is in agreement with previous findings that VEGFR-2 is expressed in cultured

arterial SMCs (Grosskreutz et al., 1999) and within intimal SMCs of denuded rabbit aorta (Hiltunen et al., 2000a). We also detected VEGFR-2 within the macrophages and ECs of atherosclerotic arteries. The observed upregulation of VEGFR-2 within the ECs of atherosclerotic arteries may be due to VEGF-A induced stimulation (Weisz et al., 2001). In contrast to VEGFR-2, VEGFR-3 expression was detected only within the ECs of adventitial vessels. Previous studies have also shown that VEGFR-3 is almost exclusively present in the lymphatic vessels and absent in the vascular system apart from vasa vasorum (Kaipainen et al., 1995; Partanen et al., 2000). Current results suggest that VEGFR-2 is the most important receptor mediating the effects of VEGF-D in large human arteries.

6.2 Gene therapy for restenosis in a rabbit model

6.2.1. Therapeutic effect on neointimal thickening

Intra-arterial manipulation, such as angioplasty, damages the vessel wall endothelium and predisposes vessels to inflammatory reactions, thrombus formation, SMC proliferation and migration which lead to the deposition of extracellular matrix, and hence restenosis (Bauters et al., 1996). It has been hypothesized that rapid re-endothelization after balloon dilatation would protect the vessel from restenosis (Rutanen et al., 2002). Accordingly, VEGFs have been used to accelerate recovery of the endothelium. Previous studies using VEGF-A and VEGF-C gene transfer have shown potential for the prevention of restenosis in animal models (Asahara et al., 1995; Hiltunen et al., 2000a; Khurana et al., 2004).

VEGF-D is constitutively expressed in normal human arteries and early-stage atherosclerotic lesions but its expression is decreased and its biological processing to the mature form is disrupted in connective tissue rich atheroclerotic lesions (I). Consequently, the vasculoprotective effects of VEGF-D may be decreased in end-stage atherosclerotic lesions that are candidates for PT(C)A. We tested the hypothesis whether intra-arterial gene transfer using the mature form of VEGF-D could be protective and prevent restenosis. It was found that AdVEGF-D$^{\Delta N\Delta C}$ gene transfer reduced neointima formation in balloon-denuded rabbit aorta via NO dependent mechanisms (II).

VEGF-D signals through two tyrosine kinase receptors, VEGFR-2 and VEGFR-3 (Achen et al., 1998). The mature form of VEGF-D$^{\Delta N\Delta C}$ binds effectively to VEGFR-2 which induces NO production from endothelium (Ferrara, 1999; Rissanen et al., 2003b). Since the therapeutic effect of intravascular AdVEGF-D$^{\Delta N\Delta C}$ gene transfer was blocked by L-NAME, the effects are mediated through NO. It is assumed that the mechanisms underlying the NO-mediated therapeutic effects are due to direct inhibition of vascular SMC proliferation and NO-induced vascular relaxation (Sarkar et al., 1997; Kumar and Lindner, 1997; Yan and Hansson, 1998).

An additional NO-dependent vascular mechanism induced by AdVEGF-D$^{\Delta N\Delta C}$ gene transfer may be the modulation of cytokine activities followed by the reduced macrophage influx into the vessel wall. NO is a known inhibitor of several cytokine-mediated processes that participate in the inflammatory response leading to restenosis (Zeiher et al., 1995; Tsao et al., 1997; Tsao et al., 1997; De Caterina et al., 1995; Qian et al., 1999; Tsakiris et al., 1999; Cipollone et al., 2001). Our study showed reduced macrophage number within the vessel wall six days after intravascular AdVEGF-D$^{\Delta N\Delta C}$ gene transfer. However, our

recent results using adenoviral gene transfer and overexpression of Nrf2, a transcription factor that activates antioxidant pathways, suggest that inhibition of macrophage influx after balloon denudation may not be sufficient per se to achieve a therapeutic reduction on I/M ratio (Inkala M et al. unpublished observation).

The therapeutic effect of AdVEGF-D$^{\Delta N\Delta C}$ gene delivery was no longer detectable four weeks after gene transfer. This also agrees with previous studies showing that the therapeutic effect is attenuated with time (Asahara et al., 1995; Hiltunen et al., 2000a). Also, in recent randomized placebo-controlled clinical trials, no reduction in restenosis was observed in target femorotibial or coronary lesions after follow up (Makinen et al., 2002; Hedman et al., 2003). Thus, combining two therapeutic approaches - simultaneous stimulation of re-endothelialization with local VEGF-based therapy and inhibition of PDGF mediated activation of SMCs - seems a rational strategy for improved results (III).

Combination therapy with AdVEGF-C and anti-PDGFR drug (STI571), in contrast to treatment with either alone, resulted in persistent reduction in I/M which was detectable six weeks after gene transfer. The prolonged therapeutic effect was a clear improvement compared to our previous studies using adenoviral gene transfer of VEGF-C (Hiltunen et al., 2000a) or VEGF-D (II) alone. Also, lesions within animals with combination treatment were characterized by fewer intimal SMCs at the six week end point. Our results highlight the major role of PDGFR-β mediated SMC migration and proliferation in restenosis process (Sirois et al., 1997; Giese et al., 1999; Davies et al., 2000). Also, AdVEGF-C gene transfer was associated with accelerated endothelial cell coverage. Because no effect on neointima formation occurred with VEGF-C treatment alone at six weeks or with VEGF-D$^{\Delta N\Delta C}$ at four weeks, it was concluded that gene therapy using adenoviral VEGFs leads only to a transient inhibition of intimal hyperplasia in a rabbit denudation model.

AdVEGF-C gene transfer significantly lowered the number of macrophages within vessel wall at six weeks (III). The reduction is assumably due to enhanced re-endothelization after VEGF-C gene transfer since an inverse relationship between macrophage infiltration and endothelial cell coverage was observed at this end point. However, VEGF-C has been reported to induce chemotaxis of macrophages in vitro and in mouse melanoma model (Skobe et al., 2001a). These controversary results suggest that the effects of VEGF-C are at least partially tissue dependent. Reduced number of macrophages in the vessel wall was also detected after three week sole treatment with anti-PDGFR drug (III). This is consistent with the recent results showing that PDGFR-β activation using PDGF-D overexpression leads to increased macrophage recruitment in tissues (Uutela et al., 2004).

The role of macrophages in different tissues and pathological or physiological situations is two-sided. In vessel wall they are clearly harmful, since inflammatory response after angioplasty and stenting is one of the key elements which leads to neointimal hyperplasia and restenosis (Serrano, Jr. et al., 1997; Kornowski et al., 1998; Brasen et al., 2001; Farb et al., 2002). However, in ischemic myocardium or peripheral muscle macrophages modulate angiogenic effects by producing cytokines and might favour the tissue survival. On the other hand, similar process in tumor or atherosclerotic plaque may lead to unwanted angiogenesis and harmful effects.

6.2.2. Efficacy and safety of the intra-arterial gene transfer

We achieved 1.91±1.32% gene transfer efficiency of intimal cells at the six days. Two weeks after the gene transfer, the amount of X-Gal positive cells was considerably lower. This is due to the transient nature of adenoviral gene transfer and continuous neointimal growth which increases the total number of intimal cells. In human arteries the medial dissection caused by the angioplasty balloon may enable transduction of deeper layers within the artery (Laitinen et al., 1998). Thus, therapeutic effects may be achieved by using adenovirus encoding secreted soluble gene products, such as VEGFs in manipulated human arteries.

Catheter-mediated intra-arterial gene transfer led to a biodistribution of adenoviral vector to ectopic organs and an increase in the serum level of transduced VEGF-$D^{\Delta N\Delta C}$. No detectable changes were found in basic clinical chemistry analysis between the treatment and control groups. VEGFs are powerful angiogenic growth factors that induce a potent angiogenic effect even in non-ischemic tissue (Rissanen et al., 2003b). However, there were no signs of unwanted angiogenesis in histological analysis of the studied organs, suggesting that local growth factor levels in ectopic tissues remained low. Earlier clinical trials have showed that intra-arterial gene transfer with a similar adenoviral dose is safe and well tolerated (Laitinen et al., 1998; Laitinen et al., 2000; Makinen et al., 2002; Hedman et al., 2003). Due to the similar size of the target arteries we chose to use the same viral dose in a rabbit model that was used in these clinical trials. Furthermore, STI571 used to block PDGF signaling has been safe in clinical trials (Capdeville et al., 2002).

6.3 Gene therapy for myocardial angiogenesis

6.3.1. Gene transfer in the myocardium

Adenoviral VEGF-$D^{\Delta N\Delta C}$ gene transfer induced a dose dependent protein production in myocardium as detected by ELISA assay. In contrast to VEGF-$D^{\Delta N\Delta C}$, VEGF-A_{165} protein could not be extracted from tissue samples for ELISA, but was readily detectable from paraffin sections by immunohistochemistry. Furthermore, transduced VEGF-$D^{\Delta N\Delta C}$, but not VEGF-A_{165}, was detected in plasma and the angiogenic effects promoted by VEGF-$D^{\Delta N\Delta C}$ were more diffuse throughout the myocardial tissue. These findings may relate to the efficient matrix-binding properties of VEGF-A_{165} (Ferrara, 1999), while there is no heparan-binding domain in the sequence of VEGF-$D^{\Delta N\Delta C}$ making it soluble and freely diffusible (Achen et al., 1998). Also, the distinct receptor-binding profiles may contribute to these differences. VEGF-A is a ligand for VEGFR-1 and VEGFR-2 while VEGF-$D^{\Delta N\Delta C}$ primarily activates VEGFR-2 and to some extent VEGFR-3 (Ferrara, 1999; Achen et al., 1998). VEGFR-1 can serve as a decoy receptor preventing VEGF-A but not VEGF-$D^{\Delta N\Delta C}$ from binding to VEGFR-2, which is the principal mediator of angiogesis (Ferrara, 1999; Rissanen et al., 2003b).

In contrast to adenoviral gene transfer, we did not find detectable protein production or biological effects with naked plasmid-mediated VEGF-$D^{\Delta N\Delta C}$ or VEGF-A gene transfer in non-ischemic pig myocardium. However, in preclinical and clinical trials naked plasmid has

been reported to stimulate therapeutic angiogenesis in ischemic heart and skeletal muscle (Tsurumi et al., 1996; Tio et al., 1999; Vale et al., 2001). Even though naked plasmid-mediated gene transfer may be enhanced by tissue damage and ischemia (Takeshita et al., 1996), collateral artery growth occurs in non-ischemic areas of the heart (Schaper and Ito, 1996). Furthermore, in order to treat patients with stable angina pectoris, who have myocardial ischemia only during exercise, the gene therapy vector should also be effective in non-ischemic tissue. Thus, the efficacy of naked plasmid DNA mediated gene transfer may not be sufficient for therapeutic angiogenesis in clinical setting.

6.3.2. Myocardial angiogenesis after adenoviral gene transfer

Intramyocardial gene transfer guided by the NOGA-system promoted transmural, epicardial weighted, effects in pig heart despite the catheter-mediated intraventricular route. This is probably due to an intramyocardial pressure gradient. The systolic subendocardial pressure is higher than the subepicardial pressure pushing the gene transfer solution and secreted growth factors towards the epicardium. Our results show that gene transfer using the NOGA catheter can be used to induce transmural therapeutic effects in the heart without the need for invasive operations, such as thoracotomy. NOGA-mediated injections themselves have not been associated with adverse effects (Kornowski et al., 1999; Vale et al., 2001). Thus, angiogenic gene therapy with a NOGA mapping catheter using the correct adenoviral dose is feasible, safe and efficient.

In agreement with previous investigations of non-ischemic skeletal muscle (Pettersson et al., 2000; Rissanen et al., 2003a; Rissanen et al., 2003b), the predominant response of microvessels to adenovirally administered VEGFs was the circumferential enlargement of pre-existing microvessels (up to 2.3-fold with AdVEGF-A_{165}) rather than an increase in the number of microvessels. In addition to microvessel dilatation, VEGFs promoted efficient proliferation of pericytes and ECs. It is commonly presumed that capillary density is increased as a result of therapeutic gene transfer. However, we did not detect any changes in the capillary density within myocardium in this study. The increase in diameter (up to 50 μm) and enhanced coverage with pericytes suggest that vessels resembling arterioles, venules or arteriovenous shunts may be developed. Similar, but perhaps more complete, blood vessel transformation was observed with long-term VEGF expression systems (Arsic et al., 2003; Springer et al., 2003). As VEGF is not a direct mitogen for SMCs, it is likely that these effects are indirect, possibly involving increased blood pressure and shear stress against the wall of the enlarged channels. Thus, it is possible that only the first step of angiogenesis, vessel enlargement, is achieved with adenoviral VEGF expression and longer expression is needed for collateral vessel formation and maturation (Dvorak et al., 1995; Pettersson et al., 2000; Dor et al., 2002; Arsic et al., 2003).

Although the role of enlarged vessels in the transfer of oxygen and nutrients to the myocardium remains unclear, perfusion was enhanced up to 2.0-fold in the treated area as compared to the apical control region at rest. Interestingly, the gene transfer-induced increase in perfusion ratio between the trasduced anterolateral and apical control regions disappeared during dobutamine stress. This is probably due to the better capacity of normal microvessels to increase their size in response to stress than those already maximally enlarged by VEGF overexpression.

As with skeletal muscle (Rissanen et al., 2003b), the largest vessels are also the leakiest to plasma proteins since a positive correlation exists between microvessel size and vascular permeability. The strong angiogenic effects induced by AdVEGFs were always accompanied by interstitial edema or even pericardial effusion at the higher doses. The edema was transient and did not cause significant tissue damage in pig myocardium. In previous animal experiments, excessive VEGF production in myocardium has been fatal for mice (Lee et al., 2000b) and pericardial fluid accumulation was associated with one death (of 10 transduced) in a dog myocardial ischemia model after AdVEGF-A_{165} gene transfer ($6x10^9$ pfu) was administered to the pericardial space (Lazarous et al., 1999). Pericardial effusion was not detected after intramyocardial AdVEGF-A121 gene transfer (titers 10^9 or 10^{10} pfu) in a pig model of myocardial ischemia (Patel et al., 1999). This is presumably because of pericardial fibrosis caused by thoracotomy or due to the reduced potency of VEGF-A_{121} in comparison with VEGF-A_{165} (Whitaker et al., 2001). Although healthy pigs in our study tolerated the transient edema and pericardial effusion well, the situation may be different in ischemic human heart and further preclinical dose-escalation studies are needed for the selection of a safe but effective adenoviral dose for subsequent use in clinical trials. In our study, the lower doses (10^{11} vp) of AdVEGFs did not induce pericardial fluid accumulation, although tissue edema was detected with the modified Miles assay. Our results indicate that the adenoviral dose, when using VEGFs, should be well below 10^{12} viral particles.

The effects of AdVEGFs on microvessel enlargement, perfusion and vascular permeability in non-ischemic myocardium were transient and not detectable three weeks after the gene transfer suggesting that demonstrable biological effects occur only during the time course of adenoviral expression. Despite pericyte recruitment, which has been shown to promote vessel maintenance (Benjamin et al., 1998; Benjamin et al., 1999), the enlarged vessels shrunk to their original size at the later time point. The marker used for pericyte and SMC detection, αSMA, is expressed both in immature and mature pericytes which makes it difficult to estimate the maturity of the vessels (Van Gieson et al., 2003). Blood perfusion usually matches the metabolic demand in healthy tissue. The regression of enlarged vessels indicates that increased blood flow alone is not sufficient to maintain enlarged microvessels in healthy myocardium. It can be hypothesized that the situation woud be different in ischemic myocardium where the increased blood flow is needed for normal metabolic needs.

7. SUMMARY AND CONCLUSIONS

It is important to further characterize basic mechanisms of atherosclerosis and other occlusive vascular diseases to identify and evaluate new therapeutic targets at the molecular level. Local gene transfer in the vascular system is a promising novel alternative to treat cardiovascular disease. Preclinical trials have demonstrated the potential of gene therapy and first clinical trials have shown that gene transfer can be safely performed in man. However, more basic information of the therapeutic mechanisms of gene transfer is needed to optimize treatment approaches for cardiovascular disease. Also, large placebo-controlled, randomized double-blind trials with hard clinical endpoints are needed to determine the true efficacy of cardiovascular gene therapy.

Our results show that VEGF-D is constitutively expressed in normal human arteries during atherogenesis, and that it is efficiently processed to its biologically active mature form in the vessel wall. However, in advanced lesions which are rich of connective tissue this process is disturbed and VEGF-D remains intracellular. This study also demonstrated the potency of adenoviral VEGF-$D^{\Delta N \Delta C}$ gene therapy to prevent restenosis in a rabbit model of intimal hyperplasia. Therapeutic effect was mediated by NO and related to reduced local inflammatory reaction in the vessel wall. Combination therapy using adenoviral VEGF-C gene transfer in conjunction with anti-PDGF drug to inhibit restenosis in a rabbit denudation-model led to prolonged therapeutic effect compared to the sole therapy. Furthermore, adenoviral gene transfer of VEGF-$D^{\Delta N \Delta C}$ and VEGF-A_{165} was used to promote efficient angiogenic effects with increased blood perfusion in pig myocardium.

The effects achieved using adenoviral gene transfer in rabbit aorta and pig myocardium were transient and no longer detectable after three weeks. Also, it was demonstrated that adenoviral gene transfer can lead to transduction of non-target organs. Our results show that too high dose of VEGFs in myocardium causes substantial edema which may result in severe side-effects, such as pericardial effusion. These results propose that for safe and efficient therapy, future experimental research should focus on the development of an efficient, long term, regulated and tissue-targeted gene transfer vector.

It is concluded that the constitutive expression of VEGF-D has a role in the maintenance of vascular homeostasis in large human arteries. VEGFs are potent factors to prevent intimal hyperplasia after balloon-denudation and to promote vascular angiogenic effects in myocardium. These results indicate that VEGF gene therapy could be further developed for therapeutic approach to inhibit restenosis after vascular manipulation or induce therapeutic vascular growth in myocardial ischemia.

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